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Insulin and Ketones: Their Roles in Brain Mitochondrial Function

Sheryl Teresa Carr

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

Master of Science

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ABSTRACT

Insulin and Ketones: Their Roles in Brain Mitochondrial Function

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Master of Science

The prevalence of both Type 2 diabetes mellitus (T2DM) and Alzheimer's disease (AD) is increasing worldwide, and the trends are unfortunately expected to continue. AD has recently been tied with mitochondrial dysfunction and insulin resistance, creating a mechanistic tie between AD and T2DM. Unfortunately, insulin resistance is often increased with aging and therefore, all individuals are at risk of brain mitochondrial dysfunction. Without proper mitochondrial function, the brain will degenerate, causing impaired cognitive function and reduced quality of life. The purpose of this study is two-fold: first, to understand the role of ceramides in insulin-induced brain mitochondrial dysfunction, and; second, to understand how ketones can restore brain mitochondrial function in aged brains.

To evaluate the role of insulin resistance and ceramides in brain mitochondrial function, we induced hyperinsulinemia in ApoE4 mice. In addition to insulin, one group received myriocin injections to inhibit ceramide biosynthesis. We observed significant increases in brain ceramides in the insulin-treated group, which correlated with disrupted brain mitochondrial function. However, the group receiving myriocin alone, and, importantly, myriocin with insulin, had normal lipid profiles and normal mitochondrial bioenergetics. Altogether, these findings support the hypothesis of the key role of ceramides in insulin resistance-induced mitochondrial dysfunction within the brain.

Next, young adult (5 months old) and old (28 months old) rats were assigned to either standard chow diets or very-low-carbohydrate, high-fat, ketogenic diets for 4 weeks. Following the treatment period, we analyzed brain mitochondrial function and oxidative stress. We found that the old rats fed the ketogenic diet had improved mitochondrial function in comparison to the old rats consuming standard rodent chow. In addition, the old rats fed a standard diet had significantly higher levels of oxidative stress than the aged rats on the very-low-carbohydrate, high-fat diet. These findings revealed that ketones can protect brain mitochondrial function in aging.

Collectively, these results suggest that insulin resistance has a role in the development of brain mitochondrial dysfunction due to ceramide accumulation, while ketones can help mitigate some of the negative consequences of aging, perhaps some due to insulin resistance, on brain mitochondrial function.

Keywords: type 2 diabetes, alzheimer's disease, mitochondria, insulin, ketones, ceramide

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CHAPTER 1: Introduction and Background

Insulin Signaling Within the Brain

The role of insulin within the brain is very different from that of peripheral tissues. Various glucose transporters (GLUTs) are found throughout the brain, but insulin only affects glucose transport in the hypothalamus, where there is the insulin-dependent transporter – GLUT4 (Ren et al., 2014). Unlike peripheral insulin receptors, insulin-mediated neuronal insulin receptors and IGF-1 receptors within the brain regulate many physiological functions such as food intake, inhibition of hepatic gluconeogenesis, counter-regulation of hypoglycemia, modulation of tau protein phosphorylation, A β PP metabolism, Amyloid- β clearance, neuronal survival, and memory (Candeias et al., 2012). Therefore, glucose uptake and metabolism within the brain is one of many actions within neuronal function regulated by insulin.

Despite insulin's lack of glucose regulation within the brain, its signaling cascade is still important for other physiological functions within the brain, especially those with neuroprotective effects. Binding of insulin or IGF-1 causes receptor auto-phosphorylation, stimulating tyrosine kinase activity, which then causes phosphorylation of the insulin receptor substrates on the tyrosine residues. This causes two main signaling cascades mediated by phosphatidylinositol 3-kinase (PI3K)/AKT and mitogen-activated protein kinase (MAPK) (Candeias et al., 2012).

AKT, along with its downstream effector CREB, has been shown to be a major contributor of neuron formation, synaptic plasticity within the brain, learning, and memory (Xiang et al., 2015). Another downstream signaling molecules of AKT is GSK3 $\alpha\beta$, and activation of AKT, protein kinase C, or c-AMP-dependent protein kinase can lead to the

inactivation of GSK-3 α β . GSK3 α increases the production of amyloid-beta while GSK3 β is the major tau kinase responsible for the hyperphosphorylation of tau and its formation of neurofibrillary tangles. In addition, the inhibition of GSK-3 β by AKT prevents apoptosis of neurons and other glial cells (Candeias et al., 2012).

The MAPK pathway is the second pathway activated by insulin within the brain. This promotes the expression of genes involved in cell and neuronal synapse growth as well as repair and maintenance of cells within the brain. Some researchers have been bold enough to conclude that insulin has vital neurotrophic and neuroprotective effects within the brain based on its downstream effects of both the PI3K/AKT and MAPK pathways (Candeias et al., 2012).

Insulin Resistance

Insulin resistance (IR) is characterized by the body's inability to effectively use the insulin it produces and, thus, insulin is unable to elicit appropriate nutrient storage after a meal in its primary target tissues, adipose tissue and the liver (Summers, 2006). Insulin resistance is not only a key contributor of Type 2 Diabetes Mellitus (T2DM), but has now emerged as a highly relevant contributor to Alzheimer's Disease.

Despite the evidence linking IR to Alzheimer's disease, there are nevertheless relevant mediators of this connection. A common mediator of IR is the sphingolipid ceramide and its downstream effects on mitochondrial dysfunction, both of which correlate with the severity of IR and cognitive decline (Haus et al., 2009; Savica et al., 2016). Ceramides are a sphingolipid that have a role in the antagonism of mitochondrial function and several different intermediates in the insulin signaling pathway. In regard to mitochondrial function, increased ceramide levels cause an increase in the production of reactive oxygen species and therefore induce more oxidative

stress within different tissues (Summers, 2006). In the brain, mitochondrial dysfunction and the resulting oxidative stress leads to increased amyloid-beta deposition, which positively regulates the oxidative stress-mediated deposition of amyloid-beta plaques (Ahmed, Mahmood, & Zahid, 2015).

The common problem with cognitive-declining and diabetic brains is a hypometabolism of glucose and deficient insulin signaling (i.e. IR). In regards to the deficient insulin signaling, the exact mechanism of how this happens is unknown, but evidence suggests it not only causes decreased glucose metabolism, but can induce neurofibrillary degeneration and tau O-GlcNAcylation, leading to tau neurofibrillary tangles (Liu, Liu, Grundke-Iqbal, Iqbal, & Gong, 2011).

Critically, as insulin resistance occurs with aging or T2DM, insulin and glucose levels slowly increase, but this inhibits ketogenesis and prevents the brain from receiving an alternate energy source to the glucose (Lange et al.). Therefore, the actual insulin response is not what disrupts the mitochondrial function of the brain, but it's the IR and disruption, or lack of insulin signaling, that causes the neurons to be more vulnerable to metabolic stress and neuronal dysfunction.

Ketones: The Alternate Fuel Source

Ketones, or ketone bodies, are made by the liver and are a family of nutrients, including acetoacetate, beta-hydroxybutyrate, and acetone. The circulating levels of ketones within the plasma is dependent on the rate of production, ketogenesis, and utilization, ketolysis. When insulin levels drop, its inhibition of free fatty acid catabolism from adipose tissue is overcome and free fatty acids are then released into the plasma. When levels of free fatty acids are high, the

production of acetyl coenzyme A increases within the liver and the enzyme is then used for ketogenesis (Fukao, Lopaschuk, & Mitchell, 2004). Ketones, a fat derived substrate, can then pass into the brain and be used in the mitochondria (Balasse & Féry, 1989).

Ketones are also beneficial for many reasons. Because free fatty acids cannot pass through the blood brain barrier, the brain is unable to use them as an alternate fuel source, but this is not the case with ketones (Pardridge, 1991). In addition, ketones can produce a larger amount of energy in comparison to glucose. This is due to the changes in mitochondrial ATP production that they induce. Lastly, ketones are able to overcome the block in glycolysis that is caused by IR (Lange et al.). Unfortunately, insulin and high glucose levels prevent both the breakdown of free fatty acids and the synthesis of ketone bodies in the liver. Because of the properties and benefits of ketogenesis, ketogenic diets and ketone supplements have been suggested as treatments for neuronal diseases such as epilepsy, autism, and Alzheimer's disease (Castro et al., 2015; Cunnane et al., 2011; Henderson et al., 2009).

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CHAPTER 2: Insulin Alters Brain Lipid Profile and Mitochondrial Function in ApoE4 Mice

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Abstract

Background

Alzheimer's disease (AD), the most common neurodegenerative disorder worldwide, is characterized by increased levels of neurofibrillary tangles and amyloid-beta plaques. Evidence has previously implicated the potent role that insulin resistance plays in the development and progression of AD, suggesting to some that AD represents a distinct type of diabetes.

Additionally, disparate lines of evidence find a role for alterations in brain mitochondrial function in AD etiology.

Methods

We sought to test a unifying hypothesis—namely, that hyperinsulinemia promotes accrual of the sphingolipid ceramide, which, as we've previously found, pathologically alters mitochondrial function. Via daily insulin injections, we induced hyperinsulinemia in ApoE4 mice. In addition to insulin, one group received myriocin injections to inhibit ceramide biosynthesis.

Results

We observed significant increases in brain ceramides in the insulin-treated group, which correlated with disrupted brain mitochondrial function. However, the group receiving myriocin alone, or, importantly, myriocin with insulin, had normal lipid profiles and apparently normal mitochondrial bioenergetics.

Conclusions

Altogether, our findings suggest a causative role for insulin in AD etiology via an insulin-induced upregulation of ceramide biosynthesis and accrual in the brain.

Background

Brain mitochondrial dysfunction is a hallmark of Alzheimer's disease (AD). With the current correlations between insulin resistance (IR) and AD, research has started to look at insulin's role within the brain and how IR alters homeostatic brain function. Unlike peripheral insulin receptors, neuronal insulin receptors and IGF-1 receptors, which also respond to insulin, within the brain regulate many physiological functions such as food intake, inhibition of hepatic gluconeogenesis, mitochondrial function, counter-regulation of hypoglycemia, modulation of tau protein phosphorylation, A β PP metabolism, Amyloid- β clearance, neuronal survival, and memory (Candeias et al., 2012). Therefore, while insulin has a minor role in glucose metabolism within the brain, it also maintains a vital role in other neuronal functions. Because of this, insulin resistance is not only a key contributor of Type 2 Diabetes (T2DM), but has now emerged as contributor to Alzheimer's Disease.

A common mediator of IR and mitochondrial dysfunction are ceramides, whose levels correlate with the severity of IR and cognitive decline (Haus et al., 2009; Savica et al., 2016).

Ceramides are a sphingolipid that have a role in the antagonism of mitochondrial function and several different intermediates in the insulin signaling pathway. In regard to mitochondrial function, increased ceramide levels cause an increase in the production of reactive oxygen species and therefore induce more oxidative stress within different tissues (Summers, 2006).

Methods

Animals

Homozygous ApoE4 mice with an average age of 4.5 months were randomly assigned into one of four treatment groups for 4 weeks: (i) PBS injections (control); (ii) insulin and PBS injections (INS); (iii) myriocin injections in order to inhibit ceramide synthesis (Myr); and (iv) insulin and myriocin injections (INS+Myr). All injections were intraperitoneal. Studies were conducted in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the IACUC (Institutional Animal Care and Use Committee) at Brigham Young University.

Insulin Tolerance Test

After 4 weeks, mice underwent intraperitoneal insulin tolerance tests. Mice were fasted overnight and received an injection of insulin (0.75 unit/kg of body weight). Blood glucose was determined at the times indicated in the figures using the Bayer Contour glucose meter.

Lipid Analysis

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 (Hansen et al., 2014).

Mitochondrial Respiration Protocol

High-resolution O₂ consumption was determined at 37°C in permeabilized cells and fiber bundles, using the Oroboros O₂K Oxygraph (Innsbruck, Austria) with MiR05 respiration buffer as described previously (Pesta & Gnaiger, 2012; Smith et al., 2013). Respiration was determined by all or parts of the following substrate-uncoupler inhibitor-titration (SUIT) protocol (Jheng et al., 2012): electron flow through complex I was supported by glutamate malate (10 and 2 mM, respectively) to determine O₂ consumption from proton leak (GML). Following stabilization, ADP (2.5 mM) was added to determine oxidative phosphorylation capacity (GMP). Succinate was added (GMSP) for complex III electron flow into the Q-junction. To determine full electron transport system (ETS) capacity over oxidative phosphorylation in cells, the chemical uncoupler carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) was added (0.05 M followed by 0.025 M steps until maximal O₂ flux was reached). Complex II-supported ETS was then measured by inhibiting complex I with rotenone (Rot; 0.5 M). Last, residual O₂ consumption was measured by adding antimycin A (2.5 M) to block complex III action, effectively stopping any electron flow. This value provides a rate of respiration that is used as a baseline.

Statistics

Values are reported as means \pm the standard errors of the means. Statistical analyses were conducted by one-way analysis of variance with treatment and set of experiment as factors.

Student's *t* test was used for comparisons between groups within each experiment, and differences were considered to be statistically significant at $P < 0.05$.

Results

Chronic Insulin Injections Increase Body Weight and Fasting Glucose Levels, While Inducing Insulin Intolerance

ApoE4 mice received injections of PBS (daily), insulin (daily; 0.75 mg/kg), myriocin (thrice weekly; 3 mg/kg; MYR), or insulin + myriocin (INS+MYR) at the same time each day for four weeks. At the end of the treatment, the INS group had the greatest increase in body weight, though no such effect was noted with the inclusion of myriocin (INS+MYR) (Figure 2.1a). INS also had decreased insulin sensitivity, based on the insulin tolerance test performed after the 4-week treatment regimen (Figure 2.1b). The PBS, MYR, and INS+MYR groups had no statistical significance between their changes in their fasting glucose levels (Figure 2.1c). In addition, fasting glucose levels were elevated in the INS group in comparison to the other three groups, and insofar as the addition of myriocin prevented this effect, the results suggest that ceramides play a role in the changing insulin sensitivity and fasting glucose levels due to myriocin preventing significant changes in these measurements (Figure 2.1c).

Insulin Increases Ceramide Accrual in the ApoE4 Brain

After tissue collection, lipid analysis was completed to determine levels of ceramides within the brains of each ApoE4 mouse. Upon analysis via shotgun lipidomics/LCMS, it was found that the INS group had the greatest amount of overall ceramides within the brain, as well as the specific sub-types of C16:1, C20, C24, and C24:1. Most importantly, myriocin was able to reduce the ceramide accrual, despite the insulin injections in the INS+MYR group (Figure 2.2).

Chronic Insulin Injections Disrupt Brain Mitochondrial Function

To measure mitochondrial function within the brain, respirations were performed on fresh brain biopsies as described in Figure 2.3b. The GM_L step determined the oxygen consumption from the proton leak and malate aspartate shuttle within the mitochondria. There was no difference in any of the groups in this first step (Figure 2.3a). Next, we determined the oxidative phosphorylation capacity, this is the GM_p , and found that insulin injected group began to fall behind while the myriocin + insulin group had comparable mitochondrial function to both the PBS and myriocin only groups (Figure 2.3a). This trend continued in the final GMS_p step where CI and CII function were evaluated (Figure 2.3a).

Therefore, overall respiration, RCR, and CII factor were reduced with insulin treatment (Figure 2.3a,c,d). The INS + MYR group also had reduced CII factor in comparison to the PBS group (Figure 2.3d).

Discussion

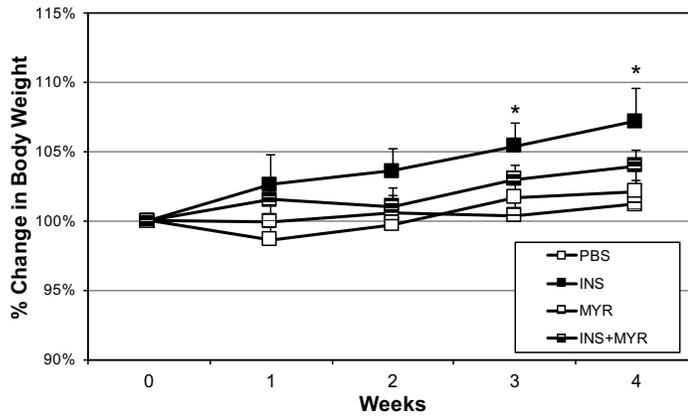
In mice, we found that prolonged insulin treatment resulted in reduced insulin tolerance, supporting previous findings that insulin alone, independent of other variables, is capable of inducing insulin resistance. This observation agrees with evidence from several other reports in both humans and rodents where hyperinsulinemia from endogenous (e.g., insulinoma) or exogenous (e.g., injections) sources causes insulin resistance (Del Prato et al., 1994; Henry et al., 1993; Hodson, Tippetts, & Bikman, 2015; Pontiroli, Alberetto, & Pozza, 1992). This insulin-desensitizing effect of prolonged hyperinsulinemia is likely partially mediated via ceramide accrual, as evidenced by our findings that ceramide inhibition via myriocin was protective

against the deleterious changes in insulin sensitivities. However, the relevance of ceramides in mediating hyperinsulinemia-induced changes extend beyond insulin sensitivity.

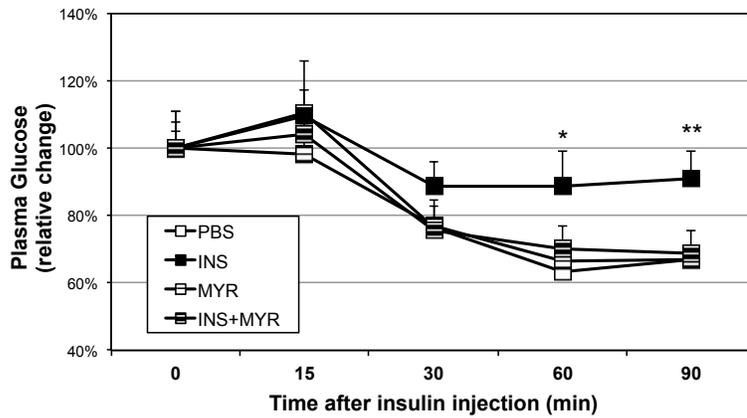
To further understand the pathology of insulin and ceramides, we looked at brain ceramide accrual and mitochondrial function insofar as insulin resistance and mitochondrial dysfunction are key markers of dementia diseases such as AD (Ahmed, Mahmood, & Zahid, 2015; Biessels & Kappelle, 2005; Readnower, Sauerbeck, & Sullivan, 2011). We conclude that ceramides are a key intermediate in insulin-induced mitochondrial dysfunction in light of the fact that the mice receiving the ceramide-inhibitor myriocin had relatively normal brain mitochondrial function, supporting our previous reports of ceramide-induced mitochondrial dysfunction (Smith et al., 2013). The impairment of energy metabolism, which includes mitochondrial dysfunction, is a significant characterization of AD and our data adds to previous data, by indicating ceramide's key role (Shenk et al., 2009).

In the last 15 years, the increasing prevalence of both AD and T2DM has led to research demonstrating insulin's critical role in AD development and the increased risk of AD caused by T2DM (Liu, Liu, Grundke-Iqbal, Iqbal, & Gong, 2011). Defective insulin signaling not only effects memory and learning, but is linked with amyloid-beta plaque formation that causes the displacement of insulin receptors from the plasma membrane, furthering the IR within the brain (Ahmed et al., 2015). The relationship between IR, ceramides, and mitochondrial function shown by our research furthers other research connecting these variables to AD, thereby suggesting a key role of both insulin and ceramides in AD. Altogether, our findings propose a causative role for insulin in brain mitochondrial dysfunction via an insulin-induced upregulation of ceramide biosynthesis and accrual in the brain.

a



b



c

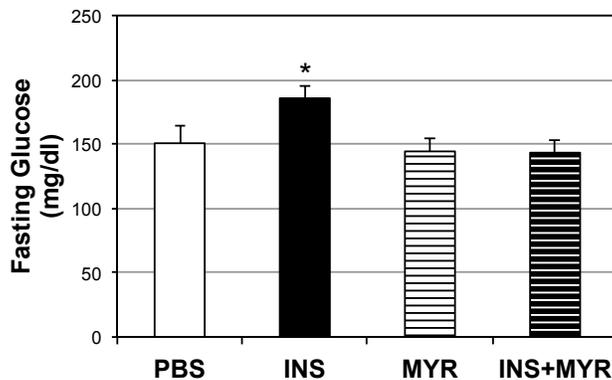


Figure 2.1: Chronic Insulin Injections Increase Body Weight and Fasting Glucose Levels, While Inducing Insulin Intolerance. ApoE4 mice received injections of PBS (daily), insulin (INS; daily; 0.75 mg/kg), myriocin (MYR, thrice weekly; 3 mg/kg), or INS + MYR. Body weight (A) was tracked weekly. Insulin tolerance (B) and fasting glucose levels (C) were measured after 30 days of treatment. *P<0.05 and **P<0.01 for INS vs. other treatments.

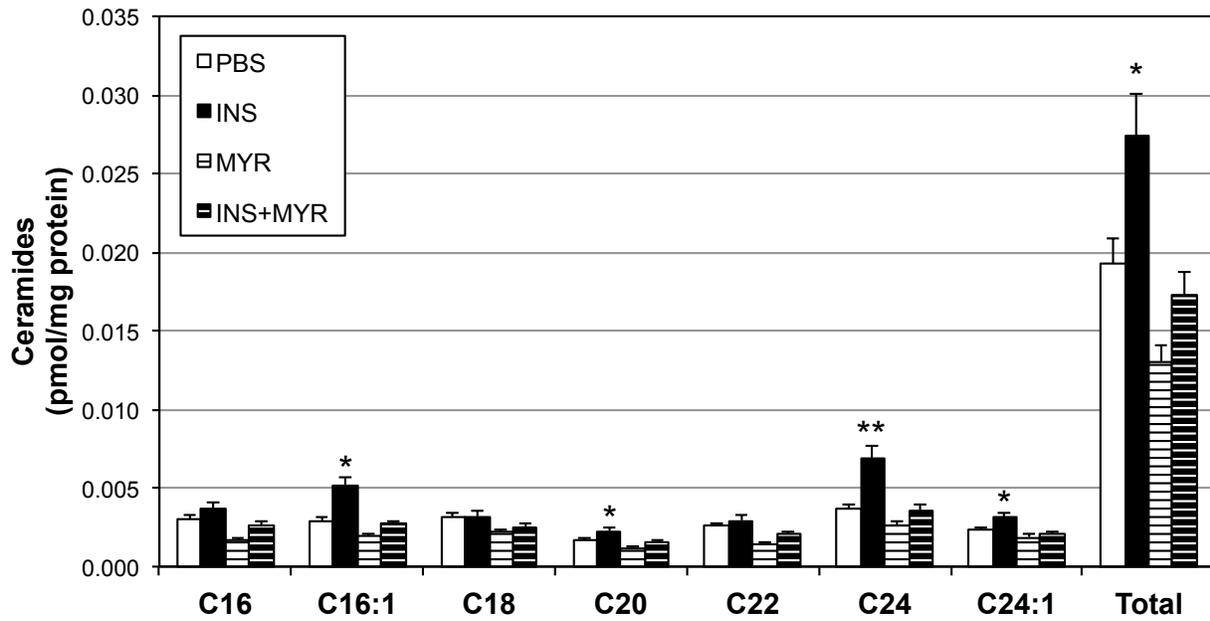
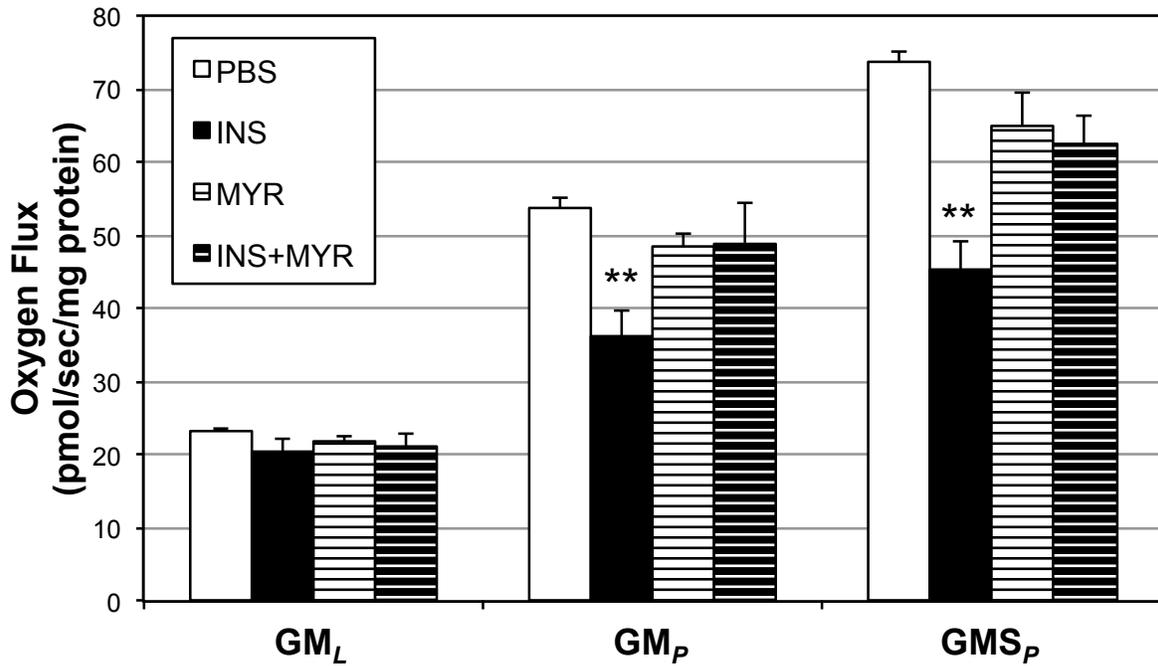


Figure 2.2: Insulin Increases Ceramides in the ApoE4 Brain. ApoE4 mice received injections of PBS (daily), insulin (INS; daily; 0.75 mg/kg), myriocin (MYR, thrice weekly; 3 mg/kg), or INS + MYR. Following the 30-day treatment, lipids were isolated from the brains for analysis of sphingolipids via LCMS. *P<0.05 and **P<0.01 for INS vs. other treatments.

a



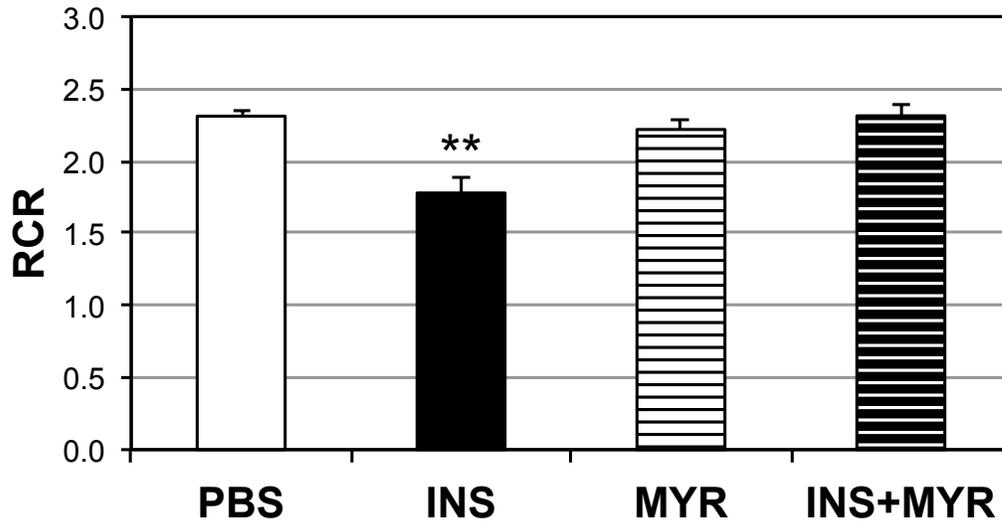
b

Abbreviation	Substrate	Condition
GM _L	Glutamate (10 mM) Malate (2 mM)	Leak (L)
GM _P	+ ADP (2.5 mM)	Phosphorylation (P)
GMS _P	+ Succinate (10 mM)	Phosphorylation (P)

Figure 2.3ab: Chronic Insulin Injections Disrupt Brain Mitochondrial Function. To measure mitochondrial respiration (A), samples were treated with: GML, Glutamate (10 mM) + Malate (2 mM); GMD, + ADP (2.5 mM); GMSD, + Succinate (10 mM). A description of the protocol is shown in B. Respiratory control ratio (RCR; C) was determined by GMP/GML. Complex II control factor (D) was used to determine the specific effect of succinate addition on respiration.

*P<0.05 and **P<0.01 for INS vs. other treatments. #P<0.05 for INS+MYR vs. PBS.

c



d

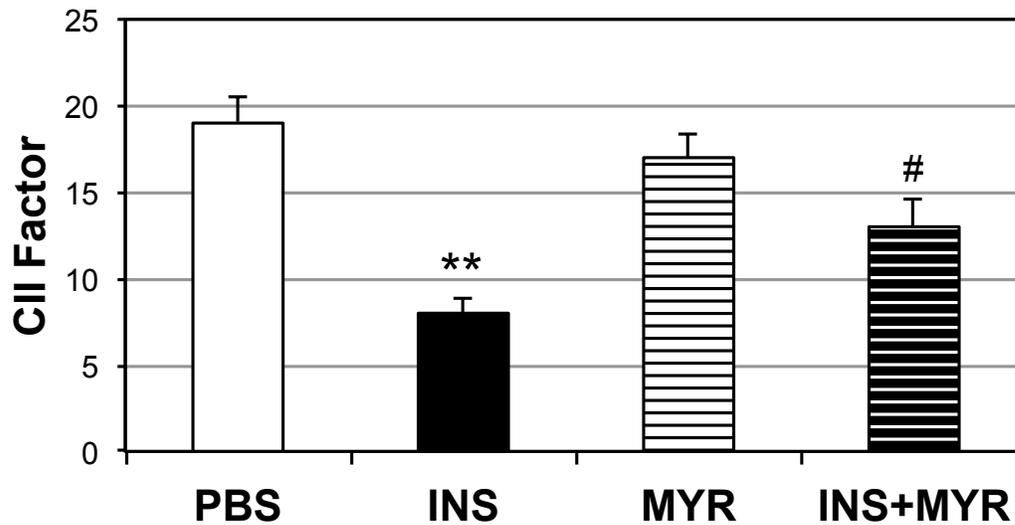


Figure 2.3cd: Chronic Insulin Injections Disrupt Brain Mitochondrial Function. To measure mitochondrial respiration (A), samples were treated with: GML, Glutamate (10 mM) + Malate (2 mM); GMD, + ADP (2.5 mM); GMSD, + Succinate (10 mM). A description of the protocol is shown in B. Respiratory control ratio (RCR; C) was determined by GMP/GML. Complex II control factor (D) was used to determine the specific effect of succinate addition on respiration.

* $P < 0.05$ and ** $P < 0.01$ for INS vs. other treatments. # $P < 0.05$ for INS+MYR vs. PBS.

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CHAPTER 3: Ketogenic Diet Improves Brain Mitochondrial Function and Reduces Oxidative Stress in Aged Rats

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Abstract

Background

Ketogenic diets (KD) have been promoted extensively for weight loss and health. Limited evidence also indicates an effect of ketones on improving multiple markers of cognitive function. However, the effect of a ketogenic diet on brain mitochondrial function remains unknown.

Methods

Young adult (YA; 5 months old) and old (O; 28 months old) rats were assigned to either standard chow diets or very-low-carbohydrate, high-fat ketogenic diets for 4 weeks. Following treatment period, we analyzed brain mitochondrial function.

Results

We observed no effect of KD on altering brain mitochondrial function in YA rats. However, KD appeared to restore mitochondrial function in O rats. Specifically, O rats fed a standard diet had significantly higher levels of oxidative stress, while KD-fed O rats had levels similar to YA rats.

Conclusions

We conclude that KD improves brain mitochondrial function in aged rats. These results possibly elucidate and compliment human trials that find improved cognition in states of elevated ketones.

Background

Unlike peripheral insulin receptors, insulin-mediated neuronal insulin receptors and IGF-1 receptors within the brain regulate many physiological functions such as: food intake, inhibition of hepatic gluconeogenesis, mitochondrial function, counter-regulation of hypoglycemia, modulation of tau protein phosphorylation, A β PP metabolism, Amyloid- β clearance, neuronal survival, and memory (Candeias et al., 2012). Therefore, insulin has a minimal role in glucose metabolism within the brain, but a vital role in other neuronal functions. When insulin resistance occurs within the brain, defects in glucose metabolism arise.

The low glucose utilization within the brain does not affect peripheral insulin levels and therefore cannot influence the production of ketones. Ketones are made by the liver and include acetoacetate, beta-hydroxybutyrate, and acetone (Fukao, Lopaschuk, & Mitchell, 2004). As insulin resistance progresses with age, insulin and glucose levels slowly increase, inhibiting the production of ketones from the liver and preventing the brain from receiving an alternate energy source to the glucose (Jose et al., 2011; Liu, Liu, Grundke-Iqbal, Iqbal, & Gong, 2011; Oya et al., 2014). Therefore, the actual insulin response is not what disrupts the mitochondrial function of the brain, but rather the insulin resistance and lack of insulin signaling that makes the neurons more vulnerable to metabolic stress and subsequent neuronal dysfunction.

With a ketogenic diet, macronutrients are altered in such a way (i.e. low-carbohydrate, high-fat) to lower inhibition, and thus, leading to a disinhibition of both lipolysis and ketogenesis. When the levels of free fatty acids are high due to lipolysis, the production of acetyl coenzyme A increases within the liver and, due to a general inhibition of pyruvate dehydrogenase and acetyl-coA carboxylase, the substrate is used for ketogenesis (Fukao et al., 2004). Ketones can then be used as nutrient energy for all cells with mitochondria, including the brain, passing through the blood-brain barrier to be readily used by the brain (Balasse & Féry, 1989). In comparison to glucose, ketones are able to produce a larger amount of ATP per unit, due to an absence of ATP use in ketone catabolism (unlike glucose catabolism). In addition, they are also able to provide ATP in spite of the block in glycolysis that is caused by IR (Lange et al.). Unfortunately, insulin and high glucose levels prevent both the breakdown of fat into free fatty acids and the synthesis of ketone bodies in the liver due to an insulin-induced inhibition of enzymes involved in β -oxidation. Because of the properties and benefits of ketogenesis, ketogenic diets and ketone supplements have been suggested as treatments for neuronal diseases such as epilepsy, autism, and Alzheimer's disease (Castro et al., 2015; Cunnane et al., 2011; Henderson et al., 2009).

Methods

Animals

Male rats were obtained from ThermoFisher (344 rats) and were split into two groups: young adult (YA; 5 month old) and old (O; 28 month old) of male Fisher 344 rats (n = 4-6/group). Rats were assigned to either standard chow (STD; Envigo Teklad Rodent Diet, 8604; 32% protein, 14% fat, 54% carbohydrate) or ketogenic diet (KETO; Envigo Teklad Custom Diet,

TD.10911; 22.4% protein, 77.1% fat, 0.5% carbohydrate) for 4 weeks, and weighed weekly. KETO rats were pair fed isocalorically with STD chow rats within each age group. Studies were conducted in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the IACUC (Institutional Animal Care and Use Committee) at Brigham Young University.

Glucose Tolerance Test

After 24 - 25 days on the assigned diet, glucose tolerance testing was performed by fasting the rats for 6 hours followed by an injection of 1 mg glucose/g BW. Blood glucose concentration was measured from tail blood immediately before and at 15, 30, 60 and 120 minutes after the glucose injection.

Ketone Measurements

Ketones were measured from tail blood prior to the necessary glucose injections for the glucose tolerance tests using a blood ketone monitor (Precision Xtra).

Brain Lipid Analysis

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 (Hansen et al., 2014).

Mitochondrial Respiration Protocol

High-resolution O₂ consumption was determined at 37°C in permeabilized cells and fiber bundles, using the Oroboros O₂K Oxygraph (Innsbruck, Austria) with MiRO5 respiration buffer as described previously (Pesta & Gnaiger, 2012; Smith et al., 2013). Respiration was determined by all or parts of the following substrate-uncoupler-inhibitor-titration (SUIT) protocol (Jheng et al., 2012): electron flow through complex I was supported by glutamate malate (10 and 2 mM, respectively) to determine O₂ consumption from proton leak (GML). Following stabilization, ADP (2.5 mM) was added to determine oxidative phosphorylation capacity (GMP). Succinate was added (GMSP) for complex III electron flow into the Q-junction. To determine full electron transport system (ETS) capacity over oxidative phosphorylation in cells, the chemical uncoupler carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) was added (0.05 M followed by 0.025 M steps until maximal O₂ flux was reached). Complex II-supported ETS was then measured by inhibiting complex I with rotenone (Rot; 0.5 M). Last, residual O₂ consumption was measured by adding antimycin A (2.5 M) to block complex III action, effectively stopping any electron flow. This value provides a rate of respiration that is used as a baseline.

Statistics

Values are reported as means \pm the standard errors of the means. Statistical analyses were conducted by one-way analysis of variance with treatment and set of experiment as factors. Student's *t* test was used for comparisons between groups within each experiment, and differences were considered to be statistically significant at $P < 0.05$.

Results

Ketogenic Diet Leads to Weight Loss in Young Rats

After four weeks on the assigned diet, it was found that the ketogenic diet only caused weight loss in the Y rats (Figure 3.1). The SD Y rats had an increase in weight at the end of the four weeks. All O rats had a decrease in weight at the end of the four weeks, despite the diet regimen.

Fasting glucose and plasma ketone levels were measured weekly over the four-week period while consuming the assigned diet. At the end of four weeks, there was not a statistically significant difference in any of the groups for either ketones or glucose levels (Figure 3.2). However, there was a trend of increased plasma ketones in the Y KD rats (Figure 3.2a).

Ketogenic Diet Improved Brain Mitochondrial Function in Old Rats

To measure mitochondria function within the brain, mitochondrial respirations were performed on fresh brain biopsies. The GM_L step determined the oxygen consumption from the proton leak and malate aspartate shuttle within the mitochondria. The O SD rats had lower mitochondrial function than the Y SD rats (Figure 3.3a). Next, we determined the oxidative phosphorylation capacity, this is the GM_p , and found that the O KD rats' brain mitochondria function began to improve significantly in comparison to the O SD rats. The O SD rats still had lower mitochondrial function than the Y SD rats (Figure 3.3a). This trend continued in the final GMS_p step where CI and CII function were evaluated (Figure 3.3a). The respiratory control ratio (RCR; GMP/GML) was a post-hoc analysis done to measure overall mitochondrial fitness (Figure 3.3b). The RCR shows that the O KD rats had better mitochondrial fitness than the O SD rats.

Lastly, oxidative stress was measured via production of H_2O_2 within the brains. The O SD rats had the highest levels of oxidative stress (Figure 3.4). However, the O KD had a statistically significant drop in H_2O_2 production within the brain in comparison to the O SD rats.

Discussion

Our most relevant finding was that a ketogenic diet (KD) improved brain mitochondrial function in a rat model of aging. Specifically, the brains from KD-fed aged rats had substantially lower rates of H_2O_2 production, indicating reduced oxidative stress. Because insulin resistance occurs with aging, the ketogenic diet is a viable therapy option in order to bypass the disrupted glucose metabolism that occurs in the mitochondria of the aged brain, and may reveal a pathway to help maintain nervous function and prevent neurodegeneration (Candeias et al., 2012; Cunnane et al., 2011; Jose et al., 2011).

These findings in the rodent model may provide a mechanism to better understand the results of related human studies. In particular, in older adults with mild cognitive impairment, 6-weeks on a low carbohydrate diet improved verbal memory performance in comparison to those on a high carbohydrate diet. This low-carbohydrate diet also reduced fasting glucose levels, fasting insulin levels, waist circumference, and weight in comparison to those eating a high carbohydrate diet (Krikorian et al.). In addition, direct administration of the ketone β -hydroxybutyrate into the brain of rats has been shown to prevent amyloid-beta plaque deposition, neuronal apoptosis, and decreased oxidative stress, therefore improving mitochondrial function and preventing markers of age-related pathologies such as Alzheimer's disease (Xie, Tian, Wei, & Liu, 2015). Our data corroborates this previous work, as we found that a low carbohydrate, ketogenic diet improved brain mitochondrial function in old rats.

In the Y rats, the KD caused a significant amount of weight loss, in comparison to the Y rats on the SD, who gained weight at the end of the 4 weeks. In contrast to Y rats, there was no difference in weight loss between the SD and KD old rats. This was surprising, as ketogenic diets have been previously shown to reduce weight over time, even in seniors (Krikorian et al.; Saslow et al., 2014; Saslow et al., 2017). A possible explanation for this is that the rats were simply too old and were already losing weight due to age-related wasting. We may have benefited from using rats that were not as old in order to better measure the effects of the KD on their weight.

In addition, it was surprising that there was no difference in plasma ketone levels in any of the groups, although there was a trend of the Y KD rats having increased plasma ketones. Previous studies have shown that rats are able to be induced into ketosis using a low-carbohydrate, high fat diet in a three to four week time period, similar to the experimental time frame we used (Bielohuby et al., 2010; Xu et al., 2010). It is possible that our protein composition of 22.4% was too high, as it has been found that only rats fed ketogenic diets very high in fat and low in protein are clearly in ketosis while rats fed high fat and normal protein diets (in comparison to SD) have no signs of ketosis (Bielohuby et al., 2010). Exogenous ketone supplements have been used to induce ketosis by increasing plasma β -hydroxybutyrate levels (Kesi et al., 2016). This has also been seen in elderly patients suffering from mild to moderate AD, where researchers administered an oral ketone supplement (AC-1202, a form of medium chain triglycerides) and found increased ketone plasma levels due to the supplement, which also correlated with increased cognitive performance scores (Henderson et al., 2009). Based on these mentioned studies, it is unclear exactly why our KD rats did not have elevated plasma ketones,

but the improved brain mitochondrial function within the O KD rats suggests that the ketones were elevated enough to overcome the decreased glucose metabolism within the aged brain.

In conclusion, these findings reveal that a carbohydrate-restricted, ketogenic diet is effective at reducing oxidative stress and improving mitochondrial function in the brain of rat model of aging. The implications of these findings may assist in directing future therapies to help mitigate the loss of cognitive function in human aging and metabolic disease.

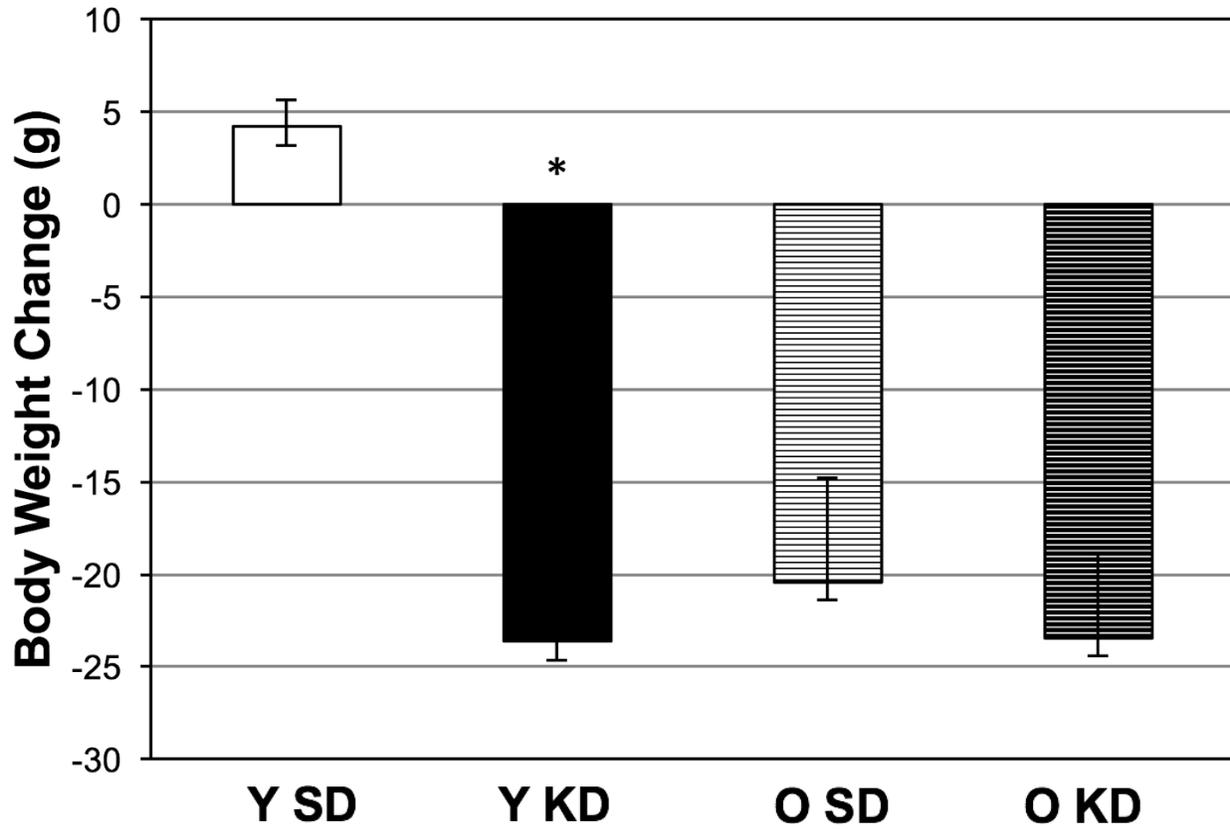


Figure 3.1: Ketogenic Diet Induced Weight Loss in Young Rats. While on the diet regimen, rats were weighted weekly and recorded. This figure represents the overall body weight change in each group.
 *P<0.05 for Y SD vs. Y KD

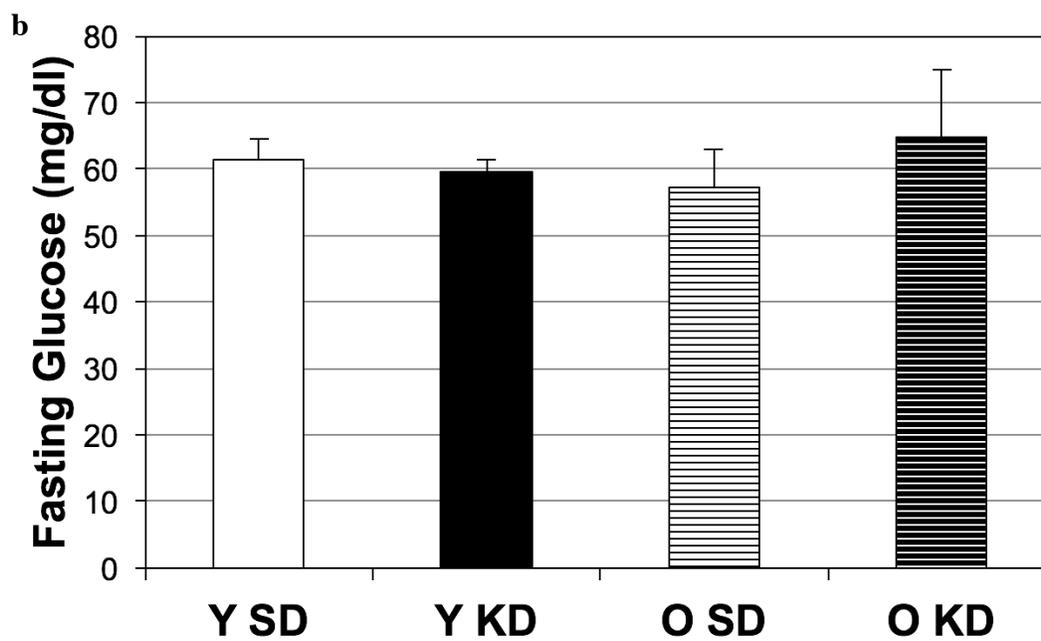
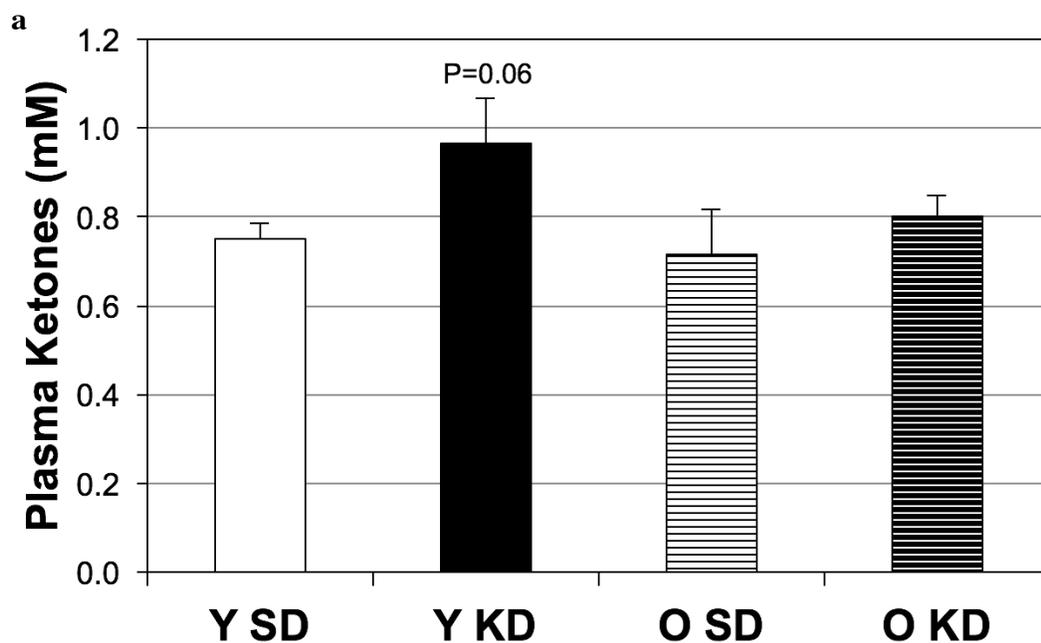
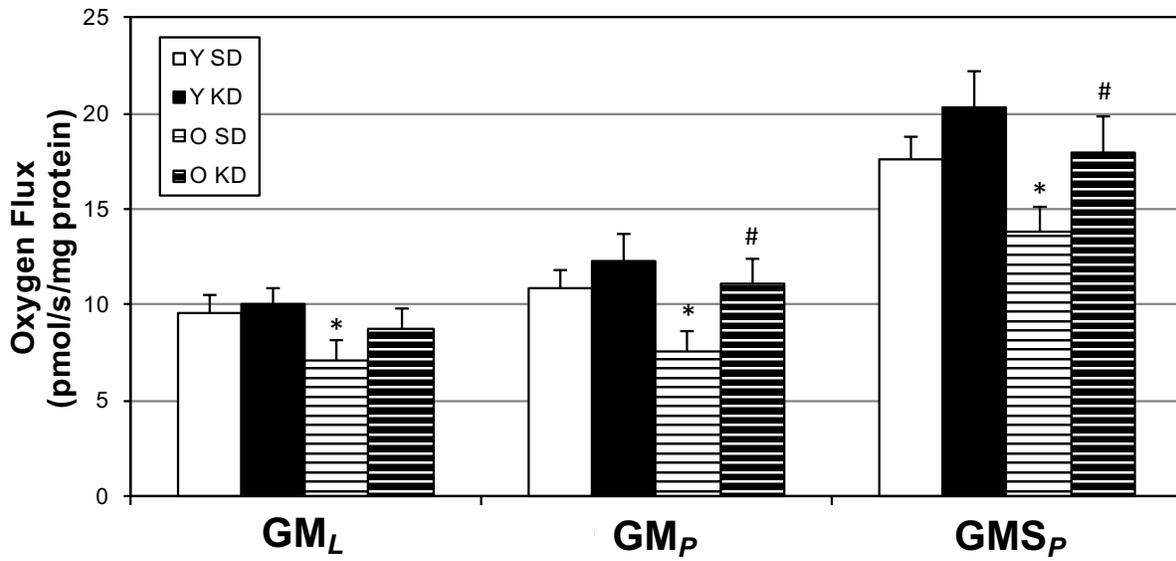


Figure 3.2: Ketogenic Diet Did Not Alter Plasma Ketones or Fasting Glucose Levels. After 24 - 25 days on the assigned diet, fasting glucose and plasma ketone levels were measured, but there was no statistically significant difference.

a



b

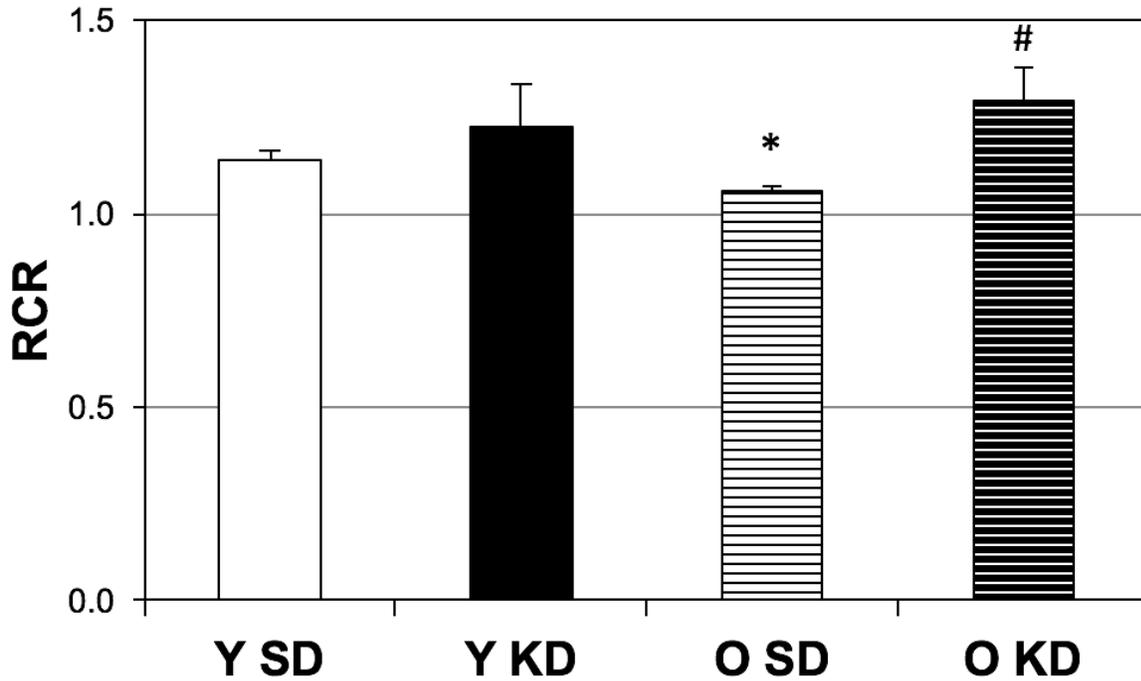


Figure 3.3: Ketogenic Diet Improved Brain Mitochondrial Function in Old Rats. To measure mitochondrial respiration (a), samples were treated with: GML, Glutamate (10 mM) + Malate (2 mM); GMD, + ADP (2.5 mM); GMSD, + Succinate (10 mM). Respiratory control ratio (RCR; b) was determined by GMP/GML. *P<0.05 for O SD vs. Y SD. #P<0.05 for O SD vs. O KD.

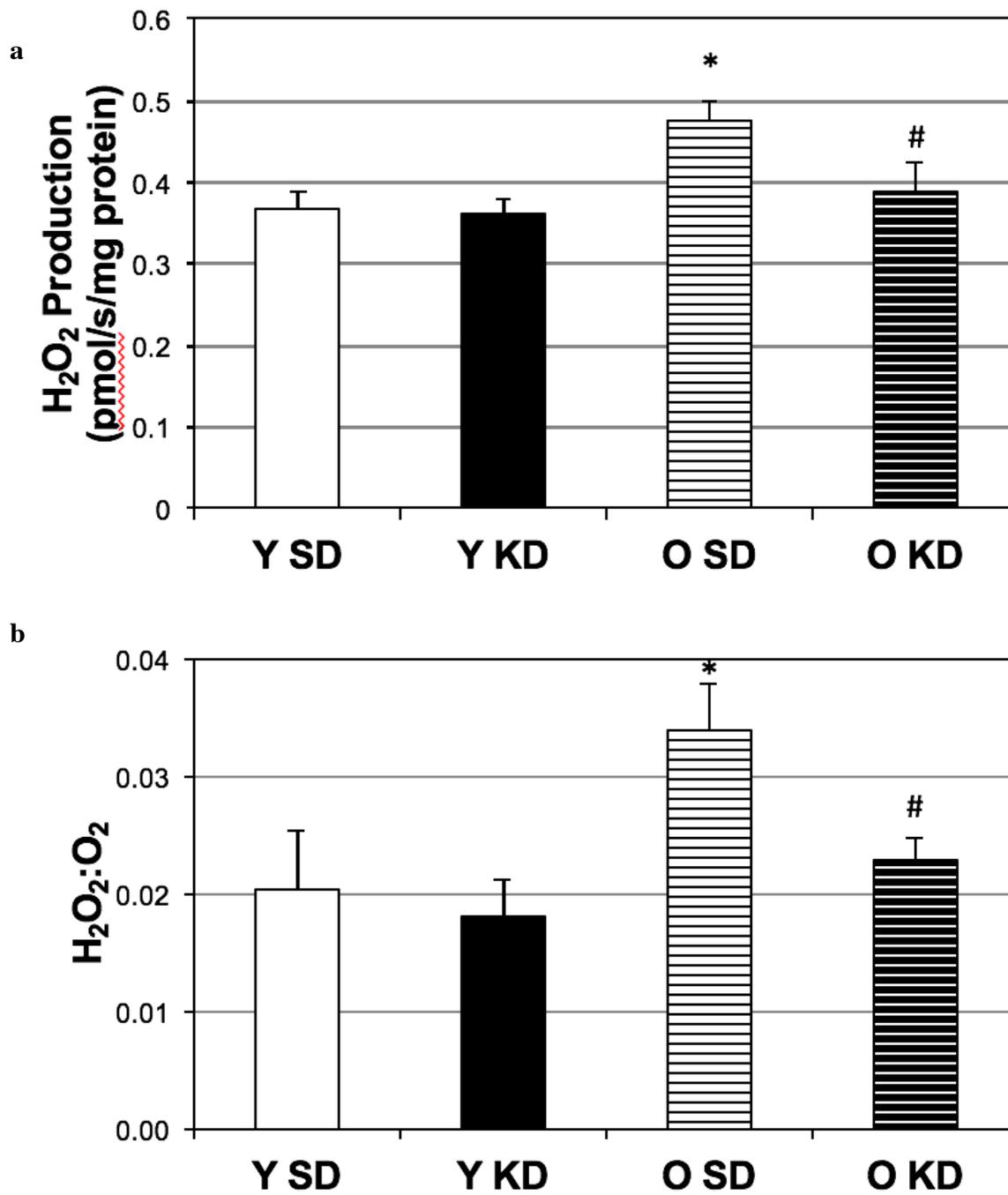


Figure 3.4: Ketogenic Diet Reduced H₂O₂ Production in Old Rats. *P<0.05 for O SD vs. Y SD. #P<0.05 for O SD vs. O KD.

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CHAPTER 4: General Discussion and Future Directions

Collectively, the data from these studies enhance the findings of existing work that not only implicate insulin resistance (IR) and ceramides as key players in brain mitochondrial dysfunction, but also reveal a growing role for ketogenesis as a pathway that may be exploited to mitigate some of the degrading effects of IR. In particular, as insulin resistance occurs with age, a ketogenic diet is a viable treatment therapy to bypass the altered brain glucose metabolism and insulin signaling, both fueling the brain and helping maintain proper mitochondrial function, as seen in Chapter 3, and prevent further neurodegeneration, such as that found in AD (Candeias et al., 2012).

We first explored how insulin resistance and its resulting ceramide accumulation can impact mitochondrial function within the brain. It has previously been found that ceramide negatively influences mitochondrial function in a variety of cells and tissues (Hodson, Tippetts, & Bikman, 2015; Smith et al., 2013; Summers, 2006). In our study, we found that chronic insulin injections induced insulin resistance and thereby lead to brain mitochondrial dysfunction in an Alzheimer's mouse model. This mitochondrial dysfunction within the brain required ceramides in order to occur, as evidence by the myriocin preventing insulin-induced mitochondrial dysfunction.

Although we did not measure how these conditions affected behavioral responses, it is reasonable to hypothesize that the insulin-induced changes in mitochondrial function would correlate with decreased cognitive function, thereby supporting the mitochondrial cascade hypothesis of AD (S. Cunnane et al., 2011; Readnower, Sauerbeck, & Sullivan, 2011; Swerdlow, 2012).

Historically, AD has been characterized by the build-up of amyloid-beta plaques and tau neurofibrillary tangles (Huang, 2006). However, with new research tying T1D and AD together, and advanced experimental techniques, the mitochondrial cascade hypothesis of AD and cognitive decline has come forth (Ahmed, Mahmood, & Zahid, 2015; Biessels & Kappelle, 2005; Hoyer, 2004; Readnower et al., 2011; Zhao & Townsend, 2009). Our research supports this mitochondrial cascade hypothesis of decreased brain mitochondrial function, especially in regard to mitochondrial dysfunction caused by IR, whether it be due to chronic insulin injections mimicking T1D or old age.

Since IR naturally occurs in aged brains, our next step was to evaluate whether not a ketogenic diet could overcome the IR and ceramide induced mitochondrial dysfunction. Using O rats, we were able to observe the benefits of a KD on brain mitochondrial function and oxidative stress levels. As mentioned previously, we did not have the ability to measure how the KD effected cognitive function or behavior, but based on our data and published studies it is reasonable to assume that the KD would improve memory and their ability to complete experimental tasks as it has in human studies, many of which used AD patients (S. C. Cunnane et al., 2016; Henderson et al., 2009; Lange et al.; Xie, Tian, Wei, & Liu, 2015).

AD is the 6th leading cause of death, responsible for mortality in one in three seniors. Unfortunately, current trends are likely to continue as the prevalence of dementia increases worldwide (Ballard et al.). A source of clinical frustration is the inability to definitively diagnose the disease in early stages, which is doubly frustrating when combined with the lack of established treatments. However, with an understanding of the interworking's of insulin and ketones within the brain, scientists and practitioners can better understand cognitive impairment and how to prevent cognitive function from worsening.

To build on our current research, we would like to determine whether a ketogenic diet can benefit the brain mitochondrial function in mouse models of AD, as well young rodents that have received chronic insulin injections. In addition, we would like to create a behavioral assay to better understand the cognitive effects of such treatments.

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CURRICULUM VITAE

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EDUCATION

M.S., Physiology and Developmental Biology June 2017
Brigham Young University, Provo, UT 84602

Thesis: Insulin and Ketones: Their Roles in Brain Mitochondrial Function
Advisor: Benjamin T. Bikman

B.S., Physiology and Developmental Biology December 2015
Department of Physiology and Developmental Biology
Brigham Young University, Provo, UT 84602

AWARDS

Brigham Young University - Graduate Research Fellowship Award (2016-2017)
Brigham Young University – Departmental Graduate Scholarship (2016 – 2017)
Graduate Retreat 2016 – 2nd Place for Excellence in Research Poster Presentation

PROFESSIONAL EXPERIENCE

Graduate Research Assistant September 2016 - Present
Mentor: Dr. Benjamin T. Bikman
Brigham Young University, Provo, UT 84602

Undergraduate Mentored Research September 2014 – April 2016
Mentor: Dr. Benjamin T. Bikman
Brigham Young University, Provo, UT 84602

Responsibilities:

- Managed the vivarium colony, which included allotting mice to research projects, checking breeding status, weaning, health checks, and coordinating the schedule for daily mice checks.
- Managed a research team for my hypothesis-based research and planned and executed experiments, often via collaboration with other professors

Pathophysiology Teacher's Assistant
Brigham Young University, Provo, UT 84602

January 2015 - April 2017

Responsibilities:

- Graded all assignments and projects
- Held office hours to meet with students individually and in groups
- Prepared and presented reviews for each exam
- Provided assistance to the professor, Dr. Bikman.

Tissue Biology Teacher's Assistant
Brigham Young University, Provo, UT 84602

August 2014 - December 2015

Responsibilities:

- Collaborated on exam development with Dr. Reynolds
- Graded research papers and weekly quizzes
- Held office hours to meet with students individually and in groups
- Prepared and presented reviews for each exam
- Provided assistance to the professor, Dr. Reynolds

Recreational Supervisor
Pleasant Grove Recreation Center, Pleasant Grove, UT 84602

December 2016 - Present

Responsibilities:

- Managed 4th and 5th grade basketball and adult volleyball leagues for the recreation department
- Set up/take down of equipment and recorded the time cards of all referees weekly
- Addressed any questions or concerns of the parents, players, and coaches.

Certified Nursing Assistant
The Crossings, League City, TX 77573

May 2016 – August 2016

Responsibilities:

- Cared for Alzheimer's residents which included assisting with dressing, eating, toileting, and other daily activities.
- Reported changes in behavior or mental state to the nurse on staff.
- Planned and executed activities throughout the day for the residents

Certified Nursing Assistant/ Med Tech
Bel Aire Senior Living, American Fork, UT 84003

March 2013 – April 2016

Responsibilities:

- Cared for Alzheimer's residents and other elderly individuals by assisting with dressing, eating, toileting, and other daily activities.
- Managed both scheduled and PRN medication administration

SERVICE

Coordinator
Anatomy Academy, Utah County, UT

September 2016 – April 2017

Responsibilities:

- Communicated with local 5th/6th grade teachers to schedule weekly lessons
- Managed 8 Anatomy Academy mentors to make sure goals were met as well individual mentor responsibilities.
- Planned and set up large group activities weekly and filled in as a mentor when needed in order to demonstrate basic anatomy and healthy lifestyle principles.

President of Marketing and Organization
BYU Diabetes Club, Provo, UT 84602

January 2017 - Present

Responsibilities:

- Planned and executed monthly activities with the help of other club leadership
- Helped create a better social media presence
- Assured that the club stayed organized according to BYU Club Rules.

PUBLICATIONS

“High-mobility Group Box 1 disrupts metabolic function with cigarette smoke exposure in a ceramide-dependent manner.” Taylor, O. J., Thatcher, M. O., Carr, S.T., ... Holland, W. L., Reynolds, P. R., Bikman, B. T. (2017). *International Journal of Molecular Sciences*

“Lipopolysaccharide Disrupts Mitochondrial Physiology in Skeletal Muscle via Disparate Effects on Sphingolipid Metabolism.” Hansen, M. E., Simmons, K. J., Tippetts, T. S., Thatcher, M. O., Saito, R. R., Hubbard, S. T., ... Bikman, B. T. (2015). *Shock. December 2015.*

MEETING ABSTRACTS

“Insulin and Its Role in Alzheimer’s Disease Pathology.” Carr, S.T., Trumbell, A., Hutchinson, B... Wisco, J.J., Bikman, B.T. *Experimental Biology – April 2017.*

"SGI-1252, a TGF- β Inhibitor, Protects Against Diet-induced Obesity and Insulin Resistance in Mice." Dallon, B.W., Carr, S.T.... Bikman, B.T. *Experimental Biology – April 2017.*

“ β -hydroxybutyrate favorably alters muscle cell survival and mitochondrial bioenergetics.” Parker, B.A., Dallon, B.W., Carr, S.T., ... Tessem, J.S., Bikman, B.T. *Experimental Biology – April 2017.*

“Effects of Ketogenic Diet in Young Adult and Old Rats.” Duplisea, M.J., Wilson, E.K., Cheung, E.C.K., Parker, B.A., Carr, S.T., Ashby, C.K., Bikman, B.T., Thomson, D.M. *Experimental Biology – April 2017.*

“Insulin Alters Brain Lipid Profile and Mitochondrial Function in ApoE4 Mice.” Carr, S.T., Trumbell, A., Hutchinson, B. ... Wisco, J.J., Bikman, B.T. *Brigham Young University Graduate Retreat – December 2016.*

REFERENCES

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