Adipose Tissue and Liver Adaptations in Exercised versus Non-exercised Rats Fed a Ketogenic Diet, Western Diet or Standard Rodent Chow

by

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ABSTRACT

We investigated the effects of a low-carbohydrate ketogenic diet (KD), versus other diets, on adipose tissue, liver and serum biomarkers in exercise-trained versus sedentary rodents. METHODS: Male Sprague-Dawley rats (~9-10 weeks of age) remained sedentary (SED) or exercised daily with resistance-loaded running wheels (EX) over 6 weeks. Rats were provided isocaloric amounts of KD (20.2% protein, 10.3% carbohydrate, 69.5% fat), Western diet (WD; 15.2% protein, 42.7 carbohydrate, 42.0% fat), or Standard Chow (SC; 24.0% protein, 58.0% carbohydrate, 18.0% fat); n=8-10 in each diet group for SED and EX rats. Upon euthanasia, body and select adipose tissue masses were recorded and preserved for analyses, and liver and serum were also removed and preserved for analyses. RESULTS: Body mass and feed efficiency was greater in WD- and SC-fed vs. KD-fed rats ($p < 0.001$). Diet (WD>KD=SC, $p < 0.05$) and activity (SED>EX, $p < 0.05$) effects existed for raw and relative (body mass-adjusted) omental adipose tissue (OMAT) masses. OMAT adipocyte diameters were lowest in KD-fed rats ($p < 0.01$). Activity effects (EX>SED, $p < 0.05$ to $p < 0.001$) existed for OMAT acetyl Co-A carboxylase (ACC), phospho/pan-p65 (Ser536), phospho/pan-AMPK (Thr172), phospho/pan-HSL (Ser563), and uncoupling protein-1 (UCP-1) protein expression patterns. Raw inguinal/subcutaneous (SQ) masses were greater in WD vs. KD-fed rats ($p < 0.01$), and diet (WD>KD, $p < 0.05$) and activity (SED>EX, $p < 0.01$) effects existed for raw subscapular brown adipose tissue (BAT) masses. Diet effects existed for liver

triglycerides (WD>KD=SC, p<0.001), and diet (WD=SC>KD, p<0.05) and activity effects (SED>EX, p=0.01) existed for liver phospho/pan-p65. Diet (WD=SC>KD, p<0.001) and activity (SED>EX, p<0.001) effects existed for serum insulin, and diet (KD>SC, p<0.01) and activity (SED>EX, p<0.01) effects existed for serum β-hydroxybutyrate (BHB). In all rats, serum insulin was positively associated with body mass (r=0.54, p<0.001), feed efficiency (r=0.57, p<0.001), relative OMAT mass (r=0.57, p<0.001), and relative SQ mass (r=0.31, p<0.05). CONCLUSIONS: While KD-fed rats weighed less and had lower adipose tissue masses compared to WD- and/or SC-fed rats, exercise did not enhance body or fat mass maintenance and/or further alter adipose tissue, liver or serum biomarkers in KD-fed rats. Notwithstanding, the association data suggest lower insulin with KD and/or exercise may facilitate attenuation of adipose tissue mass accretion in rodents.

ACKNOWLEDGMENTS

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LIST OF ABBREVIATIONS

KD	Ketogenic Diet
WD	Western Diet
SC	Standard Chow Diet
TG	Triglyceride
TNF-α	Tumor Necrosis Factor-alpha
IL-6	Interleukin 6
FGF21	Fibroblast Growth Factor
UCP-1	Uncoupling Protein-1
WAT	White adipose tissue
BAT	Brown adipose tissue
SQ	Subcutaneous adipose tissue
OMAT	Omental adipose tissue
FFA	Free fatty acid
VLDL	Very low density lipoprotein
LDL	Low density lipoprotein
HDL	High density lipoprotein
Pref-1	Preadipocyte factor 1
PPARγ	Peroxisome proliferator-activate receptor gamma
GLUT4	glucose transporter 4
FASN	fatty acid synthase
C/EBPα	CCAAT/enhancer binding protein alpha
LPL	Lipoprotein lipase
AC	Adenylate cyclase

cAMP	Cyclic AMP
PkA	Protein kinase A
HSL	Hormone sensitive lipase
ATGL	Adipose triglyceride lipase
DG	Diglyceride
MG	Monoglyceride
G_i	G inhibitory protein
CAT-1	Carnititne acyltransferase 1
ETC	Electron transport chain
ACC	Acetyl CoA Carboxylase
SREBP1	Sterol regulatory binding protein 1
FDG PET	Fluorodeoxyglucose positron emission tomography
FABP	Fatty acid binding protein
CIDEA	Cell death-inducing factor
HFD	High fat diet
SL	Small litter
NL	Normal litter
IL-8	Interleukin-8
MCP-1	Monocyte chemoattractant protein-1
CRP	C reactive protein
IGF-1	Insulin-like growth factor-1
iPTH	Intact parathyroid hormone
PDH	Pyruvate dehydrogenase
B-HB	beta- Hydroxybutyrate
AcAc	Acetoacetate
MCT-1	Monocarboxylate transporter-1
FAT	Fatty acid transporter
HMG CoA	3-hydroxy-3-methylglutaryl coenzyme A

AMPK	Adenosine monophosphate kinase
LF	Low fat
CR	Carbohydrate restricted
T4	Total thyroxine
T3	Triiodothyronine
LFHC	Low fat/high carbohydrate
IFIC	Intermediate fat/intermediate carbohydrate
HFLC	High fat/low carbohydrate
NAFLD	Non-alcoholic fatty liver disease
ER	Endoplasmic reticulum
RegD	Regular diet
SED	Sedentary
EX	Exercised
mRNA	Messenger ribonucleic acid
TBST	Tris-buffered saline tween
BSA	Bovine serum albumin
cDNA	complementary Deoxyribonucleic acid
RT-PCR	Real-time polymerase chain reaction

CHAPTER I

INTRODUCTION

Obesity is prevalent in Westernized societies and has led to numerous chronic

diseases, which subsequently feeds into increased healthcare costs (Kahn, Hull, &

Utzschneider, 2006; Must & McKeown, 2000; Van Gaal, Mertens, & De Block, 2006).

Moreover, obesity has been estimated to claim over 300,000 lives per year in the United

States (Mokdad et al., 2001). High amounts of adiposity induce inflammation, high

blood pressure, and elevated blood lipids which increase the risk for type 2 diabetes and

cardiovascular disease (Kahn et al., 2006; Van Gaal et al., 2006). While there are

pharmaceutical approaches to combating diseases associated with obesity (i.e.,

anorexiants/stimulants, incretin mimetics, and peripheral-acting obesity agents like

orlistat), different lifestyle approaches have been utilized. One controversial, yet

apparently effective approach has been the application of low-carbohydrate ketogenic

diets (KD). The KD was developed in the 1920's to imitate fasting and consequently

combat epileptic seizures (Baranano & Hartman, 2008). Similar to long-term fasting, the

KD is comprised of high amounts of fat with minimal carbohydrates (typically

<50grams/day) which sways metabolism to oxidize dietary lipids and adipose lipid stores

for energy (Baranano & Hartman, 2008). While several iterations of KDs exist, this diet

is largely comprised of: a) meats (red meat, poultry, fish, pork, etc.), dairy products

(cheeses, milk, butter, whipping cream, etc.), nuts (almonds, peanuts, cashews, etc.),

eggs, and vegetable oils (coconut oil, olive oil, etc.). The intake of grains and starch-

laden carbohydrate sources is minimal, as carbohydrate sources are usually in the form of very low-glycemic cruciferous vegetables (spinach, broccoli, kale, lettuce, etc.). While macronutrient ratios of different KD diets may vary, a typical KD diet will contain approximately 20-25% of Calories from protein, 60-65% of Calories from fat, and 10-15% Calories from carbohydrates. During dietary ketosis, partially oxidized fatty acids are converted into ketone bodies in the liver, primarily acetoacetate and β-hydroxybutyrate (β-HB), as a non-glucose energy source for the brain and other tissues (Baranano & Hartman, 2008; Fukao, Lopaschuk, & Mitchell, 2004). The utilization of fatty acids and ketone bodies for energy ultimately decreases adipose tissue depots while conserving blood glucose levels (Baranano & Hartman, 2008; Fukao et al., 2004).

The effects KD on whole-body adiposity is increasingly being studied in humans. Gardner et al. (Gardner et al., 2007) published perhaps the most highly-cited human clinical study examining the weight loss effects of a KD. In this study, the investigators examined the effects of a hypocaloric ketogenic 'Atkins diet' on body composition and serum health markers compared to other hypocaloric diets (i.e., the Ornish, Zone, and LEARN diets) over a 12-month period in 311 overweight/obese (BMI = 27-40) premenopausal women. These authors reported that average total body loss was in the Atkins group was -4.7 kg, whereas total body losses in the Zone, LEARN and Ornish groups were -1.6 kg, -2.6 kg, and -2.2 kg (-3.6 to -0.8 kg). Moreover, these authors reported that, compared to the other diets, the Atkins subjects presented more favorable alterations in body fat percentage reductions, serum triglyceride (TG) reductions, systolic blood pressure reductions, and HDL cholesterol increases. Yancy and colleagues (Yancy, Olsen, Guyton, Bakst, & Westman, 2004) also demonstrated that a 6-month KD

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induced greater fat loss and similar lean body mass loss when compared to a hypocaloric low fat diet in overweight individuals. While serum LDL levels were similar following the dietary interventions, the KD group exhibited significant reductions in TG levels while presenting greater HDL levels. Similarly, when men consumed a calorie-restricted KD versus a low-fat diet for 12 weeks, the KD group demonstrated significantly greater total body mass and fat mass losses with decreased TG and increased HDL levels compared to the low-fat group (Volek et al., 2009). A shorter-term KD of 6 weeks also facilitated significant total body mass and fat mass losses while increasing lean body mass in men compared to their counterparts who consumed a normal mixed diet (Volek et al., 2002). Volek et al. (Volek et al., 2004) demonstrated that a 4 week hypocaloric KD significantly improved HDL levels and markers of insulin resistance in moderately overweight women compared to their counterparts who consumed a hypocaloric low fat diet. In obese, type 2 diabetics, 14 days of a KD resulted in: a) significant decreases in total body mass, b) an increased insulin sensitivity by 75%, and c) a reduction in TG and total cholesterol levels (Boden, Sargrad, Homko, Mozzoli, & Stein, 2005). Thus, it is apparent that short- (weeks) to long-term (months) ketogenic dieting can promote favorable alterations in total body mass and fat mass in overweight individuals while also improving serum markers indicative of metabolic health.

Researchers are also beginning to delve deeper into the effects of a KD on adiposity and inflammation by using animal models. A 4 week study by Asrih and colleagues (Asrih, Altirriba, Rohner-Jeanrenaud, & Jornayvaz, 2015) reported that young mice fed a KD demonstrated significantly reduced the mRNA expression of inflammatory markers (TNF-α, IL-6, Emr1, CD68, Itgam, and Nlrp3) in white adipose

tissue while increasing energy expenditure compared to mice fed a standard chow diet. Also, the KD group presented an increased expression of fibroblast growth factor 21 (FGF21) and uncoupling protein-1 (UCP-1) in adipose tissue; these proteins are associated with protection from diet-induced obesity through mechanisms that increase energy expenditure and fat utilization. In contrast, a 4-week study in rats compared a KD to a low fat diet. Compared to rats consuming the low fat diet, the rats consuming the KD exhibited increased fat mass, reduced lean mass, and reduced HDL levels. Also, the KD group demonstrated increased insulin resistance and glucose intolerance (Bielohuby et al., 2013). However, it should be noted that these studies may differ in their findings due to the fact that several KDs have been utilized in human and rodent studies. For instance, a more recent rodent study examining the effects of three different KDs (fat-to-protein percentages of diet: 75/10 vs. 65/20 vs. 55/30) determined that: a) the 55/30 group presented a greater retention in whole-body nitrogen as well as an increased carcass protein composition compared to the 75/10 group, and b) the 55/30 group presented significantly less total carcass as well as liver fat composition compared to the 75/10 group. Therefore, KDs with higher percentages of protein (~25-30%) and lower percentages of fat (~55-60%) will likely result in more favorable alterations in body composition than lower-protein/higher fat diets. It is also apparent that there is inconsistency in the scientific literature regarding how ketogenic dieting affects whole body adiposity as well as general health markers.

Physical activity is also known to decrease whole-body adiposity, as this has been demonstrated both physically active humans and rodents when compared to sedentary counterparts (Booth, Chakravarthy, Gordon, & Spangenburg, 2002; Nara et al., 1999).

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While there are likely numerous mechanisms at play, increased physical activity levels have been shown to affect adipose tissue in the following manners: a) an increased fat mobilization from adipose tissue via exercise-induced catecholamine release (Booth et al., 2002), and b) a decreased activation of adipogenesis-related mechanisms (Company, Roberts, Toedebusch, Cruthirds, & Booth, 2013).

Given the aforementioned data, it stands to reason that combining an increase in physical activity levels while partaking in a KD is a very good strategy for synergizing weight loss. In this regard, ketogenic dieting increases catecholamine release which, in turn, facilitates lipolysis, and this is likely a keto-adapted mechanism which mimics a starvation state (Fukao et al., 2004). Moreover, both physical activity and KD appreciably reduce serum insulin levels (Boden et al., 2005; Brody, 1999; Volek et al., 2004). Given that insulin signals lipogenic and adipogenic mechanisms, the combinatorial effects of ketogenic dieting with exercise likely causes a synergistic effect in facilitating fat mass loss through this mechanism (Brody, 1999). Studies demonstrating the synergistic effects of KDs and exercise on body composition are very limited. In fact, only one study to my knowledge has determined how ketogenic dieting with exercise affects body composition. In this study, 16 untrained overweight women consumed either a very low carbohydrate ketogenic diet ("KD" : 6% calories from carbohydrate, 66% calories from fat, and 22% calories from protein) or a regular diet ("RegD": 41% calories from carbohydrate, 34% calories from fat, and 17% calories from protein) for 10 weeks. The subjects performed 60-100 minutes of various resistance exercise 2 times/week while on the designated diet. The KD group lost fat mass (-5.6%) without experiencing alterations in lean mass, whereas the RegD group gained lean body

mass (+1.6 kg) but did not lose fat mass Neither group showed a change in blood lipid or glucose levels (Jabekk, Moe, Meen, Tomten, & Hostmark, 2010).   However, it should be noted that the effects of the KD on adipogenic and/or lipogenic mechanisms remained unexplored.

While KDs have received more scientific consideration as an effective weight loss method, the current body of literature has not elucidated all of the mechanisms of a KD regarding the potential anti-adipogenic, anti-lipogenic and/or inflammation-related pathways that occur in whole-body adipose tissue following the dietary intervention. Also, the combined effects of a KD and physical activity in human and animal models are limited. Therefore, the primary purpose of this study was to investigate how a putative commercially-prepared KD affects adipogenic, lipogenic and/or inflammation-related pathways using a rat model. Select adipose tissue markers of adipogenesis, lipogenesis, substrate transporters, beiging, and inflammation as well as serum concentrations of insulin, glucose, and lipids were examined between three dietary interventions including a KD (5.2 kcal/g, 20.2% protein, 10.3% carbohydrate, 69.5% fat), Western diet (WD; 4.5 kcal/g, 15.2% protein, 42.7% carbohydrate, 42.0% fat; most of the carbohydrate being sucrose), and standard chow diet (SC; 3.1 kcal/g, 24.0% protein, 58.0% carbohydrate; most of the carbohydrate coming from wheat and corn). Secondarily, determination of the influence of daily physical activity, in the form of voluntary wheel running, on the proposed adipose markers for each diet were assessed to see if the combination of KD dieting with an increase in physical activity has additive effects on adipose tissue physiology.

CHAPTER II

REVIEW OF LITERATURE

Structure and function of adipose tissue

White adipose tissue (WAT), brown adipose tissue (BAT), and beige adipose

tissue are found in mammals and each has distinct functions and structures (Bjorndal,

Burri, Staalesen, Skorve, & Berge, 2011). WAT is further divided into regional depots

which include abdominal, subcutaneous, and visceral adipose tissue. Visceral is

subdivided into omental, mesenteric, retroperitoneal, gonadal, and pericardial adipose

tissues (Bjorndal et al., 2011). The expansion of omental and mesenteric adipose tissues

are associated with an increased risk for developing an obesity-related disease and

therefore are the primary visceral adipose depots studied (Bjorndal et al., 2011). Omental

adipose tissue originates near the stomach/spleen and may reach the ventral abdomen

whereas deeper, web-like mesenteric adipose tissue attaches to the intestines (Bjorndal et

al., 2011). Individual factors such as age, sex, life-style habits, and genetics contribute to

the distribution variability of subcutaneous and visceral adipose tissues (Wajchenberg,

2000).

Lipids make up 90% of adipose tissue, with 95% of the lipids in the form of

triglycerides (TGs) and the rest in the form of diglycerides, phospholipids, and

cholesterol (Brody, 1999). Adipocytes are cells that store the lipids and comprise the

majority of adipose tissue; however, adipose tissue also contains macrophages

(inflammatory white blood cells that collect circulatory debris), other immune cell subtypes, preadipocytes, fibroblasts, nerves, connective tissue, vascular tissues, and is comprised of 5-30% water by weight (Ibrahim, 2010). Interestingly, humans can survive in a fasted state on the amount of stored lipids in the body for 1-2 months while rodents may survive for only 1 week (Brody, 1999).

Adipocytes possess the ability to expand in size and number when there is a rise in the necessity for TG droplet storage (Ibrahim, 2010). After a meal, smaller adipocytes absorb more free fatty acids (FFAs) and TGs, whereas larger adipocytes may be dysfunctional and resistant to absorption (Ibrahim, 2010). Subcutaneous adipose tissue contains a greater number of small adipocytes compared to visceral adipose tissue which consists of mostly larger adipocytes (Ibrahim, 2010). This is important to note given that adipocyte size has been positively associated with the secretion of pro-inflammatory cytokines such as IL-6, TNF-α, and MCP-1 (Skurk, Alberti-Huber, Herder, & Hauner, 2007). Moreover, Western diet feeding (Boullu-Ciocca, Achard, Tassistro, Dutour, & Grino, 2008) as well as inactivity models (Ruegsegger et al., 2015) have been shown to rapidly expand visceral adipose tissue depots which, in turn, leads to a greater pro-inflammatory phenotype of this tissue.

WAT, the primary storage site for lipids, (Billon & Dani, 2012; Symonds, Pope, & Budge, 2015) has recently been determined to function as an endocrine organ as it secretes hormones such as leptin and adiponectin (Billon & Dani, 2012). Receptors on or within WAT may vary between depots, but prominently studied receptors include glucocorticoid, androgen, oestrogen, insulin, and adrenergic receptors. Visceral adipose tissue contains a high concentration of glucocorticoid receptors which regulate

metabolism and fat distribution (Joyner, Hutley, & Cameron, 2000). Visceral adipose tissue also contains more androgen receptors than subcutaneous tissue (Freedland, 2004). When testosterone levels decrease in middle-aged men, visceral adipose tissue accumulates whereas subcutaneous adipose tissue declines (Pedersen et al., 1996). Women have a greater expression of oestrogen receptors in subcutaneous adipose tissue (Pedersen et al., 1996). Post-menopausal women have decreased expression of these receptors which promotes visceral adipose tissue accumulation (Freedland, 2004). Compared to subcutaneous adipose tissue, visceral adipose tissue contains a greater level of adrenergic receptors and is more sensitive to catecholamine stimulation of β-3-adrenoreceptor and α-2-adrenergic receptors which induces lipolysis (Hellmer, Marcus, Sonnenfeld, & Arner, 1992; Imbeault, Couillard, Tremblay, Despres, & Mauriege, 2000). Lipolysis of excess visceral adipose tissue results in the accumulation of free fatty acids (FFAs) and glycerol in liver via direct portal drainage (Pedersen et al., 1996). If produced in high amounts, the high influx of FFAs and glycerol lead to an overproduction of glucose and very low density lipoproteins (VLDLs) in the liver which negatively impacts insulin sensitivity (Pedersen et al., 1996).

Although brown and beige adipose tissue are less abundant than WAT, BAT may impact the body's energy balance to a greater degree due to its higher metabolic rate and relative 'inefficiency' in ATP production which, in turn, increases energy expenditure (Symonds et al., 2015). BAT contains greater vascularization and mitochondrial density than WAT and contains multilocular lipid droplets whereas WAT contains unilocular lipid droplets (Gesta, Tseng, & Kahn, 2007). BAT is considered a thermogenic organ as brown adipocytes participate in a process called adaptive thermogenesis (Gesta et al.,

2007). This process occurs when brown adipocyte mitochondria, with the assistance of uncoupling protein-1 (UCP-1), uncouple respiration from ATP synthesis in the inner mitochondrial membrane. This ultimately leads to a greater conversion of nutrient-derived energy substrates to be converted into heat, thus, functioning to regulate body temperature (Billon & Dani, 2012; Gesta et al., 2007). Beige adipose tissue contains some UCP-1 but is located in WAT, and recent evidence suggests that exercise may convert WAT adipocytes into a 'beige' phenotype which is more metabolically-active (Billon & Dani, 2012).

Formation of Adipose Tissue

Adipogenesis begins with the formation of adipocyte progenitors from mesenchymal stem cells derived from the mesoderm and ends with the differentiation of the adipocyte progenitors into mature adipocytes (Billon & Dani, 2012). In fetal development, morphological studies suggest that the formation of capillaries in mesenchymal tissues are associated with the start of adipogenesis (Billon & Dani, 2012). During adipogenesis, mesenchymal stem cells follow a pathway of differentiation with different gene signals thereby leading to the formation of either a white or brown preadipocyte. Preadipocyte factor 1 (Pref-1) is a marker of the pre-adipocyte stage for white and brown pre-adipocytes; it is expressed in pre-adipocytes and is down-regulated once the adipocyte matures (Gesta et al., 2007; Villena, Kim, & Sul, 2002). Mature adipocyte markers include peroxisome proliferator-activate receptor γ-2 (PPARγ-2), glucose transporter 4 (GLUT4), and fatty acid synthase (FASN) (Gesta et al., 2007).

Adipose Tissue Formation during Embryogenesis and Childhood

The development of WAT occurs earlier in humans than rodents. WAT does not develop during embryogenesis in rodents but rather directly after birth (Billon & Dani, 2012). WAT gradually increases throughout life in rodents with development occurring first in the subcutaneous and perigonadal regions, followed later by omental development (Billon & Dani, 2012). Humans, on the other hand, develop WAT during embryogenesis, first in the head and gradually in all of the fat depots (Billon & Dani, 2012; Gesta et al., 2007). A human study (Landgraf et al., 2015), with 171 adipose tissue samples from lean and obese children aged 0-18 years old, found increased white adipocyte size and number, decreased lipolytic activity, and increased stromal vascular cell proliferation in the obese compared to lean children in early childhood. After 6 years of age, compared to lean children, obese children demonstrated greater macrophage infiltration and increased adipocyte size which correlated with insulin resistance (Landgraf et al., 2015).

BAT develops during embryogenesis and before WAT in both humans and rodents. BAT reaches maximal size at birth, as the need for non-shivering thermogenesis is greater during infancy, and gradually decreases with age thereafter (Cannon & Nedergaard, 2004; Symonds et al., 2015). BAT protects against childhood obesity, is more active in those with a lower BMI, and is regulated by genetic and environmental factors (Symonds et al., 2015). Figure 1 (below) demonstrates the genetic signatures for WAT and BAT during different developmental stages [modified from (Gesta et al., 2007)].

INSERT FIGURE 1 HERE

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Both WAT and BAT have transcriptional pathways that include the transcription factors C/EBPs and PPARγ but those factors may vary in function between adipose tissue subtypes. For instance, when C/EBPα is knocked out in mice, WAT becomes absent, but interscapular BAT is not affected (Linhart et al., 2001). However, when PPARγ is knocked out, both WAT and BAT are reduced (He et al., 2003).

Brown adipose progenitor cells have a similar transcription cascade as skeletal muscle but only before differentiation into a preadipocyte (Billon & Dani, 2012). Shared skeletal muscle genes during early BAT development include Myf5, MyoD, and myogenin (Billon & Dani, 2012). BAT also performs similar functions as skeletal muscle including breaking down instead of storing of lipids, participating in thermogenesis, and possessing high levels of mitochondrial activity (Billon & Dani, 2012). Similar to skeletal muscle, sympathetic nervous system activity also activates BAT expression (Billon & Dani, 2012).

Adipose tissue maintenance during adulthood via lipogenesis and lipolysis

Diet and exercise allow adults to regulate adipose tissue accumulation via lipogenesis, lipolysis, and adipogenesis. From a simplistic viewpoint these operational definitions are important to note (Boron, 2012; Brody, 1999): a) lipogenesis is the formation of TGs through acetyl CoA or FFAs within the liver or adipocytes, b) lipolysis is the breakdown of TGs into FFAs and glycerol within adipocytes or other metabolizing tissues (i.e., skeletal muscle, BAT, liver, etc.), and c) adipogenesis is the formation of

new mature adipocytes from preadipocytes in adipose tissue. These processes are described in greater detail below.

Lipogenesis

Postprandial lipogenesis in the liver consists of synthesizing fatty acids from acetyl CoA, derived from dietary glucose, in the cytosol (Brody, 1999; Horton, 2006). Specifically, dietary glucose stimulates pancreatic insulin secretion which activates pyruvate dehydrogenase and acetyl CoA carboxylase. These enzymes convert the excess glucose into acetyl CoA followed by malonyl CoA which stimulates fatty acid synthesis. The synthesized FAs esterify with glycerol to form a TG. The TGs may travel out of the liver to circulate through the blood stream via lipoproteins.

Adipocytes respond to diet-induced insulin release by translocating intracellular GLUT4 to the membrane surface to absorb glucose. Insulin also stimulates lipoprotein lipase (LPL) synthesis which is exported to the endothelial cell surface. LPL then acts in the vascular lumen by attaching to circulating lipoproteins to break down encased TGs (Brody, 1999). The resulting FFAs and glycerol are transported into adjacent adipocytes and re-esterified into TGs; accumulation of adipocyte TGs ultimately promotes increased fat mass (Boron, 2012; Brody, 1999; Horton, 2006).

Lipolysis

Exercise or fasting/starvation results in elevated blood levels of epinephrine and norepinephrine from the adrenal glands and increased glucagon secreted from the pancreas (Brody, 1999). Catecholamines aid in the stimulation of lipolysis in adipocytes

through initiating the hydrolysis of stored TGs into free fatty acids and glycerol. Conversely, when insulin is secreted from the pancreas in response to increased blood glucose, lipolysis and beta-oxidation are inhibited via a process described in greater detail below. Hence, repeated bouts of lipolysis promote a reduction in adipose tissue via a decrease in stored TGs, whereas repetitive increases in insulin likely inhibit lipolysis and, instead, promote the synthesis and storage of TGs in adipose tissue. The steps of lipolysis in an adipocyte during the fasted or exercised stated include (Brody, 1999; Horton, 2006; Spriet, 2012):

1. Increased levels of epinephrine/norepinephrine circulate through the blood stream.

2. Epinephrine binds to the β-adrenergic receptor on the adipocyte.

3. The β-adrenergic receptor stimulates the activation of the intracellular G stimulatory protein (G_s) which overrides G inhibitory protein (G_i) signaling.

4. Intracellular G_s increases adenylate cyclase (AC) activity in the adipocyte.

5. AC increases the production of cAMP concentrations in the adipocyte.

6. Through mass action, cAMP increases protein kinase A (PkA) activity in the adipocyte.

7. PkA phosphorylates perilipin A, hormone sensitive lipase (HSL), and possibly adipose triglyceride lipase (ATGL) which activates these lipases.

8. In the de-phosphorylated state, perilipin A provides a protective coating for the lipid; in the phosphorylated state, perilipin A allows the lipid to be exposed so that activated ATGL and HSL are able to catalyze lipolysis.

9. A single TG is then hydrolyzed by ATGL into a diglyceride (DG) and 1 FFA.

10. HSL hydrolyzes DG into monoglyceride (MG) and an additional FFA.

11. Monoglyceride lipase hydrolyzes MG into glycerol and a third FFA.

12. Glycerol leaves adipose tissue and travels to the liver for possible conversion into glucose via gluconeogenesis.

13. The 3 FFAs leave the adipocyte, bind to albumin in the blood, and travel to other metabolizing tissues for ATP production via mitochondrial-mediated beta-oxidation.

The resulting FFAs (FFA-albumin) from lipolysis are transported from the bloodstream into the cytosol of the tissue requiring energy. The FFA-albumin becomes activated by Fatty Acyl CoA Transferase which converts FFA into fatty acyl CoA. The fatty acyl CoA may only enter the mitochondria via the carnitine shuttle which requires the enzyme carnitine acyltransferase 1 (CAT-1). Once the fatty acid enters the mitochondria, β-oxidation can occur. β-oxidation is the process of converting the fatty acyl CoA into acetyl CoA and these steps include (Brody, 1999; Horton, 2006):

1. Oxidation Step (Q → QH$_2$): Acyl CoA → Trans-Δ^2-Enoyl CoA

2. Hydration Step: Trans-Δ^2-Enoyl CoA → L-3 hydroxyacyl CoA

3. Oxidation Step (NAD$^+$ → NADH + H$^+$): L-3 hydroxyacyl CoA → 3-ketoacyl CoA

4. Thiolase Step (HS-CoA → acetyl-CoA): 3-ketoacyl CoA → Acetyl CoA

6. Resulting: Acetyl CoA + NADH (H$^+$ carrier for electron transport chain (ETC) later).

Acetyl CoA typically enters the citric acid cycle and then the ETC resulting in energy for the cell via ATP synthesis. Insulin inhibits β-oxidation by preventing fatty acids to enter into the mitochondria from the cytosol for oxidation.

In the resting, fed or non-stressed physiological state, low levels of epinephrine and norepinephrine inhibit lipolysis by activating α-2 adrenergic receptors which stimulate the intra-adipocyte G_i protein activity (Spriet, 2012). G_i protein suppresses AC activity, and therefore reduces cAMP levels as well as the activation of PkA, HSL, ATGL, and ultimately TG hydrolysis (Spriet, 2012). Insulin also inhibits lipolysis in the adipocyte. Specifically, when insulin binds to its transmembrane receptor, the resultant intracellular signaling cascade directly inhibits AC activity by activating phosphodiesterase, which degrades intracellular cAMP (Brody, 1999; Horton, 2006; Spriet, 2012). Insulin also attenuates fatty acid β-oxidation by activating the enzyme Acetyl CoA Carboxylase (ACC) which inhibits Carnitine Transferase I (the enzyme needed to shuttle fatty acids into the mitochondria). Glucagon, on the other hand, inhibits ACC thereby providing an opportunity for fatty acids to enter the mitochondria to undergo β-oxidation. Glucagon also inhibits the pyruvate dehydrogenase complex which allows the substrates produced by lipolysis and β-oxidation to generate required energy rather than utilizing glucose for energy. Thus, based upon the aforementioned mechanisms, viable lifestyle modification strategies for reducing the cellular size of adipocytes via pulsatile TG breakdown through lipolysis includes: a) consuming a diet which keeps insulin levels relatively low as well as glucagon levels relatively higher, and/or b) engaging in daily physical activity to ensure the routine pulsatile secretion of circulating catecholamines.

Adipogenesis

As mentioned previously, adipogenesis is the formation of new adipocytes from pre-adipocytes residing in the adipose tissue milieu. Two primary adipogenic transcription factors include peroxisome proliferator-activated receptor-gamma (PPARγ) and sterol regulatory binding protein 1c (SREBP1c) (Rosen & Spiegelman, 2001). PPARγ is primarily expressed in adipose tissue and is known to: a) stimulate preadipocyte differentiation, thus leading to the increased storage of fatty acids in more mature adipocytes, and b) possess ligand binding domains which, when bound with fatty acid ligands, enter the nucleus to bind to PPAR DNA binding elements which up-regulate the expression adipogenic genes (Boon Yin, Najimudin, & Muhammad, 2008; Farmer, 2005). It is also important to note that PPARγ2 also increases the genetic expression of GLUT4 in already-existing mature adipocytes which allows glucose to enter the adipocyte and be converted into fatty acids via *de novo* FA synthesis (Wu, Xie, Morrison, Bucher, & Farmer, 1998). Thus, beyond being an important adipogenic transcription factor, PPARγ is also a lipogenic transcription factor. SREBP1c also serves as an adipogenic and lipogenic transcription factor; its prominent role is to drive the expression of PPARγ which, in turn, leads to adipocyte differentiation and/or lipogenesis in pre-existing adipocytes. Interestingly, in a study examining the adipose tissue of insulin receptor knockout mice (Bluher et al., 2002), insulin has been shown to drive the genetic expression of SREBP1c in small adipocytes (suggestive of pre-adipocytes). Thus, this again conceptually demonstrates that, in situations where in insulin levels are chronically low, an expansion of adipose tissue via adipogenesis is likely low as well.

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Adipose tissue maintenance through thermogenic mechanisms ('beiging' or 'briting')

Rodents maintain BAT throughout adulthood whereas the maintenance or physiological roles of BAT in adult humans is controversial. The use of fluorodeoxyglucose positron emission tomography (FDG PET), typically used to locate tumors, has located metabolically active tissues thought to be BAT. Rodents carry BAT in the intrascapular region whereas the FDG PET scans suggest BAT is located in the supraclavicular and neck regions in humans (Fajas et al., 1999).

BAT activity is stimulated by the sympathetic nervous system during cold exposure in both rodents and humans (Cousin et al., 1993). β-adrenergic agonists, such as circulating norepinephrine released from the sympathetic nervous system, are also thought to stimulate the differentiation of brown preadipocytes and assist in prevention of brown adipocyte apoptosis (Cannon & Nedergaard, 2004). Adrenergic agonists, as well as thyroid hormone, induce brown adipocyte UCP-1 expression which regulates the thermogenic effect of BAT through mitochondrial ETC uncoupling (Cannon & Nedergaard, 2004). The process of nonshivering thermogenesis occurs via lipolysis of BAT (Bamshad, Song, & Bartness, 1999). Cold exposure or sympathetic nervous system activity stimulates catabolism of brown adipocytes (Bamshad et al., 1999). The resulting FFAs in BAT cytosol bind to fatty acid binding proteins (FABPs), pass through the carnitine shuttle, and then undergo oxidation in the mitochondria of BAT (Bjorndal et al., 2011). The abundance of mitochondria located in BAT assists with oxidizing FFAs to generate heat via UCP-1 (Mattson, 2010). UCP-1 activity is suppressed by cell death-inducing factor (CIDEA) (Zhou et al., 2003). CIDEA is highly expressed in BAT and

when the gene is knocked out, rodents increase BAT metabolic rate, obesity-resistance, and body temperature during cold exposure (Zhou et al., 2003).

Beige adipose tissue consists of a specific type of brown adipocytes that share more similarities with WAT than with skeletal muscle, unlike classical brown adipocytes (Billon & Dani, 2012). These adipocytes are located in WAT, and are termed beige adipocytes or 'brite' adipocytes (a term linking the brown adipocyte location in WAT: brown in white = brite) (Billon & Dani, 2012). Similar to the classical brown adipocyte, brown adipocytes in WAT respond to cold exposure, and sympathetic nervous system activity via catecholamine signaling, as well as display UCP-1 expression (Billon & Dani, 2012). Interestingly, beyond cold exposure, recent highly-controversial data suggests that lifestyle modifications (such as exercise) may convert white adipocytes residing in WAT depots to a more 'beige-like' adipocyte due to the release of exercise-induced myokines (chiefly 'irisin') (Bostrom et al., 2012). Moreover, there is rodent data suggesting that ketogenic dieting in mice increases the mitochondrial size of intrascapular BAT by 60% while reducing intracellular BAT lipid droplet sizes by ~50% (Srivastava, Baxa, Niu, Chen, & Veech, 2013).

Dysregulation of fat mass maintenance

Rate of obesity and implications of being obese

Rates of individuals becoming overweight or obese (BMI>25) are increasing rapidly in the United States with half of the population now considered overweight or obese (Must & McKeown, 2000). Second to tobacco, obesity is one of the greatest environmental causes of death in the United States, claiming approximately 300,000

adult lives per year (Mokdad et al., 2001). Obesity increases the risk for cardiovascular

disease and for developing type 2 diabetes (Kahn et al., 2006; Van Gaal et al., 2006).

Increased adiposity also causes various unhealthy physiological abnormalities, such as

increased circulating lipids, high blood pressure, and inflammation, that may enhance the

risk for endothelial dysfunction, atherosclerosis, and cardiovascular disease (Van Gaal et

al., 2006).

Dietary contributions to increased adiposity

Caloric intake and dietary composition influence the structure and physiological

function of both WAT and BAT adiposity in early life. Over-feeding in early life is

associated with increased fat, WAT inflammation, and lifelong metabolic dysfunction.

For example, one study examined young mice fed a regular chow or high fat diet

(HFD=60% fat) in either small- (SL=3 mouse pups, over-fed) or normal- (NL=7 mouse

pups, not over-fed) sized litters from 5-17 weeks of age. Regular chow-fed SL mice

showed increased fat mass, but did not develop WAT inflammation. SL and NL mice fed

a HFD demonstrated similar increases in visceral adipose tissue and adipocyte

hypertrophy, but SL mice had greater inguinal adipose tissue mass compared with NL

mice. Additionally, compared to NL mice fed a HFD, SL mice fed a HFD demonstrated

greater M1 macrophages and pro-inflammatory cytokine gene expression in WAT as well

as impaired glucose homeostasis and liver function (Kayser, Goran, & Bouret, 2015). A

similar study with rat pups also demonstrated weight gain with hypercaloric

consumption. Overnourished rat pups had reduced levels of BAT UCP-1 and therefore,

reduced thermogenic capacity which may have predisposed the rat pups to become obese as they grow (Bjorndal et al., 2011).

As mentioned above, post-prandial insulin plays a central role in regulating adipose tissue physiology. A mixed meal including carbohydrate (glucose), fat, and proteins results in the storage of glucose and FAs due to increased plasma insulin concentrations (Boron, 2012). Insulin prevents futile substrate cycling as it inhibits substrate catabolism (glycogenolysis, proteolysis, and lipolysis) and promotes storage of the dietary substrates. When insulin levels are released at lower levels it only prevents the catabolism of stored substrates, whereas greater insulin levels promote the accumulation of substrate storage. Therefore, consuming a small mixed meal preserves energy stores, whereas a larger mixed meal may increase those stores. The intake of too many calories from a mixed meal may lead to increased adiposity as insulin promotes the storage of excess dietary fat and carbohydrate as TGs in adipose tissue (Boron, 2012).

Physical activity contributions to increased adiposity

Careers over the past 50 years have increasingly required employees to engage in habitual sedentary behaviors while at work, due to the progression of technology (Church et al., 2011). Occupation-related energy expenditure was assessed in adults residing in the United States and results demonstrated a decline by over 100 calories per day in the past 50 years (Church et al., 2011). Reduced occupation-related energy expenditure is suggested to contribute to the escalating rise in overweight and obesity levels (Church et al., 2011). Also, participation in physical activity during leisure/non-occupation time is dropping due to increased time spent in sedentary leisurely activities (i.e., 'screen time'

or time spent in front of a computer or television); this too is associated with greater incidence rates of the metabolic syndrome (Ford, Kohl, Mokdad, & Ajani, 2005).

Adiposity dysregulation of endocrine function and resulting systemic inflammation

The enlargement of adipocytes and adipose tissue inflammation are interdependent processes that contribute to the development of obesity-associated metabolic diseases. In obese states, adipocytes reach maximal expanding capacity, which can vary 10-15 fold (Smith, 2007), inhibiting further storage of new lipids. The dysfunctional hypertrophic adipocytes also demonstrate increased lipolysis rates (Engfeldt & Arner, 1988). Both the inhibition of storing new dietary lipids and increased lipolysis of the enlarged adipocyte lead to an accumulation of FFAs in the plasma which are eventually absorbed into the liver to form TGs (Engfeldt & Arner, 1988). Elevated TG levels in the liver lead to a condition called dyslipidemia described as increased small low density lipoproteins (LDLs), TGs, and FFAs in the plasma with reduced levels of health-promoting high-density lipoproteins (HDLs) (Engfeldt & Arner, 1988). Moreover, ectopic fat accumulation in the liver can also lead to a condition termed non-alcoholic fatty liver disease which, over time, can lead to appreciable liver damage through an increase in fibrosis and inflammation (Ong & Younossi, 2007). While this is a very serious form of liver disease that is driven by lifestyle habits, more in depth descriptions of these diseases are beyond the realm of this manuscript.

Obesity is described as a pro-inflammatory state with abnormal secretion of specific hormones and pro-inflammatory makers. Greater adiposity recruits the infiltration of macrophages and T-lymphocytes which, in turn, stimulate inflammation by

reducing the expression of anti-inflammatory adipokines (i.e, cytokines secreted by adipose tissue) and increasing the expression of pro-inflammatory adipokines (Smith, 2007). Over time, an increased adiposity can lead to an appreciable secretion of pro-inflammatory cytokines into the circulation (Vendrell et al., 2004) which can then act on other target tissues to disrupt normal physiological processes. Inflammatory cells reside more in visceral adipose tissue than subcutaneous adipose tissue, linking the observation of increased inflammatory markers with abdominal obesity (Ibrahim, 2010). Moreover, visceral adipocytes are more insulin resistant than subcutaneous adipocytes lending to the notion that smaller adipocytes are more insulin-sensitive and larger adipocytes are more resistant (Salans, Cushman, & Weismann, 1973). This point is conceptually-important given that once adipocytes reach a 'ceiling' with regards to their size, then food substrate is forced to be stored in smaller adipocytes until they reach this critical growth ceiling as well. Once all of the present adipocytes have reached a critical growth ceiling, new adipocytes must be formed in order to continue storing un-metabolized food substrate via *de novo* adipogenesis. Some critical, but not all, pro-inflammatory cytokines and adipokines related to increased adiposity include interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), IL-8, and monocyte chemoattractant protein-1 (MCP-1). Chemokines IL-8 and MCP-1 concentrations are elevated by enhanced adiposity, especially in visceral adipose tissues. (Smith, 2007) These chemokines signal macrophages to infiltrate adipose tissue which ultimately signals pro-inflammatory cytokines, such as IL-6 and TNF-α, to increase inflammation (Smith, 2007).

　　　　While IL-6 is expressed in numerous tissues, adipocyte-borne IL-6 is elevated in obese states. Considered a pro-atherogenic and pro-diabetic cytokine, IL-6 increases

vascular inflammation and reduces insulin signaling, respectively (Ibrahim, 2010). C reactive protein (CRP), an inflammatory protein produced by the liver that also promotes vascular inflammation and diabetes, is regulated by IL-6 levels (Ibrahim, 2010). Interestingly, one study examined the interstitial fluid levels of IL-6 in abdominal and femoral adipose tissues as well as systemic levels of IL-6 in the circulation of twenty healthy male human subjects (Sopasakis et al., 2004). Results showed interstitial fluid from the adipose tissue depots contained levels of IL-6 that were 100-fold greater than in the plasma. Furthermore, when IL-6 was added to human adipose tissue *in vitro*, expression of adipocyte differentiation markers, including adiponectin, adipocyte protein 2 (aP2), and PPARɣ-1, decreased (Sopasakis et al., 2004). Adipocyte size, *in vitro* and *in vivo*, correlated positively with IL-6 production (Gustafson & Smith, 2006; Sopasakis et al., 2004). Thus, enlarged adipocytes produce greater levels of IL-6 which promote impaired differentiation of pre-adipocytes, insulin resistance, and inflammation (Gustafson & Smith, 2006).

Similar to IL-6, elevated levels of TNF-α also increase vascular inflammation and insulin resistance during obesity (Ibrahim, 2010). Enlarged adipocytes are highly associated with increased levels of TNF-α when measured locally (Gustafson & Smith, 2006). TNF-α added to mouse preadipocytes prevented preadipocyte differentiation into mature adipocytes as well as lipid accumulation in adipocytes (Gustafson & Smith, 2006). Instead of allowing new adipocytes to form, TNF-α induced an inflammatory phenotype in the existing mature adipocytes which leads to insulin resistance and systemic inflammation (Gustafson & Smith, 2006; Smith, 2007).

Other hormones associated with adiposity are leptin, adiponectin, insulin, ghrelin, insulin growth factor-1 (IGF-1), and parathyroid hormone (iPTH) (Dalskov et al., 2015). Leptin functions as a feedback mechanism to regulate energy stores (Ibrahim, 2010). Obesity, primarily from elevated levels of TGs in subcutaneous adipose tissue, induces elevated leptin secretion (Tritos & Mantzoros, 1997). Leptin serves as a predicative marker for risk of cardiovascular and metabolic diseases (Ibrahim, 2010). Adiponectin is protective as it has anti-atherogenic and anti-diabetic properties (Ibrahim, 2010). Specifically, adiponectin induces the expression of anti-inflammatory cytokines such as IL-10, and improves insulin sensitivity and fatty acid oxidation (Ibrahim, 2010). Expression of adiponectin is greater in visceral adipose tissue than subcutaneous and is negatively associated with body weight (Freedland, 2004). The effects of hormones on adiposity may vary depending on age, sex, and environment. For instance, a study that included 8-11 years olds examined fasting levels of ghrelin, adiponectin, leptin, insulin, IGF-1, osteocalcin, and iPTH in association with body composition at baseline and then again 3 and 6 months later. Leptin and IGF-1 were positively associated with fat mass in girls and boys at baseline, and iPTH was for boys only. Fat free mass and adiponectin were inversely correlated in boys whereas a positive correlation for fat free mass and IGF-1 existed for girls. Over time (3-6 months) an inverse correlation for leptin and changes in fat and fat free mass occurred in girls (Dalskov et al., 2015).

The process of ketone production by the liver

When glycolysis slows down from depleted carbohydrate sources, or if the PDH complex is inhibited by glucagon, pyruvate levels are reduced and the cell cannot

produce oxoaloacetate, a citric acid cycle intermediate. Oxoaloacetate is the first step of the citric acid cycle that works with Acetyl CoA. When there is excessive Acetyl CoA from β-oxidation and not enough oxoaloacetate to carry it through the TCA cycle, acetyl CoA can be converted into ketone bodies instead to be used as energy in the muscles, intestines, and brain. Since the liver has the requisite enzymes required for ketogenesis, (Brody, 1999) ketone synthesis predominantly occurs in the mitochondria of the liver (Figure 2).

INSERT FIGURE 2 HERE

β-hydroxybutyrate (β-HB) and acetoacetate (AcAc) are the main ketone fuel sources and can be metabolized in the mitochondria of non-hepatic tissues. The liver cannot metabolize them because the liver does not possess the required enzyme succinyl CoA transferase. Synthesized ketone bodies are rapidly exported from the liver and imported into tissue such as the brain, skeletal muscle, and heart, for metabolism (Figure 3) via monocarboxylate transporters (MCTs) (Halestrap & Meredith, 2004).

INSERT FIGURE 3 HERE

Metabolism of ketone bodies down-regulates ketone synthesis via succinyl CoA transferase. This enzyme inhibits HMG CoA synthase, but only for short periods. Reactivation and deactivation frequently occur. Two acetyl CoAs are produced by ketone metabolism in the mitochondria and ready to be converted into energy via the

citric acid cycle. Ketone bodies produce less energy than fatty acids but they are water soluble unlike fatty acids, and therefore easily transported and more readily available.

Similarities between ketogenic dieting and fasting to facilitate the loss of adipose tissue mass: involved mechanisms

Starvation/fasting and the production of ketone bodies

Glycogen stores in the liver can provide glucose to the brain during the first day of fasting. Gluconeogenesis generates glucose via amino acids from muscle tissues and glycerol from adipose tissues to supply sufficient glucose to the brain once hepatic glycogen stores have depleted (Cahill, 1998). Ketone bodies and precursors, β-HB and AcAc, are also produced in the liver and can supply 60% of the brain's energy requirements, which helps reduce gluconeogenic processes and preserve muscle protein stores (outlined in Figure 4 below) (Cahill, 1998). Interestingly, ketogenesis is thought to be a metabolic adaption that is protective and cost-effective to support survival during a time of famine (Cahill, 1998).

INSERT FIGURE 4 HERE

The replacement of glucose with ketone bodies in the brain during prolonged fasting has been demonstrated in both rodents and humans. A classical rodent study fasted diabetic female rats for 48 hours and showed that increases in blood ketone levels were paralleled in cerebrospinal fluid samples (Ruderman, Ross, Berger, & Goodman, 1974). The authors further determined that the brain metabolized ketones for fuel thereby

reducing brain glucose consumption (Ruderman et al., 1974). A similar and well-cited study in humans determined that, in 3 obese human subjects undergoing prolonged starvation for 5-6 weeks (diets consisted of one multivitamin capsule per day and 1,500 ml of water), presented an increased replacement of brain glucose utilization with β-HB and AcAc as determined with carotid artery-jugular vein arteriovenous catheterization (Owen et al., 1967); of note, these 3 subjects lost ~23-25 kg over this starvation period. A longer starvation study examined patterns of fuel utilization in 5 obese humans fasted for 21 days (Owen, Smalley, D'Alessio, Mozzoli, & Dawson, 1998). The subjects lost both fat and lean mass with oxidation of skeletal muscle protein accounting for 7% of the fuel used for energy needs with the other 93% supplied by fat stores. The brain derived primarily from fat stores (through conversion into ketone bodies) and the study suggests that a normal weight human could survive starvation for 2-2.5 months due to stored fat being the primary energy fuel (Owen et al., 1998). Another human starvation study examined patterns of fuel consumption and production in forearm skeletal muscle in 8 obese men after fasting 1 night, 3 days, and 24 days. After 1 night, AcAc and β-HB consumption by the muscle was low. After 3 days, arterial AcAc and β-HB levels were significantly elevated as was consumption of those substrates. After 24 days, AcAc and β-HB levels were further elevated in arterial blood but the consumption of ketones by the skeletal muscle was not elevated from the amount consumed on day 3. Instead, FFA were the primary fuel source consumed by the forearm muscle after 24 days of fasting, with a decrease in utilization of both glucose and ketone bodies. Also, this study demonstrated that AcAc was preferentially utilized in the muscle than β-HB (Owen & Reichard, 1971). Collectively, these studies demonstrate very important concepts including:

a) During prolonged fasting and/or glucose deprivation the brain does not 'need' glucose and neurons can use alternative fuel sources (such as ketones) to function,

b) In non-overt diabetic states, nutritional ketosis elevates ketone levels modestly and this does not lead to ketoacidosis which, instead, is an elevation in β-HB, AcAc, and over 34 other organic acids that leads to a decrease blood pH and brain and kidney complications (Rosival, 2015). Of note, blood ketone concentrations in individuals regularly consuming mixed meals are ~0.1 mmoL, during nutritional ketosis blood ketone concentrations are 1.0-8.0 mmoL, and during diabetic ketoacidosis blood ketone concentrations are > 20 mmoL (http://www.ketogenic-diet-resource.com/ketoacidosis.html).

c) During prolonged fasting or starvation, while some energy to support whole-body metabolism comes from skeletal muscle catabolism and gluconeogenesis via muscle-derived amino acids, the derivation of ketones from fat stores are a large source of energy as is adipocyte-derived FFAs.

Starvation also results in the upregulation of glucagon as well as catecholamines. Glucagon is secreted by the pancreas when glucose levels are low to regulate fuel metabolism via glycogenolysis, gluconeogenesis, and ketogenesis in the liver and lipolysis in the adipose tissue. Also, less insulin is also secreted by the pancreas with low glucose levels thereby leading to a greater potential for adipose tissue fat mobilization. Glucagon promotes the oxidation of fatty acids via ACC inhibition. ACC produces malonyl CoA which synthesizes fatty acids and inhibits CAT-1. Inhibition of CAT-1 prevents fatty acids from entering the mitochondria for oxidation (Boron, 2012). Therefore, glucagon releases this inhibition of CAT-1 indirectly by inhibiting ACC. The fatty acids are then oxidized to produces ATP for the liver. Once the liver has sufficient

ATP, the fatty acids will enter β-oxidation in the mitochondria but stop further oxidation at that point. Typically acetyl CoA resulting from β-oxidation or glycolysis will enter the citric acid cycle to be converted into ATP (with the help of the ETC).

Ketogenic dieting and the production of ketone bodies

The goal of the ketogenic diet is to fuel the body via fatty acids from dietary fats and lipolysis of adipose tissue. Insulin must be consistently low to allow for an increased fat mobilization adaptation which decreases metabolic fuel reliance on exogenous glucose sources (Fukao et al., 2004). Also, the carnitine shuttle enzyme, CAT-1, is important in a ketogenic diet to allow the oxidation of fatty acids via β-oxidation. Malonyl CoA, the beginning substrate for fatty acid synthesis, inhibits CAT-1 through ACC activation (ACC produces malonyl CoA). ATP depletion via metabolic stress, such as a ketogenic diet, increases AMPK levels which phosphorylates and inhibits ACC, thus releasing the suppression of CAT-1. ACC-/- mice demonstrated greater fatty acid oxidation rates, circulating ketone body levels, and less fat than wild type mice (Abu-Elheiga, Matzuk, Abo-Hashema, & Wakil, 2001).

Interestingly, medium-chain TGs do not require CAT-1 transport into the mitochondria and consequently are favored in a ketogenic diet as they provide quick supplies of energy (Fukao et al., 2004). Ketone bodies produced in the liver from an over-abundance of acetyl CoA from fatty acid oxidation then help supply energy needs, along with fatty acids (Boron, 2012). This allows blood glucose levels and brain glucose requirements to be maintained, even with lower levels of glycogen stores. The reduced

required glucose concentrations are maintained by endogenous gluconeogenic processes (Fukao et al., 2004).

Ketogenic dieting to facilitate the loss of adipose tissue mass: empirical evidence

While several different versions of ketogenic dieting exist, KDs are largely comprised of: a) meats (red meat, poultry, fish, pork, etc.), dairy products (cheeses, milk, butter, whipping cream, etc.), nuts (almonds, peanuts, cashews, etc.), eggs, and vegetable oils (coconut oil, olive oil, etc.). The intake of grains and starch-laden carbohydrate sources is minimal, as carbohydrate sources are usually in the form of very low-glycemic cruciferous vegetables (spinach, broccoli, kale, lettuce, etc.). Hence, a typical KD diet will contain approximately 20-25% of Calories from protein, 60-65% of Calories from fat, and 10-15% Calories from carbohydrates.

Many studies have examined the effects of lower carbohydrate, higher fat diets on body composition, inflammation, and hormone levels. Gardner et al. (Gardner et al., 2007) published a highly-cited human clinical study examining the weight loss effects of a KD over a 12-month period. Specifically, the investigators examined the effects of a hypocaloric ketogenic 'Atkins diet' on body composition and serum health markers compared to other hypocaloric diets (i.e.,s the Ornish, Zone, and LEARN diets) in 311 overweight/obese (BMI = 27-40) premenopausal women. These authors reported that average total body loss was greatest in the Atkins group at -4.7 kg, whereas total body losses in the Zone, LEARN and Ornish groups were -1.6 kg, -2.6 kg, and -2.2 kg (-3.6 to -0.8 kg). Moreover, these authors reported that, compared to the other diets, the Atkins subjects presented more favorable alterations in body fat percentage reductions, serum

triglyceride (TG) reductions, systolic blood pressure reductions, and HDL cholesterol

increases. Interestingly, while all groups consumed ~1,400-1,650 kcal/d on average

throughout the duration of the study, the Atkins subjects self-reported that they were only

in a ketogenic dieting 'zone' with regards to their macronutrient intake percentages

within the first 2 months of the study (on average intakes were as follows: carbohydrates

were 17.7%, protein was 27.7%, and fat was 54.7%). Moreover, while the Atkins group

maintained a hypocaloric diet throughout the 12 month intervention, the Atkins group

began re-gaining the weight that they had lost as they were introducing more

carbohydrates back into their diets by 6-12 months of the study. Yancy and colleagues

compared a low-carbohydrate ketogenic diet ("KD" : <20grams carbohydrate/day; no

caloric restrictions) and a low-fat/low-cholesterol/reduced caloric diet ("LF" : <30% total

fat, <10% saturated fat) in 120 overweight, hyperlipidemic subjects for 6 months. Both

groups lost fat mass with the KD group losing significantly more than LF group (-9.4 kg

vs -4.8 kg, respectively). Also, the KD and low fat groups lost similar amounts of less

lean mass (-3.3 kg vs -2.4 kg, repectively), with the amounts being less than lost fat mass.

Although both groups demonstrated similar LDL levels, the KD group demonstrated

significantly reduced TG levels and greater HDL levels than the LF group (Yancy et al.,

2004). To determine if a carbohydrate-restricted diet promotes a loss in fat mass with

preservation of lean mass, as well as the possible hormones involved, 12 healthy normal

weight subjects switched from a normal diet (47% calories from carbohydrates/day) to a

carbohydrate restricted diet ("CR": 8% calories from carbohydrates/day) for 6 weeks,

while 8 subjects maintained the normal diet (Volek et al., 2002). After 6 weeks of a CR

diet, fat mass significantly decreased (-3.4 kg), lean mass significantly increased (+1.1

kg), serum insulin significantly decreased (-34%), and total thyroxine (T4) increased

(+11%). Other hormones including glucagon, testosterone, IGF-1, cortisol, and T3 did

not changed with a CR diet. No changes were found for the men that maintained a

normal diet. Volek et al (Volek et al., 2009) also reported that a carbohydrate-restricted

diet facilitates fat mass losses in humans. The study included 40 subjects with

atherogenic dyslipidemia that consumed hypocaloric diets (1500 calories/day) that were

either carbohydrate restricted ("CR" : 12% calories from carbohydrate, 59% calories

from fat, and 28% calories from protein) or low fat ("LF" : 56% calories from

carbohydrate, 24% calories from fat, and 20% calories from protein) for 12 weeks. The

CR group demonstrated significantly greater weight loss (-10.1 kg vs -5.2 kg,

respectively) with decreased fat mass than the LF group (-5.7kg vs -3.7 kg, respectively).

Also, the CR group had decreased TG (-51% vs -19%, respectively) and total TG/HDL (-

53% vs -20%, respectively) levels with increased HDL levels (+10% vs +3%,

respectively) compared to the LF group. In another study by Volek et al. (Volek et al.,

2004), blood lipids and markers of insulin resistance were compared between 13

overweight human subjects that consumed either a hypocaloric (500 kcal/day) very low-

carbohydrate diet ("LC" : <10% calories from carbohydrate) or a hypocaloric (500

kcal/day) low fat diet ("LF" : <30% fat) for 4 weeks. The LC group demonstrated

significantly increased total cholesterol (+1.0% vs -7.0%, respectively), LDL (+5% vs -

5%, respectively), and HDL (+1.0% vs -8.0%, respectively) levels and significantly

reduced glucose (-3.0% vs +2%, respectively), insulin (-9.0% vs +19%, respectively),

and insulin resistance (-14% vs +21%, respectively) compared to the LF group. Another

study that examined diet effects on insulin sensitivity in humans included 10 obese, type

2 diabetics that switched from a normal diet to consume a low carbohydrate diet (<32g carbohydrate/day) for 14 days. Compared to baseline, subjects consumed less calories/day which contributed to the significant decrease in weight loss. Also, after 14 days of a low carbohydrate diet, subjects demonstrated improved insulin sensitivity by 75% with decreases in TG and cholesterol levels (Boden et al., 2005). A study in rodents that examined the effects of a low carbohydrate/high fat diet on insulin sensitivity fed adult rats either isocaloric or hypocaloric diets of standard chow, or a low carbohydrate/high fat ("LCHF") diets for 4 weeks. The LCHF groups demonstrated acute glucose intolerance and insulin resistance. Also, LCHF group demonstrated significantly greater fat mass (177.4g vs 172.3g, respectively), less lean mass (213.0g vs 224.5g, respectively), and reduce HDL (57.6mg/dl vs 60.0 mg/dl, respectively) with increased TG (135.8 mg/dl vs 113.5 mg/dl, respectively) levels compared to the standard chow group (Bielohuby et al., 2013).

As noted by the previous studies mentioned, high fat/low carbohydrate diets have reported controversial results regarding insulin sensitivity (Bielohuby et al., 2013; Boden et al., 2005; Volek et al., 2004). To determine if the dose of dietary fat consumed in relation to carbohydrate differentially affects insulin sensitivity, one study compared a low-fat/high-carbohydrate diet ("LFHC" : 0%fat, 85% calories from carbohydrate, 15% calories from protein), an intermediate-fat/intermediate-carbohydrate diet ("IFIC" : 41% calories from fat, 44% calories from carbohydrate, 15% calories from protein), and a high-fat/low carbohydrate diet ("HFLC" : 83% calories from fat, 2% calories from carbohydrate, 15% calories from protein) in human subjects. Subjects consumed each diet for 11 days, with 8-10 weeks of their pre-intervention diet between each

experimental diet. While the diets did not show significant differences in insulin

sensitivity, the HFLC diet was glycogen-sparing as the subjects adapted to utilizing FFAs

for energy instead of carbohydrate sources (Bisschop et al., 2001).

Ketogenic dieting to facilitate the a lessened pro-inflammatory adipose tissue phenotype

Studies indicating the effects of a ketogenic dieting on adipose tissue

inflammation are limited. Aguilar et al. recently determined that that, in guinea pigs fed a

high cholesterol diet for 10 weeks, 6 weeks KD post-feeding (versus 6 weeks of high

carbohydrate feeding) period actually increased adipocyte size by 34% while non-

significantly reducing adipose tissue mass by 15% and significantly reducing the

infiltration of macrophages by 57% in the epididymal fat pad. Asrih et al. (Asrih et al.,

2015) examined the effects of a very high fat-containing KD (93% calories from fat, 2%

calories from carbohydrate, and 5% calories from protein) versus a standard chow diet

("SC": 12% calories from fat, 62% calories from carbohydrate, and 27% calories from

protein) on adipose tissue inflammation markers in 8-week old mice over a 4-week

period. Compared to the SC group, the KD group demonstrated significantly greater

energy expenditure, WAT UCP1 expression, and FGF21 levels in the plasma. Although

the KD group demonstrated greater inflammation as well as macrophage and lipid

accumulation in the liver compared to the LF group, the KD group had less inflammation

in WAT. Lipoprotein lipase (LPL) and CD36, which are associated with reduced WAT

inflammation, were also down-regulated in the KD group. Schugar and Crawford

authored a review also citing other evidence in mice demonstrating that, while KDs elicit

weight loss, liver damage and the development of NAFLD occurred (Schugar &

Crawford, 2012). However, these authors point out that many of the KD diets studied

have been very low protein diets (~5% protein) which, in the context of growing rodents,

can retard liver growth and metabolism.

Thus, these data collectively have very important implications for those looking to

engage in the KD as a form of lifestyle modification; specifically, ketogenic dieting

without exercise may be advantageous for adipose tissue inflammation while being

deleterious for the liver (at least in mice that are fed very low protein KD diets).

However, if exercise is present with concomitant ketogenic dieting, then it may be

possible that the body can oxidize ingested fat substrates and minimize liver damage.

Combined effects of a ketogenic diet and physical activity on adiposity

Studies demonstrating the synergistic effects of KDs and exercise on body

composition are very limited. In fact, only one study to my knowledge has determined

how ketogenic dieting with exercise affects body composition. In this study, 16

untrained overweight women consumed either a very low carbohydrate ketogenic diet

("KD": 6% calories from carbohydrate, 66% calories from fat, and 22% calories from

protein) or a regular diet ("RegD": 41% calories from carbohydrate, 34% calories from

fat, and 17% calories from protein) for 10 weeks. The subjects performed 60-100

minutes of various resistance exercise 2 times/week while on the designated diet. The

KD group lost fat mass (-5.6%) without experiencing alterations in lean mass, whereas

the RegD group gained lean body mass (+1.6 kg) but did not lose fat mass. Neither

group showed a change in blood lipid or glucose levels (Jabekk et al., 2010). However,

it should be noted that the effects of the KD on adipogenic and/or lipogenic mechanisms remains unexplored.

Purpose of this study

While there is ample evidence to suggest the KD as well as physical activity reduces whole-body adiposity, studies examining the potential synergy that a ketogenic diet and physical activity may have on molecular markers of adipose tissue physiology are limited. To this end, the purpose of this study was two-fold:

1) To investigate how a KD (versus a Western diet and standard chow diet) affected select adipogenic, lipogenic, thermogenic, and/or inflammation-related markers in three different adipose tissue depots [inguinal-subcutaneous WAT (termed SQ throughout), omental-viseral WAT (termed OMAT throughout), and intrascapular BAT (termed BAT throughout)] and the liver using a rat model. Markers of adipogenesis included relative fat depot weights, adipocyte cell diameter counting, C/EBPa protein expression, and SREBP1 protein expression. Markers of lipogenesis included adipocyte counts of cells with diameters of >100 μM that are putatively pre-existing adipocytes that have reached a 'growth ceiling' (Jackman et al., 2008), ACC protein expression, FASN protein expression, and CD36 protein expression. Markers of inflammation included phosphorylation levels of p65 as well the mRNA expression patterns of TNF-α, and IL-6. Markers of adipose tissue 'briting' included the histological examination of UCP-1 protein levels in all of the assayed adipose tissue depots as well as the mRNA expression patterns of UCP-1, CIDEA, and adiponectin. Finally, markers of liver health

included phosphorylation levels of p65, the mRNA expression of ChREBP and

SCD-1, the protein expression of ACC and FASN, and triglyceride levels.

2) Secondarily, determination of the influence of daily physical activity, in the form of voluntary resistance wheel running, on the proposed adipose markers for each diet were assessed to examine if the combination of KD dieting with an increase in physical activity had additive effects on the aforementioned markers of adipose tissue and liver physiology.

Of note, the three dietary interventions included a higher-protein KD (5.2 kcal/g, 20.2% protein, 10.3% carbohydrate, 69.5% fat), Western diet (WD; 4.5 kcal/g, 15.2% protein, 42.7% carbohydrate, 42.0% fat; most of the carbohydrate being sucrose), and standard chow diet (SC; 3.1 kcal/g, 24.0% protein, 58.0% carbohydrate; most of the carbohydrate coming from wheat and corn).

I hypothesized that the KD would lead to attenuated weight gain as well as less relative fat mass accumulations compared to the WD and SC diets. Additionally, I hypothesized that the animals consuming the KD would demonstrate reduced adipogenic, lipogenic, and inflammatory markers in adipose tissue and liver as well as increased adipose tissue 'briting' markers compared to the animals consuming the WD and SC diets. Finally, I hypothesized that the aforementioned dietary intervention would be further enhanced by physical activity.

CHAPTER III

METHODS

Animal Husbandry

Forty-eight male Sprague-Dawley rats ~9-10 weeks of age (~300-325 g) were

purchased (Harlan Laboratories, Indianapolis, IN, USA) and allowed to acclimate in the

animal housing facility for 1 week prior to experimentation. Animals were provided

water and fed a standard rodent chow (24% protein, 58% carbohydrate, 18% fat; Teklad

Global #2018 Diet, Harlan Laboratories) *ad libitum* in a maintained ambient temperature

and constant 12h light: 12 h dark cycle. All experimental procedures were approved by

Auburn University's Institutional Animal Care and Use Committee.

Dietary and Physical Activity Experimental Protocol

For a 6-week period after acclimation, animals were provided isocaloric amounts with

one of three diets:

1) 16 animals were provided 17 g/day of a ketogenic diet (KD) (5.2 kcal/g, 20.2%

    protein, 10.3% carbohydrate, 69.5% fat)

2) 16 animals were provided 20 g/day of a Western diet (WD) (4.5 kcal/g, 15.2%

    protein, 42.7% carbohydrate, 42.0% fat); of note, 34% of the carbohydrates come

    from sucrose which makes this a high-fat/high-glycemic chow

3) 16 animals were fed 30g/day of the healthy control standard chow diet (SC) (3.1

   kcal/g, 24.0% protein, 58.0% carbohydrate, 18% fat); of note, most of the

   carbohydrates come from wheat and corn.

Eight animals from each dietary group were doubly-housed without a running wheel and considered sedentary (SED). The other eight animals from each dietary group were individually-housed with a resistance loaded voluntary running wheel (Lafayette Instrument Company, Lafayette, IN, USA) and considered to be more physically active (EX). Daily unconsumed food weights were recorded daily and body weights were recorded every 3 days starting at baseline. The EX group performed voluntary free-wheel running for the first 2 weeks to allow the KD group to adapt to the diet. Resistance equaling 20% body weight (g) was applied to the wheel the third week, 40% body weight the fourth week, 60% body weight the fifth week, and 40% body weight the 6th week (Figure 5a). Resistance was calculated from updated weekly body weights and according to the dietary group body weight mean. Daily running distances (meters) was recorded for the EX group. After 6 weeks, the running wheels were locked 24 hours before the EX animals were sacrificed. Animals were food deprived but provided water *ad libitum* on the day of euthanasia. Animals were euthanized under CO_2 gas in a 2 L induction chamber (VetEquip, Inc., Pleasanton, CA, USA).

Necropsy

Following euthanasia, a final body mass was recorded and blood was collected from the heart using a 22-gauge syringe. The SQ, OMAT and BAT depots were dissected out and weights were recorded. The liver was also removed for analysis.

Collected blood was placed in a 6 ml serum separator tube, and centrifuged at 3,500 x g

for 10 min to separate out serum from red blood cells, white blood cells and platelets.

Serum was then aliquoted in 1.7 ml microcentrifuge tubes and stored at -80ºC until

analyte analysis. Whole blood was collected in K-EDTA tubes and refrigerated, and at

the end of each day, transported to the College of Veterinary medicine for white blood

cell analysis as described below.

Tissue preparation for biochemical assays

Approximately 1-2 cm sections were obtained from the SQ, OMAT and BAT

depots and placed in 10% formalin and preserved for histological analyses (described in

greater detail below). Another ~100 mg of tissue from the three adipose depots and the

liver was placed in 1x cell lysis buffer (Cell Signaling, Danvers MA, USA) and was

homogenized immediately upon extraction to yield adipose and liver tissue homogenates

for Western blotting (described in greater detail below). Another ~50 mg of tissue from

the three adipose depots and the liver was placed in Ribozol (Ameresco, Pelham, AL,

USA) and homogenized immediately upon extraction to yield adipose tissue

homogenates for RNA extraction for mRNA expression analysis (described in greater

detail below). Finally, the remainder from each adipose depot and the liver was wrapped

in foil and immediately frozen in liquid nitrogen and stored at -80^0C for any other

subsequent biochemical analyses.

Histology

Adipose tissue depots fixed in formalin were removed and washed in cold running tap water. The segments were placed into embedding cassettes and immediately stored in 70% alcohol for processing. The tissue processing procedure included the following steps: dehydration, clearing, and infiltration. Dehydration was accomplished by gradually increasing percentages of ethyl alcohol to replace the water content in the tissue. Hemo-De was subsequently used to clear the tissue from the ethyl alcohol to allow infiltration with paraffin. The paraffin tissue blocks were sectioned into 6 μm slices and then placed onto microscope glass slides. After the tissue on the microscope slide dries, the paraffin was removed with xylene and the mounted sections were stained with hematoxylin and eosin in order to assess adipocyte size.

Immunohistochemistry

Immunohistochemistry for adipose tissue UCP-1 protein expression (i.e., a 'briting' marker) was performed as previously described elsewhere (Oliveira et al., 2013). Briefly, additional adipose tissue sections from each adipose tissue depot previously fixed in formalin and embedded in paraffin was deparaffinized and rehydrated by boiling in a 6.0 pH solution of 10 mM sodium citrate with 0.05% Tween-20 for 30 minutes. Tissues were then incubated for 10 minutes in 3% hydrogen peroxide to inhibit endogenous HRP activity. Tissue sections were blocked in normal serum and then incubated for at least 1 hour with a primary antibody. The primary antibody included anti-uncoupling protein 1 (anti-UCP-1, #ab10983, Abcam, Cambridge, MA, USA) and a LSAB + kit (Dako Cytomation, Carpinteria, CA, USA) was used for the secondary antibody staining with

rabbit polyclonal IgG (#ab171870, Abcam) and detection occurred from the use of 3,3'-diaminobenzidine. Tissue sections were then restained with hematoxylin, dehydrated, and enclosed with a coverslip. Microscope images of stained slides were photographed using a 40x objective via a fluorescent microscope (Nikon Eclipse Ti-U) and associated software. Ten images per sample were obtained for quantification purposes. Adipocyte diameters and UCP-1 staining intensities were quantified using imageJ (National Institutes of Health, Bethesda, MD, USA).

Western blotting

Adipose tissue and liver homogenates obtained as described above were prepared for Western blotting using 4x Laemmli buffer at 2 µg/µl. Subsequently, 20 µl of prepped samples were loaded onto pre-casted 12% SDS-polyacrylamide gels (Bio-Rad, Hercules, CA, USA) and subjected to electrophoresis (200 V @ 75 min) using pre-made 1x SDS-PAGE running buffer (C.B.S. Scientific Company, San Diego, CA, USA). Proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad), and following transfer, membranes were stained with Ponceau S stain in order to ensure even loading and transfer between samples. Membranes were then blocked with 5% nonfat milk powder diluted in Tris-buffered saline with 0.1% Tween-20 (TBST) for 1 hour at room temperature. Primary antibodies directed against our proteins of interest were incubated with membranes overnight at 4º C in TBST with 5% bovine serum albumin (BSA) added. The primary antibodies used were: anti-CCAAT/enhancer binding protein α (anti-C/EBPα, #D56F10, Cell Signaling Technology), anti-fatty acid synthase (anti-FASN, #C20G5, Cell Signaling Technology), anti-acetyl CoA carboxylase (anti-ACC, #C83B10,

Cell Signaling Technology), anti-phospho-nuclear factor kappa beta p65 (anti-phospho-NF κβ p65, #93H1, Cell Signaling Technology), anti-nuclear factor kappa beta p65 (anti-NF κβ p65, #D14E12, Cell Signaling Technology), anti-CD36 (#ab78054, Abcam), anti-phospho-hormone-sensitive lipase (anti-p-HSL, #4139, Cell Signaling Technology), anti-hormone sensitive lipase (anti-HSL, #4107, Cell Signaling Technology), anti-phospho-AMP-activated protein kinase alpha (anti-p-AMPKα, #40H9, Cell Signaling Technology), and anti-AMP-activated protein kinase alpha (anti-AMPKα, #D5A2, Cell Signaling Technology). The membranes on the following day were incubated with anti-rabbit or anti-mouse IgG secondary antibodies diluted in TBST with 5% BSA added (1:1,000, Cell Signaling) at room temperature for 1 hour prior to membrane development. Membrane development was accomplished by using an enhanced chemiluminescent reagent (Amersham, Pittsburgh, PA, USA), and band densitometry was achieved with the use of a digitized gel documentation system and associated densitometry software (UVP, Upland CA, USA).

Real-time PCR

RNA was isolated from the SQ, OMAT and BAT depots as well as the liver using the Ribozol method (Ameresco) according to the manufacturer's instructions. Total RNA concentrations was analyzed using a Nanodrop Lite spectrophotometer (Thermo Scientific) and 2 μg of RNA from each of the adipose tissue depots were reverse transcribed into cDNA for real-time PCR (RT-PCR) analyses using a commercial qScript™ cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD, USA). RT-PCR was performed using gene-specific primers (Table 1) and SYBR-green-based methods in

a RT-PCR thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). Primer designer software (Primer3Plus, Cambridge, MA, USA) was employed to design the primers, and a melt curve analyses was used to show that one PCR product was amplified per reaction. Fold-change values from the SC SED condition was performed using the Livak method (i.e., $2^{-\Delta\Delta CT}$ assuming 100% primer binding efficiency), where $2^{-\Delta CT}$ = [housekeeping gene (HKG) CT – gene of interest CT] and $2^{-\Delta\Delta CT}$ (or fold-change) = [$2^{-\Delta CT}$ value/$2^{-\Delta CT}$ average of SC SED condition].

INSERT TABLE 1 HERE

Liver Triglyceride Content

Liver tissue was homogenized with chloroform/methanol (2/1) to a final volume 20 times the volume of tissue sample (50 mg in 1 mL of homogenizing buffer). After homogenizing, the mixture was agitated on the vortex 1 time every 5 minutes for 20 minutes and then centrifuged at 10,000xg for 10 minutes. The supernatant was removed and placed in a new tube. 200 µL of 0.9% NaCl solution was added to the supernatant, vortexed, and then centrifuged at a low speed (200 rpm) to separate the two phases. The upper phase was removed via siphoning and the lower chloroform phase containing the lipids was evaporated under a vacuum. The resulting dried lipids were reconstituted in 500 µL of tert-butanol:triton X114 mix stock (6 mL t-butanol: 4 mL triton X114) and vortexed. The resulting lipid mixture for each rat sample was used for the triglyceride analysis from a commercially-available kit according to manufacturer's instructions (Cayman Chemical, Ann Arbor, MI).

Ketone Body Assay and Serum Profile

A βHB colorimetric assay kit (Cayman Chemical, Ann Arbor, MI, USA) was used to determine blood ketone levels. A serum chemistry profile was provided by Auburn University's Veterinarian School. Specifically, samples were analyzed by their technical staff using an automated chemistry analyzer (Roche Cobas C311; Roche Diagnostics, Indianapolis, IN, USA) in order to derive serum, glucose, triglyceride, and total cholesterol levels. Finally, ELISAs (EMD Millipore, Billerica, MA, USA) were used to analyze serum insulin levels.

Statistics

All dependent variables were presented as means ± standard error values. Statistical comparisons of all dependent variables were run using two-way ANOVAs [diet (WD vs. KD vs. SC) x activity (SED versus EX)] using SPSS v 22.0 (IBM, Armonk, NY, USA). If a main effect for diet was observed, pairwise comparisons using Tukey *post hoc* analysis was further performed to determine which diets were statistically different. If a main effect for exercise treatment was observed, pairwise comparisons between SED vs EX were performed within each diet. If a significant Diet*treatment interaction was observed: 1) one-way ANOVAs in the SED and EX treatments were performed and a Tukey *post hoc* analysis was further performed to determine which diets were statistically different; and 2) independent t-tests between SED vs EX within each diet was performed. Because KD putatively increase serum ketones and reduce serum insulin, and both of these phenomena have been posited to facilitate adipose tissue adaptations, select bivariate correlations were performed throughout to examine if

associations existed between serum insulin or β-HB levels and various adipose tissue characteristics. For all statistics, significance was set at $p < 0.05$ but $p < 0.01$ and $p < 0.001$ were also noted throughout.

Finally, *a priori* sample-size calculations were performed. These calculations were predicated on my preliminary experiments in SED rats. Body masses were used given that body mass is related to fat mass pads in rats, and I surmised that an increase in fat mass pad size would alter the molecular variables analyzed. Specifically, WD SED rats weighed 506 ± 31 g, KD SED rats weighed 395 ± 30 g, and SC SED rats weighed 472 ± 49 g. Using a non-centrality parameter of 2.8 (alpha level of 0.05 for a two-tailed independent samples t-test, and statistical power of 0.80) dictated that n = 1 rat per condition under these circumstances may yield between-treatment significance for adipose tissue-related variables. This was likely due to the large effect size observed regarding the effects of each diet on body weight (Cohen's d = 3.682). Using a sample size calculator to determine how many KD rats were needed when comparing them to SC rats, using a non-centrality parameter of 2.8 (alpha level of 0.05 for a two-tailed independent samples t-test, and statistical power of 0.80) dictates that n = 4 rats per condition under these circumstances may yield between-treatment significance for adipose tissue-related variables. Again, this was likely due to the large effect size observed regarding the effects of each diet on body weight (Cohen's d = 1.911). Despite the apparently low number of rodents needed to observe potential effects on adipose tissue markers, I decided to study n=8-10 animals per group in order to ensure that there was adequate statistical power.

CHAPTER IV

RESULTS

Physical Activity

A one-way ANOVA indicated that total running distance over the 6-week

intervention was not different between the KD EX, WD EX or SC EX groups (p = 0.70;

Figure 5b).

INSERT PHYSICAL ACTIVITY DATA (FIGURE 5) HERE

Energy and Macronutrient Intake

Total Calories consumed during the 6 week intervention is presented in Figure 6a.

There was a main effect of diet (p < 0.001); specifically WD > SC (p < 0.001) and KD >

SC (p < 0.001), but KD = WD (p = 0.07). There was no activity effect (p = 0.40). There

was a Diet*Activity interaction (p < 0.001); specifically SED WD > SED StdChow (p <

0.001), SED KD > SED SC (p < 0.001), SED WD > EX WD (p < 0.05), SED KD > EX

KD (p < 0.01), and EX SC > SED SC (p < 0.01).

Total protein consumed during the 6 week intervention is presented in Figure 6b.

There was a main effect of diet (p < 0.001); specifically KD> WD (p < 0.001), SC > WD

(p < 0.001), and SC > KD (p < 0.001). There was no activity effect (p = 0.88). There

was a Diet*Activity interaction (p < 0.001); specifically SED KD > SED WD (p <

48

0.001), SED SC > SED WD (p < 0.001), SED WD > EX WD (p < 0.05), SED KD > EX KD (p < 0.01), and EX SC > SED SC (p < 0.01).

Total carbohydrates consumed during the 6 week intervention is presented in Figure 6c. There was a main effect of diet (p < 0.001); specifically WD > KD (p < 0.001), SC > WD (p < 0.001), and SC > KD (p < 0.001). There was no activity effect (p = 0.19). There was a Diet*Activity interaction (p < 0.001); specifically SED KD > SED WD (p < 0.001), SED SC > SED WD (p < 0.001), SED WD > EX WD (p < 0.05), SED KD > EX KD (p < 0.01), and EX SC > SED SC (p < 0.01).

Total fat consumed during the 6 week intervention is presented in Figure 6d. There was a main effect of diet (p < 0.001); specifically KD > WD (p < 0.001), KD > SC (p < 0.001), and WD > SC (p < 0.001). There was a main effect for activity (p < 0.001); specifically SED > EX. There was a Diet*Activity interaction (p < 0.001); specifically SED KD > SED WD (p < 0.001), SED KD > SED SC (p < 0.001), SED WD > SED SC (p < 0.001), EX KD > EX WD (p < 0.001), EX KD > EX SC (p < 0.001), EX WD > EX SC (p < 0.001), SED WD > EX WD (p < 0.05), SED KD > EX KD (p < 0.01), and SC EX > SC SED (p < 0.01).

INSERT MACRONUTRIENT INTAKE DATA (FIGURE 6) HERE

Body Masses

Masses throughout the 6 week study in 3-day intervals is presented in Figure 7a (SED rats only) and 7b (EX rats only). For SED animals, one-way ANOVAs at each

time point indicated that KD rats weighed less than SC and WD rats from days 4-40 ($p <$ 0.0125). For EX animals, one-way ANOVAs at each time point indicated that KD rats weighed less than SC and WD rats from days 15-40 ($p < 0.0125$).

Total Mass at sacrifice is presented in Figure 7c. There was a main effect of diet ($p < 0.001$); specifically WD > KD ($p < 0.001$) and SC > KD ($p < 0.001$), but SC = WD ($p = 0.08$). There was no activity effect ($p = 0.40$) and there was no Diet*Activity interaction ($p = 0.15$).

Feed efficiency (g body mass gain/kcal consumed) is presented in Figure 7d. There was a main effect of diet ($p < 0.001$); specifically WD > KD ($p < 0.001$) and SC > KD ($p < 0.001$), but SC = WD ($p = 0.98$). There was no activity effect ($p = 0.29$) and there was no Diet*Activity interaction ($p = 0.06$).

INSERT BODY MASS DATA (FIGURE 7) HERE

Serum Markers and White Blood Cell Differentials

Serum insulin is presented in Table 2. There was a main effect of diet ($p < 0.001$); specifically WD > KD ($p < 0.01$) and SC > KD ($p < 0.001$), but WD = SC ($p = 0.81$). There was a main effect for activity ($p < 0.001$); specifically SED > EX. There was no Diet*Activity interaction ($p = 0.58$).

Serum glucose is presented in Table 2. There was a main effect of diet ($p < 0.01$); specifically WD > KD ($p < 0.05$) and SC > KD ($p < 0.01$), but WD = SC ($p = 0.80$).

There was no activity effect (p = 0.48) and there was no Diet*Activity interaction (p = 0.06).

Serum βHB is presented in Table 2. There was a main effect of diet (p < 0.01); specifically KD > SC (p < 0.01), but KD = WD (p = 0.22) and WD = SC (p = 0.22). There was a main effect for activity (p < 0.05); specifically SED > EX. There was no Diet*Activity interaction (p = 0.34).

Serum triglycerides is presented in Table 2. There was a main effect of diet (p < 0.001); specifically WD > KD (p < 0.001), SC > KD (p < 0.01), and WD > SC (p < 0.001). There was a main effect of activity (p < 0.001); specifically SED > EX. There was a Diet*Activity interaction (p < 0.001); specifically SED WD > SED KD (p < 0.001), SED WD > SED SC (p < 0.001), SED SC > SED KD (p < 0.001), EX WD > EX KD (p < 0.05), SED WD > EX WD (p < 0.01), and SED SC > EX SC (p < 0.05).

Serum cholesterol is presented in Table 2. There was a main effect of diet (p < 0.001); specifically WD > KD (p < 0.01) and SC > KD (p < 0.001), but WD = SC (p = 0.16). There was a main effect of activity (p < 0.05); specifically SED > EX. There was a Diet*Activity interaction (p < 0.01); specifically SED WD > SED KD (p < 0.01), SED SC > SED KD (p < 0.01), EX SC > EX WD (p < 0.01), EX SC > EX KD (p < 0.01), SED WD > EX WD (p < 0.01), and SED SC > EX SC (p < 0.05).

Serum ALT is presented in Table 2. There was a main effect of diet (p < 0.001); specifically SC > KD (p < 0.001) and SC > WD (p < 0.001), but WD = KD (p = 0.95). There was no activity effect (p = 0.36) and there was no Diet*Activity interaction (p = 0.11).

INSERT SERUM MARKERS (TABLE 2) HERE

White blood cell differentials are presented in Table 3. There was a main effect of diet ($p < 0.05$); specifically WD > KD ($p < 0.05$), but SC = KD ($p = 0.30$) and WD = SC ($p = 0.43$). There was no activity effect ($p = 0.31$). There was a Diet*Activity interaction ($p < 0.05$); specifically SED WD > EX WD ($p < 0.001$), and SED WD > SED KD ($p < 0.01$).

Whole blood lymphocytes is presented in Table 3. There was a no diet effect ($p = 0.79$), there was no activity effect ($p = 0.66$) and there was no Diet*Activity interaction ($p = 0.52$).

Whole blood neutrophils is presented in Table 3. There was a no diet effect ($p = 0.72$), there was no activity effect ($p = 0.38$) and there was no Diet*Activity interaction ($p = 0.56$).

Whole blood monocytes is presented in Table 3. There was a no diet effect ($p = 0.35$), there was no activity effect ($p = 0.78$) and there was no Diet*Activity interaction ($p = 0.93$).

INSERT WHOLE BLOOD MARKER DATA (TABLE 3) HERE

OMAT masses and adipocyte diameters

Absolute OMAT masses are presented in Figure 8a. There was a main effect of diet ($p < 0.001$); specifically WD > KD ($p < 0.001$), WD > SC ($p < 0.05$), but SC = KD ($p = 0.15$). There was a main effect for activity ($p < 0.001$); specifically SED > EX. There was no Diet*Activity interaction ($p = 0.48$).

Relative OMAT masses are presented in Figure 8b. There was a main effect of diet ($p < 0.01$); specifically WD > KD ($p < 0.001$), but WD = SC ($p = 0.08$) and SC = KD ($p = 0.46$). There was a main effect for activity ($p < 0.001$); specifically SED > EX. There was no Diet*Activity interaction ($p = 0.72$).

Average OMAT adipocyte diameters are presented in Figure 8c. There was a main effect of diet ($p = 0.001$); specifically WD = SC ($p = 0.96$), but WD > KD ($p < 0.01$) and SC > KD ($p < 0.01$). There was no activity effect ($p = 0.18$) and there was no Diet*Activity interaction ($p = 0.89$).

OMAT adipocytes sized 0-19.99 microns in diameter are presented in Figure 8d (SED rats only) and 8e (EX rats only). There was a main effect of diet ($p < 0.001$); specifically SC > WD ($p < 0.001$), SC > KD ($p < 0.001$), and KD > WD ($p < 0.05$). There was a main effect of activity ($p < 0.05$); specifically SED > EX. There was a Diet*Activity interaction ($p = 0.001$); specifically, KD EX > KD SED ($p < 0.05$).

OMAT adipocytes sized 20-39.99 microns in diameter are presented in Figure 8d (SED rats only) and 8e (EX rats only). There was a main effect of diet ($p < 0.001$); specifically SC > WD ($p < 0.001$), SC > KD ($p < 0.01$), and KD > WD ($p < 0.001$). There was a main effect of activity ($p < 0.001$); specifically EX > SED. There was a Diet*Activity interaction ($p < 0.001$); specifically, KD EX > KD SED ($p < 0.05$).

OMAT adipocytes sized 40-59.99 microns in diameter are presented in Figure 8d (SED rats only) and 8e (EX rats only). There was a main effect of diet ($p < 0.001$); specifically SC > WD ($p < 0.001$), SC > KD ($p < 0.01$), and KD > WD ($p < 0.001$). There was a main effect of activity ($p < 0.001$); specifically EX > SED. There was a Diet*Activity interaction ($p < 0.001$); specifically, KD EX > KD SED ($p < 0.05$).

OMAT adipocytes sized 60-79.99 microns in diameter are presented in Figure 8d (SED rats only) and 8e (EX rats only). There was a main effect of diet ($p < 0.001$); specifically SC > WD ($p < 0.001$), SC > KD ($p < 0.001$), and KD > WD ($p < 0.05$). There was no activity effect ($p = 0.19$). There was a Diet*Activity interaction ($p < 0.01$); specifically, KD EX > KD SED ($p < 0.05$).

OMAT adipocytes sized 80-99.99 microns in diameter are presented in Figure 8d (SED rats only) and 8e (EX rats only). There was a main effect of diet ($p < 0.001$); specifically SC > WD ($p < 0.001$), and SC > KD ($p < 0.001$), but KD = WD ($p = 0.97$). There was no activity effect ($p = 0.72$) and there was no Diet*Activity interaction ($p = 0.61$).

OMAT adipocytes sized $\geq$ 100 microns in diameter are presented in Figure 8d (SED rats only) and 8e (EX rats only). There was a main effect of diet ($p < 0.001$); specifically SC > WD ($p < 0.001$), and SC > KD ($p = 0.000$), but KD = WD ($p = 0.99$). There was no activity effect ($p = 0.08$) and there was no Diet*Activity interaction ($p = 0.06$).

INSERT OMAT MASSES AND ADIPOCYTE DIAMETER DATA (FIGURE 8) HERE

SQ masses and adipocyte diameters

Absolute SQ masses are presented in Figure 9a. There was a main effect of diet ($p < 0.01$); specifically WD > KD ($p < 0.001$), but WD = SC ($p = 0.061$) and SC = KD ($p = 0.51$). There was no activity effect ($p = 0.10$) and there was no Diet*Activity interaction ($p = 0.74$).

SQ relative mass is presented in Figure 9b. There was no diet effect ($p = 0.071$), there was no activity effect ($p = 0.12$), and there was no Diet*Activity interaction ($p = 0.78$).

Average SQ adipocyte diameter is presented in Figure 9c. There was no diet effect ($p = 0.32$), there was no activity effect ($p = 0.10$) and there was no Diet*Activity interaction ($p = 0.60$).

SQ adipocytes sized 0-19.99 microns in diameter are presented in Figure 9d (SED rats only) and 9e (EX rats only). There was a main effect of diet ($p < 0.01$); specifically KD > WD ($p < 0.01$) and KD > SC ($p < 0.05$), but SC = WD ($p = 0.59$). There was no activity effect ($p = 0.90$) and there was no Diet*Activity interaction ($p = 0.61$).

SQ adipocytes sized 20-39.99 microns in diameter are presented Figure 9d (SED rats only) and 9e (EX rats only). There was a main effect of diet ($p < 0.05$); KD > WD ($p = 0.05$), but KD = SC ($p = 0.74$) and SC = WD ($p = 0.21$). There was no activity effect ($p = 0.61$) and there was no Diet*Activity interaction ($p = 0.82$).

SQ adipocytes sized 40-59.99 microns in diameter are presented in Figure 9d (SED rats only) and 9e (EX rats only). There was no diet effect ($p = 0.52$), there was no activity effect ($p = 0.11$) and there was no Diet*Activity interaction ($p = 0.09$).

SQ adipocytes sized 60-79.99 microns in diameter are presented in Figure 9d (SED rats only) and 9e (EX rats only). There was no diet effect (p = 0.89), there was no activity effect (p = 0.05) and there was no Diet*Activity interaction (p = 0.26).

SQ adipocytes sized 80-99.99 microns in diameter are presented in Figure 9d (SED rats only) and 9e (EX rats only). There was no diet effect (p = 0.50) and there was no activity effect (p = 0.74). There was a Diet*Activity interaction (p < 0.05); specifically EX WD > SED WD (p < 0.05).

SQ adipocytes sized ≥ 100 microns in diameter are presented in Figure 9d (SED rats only) and 9e (EX rats only). There was a main effect of activity (p < 0.01); specifically SED > EX. There was no activity effect (p = 0.27) and there was no Diet*Activity interaction (p = 0.50).

INSERT SQ MASSES AND ADIPOCYTE DIAMETER DATA (FIGURE 9) HERE

BAT masses and adipocyte diameters

Absolute BAT masses are presented in Figure 10a. There was a main effect of diet (p < 0.05); specifically WD > KD (p < 0.05) but SC = KD (p = 0.33) and WD = SC (p = 0.47). There was a main effect of activity (p < 0.01); specifically SED > EX. There was no effect for Diet*Activity (p = 0.94).

Relative BAT mass is presented in Figure 10b. There was a main effect of activity (p < 0.05); specifically SED > EX. There was no diet effect (p = 0.62), and there was no Diet*Activity interaction (p = 0.86).

Average BAT adipocyte diameter is presented in Figure 10c. There was a main effect of diet ($p < 0.01$); specifically SC > WD ($p < 0.01$) and SC > KD ($p < 0.05$) but KD = WD ($p = 0.83$). There no activity effect ($p = 0.12$), and there was no Diet*Activity interaction ($p = 0.35$).

BAT adipocytes sized 0-19.99 microns in diameter are presented in Figure 10d (SED rats only) and 10e (EX rats only). There was a main effect of diet ($p < 0.01$); specifically SC > KD ($p < 0.05$) and SC > WD ($p < 0.01$) but KD = WD ($p = 0.83$). There was a main effect of activity ($p < 0.05$); specifically EX > SED. There was no Diet*Activity interaction ($p = 0.35$).

BAT adipocytes sized 20-39.99 microns in diameter are presented in Figure 10d (SED rats only) and 10e (EX rats only). There was a main effect of diet ($p < 0.01$); WD > SC ($p = 0.05$) and KD > SC ($p < 0.05$), but KD = WD ($p = 0 .97$). There was no activity effect ($p = 0.14$) and there was no Diet*Activity interaction ($p = 0.64$).

BAT adipocytes sized 40-59.99 microns in diameter are presented in Figure 10d (SED rats only) and 10e (EX rats only). There was no diet effect ($p = 0.14$), there was no activity effect ($p = 0.33$) and there was no Diet*Activity interaction ($p = 0.34$).

BAT adipocytes sized 60-79.99 microns in diameter are presented in Figure 10d (SED rats only) and 10e (EX rats only). There was a main effect of diet ($p < 0.05$); specifically WD = KD ($p = 0.09$), WD = SC ($p = 0.07$) and KD = SC ($p = 1.00$). There was a main effect of activity ($p < 0.05$); specifically SED > EX. There was no Diet*Activity interaction ($p = 0.05$).

INSERT BAT MASSES AND ADIPOCYTE DIAMETER DATA (FIGURE 10) HERE

OMAT mRNA expression patterns

OMAT ChREBP1 (i.e., marker of lipogenesis) mRNA is presented in Table 4. There was no diet effect (p = 0.65), there was no activity effect (p = 0.22) and there was no Diet*Activity interaction (p = 0.64).

OMAT SREBP1 (i.e., marker of lipogenesis) mRNA is presented in Table 4. There was no diet effect (p = 0.45), there was no activity effect (p = 0.10) and there was no Diet*Activity interaction (p = 0.97).

OMAT LIPE (i.e., marker of lipolysis) mRNA is presented in Table 4. There was no diet effect (p = 0.88), there was no activity effect (p = 0.56) and there was no Diet*Activity interaction (p = 0.27).

OMAT TNFα (i.e., marker of inflammation) mRNA is presented in Table 4. There was no diet effect (p = 0.61), there was no activity effect (p = 0.07) and there was no Diet*Activity interaction (p = 0.12).

OMAT IL6 (i.e., marker of inflammation) mRNA is presented in Table 4. There was no diet effect (p = 0.79), there was no activity effect (p = 0.18) and there was no Diet*Activity interaction (p = 0.32).

OMAT ADIPOQ (i.e., marker of appetite regulation) mRNA is presented in Table 4. There was a main effect of activity (p < 0.05); specifically SED > EX. There was no diet effect (p = 0.61) and there was no Diet*Activity interaction (p = 0.60).

OMAT CIDEA (i.e., marker of white adipose tissue 'briting' or 'browning') mRNA is presented in Table 4. There was a main effect of activity (p < 0.05); specifically EX > SED. There was no diet effect (p = 0.65). There was a Diet*Activity

interaction ($p < 0.05$); specifically, SC SED > SC EX ($p < 0.05$), although there were no between-diet changes between SED and EX conditions ($p > 0.05$).

OMAT UCP1 (i.e., marker of white adipose tissue 'briting' or 'browning') mRNA is presented in Table 4. There was a main effect of diet ($p < 0.01$); specifically WD = KD ($p = 0.97$), but SC >WD ($p < 0.01$) and SC > KD ($p < 0.01$). There was no activity effect ($p = 0.62$) and there was no Diet*Activity interaction ($p = 0.50$).

OMAT Prdm16 (i.e., marker of white adipose tissue 'briting' or 'browning') mRNA is presented in Table 4. There was no diet effect ($p = 0.17$) and there was no activity effect ($p = 0.67$). There was a Diet*Activity interaction ($p < 0.05$); specifically, WD > SC ($p < 0.05$), and SC SED > SC EX ($p < 0.05$).

SQ mRNA expression patterns

SQ ChREBP1 mRNA is presented in Table 4. There was no diet effect ($p = 0.54$), there was no activity effect ($p = 0.19$) and there was no Diet*Activity interaction ($p = 0.36$).

SQ SREBP1 mRNA is presented in Table 4. There was no diet effect ($p = 0.24$), there was no activity effect ($p = 0.21$) and there was no Diet*Activity interaction ($p = 0.75$).

SQ LIPE mRNA is presented in Table 4. There was no diet effect ($p = 0.29$), there was no activity effect ($p = 0.46$) and there was no Diet*Activity interaction ($p = 0.35$).

SQ TNFα mRNA is presented in Table 4. There was no diet effect ($p = 0.42$), there was no activity effect ($p = 0.94$) and there was no Diet*Activity interaction ($p = 0.75$).

SQ IL6 mRNA is presented in Table 4. There was no diet effect ($p = 0.29$), there was no activity effect ($p = 0.09$) and there was no Diet*Activity interaction ($p = 0.79$).

SQ ADIPOQ mRNA is presented in Table 4. There was no diet effect ($p = 0.07$), there was no activity effect ($p = 0.76$) and there was no Diet*Activity interaction ($p = 0.95$).

SQ CIDEA mRNA is presented in Table 4. There was no diet effect ($p = 0.16$), there was no activity effect ($p = 0.12$) and there was no Diet*Activity interaction ($p = 0.41$).

SQ UCP1 mRNA is presented in Table 4. There was no diet effect ($p = 0.42$), there was no activity effect ($p = 0.79$) and there was no Diet*Activity interaction ($p = 0.73$).

SQ Prdm16 mRNA is presented in Table 4. There was no diet effect ($p = 0.12$), there was no activity effect ($p = 0.53$) and there was no Diet*Activity interaction ($p = 0.09$).

BAT mRNA expression patterns

BAT ChREBP1 mRNA is presented in Table 4. There was no diet effect ($p = 0.37$), there was no activity effect ($p = 0.61$) and there was no Diet*Activity interaction ($p = 0.88$).

BAT CIDEA mRNA is presented in Table 4. There was a main effect of activity ($p < 0.05$); specifically EX > SED. There was no diet effect ($p = 0.05$), and there was no Diet*Activity interaction ($p = 0.17$).

BAT ADIPOQ mRNA is presented in Table 4. There was a main effect of diet ($p < 0.005$); specifically WD = KD ($p = 0.07$) and WD = SC ($p = 0.29$), but KD > SC ($p <$

0.005). There was no activity effect (p = 0.24). There was a Diet*Activity interaction (p

< 0.05); specifically, KD EX > WD EX (p < 0.05) and KD EX > SC EX (p < 0.05),

although there were no within-diet changes between SED and EX conditions (p > 0.05).

BAT UCP1 mRNA is presented in Table 4. There was a main effect of activity (p

< 0.01); specifically EX > SED. There was no diet effect (p = 0.07) and there was no

Diet*Activity interaction (p = 0.20).

BAT Prdm16 mRNA is presented in Table 4. There was no diet effect (p = 0.63),

there was no activity effect (p = 0.96) and there was no Diet*Activity interaction (p =

0.94).

INSERT ADIPOSE TISSUE mRNA EXPRESSION PATTERNS (TABLE 4) HERE

OMAT protein expression patterns

OMAT FASN protein is presented in Figure 11a. There was a main effect of

activity (p < 0.01); specifically EX > SED. There was no diet effect (p = 0.99), and there

was no Diet*Activity interaction (p = 0.84).

OMAT ACC protein is presented in Figure 11b. There was a main effect of

activity (p < 0.01); specifically EX > SED. There was no diet effect (p = 0.79), and there

was no Diet*Activity interaction (p = 0.52).

OMAT CD36 protein is presented in Figure 11c. There was no diet effect (p =

0.68), there was no activity effect (p = 0.59) and there was no Diet*Activity interaction (p

= 0.43).

OMAT C/EBPα protein is presented in Figure 11d. There was no diet effect ($p = 0.52$), there was no activity effect ($p = 0.54$) and there was no Diet*Activity interaction ($p = 0.96$).

OMAT phospho-p65: pan p65 protein is presented in Figure 11e. There was a main effect of activity ($p < 0.01$); specifically EX > SED. There was no diet effect ($p = 0.36$), and there was no Diet*Activity interaction ($p = 0.39$).

OMAT phospho-AMPK: pan AMPK protein is presented in Figure 11f. There was a main effect of activity ($p < 0.001$); specifically SED > EX. There was no diet effect ($p = 0.74$), and there was no Diet*Activity interaction ($p = 0.84$).

OMAT phospho-HSL: pan HSL protein is presented in Figure 11g. There was a main effect of activity ($p < 0.05$); specifically SED > EX. There was no diet effect ($p = 0.87$), and there was no Diet*Activity interaction ($p = 0.78$).

INSERT OMAT PROTEIN EXPRESSION PATTERNS (FIGURE 11) HERE

SQ protein expression patterns

SQ FASN protein is presented in Figure 12a. There was no diet effect ($p = 0.08$) and there was no activity effect ($p = 0.86$). There was a Diet*Activity interaction ($p < 0.05$); specifically EX SC > EX WD ($p < 0.005$) and EX SC > EX KD ($p < 0.05$).

SQ ACC protein is presented in Figure 12b. There was no diet effect ($p = 0.60$), there was no activity effect ($p = 0.94$) and there was no Diet*Activity interaction ($p = 0.51$).

SQ CD36 protein is presented in Figure 12c. There was a main effect of diet ($p < 0.05$); specifically SC > KD ($p < 0.05$) and WD > KD ($p < 0.05$), but SC = WD ($p =$

0.69). There was a main effect of activity ($p < 0.05$); specifically EX > SED. There was no Diet*Activity interaction ($p = 0.55$).

SQ C/EBPα protein is presented in Figure 12d. There was a main effect of activity ($p < 0.001$); specifically SED > EX. There was no diet effect ($p = 0.10$), and there was no Diet*Activity interaction ($p = 0.10$).

SQ phospho-p65: pan p65 protein is presented in Figure 12e. There was no diet effect ($p = 0.85$), there was no activity effect ($p = 0.73$), and there was no Diet*Activity interaction ($p = 0.11$).

SQ phospho-AMPK: pan AMPK protein is presented in Figure 12f. There was a main effect of activity ($p < 0.05$); specifically SED > EX. There was no diet effect ($p = 0.95$), and there was no Diet*Activity interaction ($p = 0.19$).

SQ phospho-HSL: pan HSL protein is presented in Figure 12g. There was a main effect of diet ($p < 0.01$); specifically SC > KD ($p < 0.01$) but WD = KD ($p = 0.69$) and SC = WD ($p = 0.05$). There was no activity effect ($p = 0.16$) and there was no Diet*Activity interaction ($p = 0.19$).

INSERT SQ PROTEIN EXPRESSION FIGURE 12 HERE

OMAT, SQ and BAT UCP-1 Immunohistochemistry

OMAT UCP-1 histological protein expression is presented in Figure 13a. There was a main effect of activity ($p < 0.05$); specifically EX > SED. There was no diet effect ($p = 0.09$) and there was no Diet*Activity effect ($p = 0.73$).

SQ UCP-1 histological protein expression is presented in Figure 13b. There was no diet effect (p = 0.12), there was no activity effect (p = 0.20), and there was no Diet*Activity effect (p = 0.50).

BAT UCP-1 histological protein expression is presented in Figure 13c. There was a main effect of activity (p < 0.001); specifically EX > SED. There was no diet effect (p = 0.65) and there was no Diet*Activity effect (p = 0.35).

INSERT OMAT, SQ AND BFAT UCP-1 DATA (FIGURE 13) HERE

Liver triglycerides and protein expression patterns

Liver triglycerides are presented in Figure 14a. There was a main effect of diet (p < 0.001); specifically WD > KD (p < 0.001) and WD > SC (p < 0.001) but SC = KD (p = 0.51). There was no activity effect (p = 0.28) and there was no Diet*Activity interaction (p = 0.27).

Liver FASN protein is presented in Figure 14b. There was no diet effect (p = 0.57), there was no activity effect (p = 0.50) and there was no Diet*Activity interaction (p = 0.27).

Liver ACC protein is presented in Figure 14c. There was no diet effect (p = 0.67), there was no activity effect (p = 0.15) and there was no Diet*Activity interaction (p = 0.13).

Liver phospoho-p65: pan p65 protein is presented in Figure 14d. There was a main effect of diet (p < 0.05); specifically SC > KD (p < 0.01) but WD = KD (p = 0.18)

and SC = WD (p = 0.20). There was a main effect of activity (p < 0.05); specifically

SED > EX. There was no Diet*Activity interaction (p = 0.12).

Liver phospho-AMPK: pan AMPK protein is presented in Figure 14e. There was

no diet effect (p = 0.16), there was no activity effect (p = 0.08) and there was no

Diet*Activity interaction (p = 0.32).

INSERT LIVER TG AND PROTEIN EXPRESSION DATA (FIGURE 14) HERE

Liver mRNA expression patterns

Liver ChREBP1 mRNA is presented in Table 5. There was a main effect of diet

(p < 0.001); specifically WD > KD (p < 0.01) and WD > SC (p < 0.001) but KD = SC (p

= 0.08). There was a main effect for activity (p < 0.001); specifically SED > EX. There

was a Diet*Activity interaction (p < 0.01); specifically SED SC > EX SC (p < 0.001),

SED KD > EX KD (p < 0.001), SED WD > EX WD (p < 0.001), SED WD > SED KD (p

< 0.01), SED WD > SED SC (p < 0.001), and SED KD > SED SC (p < 0.05).

Liver SCD1 mRNA is presented in Table 5. There was a main effect of diet (p <

0.001); specifically WD > KD (p < 0.001) and WD > SC (p < 0.001) but KD = SC (p =

0.07). There was no activity effect (p = 0.15). There was a Diet*Activity interaction (p <

0.05); specifically SED WD > SED KD (p < 0.001), SED WD > SED SC (p < 0.001),

and EX WD > EX KD (p < 0.01).

Liver TNFα mRNA is presented in Table 5. There was no diet effect (p = 0.24)

and there was no activity effect (p = 0.76). There was a Diet*Activity interaction (p <

0.05); specifically EX WD > SED WD (p < 0.05) and EX WD > EX SC (p < 0.05).

Liver IL6 mRNA is presented in Table 5. There was a main effect of activity ($p < 0.05$); specifically EX > SED. There was no diet effect ($p = 0.06$) and there was no Diet*Activity interaction ($p = 0.75$).

INSERT LIVER mRNA EXPRESSION PATTERNS (TABLE 5) HERE

Select bivariate correlations of serum insulin or BHB with body mass and adipose tissue characteristics

Correlations between serum insulin versus select whole-body or adipose tissue parameters are presented in Figure 15. Serum insulin was positively associated with body mass ($r = 0.54$, $p < 0.001$), relative OMAT mass ($r = 0.57$, $p < 0.001$), relative SQ mass ($r = 0.31$, $p < 0.05$) and SQ adipocyte size ($r = 0.49$, $p < 0.001$); of note, serum insulin versus OMAT adipocyte size approached statistical significant ($r = 0.28$, $p = 0.054$). On the other hand, serum BHB not associated with body mass ($r = -0.14$, $p = 0.35$), relative OMAT mass ($r = -0.03$, $p = 0.83$), OMAT adipocyte size ($r = 0.02$, $p = 0.91$), relative SQ mass ($r = 0.06$, $p = 0.67$) and SQ adipocyte size ($r = -0.06$, $p = 0.68$) (*data not shown for serum β-HB and adipose tissue associations*).

CHAPTER V

DISCUSSION

The positive effects of a very low carbohydrate, ketogenic diet, on adiposity and liver health are becoming increasingly documented in scientific literature. However, molecular pathways associated with KD-related benefits in body composition have not been widely explored. Therefore, we examined adipogenic, lipogenic, lipolytic, thermogenic, and inflammatory mRNA and/or protein expression patterns in select adipose tissue depots as well as the liver of rodents consuming either a ketogenic diet, western diet, or standard chow diet with the intent of understanding the dietary-related molecular adaptations in either a sedentary or physically-active state.

The effects of KD versus WD and SC on body mass and adipose tissue characteristics

Regardless of exercise, our study demonstrated that the KD improved body mass as well as certain aspects of adipose tissue characteristics. Compared to the WD rodents, the KD rodents had significantly less absolute and relative OMAT masses. Adipose tissue may expand via hypertrophy or hyperplasia, with visceral adipocyte hypertrophy being the most deleterious regarding health consequences (Company et al., 2013). The KD rodents in our study had significantly smaller OMAT average adipocyte sizes compared to the WD and SC rodents but SQ adipocyte sizes were similar between diets. Indeed, smaller adipocytes are more insulin-sensitive compared to larger adipocytes

based upon previous research in visceral adipocytes (Salans et al., 1973). Enlarged

visceral adipocytes have also been shown to contribute to increased blood lipid

concentrations (Slawik & Vidal-Puig, 2007) and, in obese states, adipocytes reach

maximal expanding capacity (Smith, 2007) thereby inhibiting storage of new lipids and

causing a dysfunctional increase in lipolysis rates (Engfeldt & Arner, 1988). These

events lead to an accumulation of FFAs in the plasma (Engfeldt & Arner, 1988) which

may result in an increased risk for type 2 diabetes and cardiovascular disease (Dulloo,

2008; Rutkowski, Davis, & Scherer, 2009). While we did not assess adipocyte insulin

sensitivity or the direct effects of adipocytes on blood lipid levels, it is noteworthy

mentioning that KD rats, which had lower OMAT average adipocyte sizes, presented

lower levels of serum glucose, serum insulin and serum lipid levels compared to the other

dietary treatments. Hence, it will be of further interest to examine if isolated adipocyte

preparations from KD-fed rats have improved insulin sensitivity and/or reduced lipolysis

rates compared to SC- or WD-fed rats.

In hopes of determining the primary physiological factor(s) causing the reduced

fat mass that occurs with a KD, we analyzed mRNA and protein expression levels related

to lipogenic, lipolytic, inflammatory, and thermogenic processes. While most of the

assayed proteins and genes did not show a diet effect, the KD rats had lower insulin

levels and there were positive associations between serum insulin and indices of adiposity

(Fig 15). This is noteworthy given that: a) previous literature is in agreement with these

findings suggesting that reducing insulin also reduces adiposity (Boden et al., 2005;

Kennedy et al., 2007; Volek et al., 2002), and b) insulin is an anabolic hormone [i.e., it

inhibits tissue breakdown and promotes tissue storage of nutrients (Brody, 1999)].

Moreover, Jensen et al. demonstrated that moderate reductions in circulating insulin levels result in large increases in lipolysis, as metabolism is shifted toward fat oxidation (Jensen, 1989). Reducing insulin levels through carbohydrate restriction also decreases lipogenesis; a phenomenon which allows ingested fats to be metabolized for fuel rather than stored (Brody, 1999; Horton, 2006; Spriet, 2012). Therefore, our findings collectively suggest that the lower insulin levels associated with a KD may play a major role in attenuating the accumulation of adipose tissue. Alternatively stated, although we analyzed various molecular markers related to the regulation of adipose tissue lipid storage, we may not have assayed the correct insulin signaling intermediaries associated with the KD-induced improvements in adipose tissue physiology and this warrants further examination.

Effects of exercise on body mass and adipose tissue characteristics

Absolute and relative OMAT masses were reduced in the EX rodents when compared to the SED rodents, independent of diet. This physical activity-induced decrease in adiposity has been previously demonstrated in both rodents and humans (Booth et al., 2002; Nara et al., 1999). Our EX rodents also exhibited reduced circulating insulin levels but similar blood glucose levels compared to the SED rodents. Physical activity has been shown to improve insulin sensitivity; therefore, less insulin is required to signal optimal glucose uptake by tissues (Mayer-Davis et al., 1998). Moreover, physical activity results in elevated catecholamines which aid in stimulating adipocyte lipolysis (Brody, 1999). Repeated bouts of physical activity result in re-occurring lipolytic events which promote a reduction in adipose tissue (Brody, 1999; Horton, 2006;

Spriet, 2012). Although physical activity may promote increased adipose tissue lipolysis,

our EX rodents had reduced levels of circulating triglycerides compared to SED rodents;

a health benefit likely due to increased fat oxidation in the working tissues.

Despite lower OMAT masses, the EX rodents showed paradoxical increases in

select signaling intermediaries involved in lipogenesis. Specifically, after 6 weeks of

exercise training, EX rats demonstrated increased ACC and FASN (lipogenic markers)

protein levels in the OMAT depot. Similar to our findings, long-term exercise training

has previously been shown to increase phosphorylated ACC levels in visceral adipose

tissue but not subcutaneous whereas acute exercise training for 30 minutes has been

shown to decrease ACC protein levels (Takekoshi et al., 2006). This increase in

lipogenic protein expression with exercise may play a role in the mechanism of "catch-

up" fat when exercise ceases (Kump & Booth, 2005).   Alternatively stated, exercise-

induced fat loss may act in a negative feedback fashion whereby lipogenic/adipogenic

gene up-regulation occurs in order to 'prime' the tissue to store lipid as a survival

mechanism (Kump & Booth, 2005). Therefore, aside from the dietary-related themes of

this study, it appears beneficial to maintain physical activity levels in order to reduce

adipose tissue mass accretion with physical inactivity.

Interestingly, exercise increased UCP-1 protein expression in the OMAT and

BAT depots. Mechanisms involving the induction of UCP-1 expression is still not fully

known, but has been thought to stem from elevated norepinephrine levels (Nilsson,

Heding, & Hokfelt, 1975). Given that exercise increases circulating catecholamines in a

pulsatile fashion, this may be the mechanism of action related to EX-induced increases in

UCP-1 expression. Exercise has also been recently reported to stimulate the production

and secretion of the myokine irisin from skeletal muscle which, in turn, acts to induce the 'beiging' or 'browning' of white adipose tissue (Boström et al., 2012). Hence, while this mechanism of action was not examined in the current study, exercise-induced increases in irisin signaling may have also been responsible for increases in OMAT and BAT UCP-1 protein expression.

Effects of KD versus SC and WD and physical activity on liver markers

Interestingly, liver triglyceride content was greater in WD versus KD and SC rats; of note, prior literature have reported KDs to increase liver triglycerides in rodents (Garbow et al., 2011; Kennedy et al., 2007). Hepatic triglyceride storage increases when triglyceride formation is greater than clearance from the hepatocyte (Garbow et al., 2011). Hepatic triglycerides are also formed from *de novo* lipogenesis in the liver, triglyceride uptake resulting from adipose tissue lipid breakdown, and/or diet-derived fats packaged as chylomicrons in the intestines (Schugar & Crawford, 2012). Hence, while it is difficult to determine how the KD reduced liver TG accumulation, it may be due to a reduction in uptake and/or increases in liver fat oxidation rates. Additionally, with limited glucose entering the liver from the low-carbohydrate KD, it is plausible that *de novo* lipogenesis in the liver was reduced as this typically occurs from excess glucose flux (Schugar & Crawford, 2012). Thus, as with adipose tissue, we analyzed *de novo* lipogenesis-related related transcription factors and enzymes in the liver such as ChREBP1, SCD1, ACC, and FASN.

Carbohydrate feeding stimulates the glucose responsive transcription factor ChREBP1 (Sakiyama et al., 2008). In our study, the WD group demonstrated greater

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ChREBP1 mRNA expression levels in the liver versus KD and SC rats, and the SED rats

also had greater expression levels versus EX rats. ChREBP1 has been shown to induce

glycolytic and lipogenic enzyme gene expression in both liver and adipose tissues

(Filhoulaud, Guilmeau, Dentin, Girard, & Postic, 2013; Girard, Ferre, & Foufelle, 1997).

Specifically, glucose entering the hepatocyte undergoes glycolysis which activates the

enzyme glucose-6 phosphatase causing an allosteric modification that triggers ChREBP1

to enter the hepatocyte nucleus (Filhoulaud et al., 2013). Additionally, acetyl CoA

resulting from the glycolysis also enters the hepatocyte nucleus (Filhoulaud et al., 2013).

The combination of the ChREBP1 transcription factor and acetyl CoA produce a signal in

the nucleus that leads to an upregulation of lipogenic enzymes ACC, FASN, and SCD1

(Filhoulaud et al., 2013). ACC and FASN are key enzymes that convert acetyl CoA into

palmitate, and SCD1 completes the process of *de novo* lipogenesis by converting

palmitate into fatty acids (Filhoulaud et al., 2013). It has been demonstrated that

ChREBP1-null mice have decreased *de novo* lipogenesis and glycolysis as well as

attenuated obesity (Iizuka, Miller, & Uyeda, 2006). Furthermore, as observed in our

study, exercise training has been shown to combat the upregulation of ChREBP1 mRNA

expression that results from a WD (Yasari et al., 2010). SCD1, the rate-limiting enzyme

for the conversion of palmitate to monounsaturated fatty acids, is involved in the

mechanistic progression of diet-induced hepatic insulin resistance (Dobrzyn & Ntambi,

2004). Similar to Kennedy et al., our analysis of SCD1 mRNA expression in the liver

revealed greater levels in WD versus KD and SC rats (Kennedy et al., 2007). Previous

literature has also demonstrated that SCD1-deficient rodents exhibit a hepatic down-

regulation in lipogenic genes (ACC and FAS), a decrease in circulating triglycerides, but

an increase in hepatic triglycerides (Cohen et al., 2002; Gutierrez-Juarez et al., 2006). Deficiency of SCD1 has also been associated with an attenuation of obesity, hyperinsulinemia and hepatic steatosis (Cohen et al., 2002). Therefore, greater levels of hepatic ChREBP1 mRNA, SCD1 mRNA and triglyceride content observed in WD rats herein suggest that this diet contributes to the development of a fatty liver, whereas the KD and SC diets do not.

Interestingly, select markers of liver damage and inflammation (i.e., phosphorylated p65/NFκB protein and serum ALT enzyme content) was reduced in KD rodents. These findings contradict previous research that found increased liver inflammation and circulating ALT levels in KD- versus SC-fed mice (Garbow et al., 2011). A possible explanation for the KD-associated liver damage reported in the previous study, but not in our study, may be due to the differing protein contents between studies. Our ketogenic diet was made-up of 20.2% protein whereas the study of comparison fed the mice a ketogenic diet composed of 4.7% protein. Normal growth and an impairment in other organ development and function have been shown to occur when diets consist of <14% protein (Goettsch, 1948). Moreover, casein, which is common in rodent feed and was present in all of the diets studied herein, was reduced in the aforementioned mouse study; this being a phenomena which possibly caused a methionine deficiency and subsequent hepatocyte injury and inflammation (Caballero et al., 2010). Therefore, our data, as well as other similar reports, underscore the importance of examining the dietary composition of different ketogenic diets when making conclusions or extrapolating results related to changes in physiology.

Conclusions

Overall, the KD reduced body mass, reduced select adipose tissue masses, reduced OMAT average adipocyte cell size, and improved serum glucose, insulin and blood lipid markers compared to the WD and SC diets. The cellular mechanisms leading to reduced adipose tissue mass and cell size from a KD may be driven by lower levels of insulin which dampens the signal to store fat and inhibit lipolysis. In this regard, we failed to identify this protein or gene in the current study, and this is an area for future research. Moreover, we observed KD-associated improvements in liver health and reduced hepatocyte *de novo* lipogenesis, and these effects may have an additional role in regulating adipose tissue mass. Finally, independent of diet, physical activity was associated with reduced body masses, and improved serum glucose, insulin and blood lipid markers. However, exercise may 'prime' adipose tissue to store fat upon exercise cessation by up-regulating lipogenic and adipogenic genes.

Limitations

The SED rodents were not provided a voluntary resistance running wheel but were provided a companion rodent. This is a potential limitation given that companion rats play/wrestle during the dark hours and thus, increase physical activity relative to a true 'sedentary' state. Notwithstanding, our study required sedentary animals to be doubly-housed due to animal welfare, so this is an inherent limitation. Second, this study was performed in rodents and, thus, the physiological changes may be different in magnitude or time-course when related to the human condition. A final limitation was our inability to maintain an isocaloric status between diets; specifically, the SC group

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consumed fewer calories than the WD and KD groups. In spite of this limitation, body

masses in the SC group were greater than the KD group suggesting that the KD rats

demonstrated an improved feed efficiency. Hence, this unanticipated finding also

warrants further investigation.

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Appendix A

Figure 1. Gene expression lineage for BAT, and subcutaneous and visceral WAT

Mesenchymal/ mesodermal stem cell

(+)BMPR, (+)CD34, (+)CD44, (+)Sca1, (+)SCF, (+)Pref-1, (-)PPARγ2, (-)aP2, (-)Lin

Common Preadipocyte

(+)BMPR, (+)CD34, (+)Pref-1, (-)CD31 (-)PPARγ2, (-)aP2

Subcutaneous White Preadipocyte

(+)BMPR, (+)CD34, (+) Tcf21, (+)Pref-1, (-)CD31, (-)PPARγ2, (-)aP2

Visceral White Preadipocyte

(+)BMPR, (+)CD34, (+) Tcf21, (+)Pref-1, (-)CD31, (-)PPARγ2, (-)aP2

Brown Preadipocyte

(+)BMPR, (+)Prdm16, (+)FoxC2, (+)necdin, (+) PGC-1α, (+)Pref-1, (-)CD31, (-)PPARγ2, (-)aP2

Subcutaneous White Adipocyte

(+)HoxA5, (+)HoxA4, (+)Nr2f1, (+)Thbd, (+)PAPP, (+)SFRP1, (+)Angiotensinogen, (+)PPARγ2, (+)aP2, (+)Leptin, (+)Fibrillin-1, (+)RIP140, (-)Pref-1, (-)UCP-1, (-)CIDEA,

Visceral White Adipocyte

(+)HoxC9, (+)Shox2, (+)En1, (+)PPARγ2, (+)aP2, (+)Leptin, (+)Fibrillin-1, (+)RIP140, (-)Pref-1, (-)UCP-1, (-)CIDEA,

Brown Adipocyte

(+)PPARγ2, (+)aP2, (+)UCP-1, (+)CIDEA, (+)Cig30, (+)PPARα, (+)Dio-2, (+) PGC-1α, (+)Prdm, (+)HoxA1, (+)HoxC4, (-)Pref-1, (-)Leptin, (-)Fibrillin-1

Figure 2. Ketone synthesis pathway in the liver

Acetyl CoA

- *Catalytic enzyme: Acetyl CoA Thiolase*

Aceto-acyl CoA

- *Catalytic enzyme: HMG CoA Synthase*
- Regulatory step: Succinyl CoA can inhibit HMG CoA Synthase

HMG CoA

- *Catalytic enzyme: HMG CoA Lyase*

AcAc

- *Catalytic enzyme:* β-hydroxybutyrate dehydrogenase
- Or with H^+ can be converted to Acetone + CO_2

β-HB
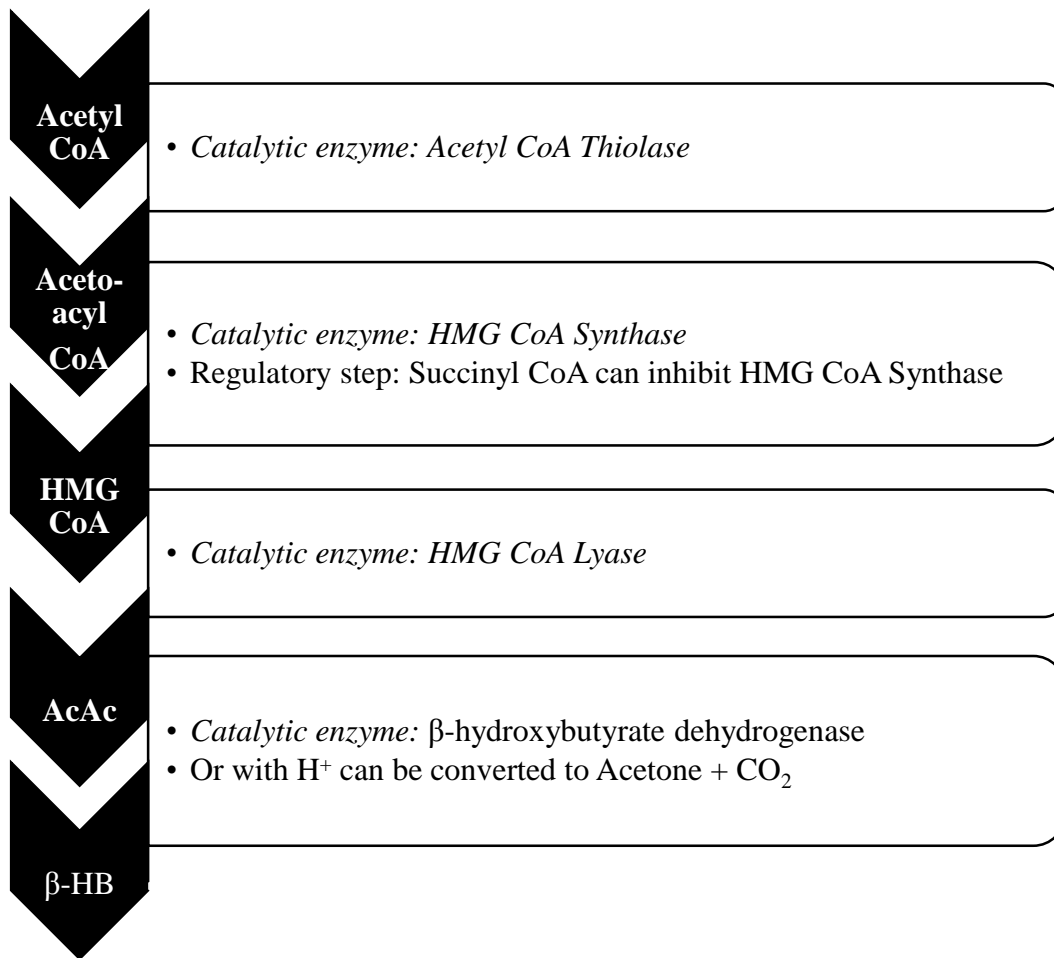
Figure 3. Ketone metabolism pathway

β-HB
- *Catalytic enzyme: β-hydroxybutyrate dehydrogenase*

AcAc
- *Catalytic enzyme: Succinyl CoA Transferase*
- Succinyl CoA → Succinate

Acetoacyl CoA
- *Catalytic enzyme: Thiolase*
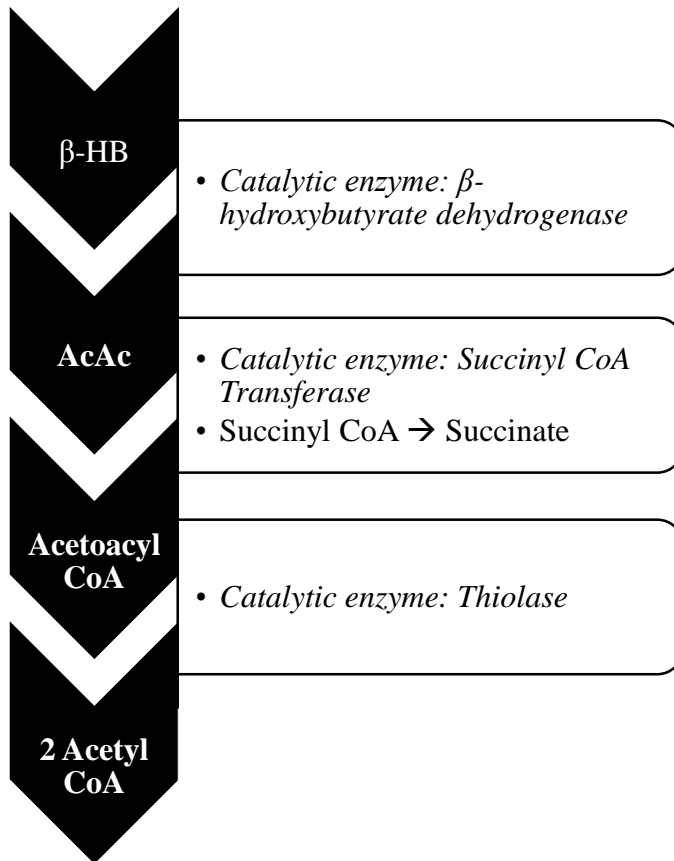
2 Acetyl CoA

Figure 4. Metabolic cascade occurring from prolonged fast

3-7 days of fast

Hypoinsulinemia	Mobilization of FA from adiopose Plasma FA levels double (then remain stable)

Low insulin/high glucagon

Increased hepatic FA oxidation	Ketogenic capacity: Liver acheives peak ketone production rates by 3rd day (~100g/day)

Low insulin progressively reduces peripheral tissue extraction of ketone bodies

Ketone body production stable but circulating ketones rise for a few weeks

CNS receives increased supply of ketones (>50% of brain energy needs)

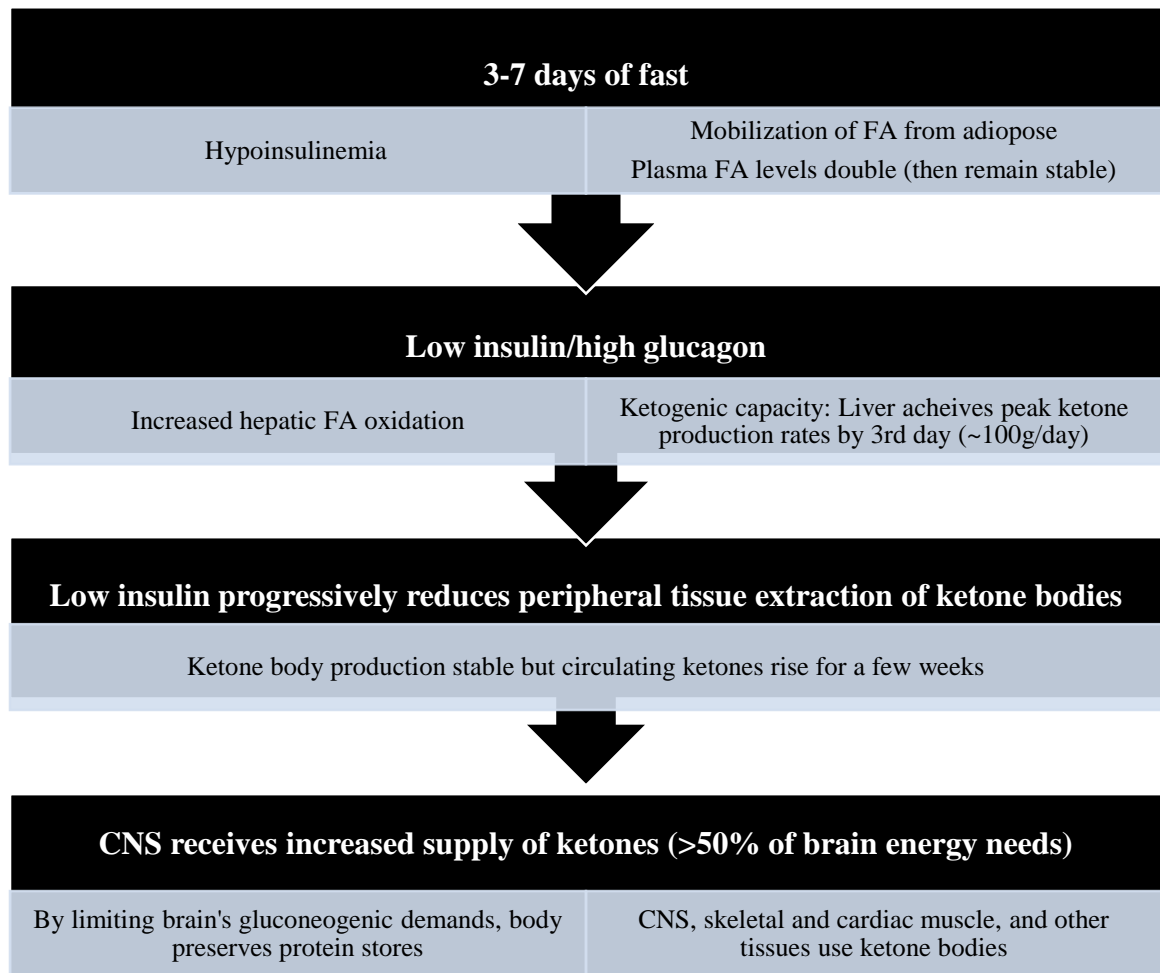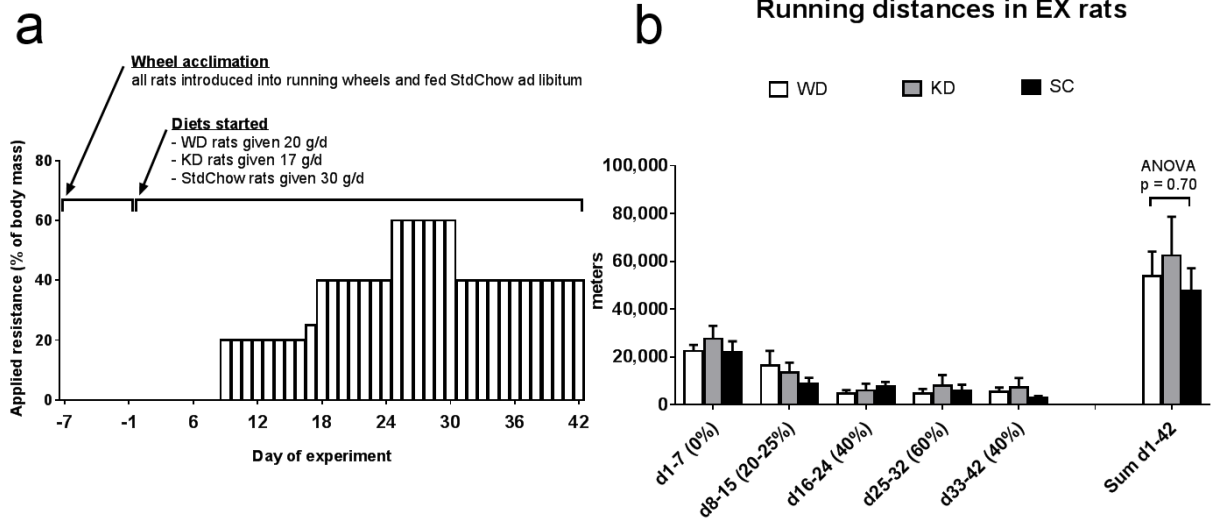| By limiting brain's gluconeogenic demands, body preserves protein stores	CNS, skeletal and cardiac muscle, and other tissues use ketone bodies

Figure 5. Study design for EX rats

Notes: for exercised (EX) rats, the running wheel scheme and resistance load is presented in A. Actual running distances are presented in B. Of note, no differences were observed for total running distances over the course of the intervention between diet groups.

Abbreviations: WD, Western diet; KD, ketogenic diet; SC, standard chow

Figure 6. Energy and macronutrient intakes over the duration of the study
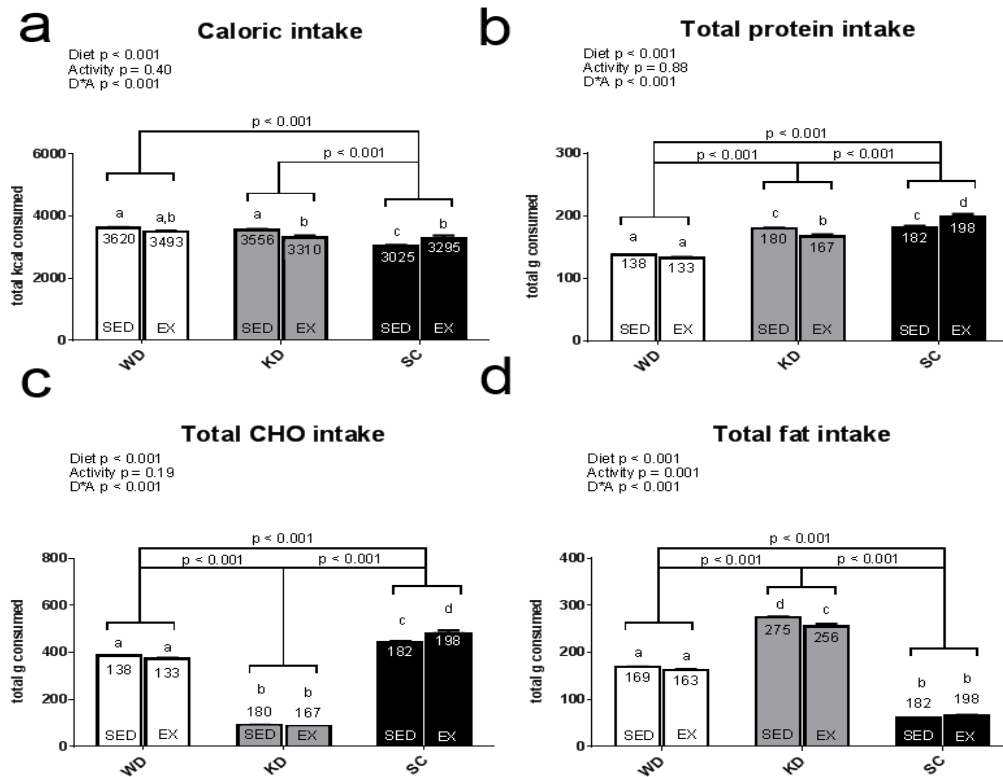
Notes: Total energy consumed is presented in A; of note, main diet effects indicated that WD = KD > SC and D*A interaction indicated that KD EX took in less energy compared to KD SED whereas SC EX took in more energy compared to SC SED. Total protein consumed is presented in B; of note, main diet effects indicated that SC > KD > WD and D*A interaction indicated that KD EX took in less protein compared to KD SED whereas SC EX took in more protein compared to SC SED. Total carbohydrate (CHO) consumed is presented in C; of note, main diet effects indicated that SC > WD > KD and D*A interaction indicated that SC EX took in more CHO compared to SC SED whereas this did not occur in the WD and KD treatments. Finally, total fat consumed is presented in D; of note, main diet effects indicated that KD > WD > SC and D*A interaction indicated that KD EX took in less fat compared to KD SED whereas this did not occur in the WD and SC treatments.

Abbreviations: WD, Western diet; KD, ketogenic diet; SC, standard chow; SED, sedentary rats; EX, exercised rats.

Figure 7. Body masses and food efficiency data
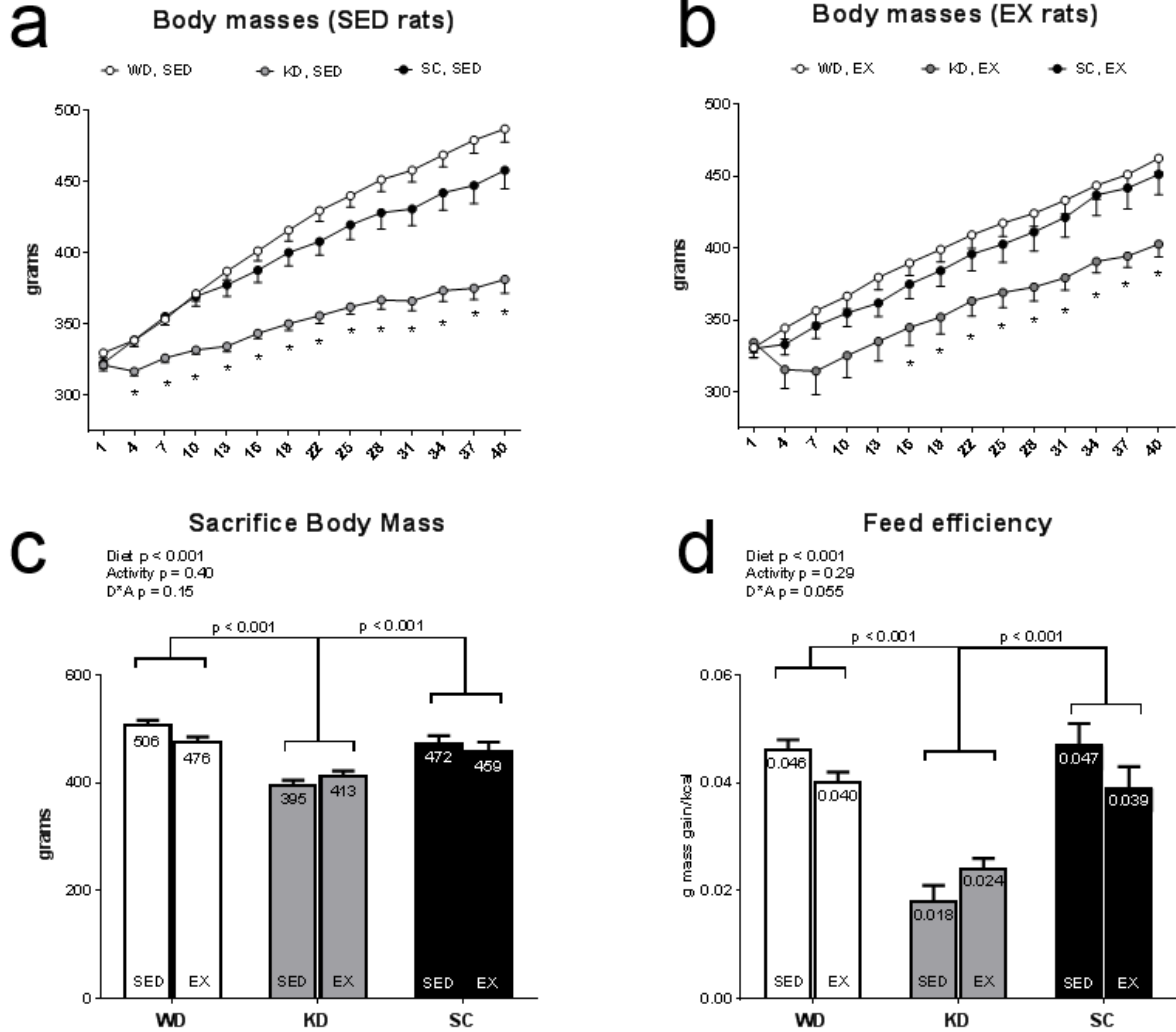
Notes: 3-d body masses for SED and EX rats are presented in A and B, respectively. Terminal body masses are presented in C; of note, there was a main effect of diet whereby WD = SC > KD. Feed efficiency data is presented in D; of note, there was a main effect of diet whereby WD = SC > KD.
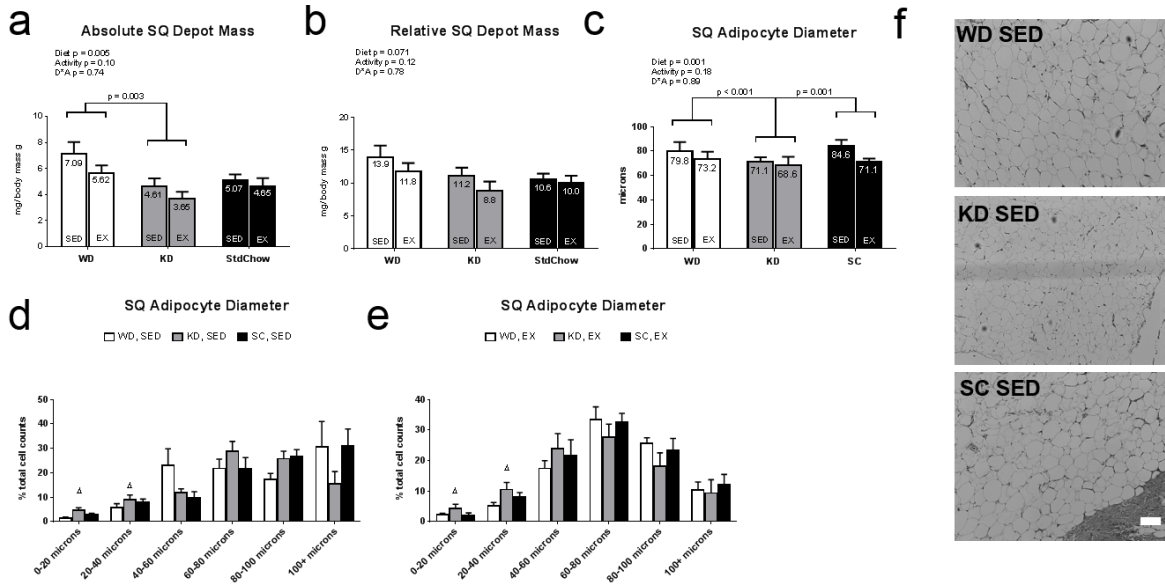
Symbols: *, WD and SC > KD ($p < 0.0125$)

Abbreviations: WD, Western diet; KD, ketogenic diet; SC, standard chow; SED, sedentary rats; EX, exercised rats.
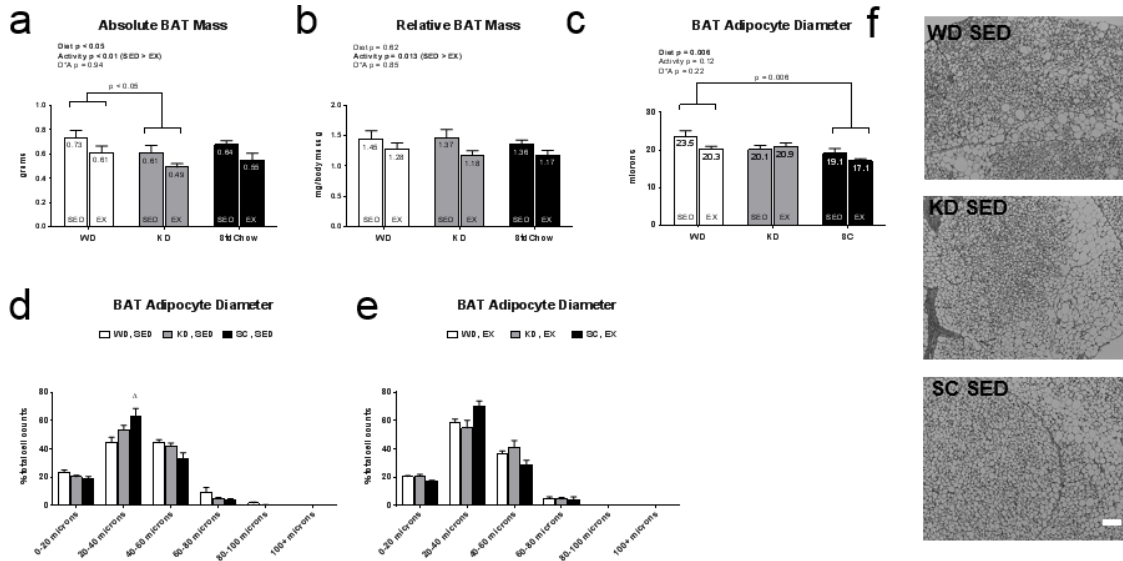
Figure 8. OMAT masses and morphology
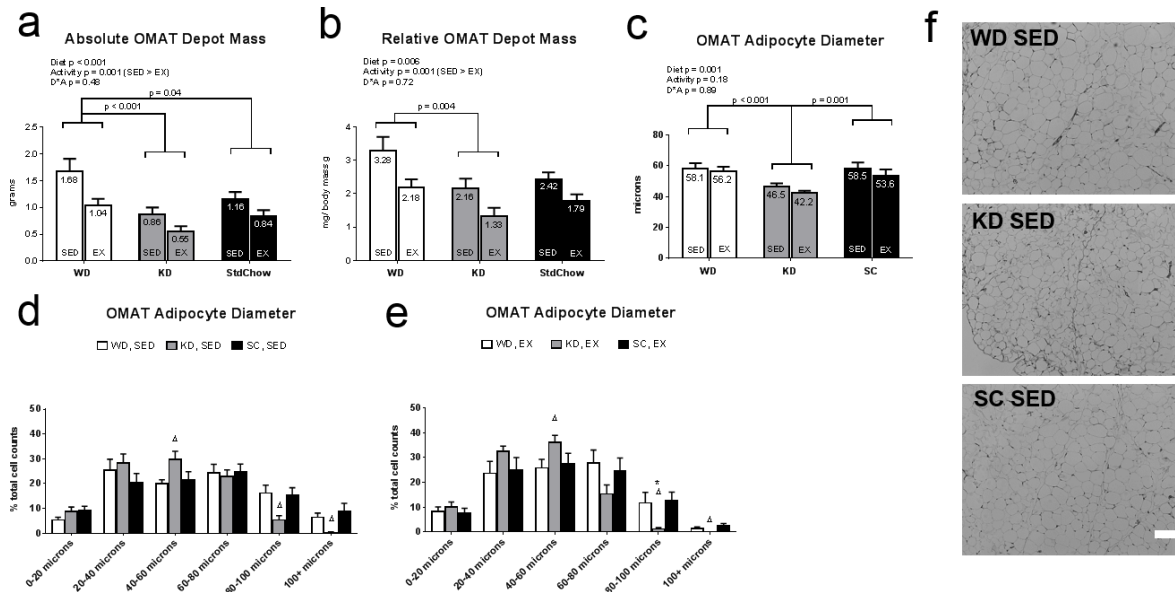
Notes: absolute OMAT mass is presented in A, relative OMAT mass corrected for body mass is presented in B, average OMAT adipocyte diameters are presented in C, OMAT adipocyte diameter ranges for SED rats are presented in D, and OMAT adipocyte diameter ranges for EX rats are presented in E. Representative images of OMAT adipocyte diameters from SED animals are presented in F (white bar = 100 microns). Symbols: Δ, diet effect indicated that KD is statistically different from WD and SC (p < 0.05); *, diet effect indicated that KD EX is statistically different from WD EX and SC EX (p < 0.05). Abbreviations: OMAT, omental; WD, Western diet; KD, ketogenic diet; SC, standard chow; SED, sedentary rats; EX, exercised rats.

Figure 9. SQ masses and morphology

Notes: absolute SQ mass is presented in A, relative SQ mass corrected for body mass is presented in B, average SQ adipocyte diameters are presented in C, SQ adipocyte diameter ranges for SED rats are presented in D, and SQ adipocyte diameter ranges for EX rats are presented in E. Representative images of SQ adipocyte diameters from SED animals are presented in F (white bar = 100 microns). Symbols: Δ, diet effect indicated that KD is statistically different from WD and SC at the indicated cell size (p < 0.05). Abbreviations: SQ, subcutaneous; WD, Western diet; KD, ketogenic diet; SC, standard chow; SED, sedentary rats; EX, exercised rats.

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Figure 10. BAT masses and morphology

Notes: absolute BFAT mass is presented in A, relative BFAT mass corrected for body mass is presented in B, average BFAT adipocyte diameters are presented in C, BFAT adipocyte diameter ranges for SED rats are presented in D, and BFAT adipocyte diameter ranges for EX rats are presented in E. Representative images of BFAT adipocyte diameters from SED animals are presented in F (white bar = 100 microns). Symbols: Δ, diet effect indicated that KD is statistically different from WD and SC ($p < 0.05$). Abbreviations: SQ, subcutaneous; WD, Western diet; KD, ketogenic diet; SC, standard chow; SED, sedentary rats; EX, exercised rats.
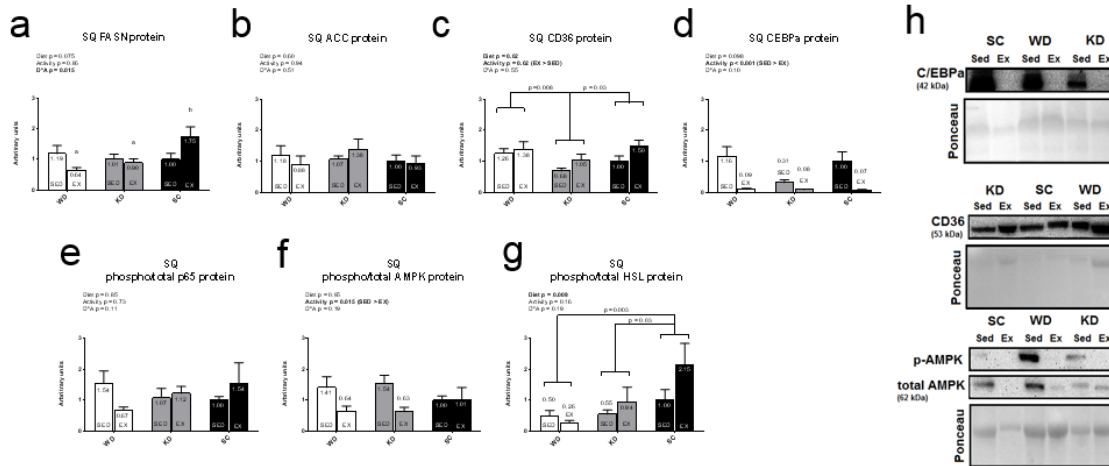
Figure 11. OMAT protein expression patterns
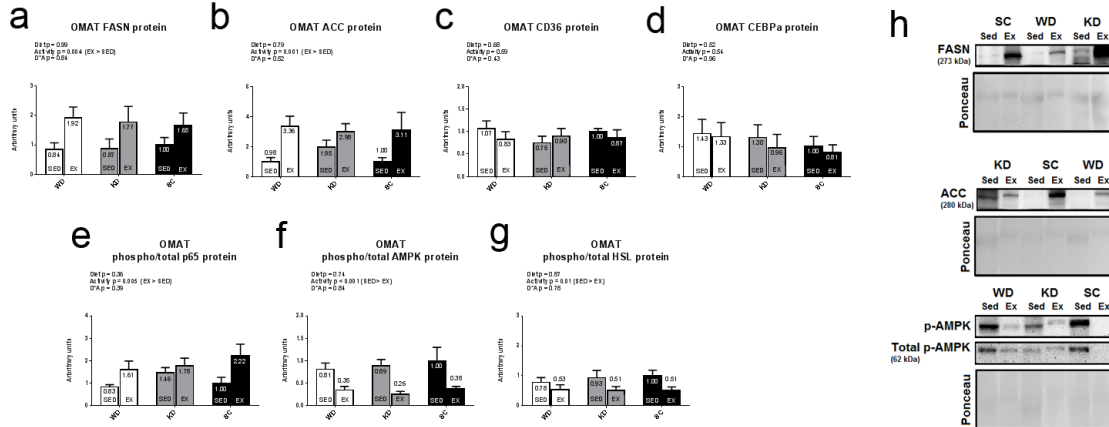
Notes: OMAT fatty acyl synthase (FASN, A), acetyl coA carboxylase (ACC, B), CD36 (C), CCAAT/Enhancer Binding Protein (CEBPa, D), phosphorylated: pan Nuclear Factor-Kappa-B p65 Subunit (E), and phosphorylated: pan 5' AMP-activated protein kinase alpha (AMPKa) subunit (F), and phosphorylated: pan hormone-sensitive lipase (HSL, G). Representative images of select targets are presented in H. Activity effects existed whereby EX > SED for FASN, ACC and phopsho: pan p65. Moreover, SED > EX for phospho: pan AMPKa and phospho: pan HSL. No diet or D*A interactions existed. Abbreviations: WD, Western diet; KD, ketogenic diet; SC, standard chow; SED, sedentary rats; EX, exercised rats.

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Figure 12. SQ protein expression patterns

Notes: SQ fatty acyl synthase (FASN, A), acetyl coA carboxylase (ACC, B), CD36 (C), CCAAT/Enhancer Binding Protein (CEBPa, D), phosphorylated: pan Nuclear Factor-Kappa-B p65 Subunit (E), and phosphorylated: pan 5' AMP-activated protein kinase alpha (AMPKa) subunit (F), and phosphorylated: pan hormone-sensitive lipase (HSL, G). Representative images of select targets are presented in H. Activity effects existed whereby EX > SED for CD36, and SED > EX for CEBPa and phospho: pan AMPKa. Diet effects existed whereby WD and SC > KD for CD36, and SC > KD and WD for phospho: pan HSL. No D*A interactions existed. Abbreviations: WD, Western diet; KD, ketogenic diet; SC, standard chow; SED, sedentary rats; EX, exercised rats.

Figure 13. Adipose tissue UCP-1 histology data
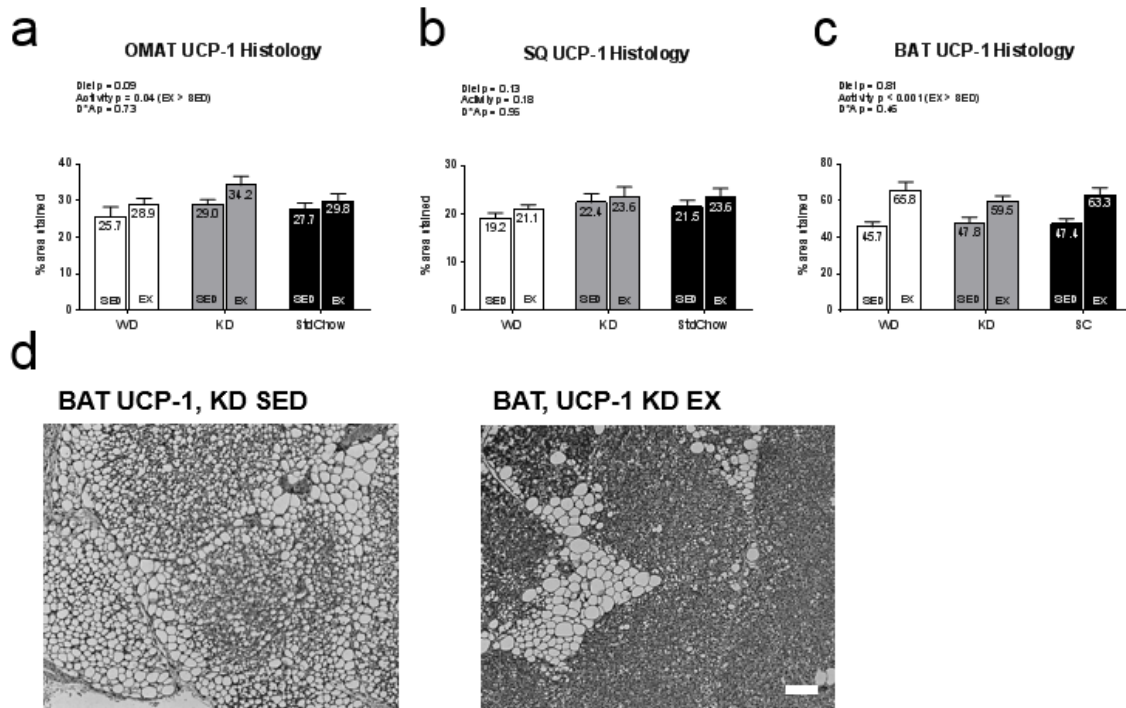
Notes: OMAT UCP-1 histology is presented in A, SQ UCP-1 histology is presented in B, BAT UCP-1 histology is presented in C. Representative images of SQ histology are presented in D (white bar = 100 microns). Activity effects existed whereby EX > SED for OMAT and BAT UCP-1 histology. No diet effects or D*A interactions existed.

Abbreviations: SQ, subcutaneous; WD, Western diet; KD, ketogenic diet; SC, standard chow; SED, sedentary rats; EX, exercised rats.

Figure 14. Liver triglyceride and protein expression patterns

Notes: Liver triglycerides (A), fatty acyl synthase (B), acetyl coA carboxylase (ACC, C), phosphorylated: pan Nuclear Factor-Kappa-B p65 Subunit (D), and phosphorylated: pan 5' AMP-activated protein kinase alpha (AMPKa) subunit (E). Representative images of select targets are presented in F. Diet effects existed whereby WD > SC and KD for liver triglycerides.   Moreover, WD and SC > KD for phospho: pan p65. No activity or D*A interactions existed. Abbreviations: WD, Western diet; KD, ketogenic diet; SC, standard chow; SED, sedentary rats; EX, exercised rats.
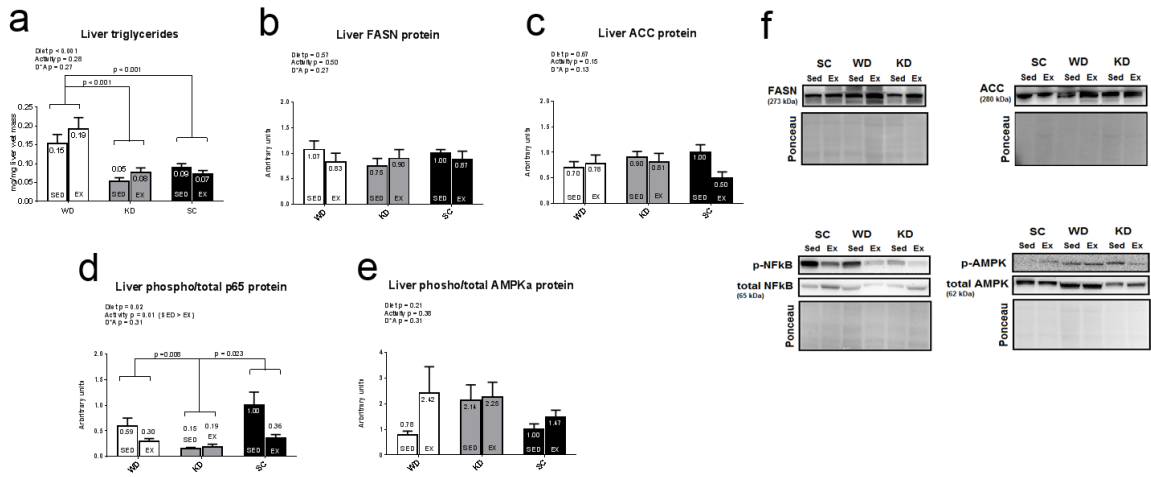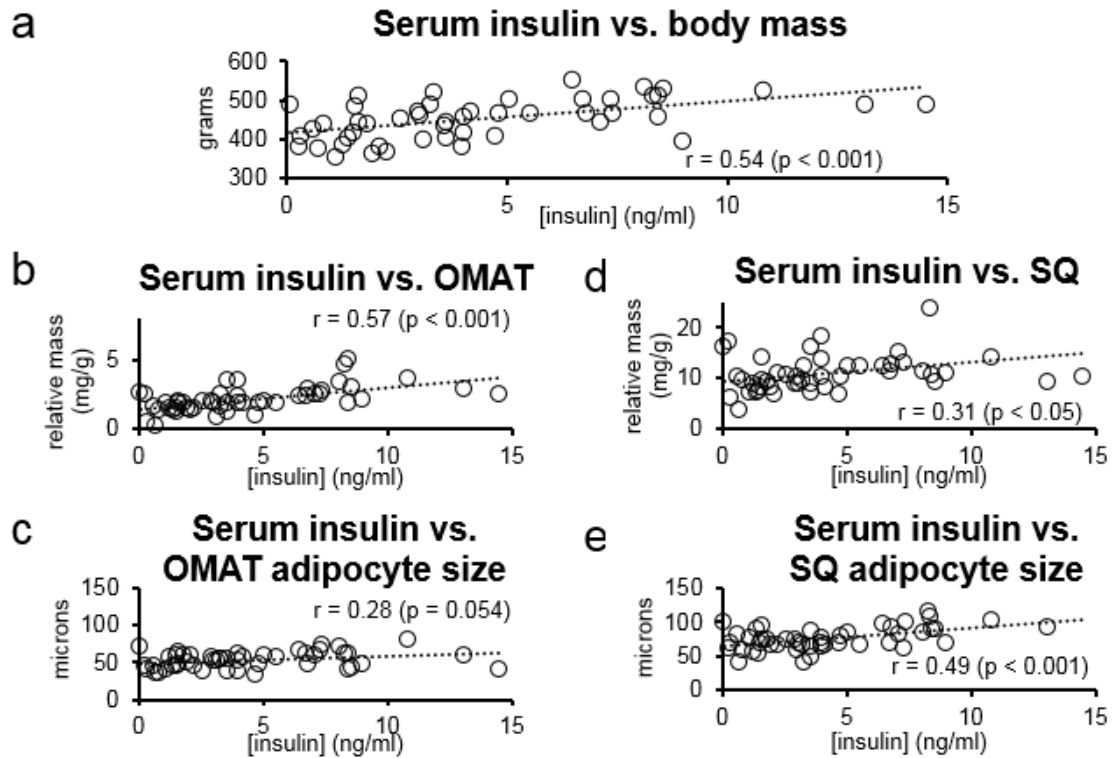
Figure 15. Select correlations between serum insulin versus body masses as well as adipose tissue characteristics

Notes: Correlations between serum insulin and body mass (A), relative OMAT mass (B), average OMAT adipocyte size (C), relative SQ mass (D) and average SQ adipocyte size (E). Significant positive associations existed between serum insulin versus body mass, relative OMAT mass, relative SQ mass and SQ adipocyte size. Abbreviations: OMAT, omental adipose tissue; SQ, subcutaneous adipose tissue.

APPENDIX B. Tables

Table 1. Gene-specific primers used for RT-PCR

Gene	Forward primer (5' → 3')	Reverse primer (5' → 3')
TNFα	GGTCAACCTGCCCAAGTACT	CTCCAAAGTAGACCTGCCCG
IL-6	ATCTGCCCTTCAGGAACAGC	GAAGTAGGGAAGGCAGTGGC
UCP-1	GGCGACTTGGAGAAAGGGAT	GCATAGGAGCCCAGCATAGG
CIDEA	GGTTTATGCGGGCGCTTATG	GGATGGCTGCTCTTCTGTGT
PRDM16	CGCACCAAGACAGTCCTCTT	CGGATCTCAGCATAGCCTGG
ADIPOQ	CAGCTAGCCAGTAAGCAGCA	CAGGCAGGAACTAACGCAGA
ChREBP1	ACAACCCCTGCCTTACACAG	GAGGTGGCCTAGGTGGTGTA
LIPE	GCATGGATTTACGCACAATG	GTATCCGTTGGCTGGTGTCT
SCD1	TCAGGAGGACACGCTGAAAC	CCTCAGAACTGCCCTTGAGG
SREBP1	CGTTTCTTCGTGGATGGGGA	TGTACAGACTCTCCTGGGGG
HDAC1*	GAGCGGTGATGAGGATGAGG	CACAGGCAATGCGTTTGTCA

Notes: *, indicates the housekeeping gene used for normalization

Table 2. Serum health markers

Gene	WD (mean ± SE)	KD (mean ± SE)	SC (mean ± SE)	Significance
Serum Markers				

Insulin (ng/ml)	SED: 7.33 ± 1.31
	EX: 3.41 ± 0.89
		SED: 0.91 ± 0.19
		EX: 2.96 ± 0.37
			SED: 7.26 ± 1.03
			EX: 4.00 ± 0.87
				Diet $p < 0.001$
				  (note: WD and SC > KD)
				Activity $p < 0.001$
				  (note: SED > EX)
				Diet*Activity $p = 0.58$

Glucose (mmol/L)	SED: 12.33 ± 0.94 EX: 12.87 ± 1.27	SED: 7.61± 0.36 EX: 11.27 ± 1.00	SED: 14.24 ± 1.53 EX: 12.12 ± 2.41	Diet $p < 0.01$ (note: WD and SC > KD) Activity $p = 0.48$ Diet*Activity $p = 0.06$

β-HB (mM)	SED: 0.45 ± 0.07
	EX: 0.30 ± 0.03
		SED: 0.65 ± 0.20
		EX: 0.40 ± 0.04
			SED: 0.27 ± 0.02
			EX: 0.16 ± 0.03
				Diet $p < 0.005$
				  (note: WD and KD > SC)
				Activity $p < 0.05$
				  (note: SED > EX)
				Diet*Activity $p = 0.34$

Triglycerides (mg/dL)	SED: 319.70 ± 34.71
	EX: 110.63 ± 15.72
		SED: 69.90 ± 6.69
		EX: 56.75 ± 6.43
			SED: 163.00 ± 21.19
			EX: 91.57 ± 16.50
				Diet $p = 0.00$
				  (note: WD > SC > KD)
				Activity $p = 0.00$
				  (note: SED > EX)
				Diet*Activity $p = 0.00$
				  (note: SED WD > SED KD, SED WD > SED SC, SED SC > SED KD, EX WD > EX KD, SED WD > EX WD, SED SC > EX SC)

Cholesterol (mg/dL)	SED: 89.90 ± 3.40
	EX: 67.63 ± 2.34
		SED: 67.70 ± 2.16
		EX: 65.75 ± 3.32
			SED: 87.00 ± 5.34
			EX: 87.71 ± 5.41
				Diet $p = 0.00$
				  (note: WD and SC > KD)
				Activity $p = 0.02$
				  (note: SED > EX)
				Diet*Activity $p = 0.01$
				  (note: SED WD > SED KD, SED SC > SED KD, EX SC > EX WD, EX SC > EX KD, SED WD > EX WD, SED SC > EX SC)

ALT (U/L)	SED: 49.30 ± 5.83 EX: 42.50 ± 1.92	SED: 46.00 ± 4.23 EX: 50.63 ± 3.05	SED: 65.00 ± 3.46 EX: 73.43 ± 5.74	Diet $p < 0.001$ (note: SC > KD and WD) Activity $p = 0.36$ Diet*Activity $p = 0.11$

Table 3. White blood cell differentials

Gene	WD (mean ± SE)	KD (mean ± SE)	SC (mean ± SE)	Significance
White Blood Cells (x10^3 cells/μL)	SED: 14.54 ± 0.84 EX: 9.01 ± 1.01	SED: 7.82 ± 0.92 EX: 8.76 ± 1.78	SED: 9.62 ± 1.32 EX: 11.03 ± 1.39	Diet p < 0.05 (note: WD > KD) Activity p = 0.31 Diet*Activity p < 0.05 (note: SED WD > EX WD, SED WD > SED KD)
Lymphocytes (x10^3 cells/μL)	SED: 88.00 ± 1.22 EX: 84.75 ± 2.35	SED: 82.13 ± 6.89 EX: 85.63 ± 2.98	SED: 87.22 ± 1.69 EX: 83.00 ± 4.08	Diet p = 0.79 Activity p = 0.66 Diet*Activity p = 0.52
Neutrophils (x10^3 cells/μL)	SED: 8.40 ± 0.21 EX: 12.50 ± 2.24	SED: 13.43 ± 3.51 EX: 12.00 ± 2.87	SED: 10.33 ± 1.23 EX: 14.00 ± 3.82	Diet p = 0.72 Activity p = 0.38 Diet*Activity p = 0.56
Monocytes (x10^3 cells/μL)	SED: 2.50 ± 0.68 EX: 2.50 ± 0.42	SED: 1.67 ± 0.33 EX: 2.00 ± 0.38	SED: 2.43 ± 0.48 EX: 2.43 ± 0.48	Diet p = 0.35 Activity p = 0.78 Diet*Activity p = 0.93

Table 4. Adipose tissue mRNA expression patterns

Gene	WD (mean ± SE)	KD (mean ± SE)	SC (mean ± SE)	Significance
SQ tissue				
ChREBP1 (lipogenic)	SED: 0.73 ± 0.21 EX: 0.60 ± 0.07	SED: 0.49 ± 0.12 EX: 0.50 ± 0.13	SED: 1.00 ± 0.32 EX: 0.43 ± 0.11	Diet p = 0.53 Activity p = 0.19 Diet*Activity p = 0.36
SREBP1 (lipogenic)	SED: 0.39 ± 0.07 EX: 0.22 ± 0.09	SED: 1.07 ± 0.43 EX: 0.45 ± 0.14	SED: 1.00 ± 0.35 EX: 0.74 ± 0.39	Diet p = 0.24 Activity p = 0.21 Diet*Activity p = 0.75
LIPE (lipolytic)	SED: 0.27 ± 0.05 EX: 0.31 ± 0.07	SED: 0.28 ± 0.04 EX: 0.38 ± 0.18	SED: 1.00 ± 0.53 EX: 0.37 ± 0.13	Diet p = 0.29 Activity p = 0.46 Diet*Activity p = 0.35
TNFα (inflammatory)	SED: 0.66 ± 0.16 EX: 0.48 ± 0.03	SED: 0.60 ± 0.13 EX: 0.88 ± 0.35	SED: 1.00 ± 0.24 EX: 0.96 ± 0.43	Diet p = 0.42 Activity p = 0.94 Diet*Activity p = 0.75
IL-6 (inflammatory)	SED: 1.30 ± 0.28 EX: 1.88 ± 0.23	SED: 1.04 ± 0.27 EX: 1.24 ± 0.28	SED: 1.00 ± 0.28 EX: 1.51 ± 0.47	Diet p = 0.29 Activity p = 0.09 Diet*Activity p = 0.79
ADIPOQ (appetite regulatory)	SED: 0.18 ± 0.03 EX: 0.10 ± 0.03	SED: 1.59 ± 0.91 EX: 1.24 ± 0.68	SED: 1.00 ± 0.44 EX: 0.77 ± 0.36	Diet p = 0.07 Activity p = 0.76 Diet*Activity p = 0.95
CIDEA (thermogenic)	SED: 0.74 ± 0.22 EX: 0.51 ± 0.08	SED: 1.70 ± 0.52 EX: 0.79 ± 0.15	SED: 1.00 ± 0.23 EX: 0.88 ± 0.24	Diet p = 0.16 Activity p = 0.12 Diet*Activity p = 0.41
UCP-1 (thermogenic)	SED: 0.98 ± 0.18 EX: 1.18 ± 0.14	SED: 0.86 ± 0.18 EX: 0.84 ± 0.19	SED: 1.00 ± 0.20 EX: 0.93 ± 0.19	Diet p = 0.42 Activity p = 0.79 Diet*Activity p = 0.73
Prdm16 (thermogenic)	SED: 1.33 ± 0.36 EX: 0.52 ± 0.21	SED: 1.13 ± 0.26 EX: 1.79 ± 0.52	SED: 1.00 ± 0.35 EX: 1.01 ± 0.06	Diet p = 0.12 Activity p = 0.53 Diet*Activity p = 0.09
OMAT tissue				
ChREBP1 (lipogenic)	SED: 0.85 ± 0.85 EX: 1.28 ± 0.26	SED: 0.52 ± 0.51 EX: 1.06 ± 0.56	SED: 1.00 ± 0.33 EX: 0.98 ± 0.24	Diet p = 0.65 Activity p = 0.22 Diet*Activity p = 0.64
SREBP1 (lipogenic)	SED: 1.44 ± 0.78 EX: 0.88 ± 0.24	SED: 1.00 ± 0.28 EX: 0.55 ± 0.13	SED: 1.00 ± 0.24 EX: 0.37 ± 0.12	Diet p = 0.45 Activity p = 0.10 Diet*Activity p = 0.97
LIPE (lipolytic)	SED: 0.60 ± 0.21	SED: 0.93 ± 0.21	SED: 1.00 ± 0.21	Diet p = 0.88 Activity p = 0.56 Diet*Activity p = 0.27

	EX: 0.89 ± 0.24	EX: 0.76 ± 0.21	EX: 0.55 ± 0.27	
TNFα (inflammatory)	SED: 0.39 ± 0.15 EX: 0.45 ± 0.13	SED: 0.51 ± 0.13 EX: 0.36 ± 0.05	SED: 1.00 ± 0.86 EX: 0.22 ± 0.11	Diet p = 0.61 Activity p = 0.07 Diet*Activity p = 0.12
IL-6 (inflammatory)	SED: 0.47 ± 0.08 EX: 1.41 ± 0.32	SED: 1.17 ± 0.37 EX: 1.08 ± 0.48	SED: 1.00 ± 0.27 EX: 1.32 ± 0.37	Diet p = 0.79 Activity p = 0.18 Diet*Activity p = 0.32
ADIPOQ (appetite regulatory)	SED: 0.52 ± 0.27 EX: 0.11 ± 0.05	SED: 0.70 ± 0.30 EX: 0.27 ± 0.07	SED: 1.00 ± 0.34 EX: 0.12 ± 0.03	Diet p = 0.61 Activity p < 0.05 (note: Sed > Ex) Diet*Activity p = 0.60
CIDEA (thermogenic)	SED: 0.54 ± 0.03 EX: 0.73 ± 0.18	SED: 0.74 ± 0.18 EX: 0.38 ± 0.11	SED: 1.00 ± 0.16 EX: 0.38 ± 0.10	Diet p = 0.65 Activity p < 0.05 (note: Sed > Ex) Diet*Activity p < 0.05 (note: Sed > Ex for KD and SC; Ex > Sed for WD)
UCP-1 (thermogenic)	SED: 0.41 ± 0.06 EX: 0.62 ± 0.19	SED: 0.44 ± 0.10 EX: 0.53 ± 0.43	SED: 1.00 ± 0.17 EX: 0.88 ± 0.64	Diet p < 0.01 (note: SC > KD = WD) Activity p = 0.62 Diet*Activity p = 0.50
Prdm16 (thermogenic)	SED: 0.68 ± 0.15 EX: 1.05 ± 0.32	SED: 0.42 ± 0.19 EX: 0.57 ± 0.10	SED: 1.00 ± 0.16 EX: 0.28 ± 0.09	Diet p = 0.17 Activity p = 0.67 Diet*Activity p < 0.05 (note: Ex > Sed for WD; Sed > Ex for SC)
BFAT				
ChREBP1 (lipogenic)	SED: 1.08 ± 0.19 EX: 1.00 ± 0.35	SED: 0.67 ± 0.17 EX: 0.69 ± 0.26	SED: 1.00 ± 0.22 EX: 0.75 ± 0.33	Diet p = 0.37 Activity p = 0.61 Diet*Activity p = 0.88
CIDEA (thermogenic)	SED: 13.49 ± 8.98 EX: 6.78 ± 6.44	SED: 33.60 ± 13.33 EX: 79.25 ± 34.17	SED: 1.00 ± 0.47 EX: 64.23 ± 25.90	Diet p = 0.05 (note KD > WD) Activity p < 0.05 (note: Sed > Ex for WD; Ex > Sed for KD and SC) Diet*Activity p = 0.17
UCP-1 (thermogenic)	SED: 1.35 ± 0.62 EX: 6.91 ± 6.80	SED: 15.16 ± 6.21 EX: 28.65 ± 12.08	SED: 1.00 ± 0.50 EX: 34.05 ± 13.13	Diet p = 0.07 Activity p < 0.01 (note: Ex > Sed) Diet*Activity p = 0.20
Prdm16 (thermogenic)	SED: 0.59 ± 0.12 EX: 0.71 ± 0.01	SED: 0.89 ± 0.21 EX: 0.75 ± 0.29	SED: 1.00 ± 0.44 EX: 0.97 ± 0.24	Diet p = 0.63 Activity p = 0.96 Diet*Activity p = 0.94

Table 5. Liver mRNA expression patterns

Gene	WD (mean ± SE)	KD (mean ± SE)	SC (mean ± SE)	Significance
ChREBP1 (lipogenic)	SED: 2.61 ± 0.23 EX: 0.85 ± 0.24	SED: 1.75 ± 0.17 EX: 0.54 ± 0.12	SED: 1.00 ± 0.07 EX: 0.54 ± 0.07	Diet $p < 0.01$ (note: WD > KD and SC) Activity $p < 0.01$ (note: Sed > Ex) Diet*Activity $p < 0.01$ (note: Sed SC > Ex SC; Sed KD > Ex KD; Sed WD > Ex WD; Sed WD > Sed KD; Sed WD > Sed SC; Sed KD > Sed SC)
SCD1 (lipogenic)	SED: 4.03 ± 0.32 EX: 2.66 ± 0.63	SED: 0.45 ± 0.10 EX: 0.34 ± 0.09	SED: 1.00 ± 0.20 EX: 1.34 ± 0.27	Diet $p < 0.01$ (note: WD > KD and SC) Activity $p = 0.21$ Diet*Activity $p < 0.05$ (note: Sed WD > Sed KD; Sed WD > Sed SC; Ex WD > Ex KD)
TNFα (inflammatory)	SED: 0.68 ± 0.08 EX: 1.19 ± 0.18	SED: 1.09 ± 0.20 EX: 1.05 ± 0.08	SED: 1.00 ± 0.12 EX: 0.63 ± 0.13	Diet $p = 0.24$ Activity $p = 0.76$ Diet*Activity $p < 0.05$ (note: Ex WD > Sed WD; Ex WD > Ex SC)
IL-6 (inflammatory)	SED: 0.55 ± 0.09 EX: 1.55 ± 0.37	SED: 1.73 ± 0.52 EX: 2.16 ± 0.43	SED: 1.00 ± 0.20 EX: 1.66 ± 0.43	Diet $p = 0.06$ Activity $p < 0.05$ (note: Ex > Sed) Diet*Activity $p = 0.75$

APPENDIX C. Anticipatory publication details.

Manuscript title: Effects of a putative ketogenic diet with and without exercise training on adipose tissue and liver physiology in rodents

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