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# The Role of Beta-Hydroxybutyrate in Altering Adipose Mitochondrial Bioenergetics

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Master of Science

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ABSTRACT

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The rampant growth of obesity worldwide has stimulated explosive research into human metabolism. Metabolic rate has been shown to be altered by diets differing in macronutrient composition, with low-carbohydrate, ketogenic diets eliciting a significant increase over other interventions. The purpose of this study was to determine the effects of the ketone β-hydroxybutyrate (βHB) on mitochondrial respiration and coupling status in adipose tissue. To explore this, we employed three distinct systems, namely cell, rodent, and human models. In every model, βHB robustly increased mitochondrial respiration. Furthermore, in cultured adipocytes and rodent adipose, we quantified the expression of genes involved in mitochondrial biogenesis and coupling status. We observed that genes involved in mitochondrial biogenesis and uncoupling were significantly higher in models exposed to ketone treatments.

In conclusion, ketones increase mitochondrial respiration in cells and mammalian adipose tissue, but not ATP production, indicating greater mitochondrial uncoupling. These findings may partly explain the increased metabolic rate evident in states of elevated ketones and may facilitate the development of novel obesity interventions in the future.

Keywords: ketones, mitochondrial uncoupling, adipose, metabolism

ACKOWLEDGEMENTS

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# INTRODUCTION

In the United States and beyond, obesity has reached epidemic proportions. By the World Health Organization standards, 33% of US adults are overweight, and 37.7% are obese (Ogden, Carroll, Fryar, & Flegal, 2015), with an estimated 88% of US adults being considered metabolically unhealthy (Flegal, Kruszon-Moran, Carroll, Fryar, & Ogden, 2016). The dramatic increase in the prevalence of obesity, and related metabolic disorders, has occurred in just the last few decades, and if this trend persists, almost the entire US population will be overweight within a few generations (Foreyt & Goodrick, 1995). Unfortunately, this prevalence of obesity is not unique to adults, affecting over 25% of children (Hill & Trowbridge, 1998).

Naturally, the explosion in obesity has given rise to intensive research to better understand its etiology. These efforts have revealed, among other things, that humans store fat in two distinct depots that differ by function: white adipose tissue (WAT) and brown adipose tissue (BAT). Whereas WAT primarily acts as an energy and fuel storage, with a very low energy expenditure, BAT contains high levels of mitochondria that acts as a heat source, with a very high energy expenditure and a high degree of mitochondrial uncoupling.

In higher vertebrates, WAT stores vast amounts of nutrients as lipids in unilocular lipid droplets within adipocytes, which can then be released as fatty acids when necessary. However, the excess WAT accumulation that occurs in obesity is a significant risk factor for developing insulin resistance, type 2 diabetes mellitus (Despres, 1993), cardiovascular disease (Liu et al., 2019), and some cancers (Calle & Kaaks, 2004). In stark contrast, BAT has a limited lipid storage, in multi-locular lipid droplets, with a substantial amount of the cell occupied by mitochondria (giving rise to the distinct reddish-brown color). Increased BAT levels and activation are associated with a resistance to obesity and related disorders (Cinti, 2006; Farmer &

Boss, 2012). Perhaps the most unique aspect of BAT is the nature of its mitochondria—they express a proteome that fosters energy wasting by uncoupling phosphorylation from electron transport.

As nutrient shortages and cold environments can occur momentarily and independently from each other, both WAT and BAT regularly undergo adaptive and dynamic changes in response to starvation and overfeeding, as well as cold and thermoneutral environments (Rosenbaum & Leibel, 2010). The initial changes within the first 24-hours may only involve an altered expression of proteins. However, after 2-3 days, dietary and environmental stimuli can induce marked tissue remodeling, which results in altered adipose tissue morphology and possibly also modified functional properties (Choe, Huh, Hwang, Kim, & Kim, 2016). In particular, WAT is able to manifest characteristics of BAT, including mitochondrial biogenesis and uncoupling, in response to various external stimuli, such as cold exposure, in a process known as "beiging" (Bartelt & Heeren, 2014; Gantner, Hazen, Conkright, & Kralli, 2014). This responsiveness of WAT is essential, as, during evolution, animal species were repeatedly challenged by limited food supplies and cold temperatures and needed to be able to respond to both changes in nutrient supply and ambient temperature (Bartelt & Heeren, 2014).

BAT and WAT are regulated via complex molecular mechanisms. Recent discoveries have revealed that ketones, such as acetoacetate and its more prevalent metabolite β-hydroxybutyrate (βHB), are not only viable fuel sources for all cells with mitochondria (Parker et al., 2018), but are also legitimate signaling molecules, eliciting advantageous changes in inflammation (Youm et al., 2015), cognition (Frey et al., 2017; Kim & Rho, 2008; Yuen, Walcutt, & Sander, 2017), oxidative stress (Kim et al., 2007) and more. In mammals, acetoacetate and β-hydroxybutyrate have been shown to play a role by connecting fat stores in

adipocytes to adenosine triphosphate (ATP) production in peripheral tissues, allowing for these energy metabolites to sustain energetic requirements for basic cellular functions during starvation periods (Cahill Jr, 2006; McGarry & Foster, 1980; Robinson & Williamson, 1980). Other findings suggest that βHB is not solely a metabolic intermediate, but also acts as a signal to directly regulate metabolism and maintain energy homeostasis during nutrient deprivation (Rojas-Morales, Tapia, & Pedraza-Chaverri, 2016).

Previous findings of mitochondrial adaptations to ketones may offer insight into the benefits of ketosis, a state of mildly elevated blood ketones. Whether through the use of a ketogenic diet or the consumption of exogenous ketones, limited evidence suggests a generally favorable metabolic milieu that should be considered as a tool in our collective efforts to address human obesity (Parker et al., 2018). Thus, with the complex mechanistic regulation of adipose metabolism in mind, and the role of ketones as a metabolic intermediate and signaling molecule, the purpose of this study is to explore the effects of βHB on adipose tissue mitochondrial bioenergetics.

## MATERIALS AND METHODS

### Cell Culture

3T3-L1 MBX murine fibroblast cells were maintained in DMEM (Dulbecco's modified Eagle's medium; D6546, Sigma-Aldrich, Saint Louis, MO, USA) plus 10% FBS (Invitrogen, Carlsbad, CA, USA) and 1% Penicillin-Streptomycin (P/S) (ThermoFisher, Waltham, MA, USA) . The cells were grown in 10cm culture dishes at 37°C in a humidified atmosphere with 5% CO2. According to ATCC recommendations, the cells were strictly subcultured before they reached a density of $6 \times 10^6$ viable cells/$cm^2$.

For differentiation into adipocytes, 3T3-L1 MBX fibroblasts were grown to confluency and the medium was replaced with DMEM containing 10% fetal bovine serum (FBS, no. S 0615, Biochrom), 1% P/S, 0.5 mM IBMX, 0.25 µM dexamethasone, 2 µM rosiglitazone, and 1 µg/ml insulin. After 48 h, the medium was changed to DMEM containing 10% FBS, 1% P/S, and 1 µg/ml insulin for 48 hours. On day 7, the medium was changed to DMEM containing 10% FBS and 1% P/S. This medium was refreshed on days 8, 10, 12, and 13. For treatments, cells were either incubated with EtOH (for control) or βHB at 5 mM (54965, Sigma-Aldrich) for 24 h following completion of the differentiation protocol.

### Animals

All animal procedures were approved (18-0101, approved 1-26-2018) by the institutional animal care and use committee at Brigham Young University. 20-week-old male and female Sprague-Dawley rats were acclimatized for 1 week after arrival at the animal facility and were then pair-fed with either standard diet (STD; Envigo Teklad Rodent Diet, 8604; 32% protein, 14% fat, 54% carbohydrate) or ketogenic diet (KD; Envigo Teklad custom diet, TD.10911; 22.4% protein, 77.1% fat, 0.5% carbohydrate) for 4 weeks. Each day the chow remaining in the

cage was weighed and replaced with fresh chow. Body weight for the mice was recorded weekly. At 4 weeks, the mice were euthanized and blood, perirenal, intrascapular, and inguinal adipose depots were removed. Plasma ketone levels were measured using the Abbott Precision Xtra Ketone Monitoring System. Adipose not used for respiratory analysis was frozen at the temperature of liquid nitrogen and stored at −80 °C for later analysis.

## Mitochondrial Respiration

Cells and tissue were prepared for mitochondrial respiration as described previously (Parker et al., 2018) before being transferred to respirometer chambers using the Oroboros O2K oxygraph (Oroboros, Innsbruck, Austria). Electron flow through complex I was supported by glutamate + malate (10 mM and 2 mM, respectively) to determine leak oxygen consumption (GM). Following stabilization, adenosine diphosphate (ADP) (2.5 mM) was added to determine oxidative phosphorylation capacity (D). Succinate was added (S) for complex I + II electron flow into the Q-junction. Lastly, residual oxygen consumption was measured by adding antimycin A (2.5 μM) to block complex III action, effectively stopping any electron flow, which provides a baseline rate of respiration. Following respiration protocol, samples were removed from the chambers and used for further analysis, including protein quantification. As a measure of mitochondrial uncoupling, the ratio of ATP produced to oxygen consumed was used as an indicator to determine the coupling the oxygen transport to oxidative phosphorylation.

## Tissue Homogenization

Adipose samples were ground-glass homogenized in lysis buffer (50 mM Tris-HCl, pH 7.4; 250 mM mannitol; 50 mM NaF; 5 mM sodium pyrophosphate; 1 mM EDTA; 1 mM EGTA; 1% Triton X-100; 50 mM β-glycerophosphate; 1 mM sodium orthovanadate; 1 mM DTT; 1 mM benzamidine; 0.1 mM phenylmethane sulfonyl fluoride; 5 μg/mL trypsin inhibitor). The sample

was then centrifuged at 10,000 × g for 10 min. The supernatant was retained for western blotting analysis.

ATP Measurements in Cell Culture

Following the culture period, cells were transferred to 2.5 mM glucose in SAB buffer for 2 h, followed by transfer to either 2.5 mM glucose SAB buffer or 16.7 mM glucose SAB buffer for 1 h. Cells were washed with PBS, harvested by trypsinization and pelleted by centrifugation. The cells were lysed in 150 μL 1M perchloric acid on ice to precipitate cellular proteins. Lysate was centrifuged at 20,000× g for 10 min, after which 150 μL supernatant was transferred to a new tube with 150 μL 1M KOH. ATP was quantified using the ATP assay kit (Life Technologies, Carlsbad, CA, USA).

ATP Measurements in Tissue Samples

Tissue samples were thawed from frozen, and 6mg of tissue were placed into 1mL of mammalian lysis solution. Samples were then homogenized using a glass pestle and mortar. Post-homogenization, 450uL of each sample was aliquoted into a 96-well dish. ATP was quantified using an ATP assay kit (Perkin Elmer, Waltham, MA, USA).

RT-qPCR

Total RNA was isolated from tissue samples using RNeasy Lipid Tissue Mini Kit (QIAGEN) and measured on a NanoDrop 2000c spectrophotometer (Thermo Scientific). mRNA in 2 μg of total RNA was converted to cDNA using oligo(dT) primer and random hexamers according to the manufacturer's instructions (Clontech EcoDry Premix). The potential primers and probes were analyzed for the requirements imposed by real-time PCR using the PrimerQuest program. Finally, the chosen primers and probes were analyzed for specificity against GenBank

sequences with the BLAST program package (Altschul, Gish, Miller, Myers, & Lipman, 1990). Primer sequences used can be found in Table 1. For thermal cycling and fluorescence detection, a LightCycler 480 machine was used. The threshold (Ct) values of fluorescence for each readout was normalized to corresponding Ct values from β2m and Ppia (Gong et al., 2016), and fold change was calculated using the ΔΔCt method (Pfaffl, 2001).

Western Blotting

Adipose homogenates were analyzed for protein concentration using a modified Lowry assay (DC Protein Assay; Bio-Rad Laboratories, Hercules, CA, USA) according to manufacturer's protocols. Equal amounts of protein were then separated by electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes. Proper transfer and equal protein loading were verified by Ponceau-S staining of the PVDF membranes after transfer. Primary antibodies [OXPHOS (Invitrogen #457999), cytochrome C (Santa Cruz #sc-13156), MFN2 (EMD-Millipore #ABC42), DRP1 (Cell Signaling, #8570)] were applied overnight at 4 °C. Membranes were exposed to autoradiographic film and resulting band intensities were determined using Gel-Pro software (Media Cybernetics, Rockville, MD, USA).

Indirect Calorimetry

To quantify energy expenditure of mice, the Columbus Instruments Comprehensive Lab Animal Monitoring System (CLAMS) was used. CLAMS is the industry standard for quantifying numerous metabolic parameters in rodents, enabling the measurement of metabolic rate (via indirect calorimetry) and activity. CLAMS was used as previously described by Thomson et al. (Thomson et al., 2010).

## Human Diet Treatments

Subjects were recruited based upon extended ongoing adherence either a ketogenic or standard American diet. Subjects were encouraged to adhere to a simple rule of "control carbohydrates" (~5% calories), "prioritize protein" (~20-25% calories), and "fill with fat" (~70-75% calories). The key aspect of the dietary intervention was to control carbohydrates by consuming no more than 30 grams per day, coming mostly from nontuberous vegetables and berries. Subjects were encouraged to freely consume protein and fat, including meats, eggs, and cheese. Adherence to the intervention was confirmed with weekly tests to ensure plasma ketones (KETO-MOJO, Auburn, CA, USA) were at or above 0.5 mmol/L in the KD group.

## Human Fat Biopsies

Percutaneous needle biopsies were taken from subcutaneous fat tissue near the navel, the area of most subcutaneous fat. To perform the biopsy, a small area on the skin near the navel was shaved with an electric razor (when needed) and then cleaned with the antiseptic chlorhexidine. After the area was sterilized, injection of a local anesthesia (1 % lidocaine with epinephrine) was used to numb the area. After the participant was able to report no sensation in the area in response to gentle probing, a small incision ( ~ 1 cm) was made into the skin. Following this, the biopsy needle was inserted into the fat about 3 cm at a shallow ( ~30 degree) angle into the fat. Using manual suction, approximately 75 mg (about the size of a pencil eraser) of fat tissue was withdrawn. The sample was immediately placed in a cell buffer solution for analysis of mitochondrial respiration. Additional portions of tissue designated for ATP analysis were snap frozen in liquid nitrogen.

Statistical Methods

Data are presented as means ± SEM. Data were compared with Student's t-test (Graphpad Prism; Microsoft Excel). Significance was set at $p < 0.05$.

## RESULTS

### *β*-Hydroxybutyrate Stimulates Increased Mitochondrial Respiration in Adipocytes

Following 24 h of treatment with β-hydroxybutyrate (βHB), adipocytes exhibited an increased rate of respiration. These significant elevations in mitochondrial respiration rate (Figure 1A), only occurred after the addition of ADP (D) and continued with the addition of succinate (S). There was no significant difference in respiratory control ratio (RCR), a general indicator of mitochondrial "fitness" (Figure 1B).

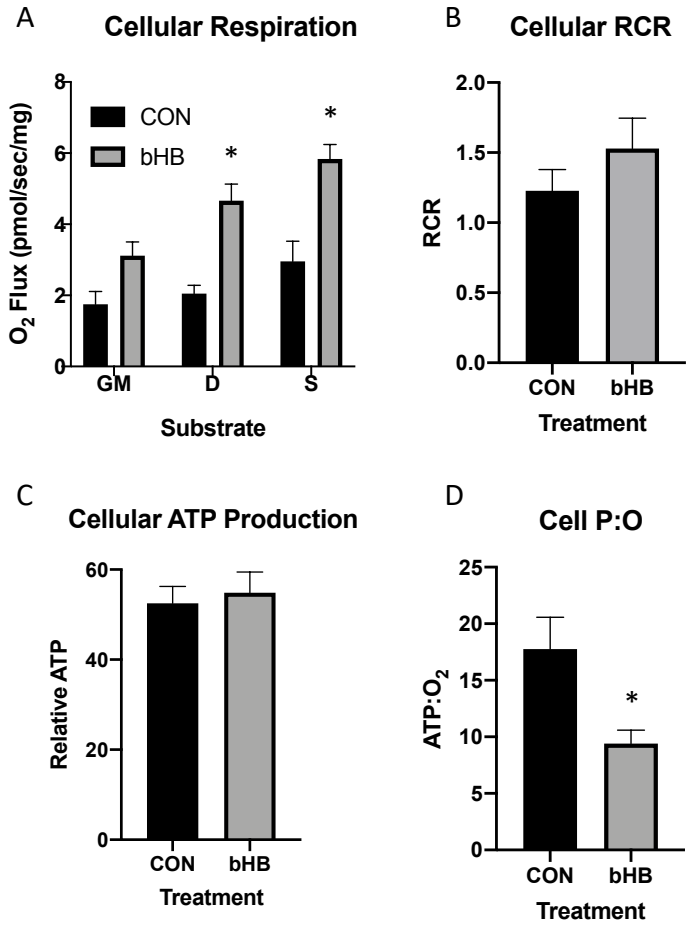


Figure 1: *β*-Hydroxybutyrate Stimulates Increased Mitochondrial Uncoupling in Adipocytes.

*A)* β-Hydroxybutyrate increased mitochondrial respiration in adipocytes differentiated from 3T3-L1 cells. *B)* There was no significant difference in respiratory control ratio (RCR) between treatments. *C)* The was no significant difference in ATP production between treatments. *D)* Adipocytes had a significantly lower ratio of oxygen transport to oxidative phosphorylation relative to control, revealing increased uncoupling in β-Hydroxybutyrate-treated adipocytes.

### *β*-Hydroxybutyrate Does Not Change ATP Production in Adipocytes

We sought to better understand the functional effects of ketones in altering oxygen use by measuring ATP production. Although the analysis of mitochondrial respiration indicated an increase rate of oxygen use, we found that βHB had no significant effect on ATP production in cells (Figure 1C).

### *β*-Hydroxybutyrate Treatment Results in a Significant Increase in Adipocyte Mitochondrial Uncoupling

The ratio of oxygen transport to ATP generation was utilized as a measure of mitochondrial uncoupling. Following 24 h of treatment with βHB, adipocytes had a significantly lower ratio of oxygen transport to oxidative phosphorylation relative to control, revealing increased uncoupling in βHB-treated adipocytes (Figure 1D).

### *β*-Hydroxybutyrate Stimulates Changes in the Genetic Expression of Adipocytes

In addition to meaningful changes in mitochondrial coupling, βHB-treated adipocytes presented with an increased expression of genes associated with mitochondrial uncoupling. Namely, we detected significantly higher levels of PRDM16, PGC1a and UCP1 when compared with control conditions (Figure 2).

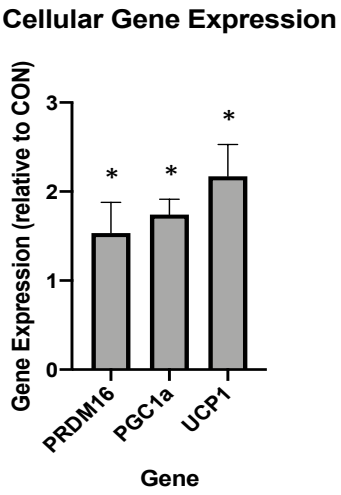


Figure 2: *β*-Hydroxybutyrate Stimulates Changes in Adipocyte Gene Expression.

Treatment with β-Hydroxybutyrate resulted in significantly increased levels of genes associated with metabolism (PRDM16, PGC1a, and UCP1).

A Ketogenic Diet Alters Specific Parameters of Adipose Physiology

Rats on a ketogenic diet (KD) diet were pair-fed based on the consumption of standard (SD)-fed rats, such that caloric intake was not different between diets. Through the 4-week experimental period there was no significant differences in body weight, regardless of treatment or time (Figure 3A). Interestingly, only select depots of adipose tissue were affected by the ketogenic diet. The perirenal (PRF) and subcutaneous (SUB) adipose significantly decreased in rats fed a KD, while there was no significant difference in interscapular (INT) fat or gastrocnemius (GTC) (Figure 3B).

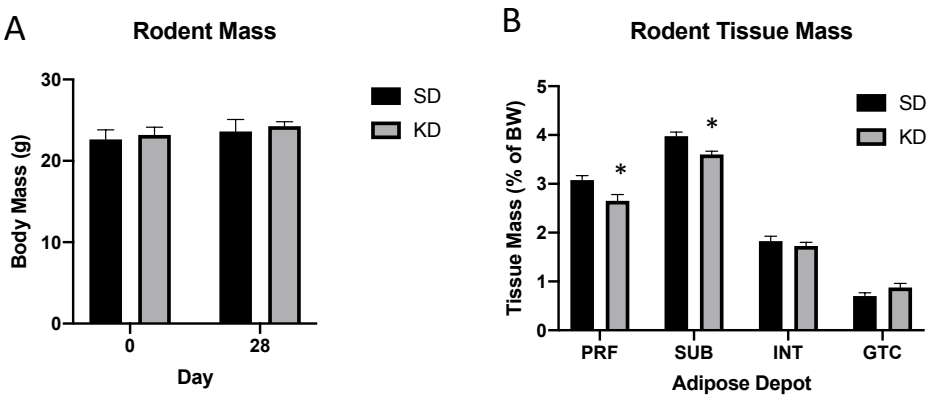


Figure 3: A Ketogenic Diet Alters Fat Mass of Specific Adipose Depots.

*A)* Mice on a ketogenic diet did not have any difference in weight between starting and end date between treatments. *B)* Ketogenic diets altered specific tissue masses, namely perirenal and subcutaneous, but not intrascapular or gastrocnemius tissues.

In rodents fed a KD, interscapular respiration was not significantly different for GM or D, but was significantly increased in the S (Figure 4A). Interscapular adipose tissue did not have any significant difference of the ratio of oxygen transport to oxidative phosphorylation between treatment groups (Figure 4B).

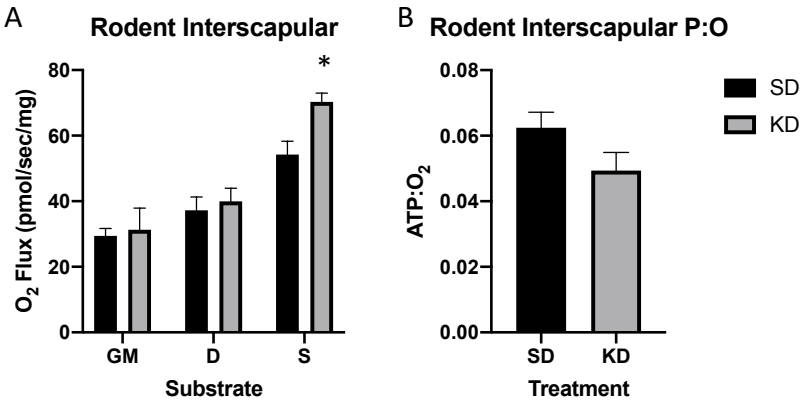


Figure 4: *β*-Hydroxybutyrate Does Not Stimulate Changes in Interscapular Adipose.

*A)* Ketogenic diets did not increase mitochondrial respiration of interscapular adipose tissue with the addition of glutamate, malate (GM) or ADP (D), but did increase with the addition of succinate (S). *B)* Interscapular adipose tissue did not have any significant difference of the ratio of oxygen transport to oxidative phosphorylation between treatment groups.

In rodents fed a KD, subcutaneous fat depots had significant elevations in mitochondrial respiration rate starting with the proton leak (GM), and continuing through the addition of ADP (D) and succinate (S) (Figure 5A). When compared with control, the subcutaneous adipose of KD mice did not have any significant difference of the ratio of oxygen transport to oxidative phosphorylation (Figure 5B).

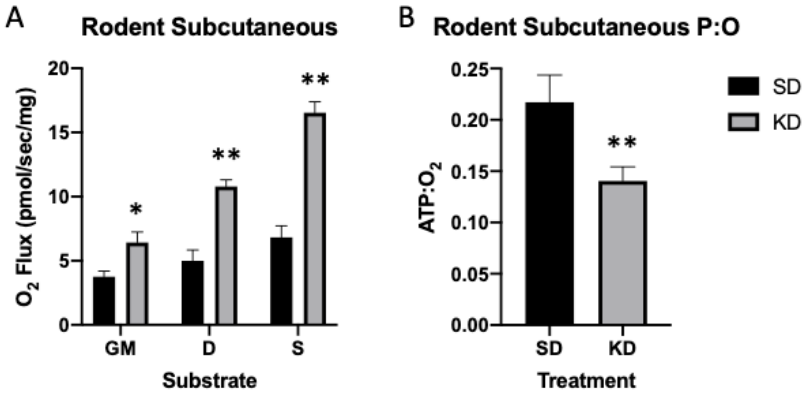


Figure 5: β-Hydroxybutyrate Stimulates Mitochondrial Uncoupling in Subcutaneous Adipose.

*A)* Ketogenic diets increased mitochondrial respiration in subcutaneous adipose tissue with the addition of glutamate, malate (GM), ADP (D), and succinate (S). *B)* Subcutaneous adipose tissue also had a significant difference in the ratio of oxygen transport to oxidative phosphorylation between treatment groups.

Perirenal adipose did not express any significant difference in respiration between KD or SD-fed groups (Figure 6A). Perirenal adipose tissue from the KD mice also did not have a significant difference in the ratio of oxygen transport to oxidative phosphorylation relative to control (Figure 6B).

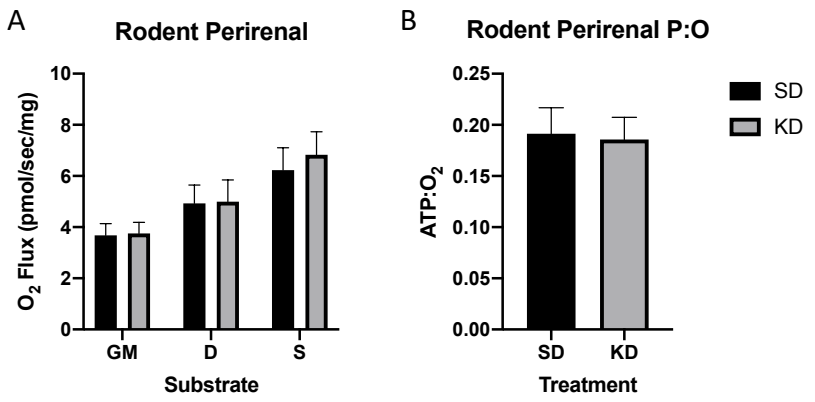


Figure 6: *β*-Hydroxybutyrate Does Not Stimulate Changes in Perirenal Adipose.

*A)* Ketogenic diets did not increase mitochondrial respiration in perirenal adipose tissue with the addition of glutamate, malate (GM), ADP (D), or succinate (S). *B)* Perirenal adipose tissue did not have any significant difference of the ratio of oxygen transport to oxidative phosphorylation between treatment groups.

There was no significant difference in ATP production across treatments in any of the adipose depots (Figure 7A-C).

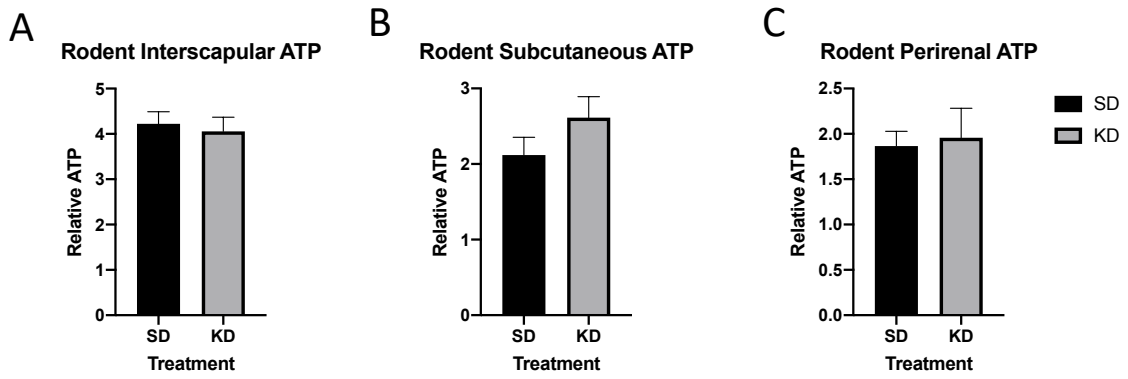


Figure 7: Ketogenic Diet Does Not Alter ATP Production in Adipose Tissue.

Ketogenic diets did not affect ATP production in A) interscapular adipose, B) subcutaneous adipose or C) perirenal adipose tissues.

Ketogenic Diets Stimulate Changes in the Genetic Expression of White Adipose Depots, but not Brown Adipose Depots

Following 4-weeks of treatment, the three adipose depots (PRF, SUB, INT) exhibited different responses in the genes associated with browning and mitochondrial uncoupling. Neither the INT or PRF depots expressed any significant difference between treatments in PGC1a,

PRDM16, or UCP1 (Primers for genes can be found in Table 1). However, in KD-fed rats, SUB adipose tissue expressed significantly higher levels of PGC1a and UCP1, but had no significant difference in PRDM16 (Figure 8).

Table 1: Primer Information.

Information concerning forward and reverse primers for genes of interest.

| Gene | Forward Primer (+) | Reverse Primer (–) |
|---|---|---|
| PGC1a | GACAATCCCGAAGACACTACAG | AGAGAGGAGAGAGAGAGAGAGA |
| Prdm16 | ACCTGCCACAGCAAAGAA | CCATCCAAGCAGAGAAGTAGAC |
| UCP1 | GAGGTCGTGAAGGTCAGAATG | AAGCTTTCTGTGGTGGCTATAA |

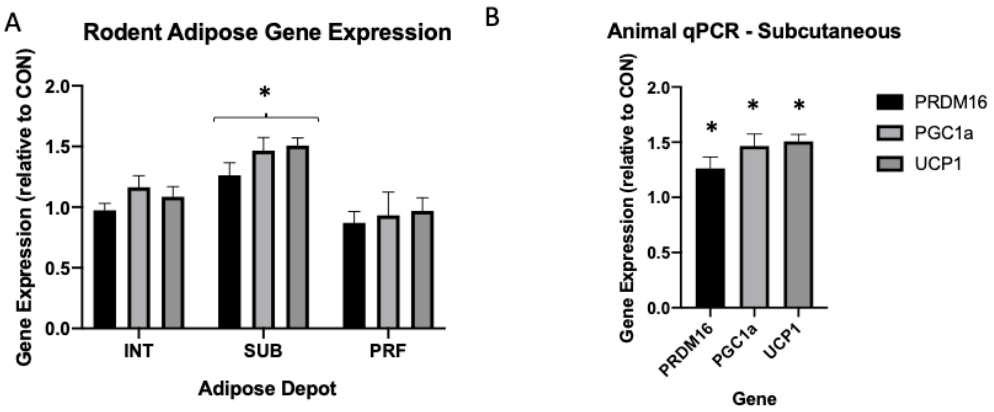


Figure 8: Ketogenic Diet Stimulates Distinct Genetic Expression in White Adipose Tissue.

*A)* Ketogenic diets did not result in any significant difference of gene expression in interscapular or perirenal adipose tissue but did result in a significant increase expression of genes associated with metabolism in subcutaneous adipose tissue. *B)* Ketogenic diets resulted in increased expression of metabolic genes within subcutaneous adipose tissue.

Ketogenic Diets Result in Elevated Basal Metabolic States

Mice on the KD exhibited higher basal metabolic states than their standard chow-fed counterparts as measured with Comprehensive Lab Animal Monitoring System (CLAMS; Figure 9).

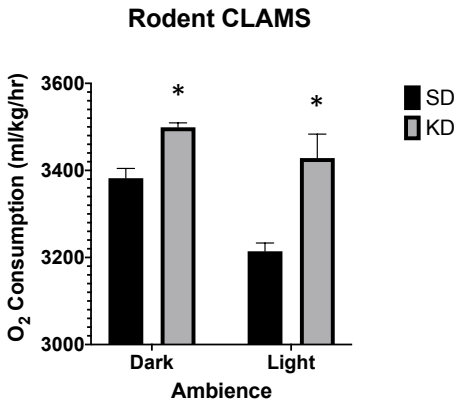


Figure 9: Ketogenic Diet Results in Elevated Basal Metabolic States.
Mice on the KD exhibited higher basal metabolic states than their standard chow-fed counterparts.

Shifts in Ketone-induced Adipose Mitochondrial Bioenergetics are Conserved in Human Adipose Tissue

To test this phenomenon in humans, volunteers adhered to a standard (SD) or ketogenic diet (KD) to induce ketosis (Figure 10A). Similar to the subcutaneous adipose tissue of rodents, subcutaneous fat from volunteers in ketosis had significant elevations in mitochondrial respiration rate through all measurements, including following the addition glutamate and malate (GM), and continuing through the addition of ADP (D) and succinate (S) (Figure 10B). There was no significant difference in RCR between treatments (Figure 10C), indicating generally similar mitochondrial functionality. Importantly, the adipose from people in ketosis revealed comparable levels of ATP production (Figure 10D), which, when combined with the disparate respiration rates, indicates greater mitochondrial uncoupling in states of ketosis (Figure 10E).

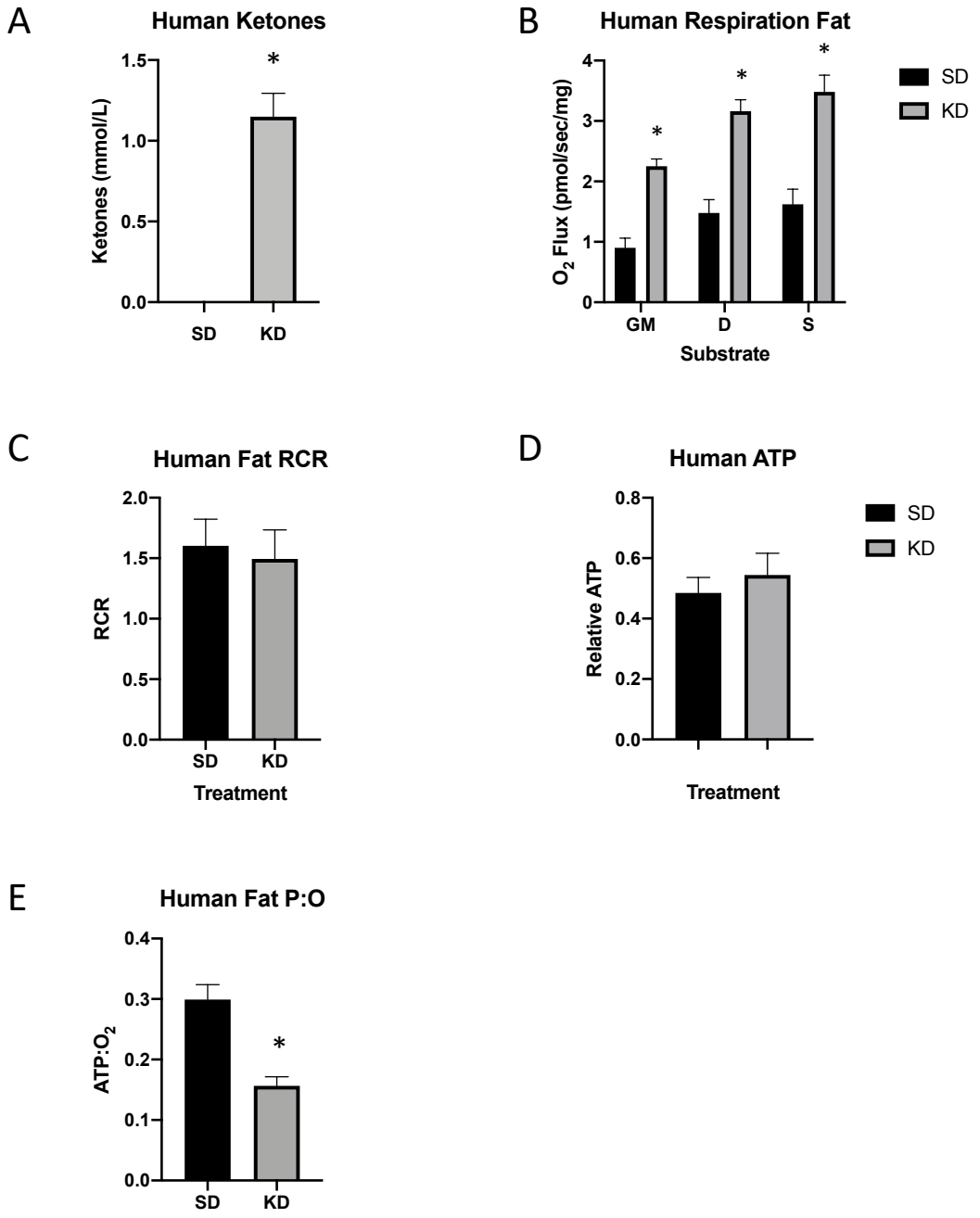


Figure 10: Ketosis Stimulates Mitochondrial Uncoupling in Human Adipose Tissue.

*A)* Human subjects on the ketogenic diet had significantly elevated levels of ketones. *B)* Biopsies of human adipose tissue had significantly higher levels of mitochondrial respiration with the addition of glutamate, malate (GM), ADP (D), or succinate (S). *C)* There was no significant difference in respiratory control ratio (RCR) between treatments. *D)* The was no significant difference in ATP production between treatments. *E)* Human adipose tissue also had a significant difference in the ratio of oxygen transport to oxidative phosphorylation between treatment groups.

# DISCUSSION

As with all nutrients, ketones are both a source of energy, as well as a signaling molecule. However, while once considered "metabolic garbage", ketones are becoming established as crucial regulators of metabolic health in light of their ability to elicit beneficial epigenetic changes. Ketogenic diets have been shown to alter insulin signaling (Badman, Kennedy, Adams, Pissios, & Maratos-Flier, 2009), fatty acid metabolism (Douris et al., 2015), histone deacetylases (Shimazu et al., 2013), and numerous mediators of cell growth and autophagy (Freedland et al., 2008; Kennedy et al., 2007; McDaniel, Rensing, Thio, Yamada, & Wong, 2011).

The key and novel finding of this study is that ketones elicit a mitochondrial uncoupling effect in white subcutaneous adipose, but not in other white adipose depots or brown adipose tissue. This finding is supported by a significant increase in mitochondrial respiration of inguinal subcutaneous adipose tissue in rodents and humans, as well as increased expression of UCP-1 and PGC-1α gene and protein expression in cell-autonomous and mouse models. Overall, our collective findings suggest an important role for ketones in mediating a profound characteristic shift in subcutaneous white adipose mitochondria that may provide a metabolic advantage to those looking to leverage dietary interventions to facilitate weight loss.

These results corroborate those of previous studies. Kekwick et al. revealed that differences in macronutrients between diets can elicit shifts in metabolic rates (Kekwick & Pawan, 1956). Kekwick attributed the significantly faster weight loss from high-fat diets, when compared to the less rapid weight loss of high-carbohydrate diets, to metabolic shifts in response to macronutrient availability. Similar to this and other studies, we hypothesized that the increased resting energy expenditure was due to alterations in metabolism, and that ketones may partly mediate this effect. However, rather than attributing these metabolic shifts to the relative

efficiencies of different macronutrients, we reveal these metabolic alterations to occur via ketones eliciting an uncoupling effect. Our current study showed rodents and humans on a ketogenic diet had increased mitochondrial uncoupling within the subcutaneous, but not other, fat depots.

The mitochondrial response to ketones within adipose tissue corroborates our previous research regarding adipose responsivity to shifts in the metabolic milieu. Our previous study showed that hyperinsulinemia elicited a disparate effect on adipocyte mitochondrial function, driving subcutaneous and brown adipose to become more tightly coupled. Indeed, our previous work on insulin served as a justification for this current project; ketones are exquisitely controlled by insulin levels, wherein elevated insulin inhibits ketogenesis and slows metabolic rate (Georgopoulos et al., 2009; Parker et al., 2018).

Although further studies are needed to establish an increased sensitivity to insulin post-ketone exposure, and the roles of ketones in insulin-controlled environments, our *in vitro* models included the administration of insulin with beta-hydroxybutyrate. We observed increased levels of UCP-1 and PRDM16, in addition to an elevated metabolic rate. This suggests that βHB plays a key regulatory role in increasing UCP-1 expression, even in the presence of insulin. Although the gene and protein data were not available from human samples, we nevertheless observed an increased level of mitochondrial respiration in human subcutaneous adipose samples.

These findings are relevant to our general understanding of obesity. The prevailing calorie-centric focus on obesity has several relevant considerations in the determination of energy balance, including basal metabolic rate, physical activity, the thermic effect of food, and, more recently, metabolically active adipose tissue (Lee et al., 2016; Virtanen et al., 2009). Our

study reveals a distinct tissue-specific impact of macros on mitochondrial function, which can potentially influence the overall basal metabolic rate.

While the focus on obesity as a caloric imbalance certainly has merit, the endocrine component of the condition must also be considered to understand the condition and improve interventions fully. Prolonged insulin treatment induces weight gain and insulin resistance in animals (Guo et al., 2015), an effect similar to that seen in humans (Boden, 2011). Importantly, those with highest insulin levels appear to benefit the most from carbohydrate restriction, even when calories are kept constant (Ebbeling et al., 2018; McClain, Otten, Hekler, & Gardner, 2013).

These results are relevant in the rich history of research exploring the effects of insulin and ketones on metabolism. Specifically, noteworthy diabetes and metabolic scientists Elliot P. Joslin and Francis G. Benedict noted in 1912 that metabolic rate in untreated insulin-deficient diabetes was roughly 15% higher compared with similar body weight subjects with normal insulin (Benedict & Joslin, 1912). Our data suggest that at least part of this metabolic shift is attributable to the elevated ketones in the insulin-deficient diabetic. Thus, in addition to insulin, ketones may represent a unifying paradigm that considers both the caloric and endocrine aspects of obesity.

In conclusion, these results indicate that ketones elicit a pronounced and perhaps even meaningful shift in mitochondrial function in adipose tissue. Whereas adipose tissue is generally a low metabolic rate organ that stores excess energy, ketones enable a fundamental and uncharacteristic shift towards energy wasting via the initiation of a futile cycle in the form of uncoupling electron transport from ATP synthesis. These findings shed light on previous

observations of enhanced fat loss in ketogenic states (via dietary manipulation) and may provide novel interventions in the future to help combat the growing trend of obesity.

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CURRICULUM VITAE

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Education

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|---|---|
| Brigham Young University – College of Life Sciences | Provo, UT |
| Master of Science in Physiology and Developmental Biology, Apr 2020 | |
| Research Advisor: Benjamin Bikman, Ph.D., Associate Professor | |
| Brigham Young University – College of Life Sciences | Provo, UT |
| Bachelor of Science in Physiology and Developmental Biology, Aug 2018 | |

Work Experience

| | |
|---|---|
| Advanced Physiology Assistant – Dr. Allison Woods – BYU | Aug 2018 – Present |
| Research Assistant – Dr. Benjamin Bikman – BYU | Aug 2018 – Present |
| Pathophysiology Teaching Assistant - Dr. Benjamin Bikman – BYU | Aug 2017 – Present |
| Lab Technician – Dr. Benjamin Bikman – BYU | Apr 2017 – Aug 2018 |
| Histology Lab Teaching Assistant - Dr. Paul R. Reynolds – BYU | Jan 2016 – Aug 2018 |
| Teacher – English as a Second Language – LDS Church | Jan 2016 – Dec 2016 |

Research

| | |
|---|---|
| Dr. Benjamin Bikman – Brigham Young University | Jan 2017 – Present |
| Dr. Jonathan Jayme Wisco – Brigham Young University | Jun 2016 – August 2018 |

Publications

*Improvement in glycemic and lipid profiles in diabetic subjects with a 90-day ketogenic diet: A pilot study*
Walton, C. M., Perry, K., Hart, R. H., Berry, S. L., & Bikman, B. T. (2019). Improvement in Glycemic and Lipid Profiles in Type 2 Diabetics with a 90-Day Ketogenic Diet. Journal of Diabetes Research, 2019.

*Diesel Exhaust Particle Exposure Compromises Alveolar Macrophage Mitochondrial Bioenergetics*
Gibbs, J. L., Dallon, B. W., Lewis, J. B., Walton, C. M., Arroyo, J. A., Reynolds, P. R., & Bikman, B. T. (2019). Diesel Exhaust Particle Exposure Compromises Alveolar Macrophage Mitochondrial Bioenergetics. International Journal of Molecular Sciences, 20(22), 5598.

*β-hydroxybutyrate elicits favorable mitochondrial changes in skeletal muscle.*
Parker BA, Walton CM, Carr ST, Andrus JL, Cheung ECK, Duplisea MJ, Wilson EK, Draney C, Lathen DR, Kenner KB, Thomson DM, Tessem JS, Bikman BT. International Journal Molecular Science; 19(8); 10.3390/ijms19082247, 2018.

Invited Presentations

*Effect of Beta-hydroxybutyrate on Myoblast Proliferation and Differentiation.*
Oral presentation at Experimental Biology 2019, Orlando, Florida

*Ketones Drive Mitochondrial Uncoupling in Adipose Tissue*
Oral presentation at Experimental Biology 2018, San Diego, California

*Assessment of Anatomical Variation in the Glenohumeral Joint of Swimmers*
Oral presentation at AACA 2018, Atlanta, Georgia

*Isolation of RNA and Bioenergetics of High-lipid Tissue*
Oral presentation at Grad Fad 2018, Provo, UT

*Manipulating Mitochondrial Physiology in Human Adipose Tissue*
Oral presentation at Graduate Seminar, April 2019, Provo, UT

*The Role of Beta-Hydroxybutyrate in Altering Adipose Mitochondrial Bioenergetics*
Oral presentation at Graduate Seminar, December 2019, Provo, UT

*Selective androgen receptor modulation with MK-2866 favorably alters muscle*
Poster presentation at Experimental Biology, Pathology, 2020, San Diego, California

*Yerba mate increases mitochondrial uncoupling in adipose tissue.*
Poster presentation at Experimental Biology, Physiology, 2020, San Diego, California

*4-HNE Unfavorably Alters Muscle Mitochondrial Bioenergetics and Cell Viability.*
Poster presentation at Experimental Biology, Pathology, 2020, San Diego, California

*Full body loss of the nuclear hormone receptor Nr4a3 results induces obesity and glucose intolerance*
Oral presentation at Experimental Biology, Physiology, 2020, San Diego, California

Academic Honors

*Dean's List*

- Dean's List
- Summer 2013
- Summer 2016
- Fall 2016
- Winter 2017
- Fall 2017
- Winter 2018
- Summer 2018
- Fall 2018

*Scholarships*

2016-2017: Half Tuition, BYU Scholarship
2017-2018: Marigold Saunders
2018-2019: Marigold Saunders
Teaching Assistant, Advanced Physiology
Teaching Assistant, Pathophysiology
Research Assistant, Bikman Lab

Extracurricular/Community/Professional Activities

| | |
|---|---|
| Sugar Rush 5K – Dr. Benjamin Bikman – BYU | Dec 2017 – Present |
| Hospice – Cheryl Dalton – Spanish Fork, UT | Jul 2017 – Present |
| Surgical Services – Utah Valley Hospital – Provo, UT | Jan 2017 – Present |
| Guitar/Piano – Bands/Performances/Leisure – Personal | Aug 2012 – Present |
| Anatomy Academy Coordinator – Dr. Jonathan Wisco – BYU | Jan 2017 – Apr 2018 |
| Adaptive Aquatics – Dr. Jonathan Wisco – BYU | Aug 2016 – Apr 2017 |
| Wound Care – Utah Valley Hospital – Provo, UT | Apr 2016 – Dec 2016 |
| Anatomy Academy Mentor – Dr. Jonathan Wisco – BYU | Jan 2016 – Dec 2016 |
| Visitor Services – Utah Valley Hospital – Provo, UT | Aug 2015 – Dec 2016 |
| LDS Mission – Retalhuleu, Guatemala | Sept 2013 – Sept 2015 |
| Eagle Scout – Boy Scouts of America – Cody, Wyoming | June 2010 |