EFFECTS OF β-HYDROXYBUTYRATE (βHB) ON L6 MUSCLE CELL GLUCOSE AND FAT METABOLISM

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Abstract

This study investigated the effects of the ketone body β-hydroxybutyrate (βHB) on glucose and fat metabolism in skeletal muscle cells. To accomplish this, L6 muscle cells were incubated with various βHB concentrations (0.5, 1.0, 1.5, 20, and 2.5 mM) for 1, 3, and 5 days. Here, we report that βHB significantly increased glucose uptake and its metabolism in L6 muscle cells. Time course analysis revealed that as early as 1 day of exposure to 1mM βHB significantly enhanced basal and insulin-stimulated glucose uptake (1.6-fold), glycogen synthesis (1.3-fold), glucose oxidation (1.5-fold), and lactate production (1.3-fold) in skeletal muscle cells. Even though carnitine palmitoyl transferase 1 (CPT1) content and AMP-activated protein kinase (AMPK) phosphorylation significantly increased, no alteration was found in palmitate oxidation with βHB treatment. Surprisingly, protein kinase B (AKT) phosphorylation was significantly reduced in muscle cells exposed to βHB. In summary, βHB increased AMPK phosphorylation and enhanced glucose uptake without altering fatty acid oxidation in skeletal muscle cells.

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Manuscript

List of Acronyms

IR	Insulin resistance
FOXO	Forkhead box O
PGC1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
GLUT4	Glucose transporter type 4
FFA	Free fatty acids
CNS	Central nervous system
βHB	β-hydroxybutyrate
AcAc	Acetoacetate
TCA cycle	The tricarboxylic acid
HMGCS2	3-hydroxy-3-methylglutaryl-CoA synthase 2
BDH1	3-Hydroxybutyrate Dehydrogenase 1
SLC16A6	Monocarboxylate transporter 7
MCT1	Monocarboxylate transporter 1
PPARα	Peroxisome proliferator-activated receptor alpha
OXCT1	3-oxoacid CoA-transferase 1
PDC	Pyruvate Dehydrogenase Complex
PDPs	Pyruvate dehydrogenase phosphatases
PDK4	Pyruvate dehydrogenase kinase 4
HDAC	Histone deacetylases
PI3K	Phosphatidylinositol 3-kinase
AKT	Protein kinase B
IRS	Insulin receptor substrate 1
GSK3	Glycogen synthase kinase 3
GS	Glycogen Synthase
AMPK	5′ AMP-activated protein kinase
AS160	Akt substrate of 160 kDa
ACC	Acetyl-CoA carboxylase
FAS	Fatty acid synthase
SIRT1	Sirtuin 1
FGF21	Fibroblast growth factor 21
IGF	Insulin-like growth factor
mTOR	mechanistic target of rapamycin
GTP	Guanosine triphosphate
NAD	Nicotinamide adenine dinucleotide
CoQH	Coenzyme Q
ATP	Adenosine Triphosphate
HMG-CoA	3-hydroxy-3-methyl-glutaryl-coenzyme A reductase
MYOD1	myogenic differentiation 1
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
TP53	Tumor suppressor 53
TGFβ	Transforming growth factor beta 1
ChIP	Chromatin immunoprecipitation
GPCRs	G-protein coupled receptors
HCAR2	Hydroxycarboxylic acid receptor 2

FFAR3	Free fatty acid receptor 3
PARP	Poly ADP ribose polymerase
$PI(3,4,5)P_3$	Phosphatidylinositol (3,4,5)-trisphosphate
$PI(4,5)P_2$	Phosphatidylinositol (4,5)-trisphosphate
PDK1	3-phosphoinositide-dependent protein kinase 1
mTORC2	Mammalian target of rapamycin complex 2
LKB1	Serine/Threonine Kinase 11
CAMKK	Calcium/calmodulin-dependent protein kinase kinase
PP2A	Protein Phosphatase 2A
PP1	Protein phosphatase 1
PKA	Protein Kinase A
CPT1	Carnitine palmitoyltransferase I
NAFLD	Nonalcoholic fatty liver disease
HOMA-IR	Homeostatic model assessment for insulin resistance
LDL	Low-density lipoproteins
HDL	High-density lipoproteins
KE	Ketone ester
VT	Ventilatory threshold

Introduction

Ketogenic diets (KDs) are characterized by high and low contents of fat and carbohydrates, respectively[1]. These diets have been known for their therapeutic value in the treatment of neurological disorders such as epilepsy[2]. In recent years, the popularity of KDs has increased due to the fact that they can also be beneficial in the treatment of metabolic diseases primarily through improvements in whole-body glucose control[2]. Although KDs have been shown to have broad therapeutic potential, patient compliance can be challenging[2,3]. This is because along with severe dietary restriction and unpalatability of meals associated with KDs, those who are on a KD deal with possible hypoglycemic episodes and intolerance to high fat ingestion[3,4]. In this context, it has been suggested that oral supplementation of ketones may mimic a metabolic state of ketosis without any of the shortcomings of a typical KD, therefore serving as a practical alternative[3,5]. β-Hydroxybutyrate (βHB), acetoacetate, and acetone are the ketones produced by the body. However, βHB considered the most functionally relevant because of its signaling capabilities and the fact that it is less likely to undergo degradation and be eliminated relative to than acetoacetate and acetone[6,7].

Therefore, chronic supplementation of βHB has been suggested as an alternative approach to create ketogenic conditions without having to adhere to a typical KD. This has been tested both in rodent and human models[8]. Initial studies established that a state of ketosis can be achieved and maintained in humans and rodents with various types of ketone/βHB supplements without the need for dietary changes[5,9]. Significant metabolic effects of βHB supplementation were demonstrated by a study in which βHB salts were administered orally to rats[5]. The rats experienced an improved plasma lipid profile as well as an overall decrease in adipocyte volume[5]. Interestingly, high-fat-fed mice receiving a βHB-ester supplement displayed significant weight loss and an increase in

energy expenditure compared to high-fat and calorie-restricted mice[10]. In humans, supplementation of βHB salts resulted an overall increase in fat oxidation and a reduction in respiratory exchange ratio (RER), mimicking what is observed in individuals on a KD[11].

Even though several studies report that βHB supplementation can lead to alterations in substrate utilization and affect exercise performance, the molecular mechanisms responsible for the results described above remains unknown, although some hypotheses have been proposed. Once taken up by peripheral tissues, βHB is thought to affect cellular metabolism in two ways: a) by inhibiting class 1 histone deacetylases (HDACs), which regulate gene expression[12,13], and b) through post translational protein modifications including acetylation, succinylation, and β-Hydroxybutyrylation[1,12]. One of the most important peripheral tissues that utilize βHB is skeletal muscle, which accounts for up to ~40% of body weight in men and ~30% in women. Additionally, skeletal muscles are the primary site of insulin-stimulated glucose uptake and play a major role in the regulation of glucose and fat homeostasis in the body[14,15]. Therefore, any potential effect of βHB on muscle could be of great physiological and therapeutic relevance and worthy of further investigation.

In this context, the research described in this thesis was designed to investigate the effects of βHB on skeletal muscle metabolism. To accomplish that, an in vitro model using L6 rat skeletal muscle myoblast cells were used. Using L6 myoblasts allowed for direct assessment of βHB on skeletal muscle without potential confounding systemic influences on skeletal muscle metabolism. Alterations to important components of glucose and fat metabolism specifically, were analyzed using various functional assays including: glucose uptake, glucose oxidation, glycogen synthesis, lactate production, and palmitate oxidation in myoblasts. Western blot analysis was used to assess alterations in signaling pathways related to glucose and fat metabolism. These included

phosphorylation protein kinase B (AKT), AMP-activated protein kinase (AMPK), glycogen synthase (GS), and glycogen synthase kinase 3 (GSK3), as well as content of carnitine palmitoyl transferase 1B (CPT1B). Using these data, the role of βHB in the regulation of skeletal muscle cell metabolism was evaluated.

Literature Review

Metabolism of ketones

A typical KD consists of a 4:1 ratio of grams of fat to carbohydrates, while reducing total carbohydrate intake to less than 50 grams per day. More restricted KDs can reduce carbohydrate consumption to as low as 20 grams per day. Generally, KDs suggest obtaining 70-80% of total daily calories from fat, 5-10% from carbohydrates, and 10-20% from protein. The amount of protein is kept moderate as too little protein intake can lead to muscle loss and decreased satiety. Too much protein intake can prevent ketosis as gluconeogenic amino acids can be converted into glucose[16,17,18,4,19].

KDs induce metabolic changes in the organism by lowering blood glucose levels as a result of reduced carbohydrate intake[20,21]. This leads to a reduction in insulin release and increased glucagon secretion from the pancreatic β and α-cells, respectively[21,6]. The low insulin: glucagon ratio increases hepatic gluconeogenesis to provide glucose to tissues such as brain and red blood cells that rely on glucose for energy production[20,21,6]. Low levels of circulating insulin enhances lipolysis and allows the body to mobilize fat stores within adipose tissue[6,22]. Stored triglycerides within adipocytes are actively hydrolyzed into non-esterified fatty acids (NEFAs) and glycerol, which are then released into the bloodstream[22,23]. Glycerol is taken up by the liver where it is used as a substrate for gluconeogenesis[22,23]. With the exception of erythrocytes and the brain, peripheral tissues uptake NEFAs where they enter mitochondria, peroxisomes, or endoplasmic reticulum to undergo oxidation[6,22,23]. An increase in hepatic uptake of NEFAs leads to high rates of β-oxidation resulting in surplus acetyl-CoA production, which is directed towards ketogenesis[24]. The ketone bodies, namely β-hydroxybutyrate (βHB), acetoacetate (AcAc), and acetone are formed (Fig. 1)[6,25]. In addition to increased acetyl-CoA produced from β-oxidation, the formation of ketone bodies is exacerbated by the reduction in hepatic mitochondrial oxaloacetate. This component of

the TCA cycle is diverted to gluconeogenesis, which reduces its availability for condensation with acetyl-CoA and formation of citrate[21]. As a consequence, more acetyl-CoA is diverted toward the formation of ketone bodies[6,25].

Acetoacetate (AcAc) is initially formed by the condensation of two acetyl-CoA molecules and a small fraction of AcAc enters the circulation to be taken up by peripheral tissues for energy production[26-29] (Fig. 1-2). Another fraction spontaneously decarboxylates into acetone, which is volatile and can be eliminated through breathing. In fact, it is acetone that confers the "fruity odor" to the breath of individuals in ketosis[27,28]. Most of the AcAc produced however, is reduced to βHB, which is the primary circulating ketone[29]. Under conditions of low carbohydrate intake, AcAc and primarily βHB play a key role in sparing glucose for use by the brain and erythrocytes, which cannot utilize fatty acids for energy[26]. In the case of the brain, βHB can function as an alternative energy source accounting for up to two thirds of its energy needs while on a KD or during periods of prolonged fasting or starvation[29].

Ketone bodies are present in small amounts in the circulation of healthy individuals with basal serum levels of βHB typically in the low μM range (25-150μM) rising up to a few hundred μM (150-900μM) with 12-16 hours of fasting[2,30]. Significant elevations in circulating ketone body levels are seen in specific physiological states[29]. For example, two days of fasting can bring serum levels of βHB up to 1-2mM, where prolonged starvation can result in βHB levels reaching 6-8mM[2,30]. compared to basal levels. Variations in βHB levels are thought to be caused by differences in hepatic glycogen stores, basal metabolic rate, and mobilization of amino acids from muscle protein break down[29]. Similar levels can be attained with 90 minutes of intense exercise or by adopting a KD[2,30]. Infants can achieve serum βHB levels of 0.5-2.5mM while on diet typical for a child of that age. Within 8-10 hours, younger children have been found to enter a state of

ketosis and achieve ketone body turnover rates similar to adults who have fasted for several days[2,30]. This relatively efficient ketone body production and utilization is especially important in the days following birth when the brain relies on βHB as its primary source of energy[2,30]. The elderly can generate levels similar to young children after a fast or when adopting a KD[29].

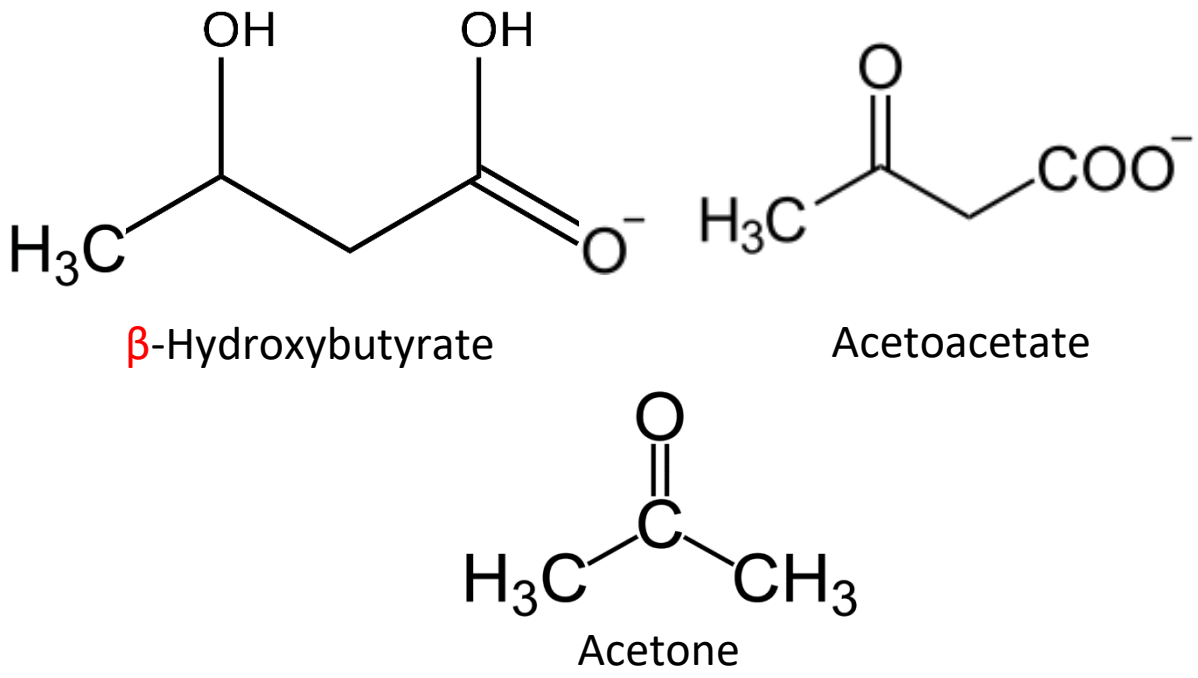

Figure 1: Molecular structure of ketone bodies produced in the body.

Ketone production, transport, and utilization

Ketogenesis primarily occurs in the mitochondria of perivenous hepatocytes, with scarce amounts being produced in other tissues[12]. Under typical aerobic conditions, fatty acids and glucose are converted to acetyl-CoA by β-oxidation and glycolysis, respectively[31,28]. Typically, acetyl-CoA enters the TCA cycle where it undergoes two decarboxylations and two molecules of CO_2 are formed[26]. Each round of the cycle releases high energy molecules including GTP, 3 NADH, $FADH_2$, and $CoQH_2$, which produce ATP through oxidative phosphorylation[26]. In a state

of ketosis, the lack of oxaloacetate available for the TCA cycle is reduced, thereby further driving acetyl-CoA towards ketogenesis.

The process begins with the formation of acetoacetyl-CoA by the condensation of two acetyl-CoA molecules in a reversible reaction catalyzed by acetoacetyl-CoA thiolase (AcAcT)[29] (Fig. 2). Mitochondrial HMG-CoA synthase (HMGCS2) then condenses acetoacetyl-CoA with another acetyl-CoA to form β-Hydroxy β-methylglutaryl-CoA (HMG-CoA)[26,31]. Acetoacetate is then formed when HMG-CoA lyase (HMGCL) cleaves an acetyl-CoA off of HMG-CoA. AcAc can then be reduced to βHB via the catalytic enzyme β-hydroxybutyrate dehydrogenase (BDH1)[26]. Once produced, βHB and a small fraction of AcAc are thought to be transported out of hepatocytes by the monocarboxylate transporter SLC16A6[26]. As small (4 carbon) polar molecules, βHB and AcAc are readily soluble in the blood stream. Various other monocarboxylic transporters including MCT1 and MCT2 are responsible for uptake into target tissues as well as movement of ketones across the blood brain barrier[1,12] .

Once taken up by peripheral tissues, ketone bodies undergo ketolysis, which is essentially the reversal of ketogenesis[27,12]. Ketolysis begins with the oxidation of βHB back to AcAc by BDH1, which was the last reaction in ketogenesis. At this point, ketolysis diverges from ketogenesis and avoids the irreversible reaction catalyzed by HMGCS2 in ketogenesis where HMG-CoA is produced[12,28]. Instead succinyl-CoA donates its CoA to AcAc to form acetoacetyl-CoA, a reaction catalyzed in most peripheral tissues by 3-ketoacid coenzyme A transferase (OXCT1)[12,28]. These differing enzymatic routes of production and utilization along with the fact that OXCT1 is not expressed in the liver, prevent a futile cycle of βHB production and synthesis[27]. Acetoacetyl-CoA thiolase then converts acetoacetyl-CoA along with a free CoA into two molecules of acetyl-CoA, which then enter the TCA cycle for ATP production[28,31]. The rate

limiting step in ketolysis involves Acetoacetyl-CoA thiolase which uses succinyl CoA from the TCA cycle as its CoA donor. Its activity is down regulated by AcAc levels equal to or higher than 5 mM[26].

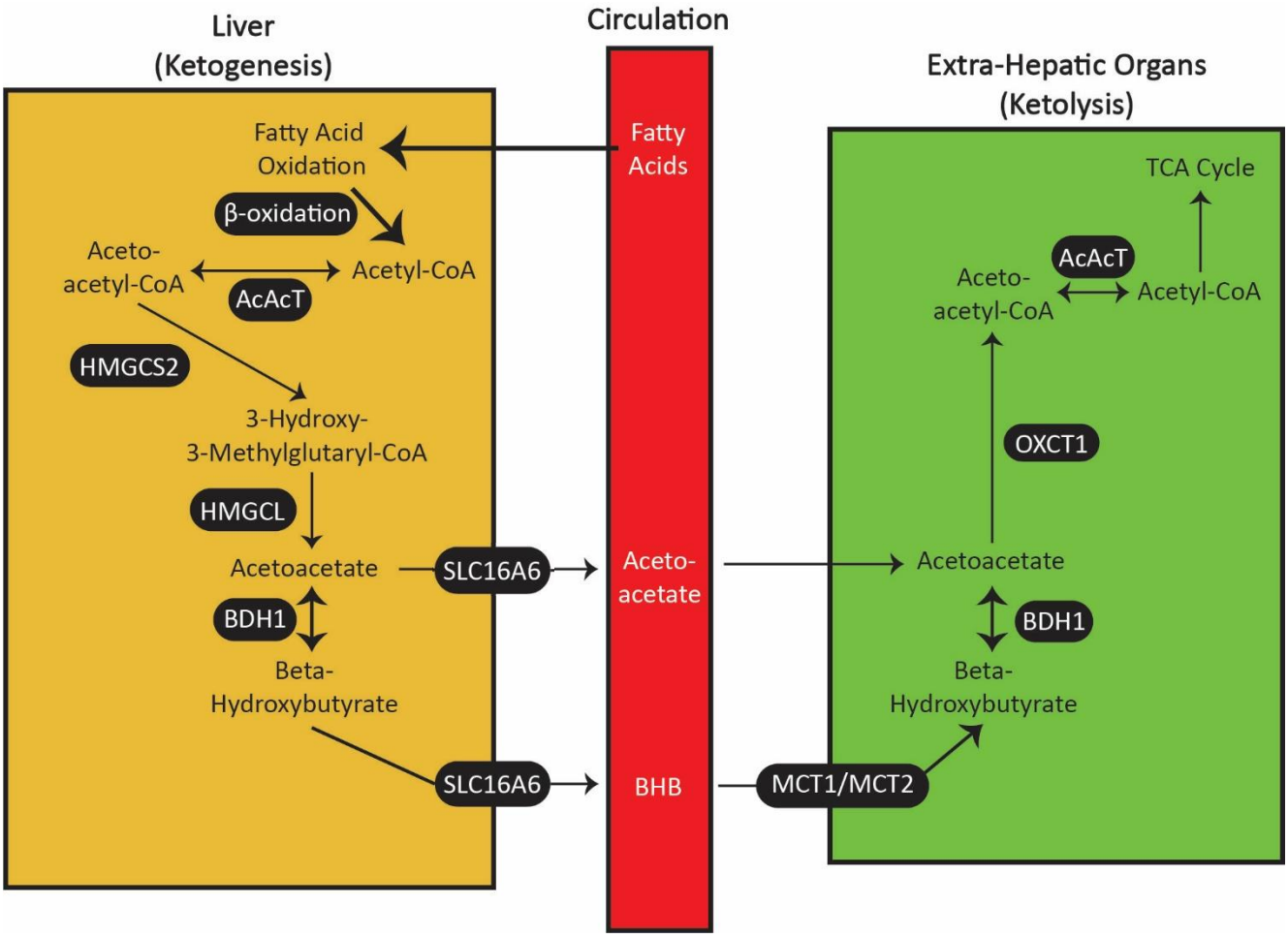

Figure 2: Outline of ketone body production and utilization. Increases in hepatic NEFA uptake leads to high rates of β-oxidation, which creates a surplus of acetyl-CoA that is directed towards ketogenesis. Ketone body formation is exacerbated by the reduction in hepatic mitochondrial oxaloacetate of the TCA cycle, which is directed towards gluconeogenesis, reducing its availability for condensation with acetyl-CoA. A small fraction of Acetoacetate production enters circulation while most of it is converted to β-hydroxybutyrate, which then enters circulation. β-hydroxybutyrate is taken up by peripheral tissues such as the brain and skeletal muscle for energy production.

Regulation of Ketone Metabolism

Understanding the regulation of ketone body metabolism which includes aspects such as production and utilization is crucial for the understanding of βHB signaling properties[12]. An important point in regulation occurs at the enzyme BDH1, which is responsible for interconverting AcAc and βHB in the final step of ketogenesis and the first step of ketolysis[31]. Ketogenesis is regulated through two mechanisms: 1) substrate availability in the form of fatty acids, and 2) through the expression of the enzyme HMGCS2 responsible for the synthesis of HMG-CoA[32]. Both mechanisms are regulated by the insulin: glucagon ratio, which controls NEFA mobilization from adipose tissue and HMGCS2 expression via transcription factor FOXA2 regulation[12,27,28]. Insulin and glucagon regulate FOXA2 in an opposing manner with insulin signaling leading to the inactivation of FOXA2 via phosphorylation and nuclear export. Glucagon activates FOXA2 via p300 acetylation allowing it to bind to the HMGCS2 promoter and activate transcription[33,34]. FOXA2 acetylation/activation is regulated in part by SIRT1, a NAD-dependent enzyme along with other class 1 and 2 HDACs[35]. HMGCS2 gene expression is also indirectly regulated by the protein complex mammalian target of rapamycin complex 1 (mTORC1)[6,36]. The inhibition of mTORC1 is necessary for the activation of fibroblast growth factor 21 (FGF21) and peroxisome proliferator-activated receptor alpha (PPARα), both of which are needed to induce ketogenesis and increase HMGCS2 gene expression[6,36]. Both PPARα and its target gene FGF21 are dramatically up-regulated in liver after fasting or by ketogenic diets, and mice lacking either PPARα or FGF21 have reduced levels of ketogenesis[6,36].

Post-translational regulation of HMGCS2 activity occurs through acetylation and succinylation, which the mitochondrial deacetylase SIRT3 and desuccinylase SIRT5 carry out[37]. SIRT3, which is NAD dependent, has been found to regulate many pathways involved in fasting

metabolism. In fact, mice lacking SIRT3 displayed lower levels of βHB production upon fasting[37,38]. Many of the enzymes involved in ketogenesis undergo acetylation and contain at least one site of interaction with SIRT3 for deacetylation[28]. Utilization of βHB in peripheral tissues involves the transfer of CoA from succinyl-CoA to AcAc to form acetoacetyl-CoA. This process therefore reduces the amount of succinyl-CoA available for post translational protein modification. SIRT5, a desuccinylase, regulates various mitochondrial pathways related to fasting metabolism. The enzyme HMGCS2 has been known to have reduced activity as a result of being succinylated[2,39]. The mechanism driving succinylation is not yet clear, but it's been suggested that there is some level of dependence on succinyl-CoA levels[2,39]. This is based on the fact that both HMGCS2 succinylation and succinyl-CoA abundance in the liver is reduced following treatment of rats with glucagon[2,39]. Succinyl transferase is also not known to be present in mammalian cells indicating that succinylation is primarily a non-enzymatic process dependent on local concentrations of succinyl-CoA[12] .

βHB and Carbohydrate Restricted Diets

Metabolic states where energy intake is restricted or modified in terms of macronutrients (*e.g.* caloric restriction and intermittent fasting) have been shown to metabolically benefit individuals suffering from metabolic disease[40]. These metabolic states are associated with the production of ketones and achieving a state of ketosis. Adhering to a ketogenic diet, which restricts carbohydrates to just 5% of caloric intake, is the most effective way to achieve ketosis[12,40]. This was demonstrated by a study that placed high-fat-fed mice in one of three conditions: control (continued high-fat diet), calorie restriction (25% restriction), or continued high-fat diet with ketone supplement replacing 30% of caloric intake to achieve ketosis[10]. Mice supplemented with ketones experienced significantly greater weight loss and increase in energy expenditure compared

to the calorie-restriction condition[10]. This indicates that the most beneficial effects are observed with achieving a state of ketosis through adequate carbohydrate restriction or in the case above, through adequate ketone supplementation to simulate ketosis[31]. As we continue to develop a greater understanding of the mechanisms involved with ketogenic diets and ketone supplementation, interest in using these potential therapies for metabolic issues has grown significantly.

Most of the understanding on the biological effects of KDs originated from rodent studies, which observed effects on metabolism resembling those of typical high-fat or western diets (high in fat and carbohydrates)[41]. This resemblance was demonstrated by a study that subjected lean healthy mice to a KD, which experienced changes in metabolism similar to mice subjected to a high fat diet[42]. These changes included; increased fasting leptin, hyperglycemia with insulin resistance, and a strong induction of genes involved in fatty acid oxidation such as acly-CoA dehydrogenase[42,43]. KDs differ from high fat diets because they cause additional changes to metabolism which include; suppressing hepatic expression of enzymes involved in fatty acid synthesis such as stearoyl-CoA desaturase 1, low insulin levels, and reduced IGF signaling[43–45]. KDs were also associated with increased activity of PPARα, FGF21, PGC1α, and AMPK, along with mTOR repression. A net result of these changes is improved glucose tolerance and insulin sensitivity, which is especially apparent in diabetic and obese mice[43–45].

In comparison to rodent studies, there is a relatively limited amount of data on the use of KDs in adult humans[46]. Data gathered so far suggests that individuals on a KD experienced weight loss and improved insulin sensitivity[47]. This is in comparison to other dietary conditions, which includes caloric restriction, intermittent fasting, and low fat – high carbohydrate diets[47,48]. These conditions were examined in obese, type 2 diabetic humans, which were randomized to either a

low carbohydrate, ketogenic diet (<20 g of carbohydrate daily; KD-fed), or caloric restriction (500 kcal/day deficit from weight maintenance diet)[48]. Both groups experienced improvements in hemoglobin A1c, fasting glucose, fasting insulin, blood lipid profile, and weight loss[48]. However, the KD-fed group experienced significantly greater improvements in these measurements, which led to 95.2% of KD-fed participants to reduce or eliminate diabetic medications compared to 62% of participants in the caloric restriction group[48].

Absolute and Optical Configuration of βHB

The absolute configuration of βHB or chirality is based on the presence of an asymmetric carbon with 4 unique substituents is one of a few key features that give a molecule this property. Mirror images of a chiral molecule are called optical isomers or enantiomers which can either be clockwise (R) or counterclockwise (S)[49,50]. Directionality is determined based on the prioritized order of the 4 unique groups on the chiral carbon. βHB is a chiral molecule at the 3' hydroxyl group, which is an important property to consider when examining its signaling properties and possible therapeutic applications[1]. The R enantiomer (R-βHB) is the usual product in human metabolism. The enzyme BDH1 which catalyzes the final step in ketogenesis and the first step in ketolysis, introduces the R- βHB specificity. BDH1 does this by catalyzing the reduction of the non-chiral 3' carbonyl on acetoacetate to a chiral 3' hydroxyl group on βHB. This way BDH1 ensures that only R- βHB is produced during endogenous βHB production, and only R-βHB can be metabolized into acetyl-CoA and ATP[49,50]. Although S-βHB is not a normal product of βHB production, a version of it, S-βHB-CoA has been found in the final step of β-oxidation of fatty acids as a transient intermediate. Normally, it does not persist long enough for it to leave the mitochondria and enter circulation[49,50]. Previous studies involving the infusion of radiolabeled S-βHB, R-βHB, or a mixture of the two into pigs and rats found that most of the S- βHB was

converted into R- βHB through an unknown molecular pathway[49]. There is evidence that S-βHB can be metabolized by BDH1 into acetyl-CoA, but at a significantly slower rate compared to R-βHB[51]. Infusion of S-βHB or a racemic mixture would result in higher levels of S-βHB being measured in circulation and being sustained for longer periods of time compared to R-βHB infusions[49,50].

The relative configuration designated by either a D (+) or L (-) is used to describe the form of βHB as well. Configuration is based on the direction in which the compound rotates the plane of polarized light. Rotation in the clockwise direction designates a D configuration, while rotation in the counter-clockwise direction designates a L configuration.

Research examining the more physiologically active form of βHB refer to it as either R-βHB or D-βHB[1]. Configurations become important when designing studies that involve supplementing subjects with βHB. The various forms of βHB supplementation include those in the ester, salt, and acid form. These forms typically come in a racemic mixture meaning that half of the supplement is significantly less active physiologically. This needs to be taken into account when creating solutions to attain a desired serum concentration of βHB within a subject[1]. Most studies however will specify the form of supplement and its makeup.

Regulation of metabolism through βHB signaling

The ability of an organism to completely adapt to a fasting or low-carbohydrate state appears to be in part due to the integration of the various direct and indirect signaling functions of βHB. The transition of the body into ketosis is brought on by a two-part mechanism comprised of a high fat – low carbohydrate diet, and the resulting production of ketones, primarily βHB. This alters the metabolism of peripheral tissues as well as whole-body glucose and fat metabolism[12]. Regulation of gene expression and protein signaling are common themes mentioned when

considering the direct and indirect effects of βHB. Histones are at the center of these themes due to the role they play in regulating gene expression when subjected to post-translational modifications, which include acetylation, succinylation, and β-hydroxybutyrylation[1]. Histone acetylation is a well understood phenomenon capable of broad and specific gene regulation. βHB can alter acetylation through direct inhibition of histone deacetylases (HDACs), and indirectly through increased acetyl-CoA flux thereby promoting acetyltransferases[1].

Class 1 HDACs are a family of proteins that play a crucial role in regulating gene expression of histone and non-histone proteins through deacetylation of their lysine residues[1,52]. Class 1 HDACs which include HDAC1, HDAC2, HDAC3, and HDAC8, are small proteins mainly located in the nucleus. They are largely made up of a deacetylase domain and are regularly found in large multi-protein regulatory complexes[27,28]. Increases in the expression of genes regulated by class 1 HDACs have been correlated with histone hyperacetylation, and so increases in class 1 HDAC activity has been shown to suppress gene expression. Numerous non-histone proteins such as MYOD1, NF-κB, TP53, MYC, etc., also undergo HDAC regulation[52,53]. βHB has been found to inhibit HDAC1, HDAC3, and HDAC4 which are a part of classes 1 and 2a deacetylases. Treatment of certain cell culture lines such as macrophages and neurons with βHB have shown a dose-dependent induction of hyperacetylation, specifically at lysine residues 9 and 14 on histone H3[31,39]. Studies involving fasting mice showed that increases in histone acetylation in numerous tissues including the liver, correlated with increases in plasma βHB levels. βHB treatment of mice using an osmotic pump resulted in histone hyperacetylation, most notably in the kidney, as well as changes in the expression of genes such as forkhead box O3 (Foxo3a)[44,54]. HDAC1 and HDAC2 have been found on the promotor of Foxo3a, indicating that its induction is related to HDAC inhibition. Knockdowns of both HDAC1 and HDAC2 relieved HDAC-mediated suppression on

Foxo3a with βHB treatment resulting in hyperacetylation of histones at the Foxo3a promoter[55,56]. The mechanism controlling HDAC inhibition is considered to center around competitive inhibition of the catalytic site. Studies examining the inhibition of human class 1 HDAC8 found that various hydroxamic and carbonic acid inhibitors bound to the catalytic zinc found in the hydrophobic active site[1,57]. βHB is structurally similar to butyrate, a well-known inhibitor of HDACs, differing only in the oxidation state of the 3'carbon. With similar kinetics to competitive inhibitors, the carboxylic acid of βHB is thought to chelate the catalytic zinc in a manner similar to the acidic groups on other HDAC inhibitors[1,57]. Increasing oxidation may be a barrier to binding within the hydrophobic channel of the HDAC active site, and as expected, the IC50 of βHB for HDAC1 increases with the oxidation state[53].

HDAC inhibition by βHB could affect the pathogenesis of type 2 diabetes through direct regulation of HDAC-dependent glucose metabolism and by promoting resistance to oxidative stress[58]. Studies in knockout mice have shown that class 1 HDACs play a key role in regulating metabolism, especially in models of metabolic disease[1,12]. HDAC3 for example, regulates gluconeogenic genes and HDAC3 knockout mice displayed reduced insulin and fasting glucose levels[44,52]. Mice treated with βHB resembled HDAC3 knockout mice and remained essentially normal metabolically while on a high fat diet[59]. This supports the idea that βHB is associated with lower insulin and glucose levels, improved glucose tolerance, and prevent weight gain[60,61]. βHB can also provide these benefits to mice already obese due to a high fat diet[62].

SAHA, another class 1 HDAC inhibitor, administered to a diabetic mouse model improved insulin sensitivity, lowered serum glucose levels, and reduced body fat[63]. The mechanism underlying class 1 HDAC inhibition and these metabolic effects could be an up-regulation of PGC1α in various tissues by relief of HDAC3-mediated transcriptional repression[62,63]. FGF21

transcription is up-regulated in a similar fashion through HDAC3 inhibition via βHB, thereby triggering ketogenesis in obese mice[64]. The effects of HDAC3 inhibition has not been fully explored yet, let alone inhibition of other class 1 HDACs in relation to metabolic regulation[65].

The micro and macro vascular complications of type 2 diabetes such as cardiovascular disease is thought to be partially caused by increased oxidative stress brought on by various pathways such as the activation of protein kinase C[66]. With this in mind, the newly discovered role of βHB in possibly suppressing oxidative stress could be relevant to managing the complications of type 2 diabetes[66]. As previously described, βHB activates FOXO3 transcription and increases downstream antioxidant gene expression via HDAC inhibition[44]. This is all associated with protection of the mouse kidney from oxidative stress and damage, which is in accordance with studies that have suggested a protective role for βHB and HDAC inhibition against oxidative and/or ischemic stress[44]. Specifically HDAC 2, which is a class 1 HDAC, has abnormally increased activity in the kidney of diabetic mice to protect against the oxidative stress caused by transforming growth factor beta 1 (TGFβ)[67]. Similar to a ketogenic diet, βHB administration has shown protective effects against ischemic neuronal death, myocardial damage, and the effects of hydrogen peroxide, which is a source of free radicals[67,68]. A possible mechanism for the protective antioxidant effects of βHB in type 2 diabetes and associated vascular complications could be an induction of antioxidant genes through βHB-induced HDAC inhibition[1,69]. Even with the wide spread role that HDACs play in regulating changes in gene expression in response to stimuli, the exact mechanisms behind this regulation have yet to be studied systematically[1].

Succinylation of lysine residues is another post translation protein modification controlled indirectly by βHB. During the process of ketolysis through which βHB is converted to acetyl-CoA, succinyl-CoA donates its CoA to AcAc to form acetoacetyl-CoA. This process thereby reduces

the amount succinyl-CoA available for succinylation[1,31]. Histone β-hydroxybutyrylation remains largely unstudied, but should receive strong interest because of its ability to respond to changes in metabolism by modifying gene expression accordingly[31].

β-Hydroxybutyrylation of proteins

In addition to inhibiting regulatory enzymes involved in post-translational modifications such as HDACs, βHB can itself directly modify proteins at the post-translational level[1,69]. Histones that have undergone β-hydroxybutyrylation at specific lysine residues are referred to as KβHB, and they have been detected by mass spectrometry in yeast, fruit fly, mouse, and human cells[1,69]. Western blot analysis revealed increased in histone KβHB in human cells, an effect that was proportional to exogenous βHB treatment[1,69]. This was also detected in mouse liver, which was proportional to increases in plasma βHB levels under conditions of diabetic ketoacidosis. In the case of both humans and mice, a 40-fold increase in the relative abundance of KβHB was caused by fasting[69]. At least 40 histone lysine sites have been identified that are capable of β-hydroxybutyrylation, some of which are crucial for transcriptional regulation such as on the histone H3K9[1,69]. Histone H3K9 has been shown to be associated with the promotors of actively transcribed metabolic genes through chromatin immunoprecipitation (ChIP)[1,69]. When RNA gene expression data was compared with ChIP data from fasted mouse livers, there was a strong correlation between genes with the greatest increase in KβHB at the promoter and those with the greatest increase in expression. H3K9 KβHB has been associated with increased gene expression independent of other activation markers such as acetylation and methylation, suggesting an independent function which isn't yet clear[69,54]. It is unknown whether non-histone proteins can undergo β-Hydroxybutyrylation and whether this only occurs in the liver. It is also unclear if any enzymes participate in the addition and/or removal of βHB from lysine residues, which would

provide an opportunity for fine-tuned regulation and specific targeting of β-hydroxybutyrylation. Overall, β-hydroxybutyrylation may be a key point in gene regulation associated with fasting and other conditions linked to increased βHB levels[1,54,69].

Acetyl-CoA as a substrate for protein acetylation

The catabolism of βHB ends in its conversion to acetyl-CoA, which would overall raise acetyl-CoA levels in target tissues and facilitate enzymatic and non-enzymatic protein acetylation[12]. This post-translational protein modification would complement the HDAC inhibition by βHB and together may have broader effects on various cellular components and functioning[12]. Acetylation of proteins in the mitochondria is especially sensitive to increases in acetyl-CoA, as conditions associated with increased lipid utilization such as high-fat diets, fasting, and dietary restriction, increase mitochondrial protein acetylation[70]. This increase in mitochondrial protein acetylation occurs despite the fact that neither acetyltransferases nor βHB inhibited HDACs entering the mitochondria[70].

Increased acetyl-CoA pools also affect acetylation of nuclear proteins. Mitochondrial acetyl-carnitine is the source of acetyl-CoA used for nuclear histone acetylation[71]. The citrate shuttle, regulated by citrate synthase inside the mitochondria and ATP citrate lyase in the cytoplasm, is responsible for the export of acetyl-CoA from the mitochondria[72]. An alternate pathway for mitochondrial export of acetyl-CoA is via the enzymes carnitine acetyl transferase and carnitine/acyl carnitine translocase[72]. A muscle specific knockout of carnitine acetyl transferase in mice resulted in reduced glucose tolerance and insulin sensitivity[73]. This study provided a clear example of the importance of intracellular acetyl-CoA transport to overall metabolic health as well as the potential impact βHB has on acetyl-CoA levels[72,73].

βHB receptors

Of the various G-protein coupled receptors (GPCRs) that bind to fatty acid ligands and, there are at least two that also bind βHB [12,74]. One is hydroxycarboxylic acid receptor 2 (HCAR2), which has been shown to bind and be activated by βHB leading to reduced lipolysis in adipocytes[12,75]. This may represent a type of feedback mechanism to aid in regulating the availability of non-esterified fatty acids (NEFAs) in the circulation. Without this mechanism, unregulated lipolysis in adipocytes has been shown to greatly elevate NEFAs in the circulation and contribute to insulin resistance[75,76]. HCAR2 activation has also been shown to decrease plasma glucose, thereby contributing to improve glycemic control and alleviate some of the vascular complications in type 2 diabetic patients[75,76].

Free fatty acid receptor 3 (FFAR3) is another GPCR highly expressed in sympathetic ganglions[31,77]. Some studies have reported that βHB binding to FFAR3 suppressed sympathetic tone and reduced heart rate, whereas other studies have found the opposite[77,78]. Thus, it may be that βHB binding to FFAR3 is responsible for sympathetic depression during fasting, although further studies are required to test this hypothesis.

Cytoplasmic and mitochondrial NAD: NADH equilibrium

The ratio between NAD and NADH in cytoplasmic and mitochondrial pools is emerging as an important aspect of cellular metabolism[1,79]. This ratio is first altered when βHB is utilized in peripheral tissues such as skeletal muscle, where it is converted to AcAc by mitochondrial BDH1 using 2 NAD as a cofactor, which generates 2 NADH[31]. This differs from the metabolism of glucose which converts 4 NAD into NADH, 2 of which are in the cytosol[31]. High glucose utilization would lead to a decrease in NAD relative to NADH. Combined with the fact that cytoplasmic NADH is shuttled into the mitochondria for ATP generation, the NAD: NADH ratio

may be further imbalanced[79]. With mitochondrial and cytoplasmic pools being relatively distinct, preservation of the cytoplasmic pool can have important effects on cellular metabolism[80,81]. Preservation of these discrete pools and maintain the NAD: NADH balance would affect the functioning of enzymes that use NAD as a cofactor[80,81]. An example of this is the enzyme PARP that uses NAD in the process of repairing single strand DNA breaks. Depleted cytoplasmic pools of NAD would leave PARP with an inadequate NAD supply, which has been associated with metabolic and age-related diseases[79,82]. This includes atherosclerosis, other cardiovascular disorders, dementia, diabetes, arthritis, and osteoporosis[79,82].Conversely, replenishment with nicotinamide mononucleotide improves glucose tolerance in high-fat fed mice[80,81]. This indicates that NAD sparing through βHB metabolism may have important consequences for metabolic diseases and diabetes through changes in cellular metabolism[12].

Protein Kinase B (AKT)

Skeletal muscle plays an important role in glucose metabolism due to the fact that it is the primary site of insulin mediated glucose uptake and makes up approximately 40% of body weight in humans. With this in mind, it is crucial that the cellular metabolic pathways related to glucose metabolism and how they may be affected by βHB, be examined. Two major metabolic pathways related to insulin signaling and glucose homeostasis[83,84]. The first one is the PI3K-AKT pathway that is activated by insulin signaling[83]. The PI3K-AKT pathway is considered to be the primary pathway directly stimulated by insulin signaling that mediates energy metabolism and insulin sensitivity(fig. 3)[83,84]. The phosphatidyl inositol 3 kinase (PI3K)-AKT pathway is engaged upon insulin binding to its receptor on the plasma membrane. This leads to auto-phosphorylation and a conformational change of the insulin receptor (IR) with subsequent recruitment and phosphorylation of the insulin receptor substrate (IRS) on key tyrosine residues[83,84].

These residues are recognized by PI3K via its p85 regulatory subunit, which allows its catalytic subunit, p110 to phosphorylate phosphatidylinositol (4,5) bisphosphate ($PI(4,5)P_2$). As a result, phosphorylate phosphatidylinositol (4,5) triphosphate ($PI(3,4,5)P_3$) formed and anchored to the plasma membrane[83,84]. $PI(3,4,5)P_3$ is then able to bind directly to proteins with a pleckstrin homology, which include AKT and 3-phosphoinositide-dependent protein kinase 1 (PDK1). The binding of AKT leads to a conformational change which exposes phosphorylation sites Thr308 and Ser473. PDK1 which is also bound to $PI(3,4,5)P_3$, phosphorylates AKT at Thr308[85]. Complete activation of AKT requires phosphorylation at Ser473 which is regulated by mammalian target of rapamycin complex 2 (mTORC2)[86]. Once activated, AKT phosphorylates and inhibits AS160[86]. AS160 is a Rab GTPase activating protein that converts the active GTP bound form of Rab into the non-active GDP bound form. Rab proteins regulate many steps of membrane trafficking, including vesicle formation, vesicle movement along actin and tubulin networks, and membrane fusion[85,86]. RabGTPs stimulate the translocation of vesicles carrying GLUT4 transporters to the plasma membrane where they fuse[85,86]. This allows GLUT4 transporters to localize to the plasma membrane and increasing glucose uptake[85,86].

5′ AMP-activated protein kinase (AMPK)

AMP-activated protein kinase (AMPK) has wide range of functions ranging from mitochondrial biogenesis and angiogenesis, control of food intake and energy expenditure, to regulation of gene transcription[85,87]. It is an energy sensing enzyme that is activated by anabolic processes that increase the intracellular AMP:ATP ratio, which can occur due to either increased of energy expenditure or limited ATP production[88]. Once activated, catabolic processes that increase ATP production are stimulated (e.g. glucose and fatty acid oxidation), whereas ATP-consuming anabolic pathways are suppressed (e.g. cell growth and proliferation)[85,88]. AMPK

activation has been shown to counteract many physiological abnormalities such as insulin resistance, inflammation, and ectopic lipid deposition, especially in skeletal muscles[87,89]. These abnormalities are typically dealt with in part by diet regiments including caloric restriction, intermittent fasting, and high fat-low carbohydrate (ketogenic) diets, which have been associated with AMPK activation in rodent and human models[1,87]. This is demonstrated in a study in which rats were subjected to either caloric restriction, the AMPK activator AICAR, or regular exercise[87,89]. Caloric restriction was found to increase AMPK activity to a degree resembling that of the AICAR and regular exercise conditions[87,89]. Compared to caloric restriction, KDs also increase AMPK activation. Human work has found that KD induced activation occurs in various tissues, notably skeletal muscle via an increase in SIRT1 expression[87,90]. βHB has been shown to increase SIRT1 expression, which would become significant while on a KD. LKB1 is one of the proteins that are deacetylated by SIRT1, which activates it allowing it to phosphorylate and activate of AMPK[87,90].

AMPK is a heterotrimeric enzyme comprised of one catalytic subunit (α), and two regulatory subunits (β and γ)[88]. Its activation can be achieved by allosteric and covalent mechanisms[88,87]. Under conditions of lower ATP levels, AMPK is allosterically activated by ADP or AMP binding to the γ subunit. Bindings results in a conformational change, which opens AMPK up to phosphorylation[88,87]. AMP and ADP are also capable of binding to sites meant for nucleotides further allowing AMPK to respond to a variety of physiological changes[88,87]. AMPK activation is also regulated by NAD+ levels with SIRT1 being key to this pathway[88,87]. SIRT1 is a deacetylase that uses NAD+ as a cofactor[88,87]. Under conditions of an increased NAD+/NADH ratio, NAD+ allosterically activates SIRT1 which goes on to deacetylate the nuclear bound serine/threonine kinase LKB1[88,87]. After deacetylation, LKB1 moves into the

cytoplasm where it forms a complex with its allosteric activators, STRAD and MO25[91]. This leads to the second mechanism of AMPK activation, which is covalent activation through the phosphorylation of Thr172 by LKB1 or CAMKK (fig. 3)[91]. LKB1 is able to phosphorylate AMPK only once it is complexed and in the cytoplasm[91]. Intracellular Ca^{+2} levels are another regulator of metabolic pathways in muscle cells acting as an allosteric activator of AMPK. Protein kinases can be regulated by Ca^{+2} levels through calmodulin (Cam) kinases[88,87]. These Cam-dependent protein kinases (CamKs) are regulated by other CamKs referred to as (CAMKKs) which also possess the ability to phosphorylate AMPK at Thr172[88,87]. Once active, AMPK, like AKT, phosphorylates AS160 thereby deactivating it and allowing Rab to stay in its active GTP bound state and promote GLUT4 translocation and fusion to the plasma membrane[88,87].

Glycogen Synthase Kinase 3 (GSK3) and Glycogen Synthase (GS)

Glycogen synthesis begins with the phosphorylation of glucose to glucose-6-phosphate (G6P) via hexokinase. G6P is then converted to glucose-1-phosphate (G1P) by phosphoglucomutase. G1P can then be activated via the addition of a uridine nucleotide to form UDP-glucose, which is catalyzed by UDP-glucose pyrophosphorylase 2 (UGP2). Glycogen synthase (GS) then catalyzes the addition of the glucose within UDP-glucose to glycogen polymers[92]. GS is upregulated allosterically by glucose-6-phosphate while phosphorylation by glycogen synthase kinase 3 (GSK3) leads to inactivation[92,93]. GSK3 itself is a downstream target of the AKT/PI3K pathway and is therefore regulated in part by insulin signaling (fig. 3)[84]. Insulin signaling leads to the activation of AKT which phosphorylates Ser21 of GSK3α and Ser^{9} of GSK3β resulting in transient inactivation[93]. In the absence of insulin, phosphorylation of Ser^{21} and Ser^{9} is reversed by the protein phosphatases PP2A and PP1 allowing GSK-3 to remain active until further stimulation of the tissue by insulin or other PI3K agonists[84,93]. With GSK3 inactivated, it

is unable to phosphorylate GS allowing it to remain active and catalyze glycogen synthesis (fig. 3)[84,93]. In the case of GS being phosphorylated, the same phosphatases that act on GSK3 (PP2A and PP1) act on GS thereby activating it[84,93]. GS is also capable of being phosphorylated by AMPK and protein kinase A (PKA). Insulin signaling leads to PK1 activation which is responsible for the phosphorylation and activation of PKA[84,93].

Insulin signaling along with the glucose metabolism is affected by dietary changes including caloric restriction, intermittent fasting, and high fat-low carbohydrate (ketogenic) diets. KDs for example have been shown to improve signaling, glucose tolerance, and overall glucose metabolism. Considering the signaling capabilities of βHB and its potential to play a role in altering glucose metabolism, changes in glycogen synthesis as a result of βHB are possible. Through HDAC regulation or post-translational modification, βHB supplementation may alter the gene expression or activity of any one of the proteins involved in the glycogen synthesis pathway. An example of such targets are the phosphatases PP2A and PP1 which help regulate the activity of GS and GSK3 through dephosphorylation[84,93].

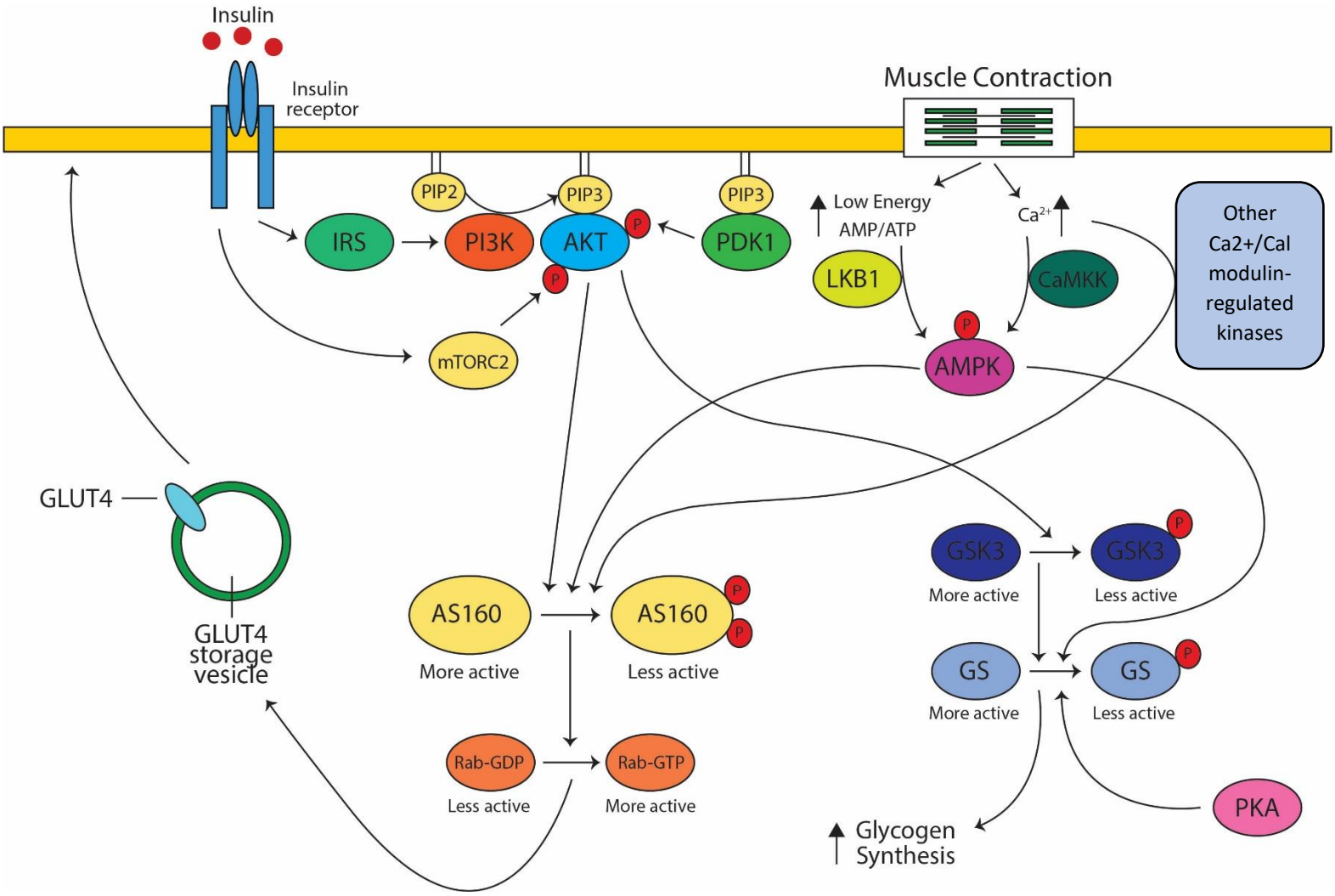

Figure 3: Signaling pathways for insulin and contraction stimulated glucose transporter 4 (GLUT4) translocation in muscle. Insulin stimulates AKT through phosphoinositide-dependent protein kinase-1 (PDK1) and mammalian target of rapamycin complex-2 (mTORC2). Activated AKT phosphorylates AS160, which inhibits its Rab-GTPase-activating protein (GAP). Inhibition of GAP promotes conversion of less active GDP-loaded Rab to more active GTP-loaded Rab. The more active GTP-loaded Rab then allows GLUT4 storage vesicles to move to and fuse with the plasma membrane. Energy depletion (elevated AMP/ATP ratio) through contraction and elevated intracellular [Ca^{2+}] leads to activation of AMP-activated protein kinase (AMPK) via LKB1 and Ca^{2+}calmodulin-dependent protein kinase kinase (CaMKK), respectively (and other Ca^{2+}-regulated protein kinases). Activated AMPK phosphorylates AS160 adding further regulation to GLUT4 membrane fusion. GSK3 is regulated by insulin signaling via phosphorylation by AKT, which leads to its inactivation. It is unable to phosphorylate GS allowing it to remain active and catalyze glycogen synthesis. AMPK is also capable of phosphorylating GS thereby deactivating it and reducing glycogen synthesis.

Carnitine Palmitoyl Transferase 1 (CPT1)

Carnitine palmitoyltransferase I (CPT1) is an integral membrane protein with three isoforms, CPT1A, CPT1B, and CPT1C. Isoform CPT1A is found on the mitochondria of all cells throughout the body, except for skeletal muscle and brown adipose tissue[94,95]. CPT1B is the isoform found in brown adipose tissue as well as heart and skeletal muscle. Little is known about the CPT1C isoform, which is expressed in the brain and testes. CPT1 is an integral membrane protein that possesses transmembrane regions allowing it to interact with the outer mitochondrial membrane (Fig. 4)[94]. CPT1B is a part of the carnitine palmitoyl transferase system which is essential for the beta-oxidation of fatty acids[94,95]. The system is responsible for transporting activated fatty acids outside the mitochondria into the mitochondrial matrix where they are oxidized[94,95]. Long chain fatty acids such as palmitoyl-CoA, unlike short and medium chained fatty acids, are unable to freely diffuse through the inner mitochondrial membrane thus requiring the shuttle system[94,95]. CPT1B is the first component of the carnitine palmitoyl transferase system and is responsible for catalyzing the rate limiting step of forming palmitoylcarnitine by transferring the acyl group from coenzyme-A to carnitine[96]. At this point palmitoylcarnitine is in the intermembrane space where a translocase then shuttles it into the mitochondrial matrix[95,96]. Once in the matrix, palmitoylcarnitine is converted back into palmitoyl-CoA. CPT1 activity is regulated by malonyl-CoA with the CPT1B isoform being significantly more sensitive than the CPT1A isoform[95,96]. Acetyl-CoA carboxylase (ACC) is the enzyme that catalyzes the formation of malonyl-CoA from acetyl-CoA making it an important regulator of fatty acid metabolism[95,96].

The reaction catalyzed by ACC is comprised of two half reactions; the ATP-dependent carboxylation of biotin with bicarbonate to form carboxybiotin, followed by transfer of the carboxyl group from carboxybiotin to acetyl-CoA to form malonyl-CoA[97]. Studies have suggested that diverse physiological conditions which include various diets, can affect fatty acid synthesis

via alterations in ACC activity[98]. AMPK for example is activated by AMP signifying a low energy state in the cell. This triggers a response by AMPK that maximizes ATP production and reduces anabolic processes such as fatty acid synthesis via phosphorylation and deactivation of ACC[6]. As mentioned above, AMPK activity can be upregulated in a ketogenic condition, possibly due to the activity of βHB.

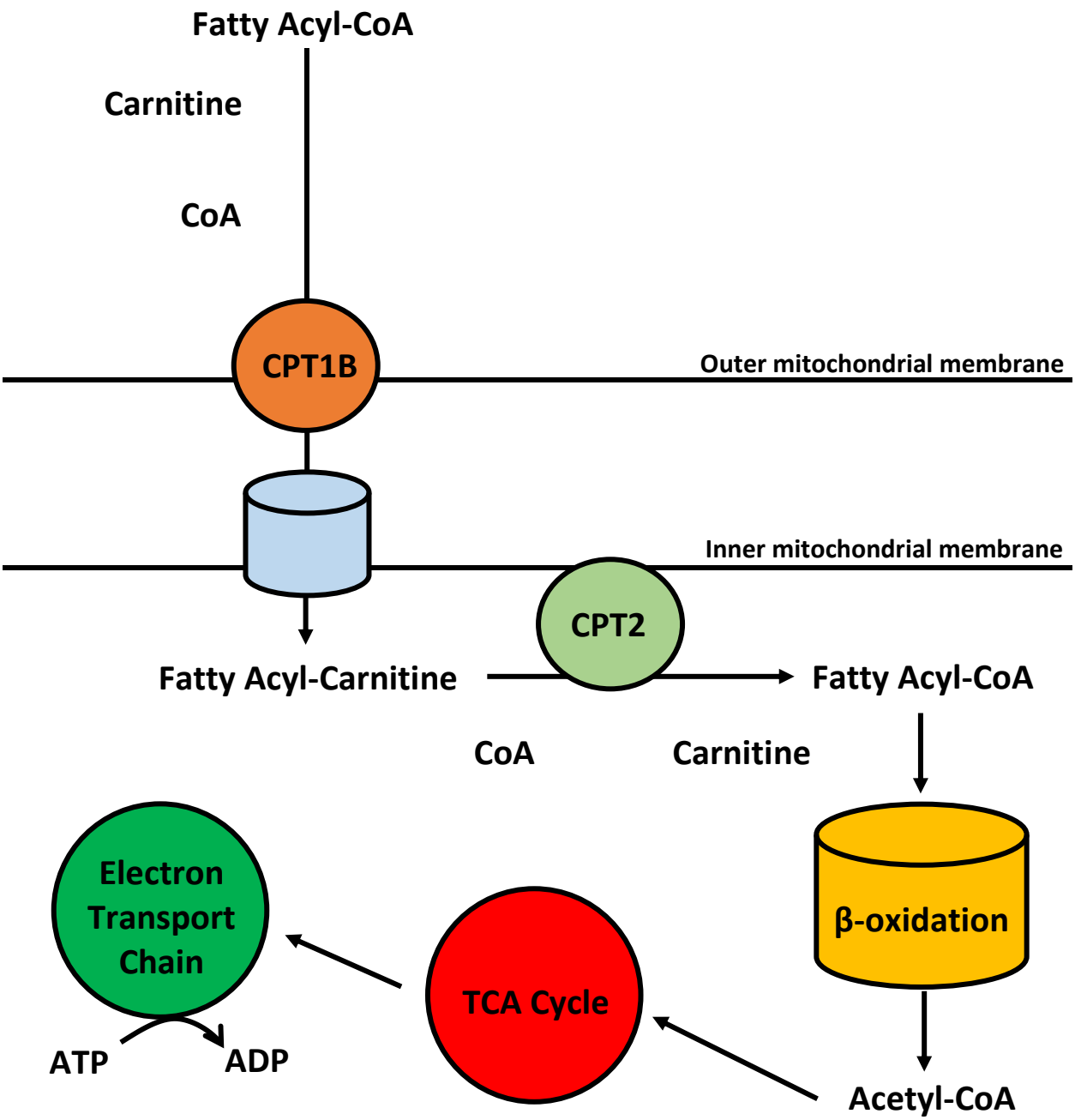

Figure 4: Schematic representation of b-oxidation pathway. The rate-limiting step in b-oxidation is the transport of activated fatty acids (fatty acyl-CoA) into mitochondria. This is catalyzed by CPT1B, which transfers carnitine for CoA, forming fatty acyl-carnitine. Once inside the mitochondrial matrix, CPT2 reforms fatty acyl-CoA, which enters the b-oxidation cycle, generating acetyl-CoA molecules from which ATP is generated through the TCA cycle and electron-transport chain.

Whole-body effects of a KD and exogenous ketone supplementation in rodents

The effectiveness of the KD as a treatment for various aspects of metabolic disease and ketone supplementation as a possible KD substitute has only recently begun to be investigated. Work focused on the cellular mechanisms responsible for the observed effects of KDs and ketone supplementation relative to work done with human and animal models. **S**tudies in rodents found that KD effectively stimulated weight loss along with changes in body composition. Garbow et al. found that after 12 weeks of KD, there was significantly lower weight gain compared to mice on various diets including chow-fed, high carbohydrate, and high-fat western diet. Lean mass was found to decrease significantly in KD-fed mice compared to mice on the other diets. The decrease in lean mass could be attributed to the protein content of the KD comprising only 4.5% of the caloric intake leading to muscle catabolism.

Jornayvaz et al. showed that mice fed a KD for 5 weeks gained significantly less weight than regular chow-fed mice due to increased energy expenditure. KD-fed mice also suffered from hepatic insulin resistance reflected by decreased suppression of hepatic glucose production during a hyperinsulinemic-euglycemic clamp[99]. The clamp indicated that the KD-fed mice required glucose infusion rates that were 47% lower than in controls. Hepatic insulin resistance could be attributed to various factors, including more than a 3-fold increase in hepatic DAG content resulting in protein kinase C activation (PKC). This in turn decreases insulin signaling through IRS-2 phosphorylation regulation. KD-fed mice developed a decrease in whole-body glucose disposal, which was likely a result of a change in body composition[99]. Although these mice had lower body weights compared to controls, they had higher body fat content[99]. There was also a decrease in lean body mass, which would directly lead to a decrease in insulin stimulated glucose uptake. Lean muscle loss could be attributed to the low protein (4.5%) and carbohydrate (0.4%)

content of the KD. This would lead to increased proteolysis where a portion of amino acids would be used for gluconeogenesis[99]. Other measures of insulin sensitivity were used as well including the homeostasis model assessment of insulin resistance (HOMA-IR) (40) and the quantitative insulin sensitivity check index (QUICKI), which both rely on fasting concentrations of glucose and insulin[99]. With the KD-fed mice exhibiting lower basal insulin and glucose levels, the HOMA-IR and QUICKI suggested improved insulin sensitivity. This is in comparison to the hyperinsulinemic-euglycemic clamp which administered insulin and glucose in order to balance glucose uptake and infusion[99]. The possible role of KD in inducing insulin resistance is nevertheless controversial. Indeed, several authors reported that long term KD fed mice had normal glucose tolerance, lower baseline insulin levels and improved insulin sensitivity[100].

Another study examined the long term effects of KD feeding in mice over the course of 80 weeks[101]. Weight loss was seen in the first 18 weeks, but returned to baseline and increased afterwards[101]. Weight gain was less than controls as a result of overall reduced lean and fat mass. The KD-fed mice also increased energy expenditure and RER, which indicated enhanced use of fatty acids as an energy substrate[101]. A recent study looked at the effects of KD on exercising and sedentary rats compared to standard chow-fed control animals[102]. After 6 weeks on the diet, KD-fed rats had smaller adipocytes in visceral fat, 25% lower body mass, 80% lower levels of serum insulin, 50% lower levels of plasma glucose, 55% lower levels of plasma triglycerides, and 20% lower levels of total cholesterol relative to levels seen before beginning the KD feeding[102]. Despite the KD-fed rats being separated into sedentary and exercised groups, they both displayed similar results[102]. Exercising KD-fed rats did show 40% lower serum βHB levels compared to KD-fed sedentary rats possibly due to low intensity exercise having a greater reliance on ketone and fat oxidation for fuel[102].

These results suggest that some of the physiological changes brought about by the KD such as body fat regulation through changes in adipose tissue mass and cell size, may be similar to the effects of exercise. Increased serum ketone levels may play a role in these changes although it is unclear how significant[102]. Another study determined whether orally ingesting βHB salts could increase ketonemia in Wistar rats without any other dietary changes[5]. βHB salts were administered acutely by gavage or chronically by drinking water over 4 weeks. Glucose overload was done at the same time to suppress any potential endogenous ketogenesis to isolate the effects of the βHB salts[5]. In both conditions, ketonemia increased along with increased HDL concentrations, decreased LDL/HDL ratio culminating in an improved blood lipid profile. An overall decrease in adipocyte volume and serum lipolysis byproducts, which include; triacylglycerol (TAG), HDL, LDL, and NEFAs was also found[5]. These results support the idea that ketonemia can be induced through βHB supplementation and as a result, physiological changes are possible.

Whole-body effects of a ketogenic diet and exogenous ketone supplementation in humans

In humans, the KD has been shown to be an effective weight loss therapy, although the mechanisms at work are still unclear. Proposed mechanisms include reduced caloric intake, increased use of proteins as a source of fuel, energy demanding gluconeogenesis, and increased satiety[36]. Investigating these mechanisms often involved comparing the KD to other diets such as one that is hypocaloric. Studies have shown KD reduces ghrelin levels and appetite, whereas a hypocaloric diet typically causes an increase in these levels[103]. This was confirmed by a study conducted on 132 severely obese patients with a high prevalence of T2D or metabolic syndrome where the participants lost more weight while on the KD compared to other diets[104]. Other authors compared different diets in the same group of patients eating alternatively a KD, a low-carbohydrate non-KD, and a normal Mediterranean diet[105]. Significant weight loss and decreases

in body fat percentage was found in KD-fed participants compared to participants on the other two diets[105]. Moreover, if the patients were compliant to the prescribed Mediterranean diet, which was a relatively strict 1800 kcal/day during the maintenance period, no weight regain was observed at 12 months[105]. Another study compared a low-carbohydrate high unsaturated fat diet to a high-carbohydrate low-fat diet in addition to a structured exercise with obese type 2 diabetes patients[106]. Both groups experienced similar weight loss of approximately 9% with a trend towards regaining the weight seen only in the low-carbohydrate group at 52 weeks[106]. Jabekk et al., examined the effects of resistance training in combination with either a KD or a control diet, on body composition in overweight women[107]. The KD group lost fat mass without experiencing a significant alteration in lean mass, while the other group gained lean mass without a significant change in fat mass[107]. In terms of effects on glucose metabolism, a study in obese type 2 diabetic patients fed a KD saw significant improvement in fasting glucose levels was seen after 12 weeks and continued after 56 weeks[108]. A similar study only saw a short-term decrease in HbA1c at 6 months, which was not sustained at 24 months[109]. Comparing the diets used in these two studies, the latter used a less intense KD with a slightly higher carbohydrate intake. This suggests that lower intensity KD can be adapted to leading to a decrease in the benefits seen over time.

Stubbs et al., examined whether exogenous ketones were an effective way to achieve ketosis as an alternative to a high-fat, low-carbohydrate "ketogenic" diet that are typically difficult to adhere to. Healthy human volunteers took part in three randomized metabolic studies of drinks containing a ketone ester (KE); (R)-3-hydroxybutyl (R)-3-hydroxybutyrate, or ketone salts (KS); sodium plus potassium βHB[110]. In the first study 15 participants consumed KE or KS drinks that delivered ~12 or ~24 g of βHB both of which elevated plasma βHB concentrations and returned to baseline within 3 to 4 hours[110]. Urinary excretion of βHB was less than 1.5% of the total βHB

ingested. In the second study, the effect of a meal before a KE drink on plasma βHB concentrations was determined. Food lowered peak plasma βHB concentration by 33% but did not alter acetoacetate or breath acetone concentrations. All ketone drinks lowered blood glucose, free fatty acid and triglyceride concentrations, and had similar effects on blood electrolytes[110]. The final study compared different methods of βHB administration. Patients were given KE over 9 hours using three drinks at 3-hour intervals or through continuous nasogastric infusion after an initial bolus. Both results methods were found to be nearly identical in terms of total appearance of βHB in the blood[111].

One study investigated the effects of raising plasma βHB levels through ingestion of salts on cycling performance and substrate oxidation[11]. Ten healthy adult males were recruited and on experimental days, fasted participants consumed either 0.3 g/kg βHB ketone salts or a flavor-matched placebo at 30 min prior to engaging in cycling exercise[11]. Subjects completed steady-state exercise at 30%, 60%, and 90% ventilatory threshold (VT) followed by a 150-kJ cycling time-trial. Plasma βHB levels were elevated starting from baseline throughout the entire ketone condition[11]. RER was lower at 30% and 60% VT in the ketone compared with control condition with total fat oxidation being greater in the ketone condition versus the control[11]. Average time trial power output was 7% lower in the ketone condition. Overall these results indicated that ingestion of βHB salts prior to exercise increased fat oxidation during steady-state exercise while impairing high intensity exercise performance[11].

Little to no work done to examine the tissue specific changes or cellular mechanism responsible for physiological changes seen when on a KD. Little has attention has been given to the ketones such AcAc and especially βHB that are produced when individuals enter a state of

ketosis when on a KD. βHB for example has been found to possess signaling potential worth exploring on a cellular level where the metabolism of cells can be affected.

Objectives and Hypothesis

The general objective of this study was to determine if βHB can mimic the effects of a KD on skeletal muscle glucose and fat metabolism. To accomplish this, an *in-vitro* model with L6 muscle cells supplemented with various βHB in a dose response treatment will be used. This will determine which physiologically relevant concentrations of βHB would be capable of inducing changes in glucose and fat metabolism by measuring glucose uptake. The minimum effective dose of βHB would then be examined in subsequent functional assays done on cells subjected to a time course treatment. Based on the literature, we know that insulin sensitivity and whole-body glucose clearance improves when on KD. Therefore, we hypothesize that glucose uptake would increase. In addition to an increase in glucose within the cell, we expect glucose utilization pathways such as glycogen synthesis, glucose oxidation, and lactate production to also be elevated.

According O'Malley et al., we know βHB salt supplementation increased fat oxidation in human males despite differences in exercise routines. Therefore, we expected that palmitate oxidation would increase with βHB supplementation.

In addition to looking at the functional implications of βHB supplementation, we wanted to examine key proteins involved in glucose and fat metabolism. Using western blot analysis, we would measure AKT and AMPK (phosphorylated and unphosphorylated states) which play a role in glucose uptake. We expect an elevation in PAKT and PAMPK levels to explain an increase in GLUT4 translocation to the plasma membrane and therefore glucose uptake. Proteins involved in the glycogen synthesis pathway such as GSK3 and GS (phosphorylated and unphosphorylated states) would be measured. Changes in the expression levels would cause a change in the activity

levels and impact the ability of the cells to synthesis glycogen. Finally, we will be examining CPT1β which we expect to see an increase, as it is involved in the rate limiting step of fatty acid oxidation. These experiments would provide a better understanding of the mechanisms involved in the various pathways that βHB would impact. With better insight, we can evaluate the potential of βHB supplementation as a viable alternative to the KD.

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EFFECTS OF β-HYDROXYBUTYRATE (βHB) ON L6 MUSCLE CELL GLUCOSE AND FAT METABOLISM

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Keywords: β-hydroxybutyrate (βHB), Ketogenic diet (KD), glucose, glycogen, lactate, palmitate, AMPK, AKT, GSK3, GS, CPT1B, supplementation

Figures: 11

Abstract

This study investigated the effects of the ketone body β-hydroxybutyrate (βHB) on glucose and fat metabolism in skeletal muscle cells. To accomplish this, L6 muscle cells were incubated with various βHB concentrations (0.5, 1.0, 1.5, 20, and 2.5mM) for 1, 3, and 5 days. Here, we report that βHB significantly increased glucose uptake and its metabolism in L6 muscle cells. In fact, time course analysis revealed that as early as 1 day of exposure to βHB (1mM) significantly enhanced basal and insulin-stimulated glucose uptake (1.6-fold), glycogen synthesis (1.3-fold), glucose oxidation (1.5-fold), and lactate production (1.3-fold) in skeletal muscle cells. Even though carnitine palmitoyl transferase 1 (CPT1) content and AMP-activated protein kinase (AMPK) phosphorylation significantly increased, no alteration was found in palmitate oxidation with βHB treatment. Surprisingly, protein kinase B (AKT) phosphorylation was significantly reduced in muscle cells exposed to βHB. In summary, βHB increased AMPK phosphorylation and enhanced glucose uptake without altering fatty acid oxidation in skeletal muscle cells.

Introduction

Ketogenic diets (KDs) are characterized by high and low contents of fat and carbohydrates, respectively[1]. These diets have been known for their therapeutic value in the treatment of neurological disorders such as epilepsy[2]. In recent years, the popularity of KDs has increased due to the fact that they can also be beneficial in the treatment of metabolic diseases primarily through improvements in whole-body glucose control[2]. Although KDs have been shown to have broad therapeutic potential, patient compliance can be challenging[2,3]. This is because along with severe dietary restriction and unpalatability of meals associated with KDs, those who are on a KD deal with possible hypoglycemic episodes and intolerance to high fat ingestion[3,4]. In this context, it has been suggested that oral supplementation of ketones may mimic a metabolic state of ketosis without any of the shortcomings of a typical KD, therefore serving as a practical alternative[3,5]. β-Hydroxybutyrate (βHB), acetoacetate, and acetone are the ketones produced by the body. However, βHB considered the most functionally relevant because of its signaling capabilities and the fact that it is less likely to undergo degradation and be eliminated relative to than acetoacetate and acetone[6,7].

Therefore, chronic supplementation of βHB has been suggested as an alternative approach to create ketogenic conditions without having to adhere to a typical KD. This has been tested both in rodent and human models[8]. Initial studies established that a state of ketosis can be achieved and maintained in humans and rodents with various types of ketone/βHB supplements without the need for dietary changes[5,9]. Significant metabolic effects of βHB supplementation were demonstrated by a study in which βHB salts were administered orally to rats[5]. The rats experienced an improved plasma lipid profile as well as an overall decrease in adipocyte volume[5]. Interestingly, high-fat-fed mice receiving a βHB-ester supplement displayed significant weight loss and an increase in

energy expenditure compared to high-fat and calorie-restricted mice[10]. In humans, supplementation of βHB salts resulted an overall increase in fat oxidation and a reduction in respiratory exchange ratio (RER), mimicking what is observed in individuals on a KD[11].

Even though several studies report that βHB supplementation can lead to alterations in substrate utilization and affect exercise performance, the molecular mechanisms responsible for the results described above remains unknown, although some hypotheses have been proposed. Once taken up by peripheral tissues, βHB is thought to affect cellular metabolism in two ways: a) by inhibiting class 1 histone deacetylases (HDACs), which regulate gene expression[12,13], and b) through post translational protein modifications including acetylation, succinylation, and β-Hydroxybutyrylation[1,12]. One of the most important peripheral tissues that utilize βHB is skeletal muscle, which accounts for up to ~40% of body weight in men and ~30% in women. Additionally, skeletal muscles are the primary site of insulin-stimulated glucose uptake and play a major role in the regulation of glucose and fat homeostasis in the body[14,15]. Therefore, any potential effect of βHB on muscle could be of great physiological and therapeutic relevance and worthy of further investigation.

In this context, the research described in this thesis was designed to investigate the effects of βHB on skeletal muscle metabolism. To accomplish that, an in vitro model using L6 rat skeletal muscle myoblast cells were used. Using L6 myoblasts allowed for direct assessment of βHB on skeletal muscle without potential confounding systemic influences on skeletal muscle metabolism. Alterations to important components of glucose and fat metabolism specifically, were analyzed using various functional assays including: glucose uptake, glucose oxidation, glycogen synthesis, lactate production, and palmitate oxidation in myoblasts. Western blot analysis was used to assess alterations in signaling pathways related to glucose and fat metabolism. These included

phosphorylation protein kinase B (AKT), AMP-activated protein kinase (AMPK), glycogen synthase (GS), and glycogen synthase kinase 3 (GSK3), as well as content of carnitine palmitoyl transferase 1B (CPT1B). Using these data, the role of βHB in the regulation of skeletal muscle cell metabolism was evaluated.

Experimental Design & Methods

Reagents

Minimum essential medium (α-MEM) with and without phenol red, fetal Bovine Serum (FBS), trypsin, phosphate buffer saline (PBS) suitable for cell culture, and 1% (v/v) antibiotic antimycotic were obtained from Wisent Bioproducts (ST-BRUNO, Quebec, Canada). DL-β-hydroxybutyric acid sodium salt (βHB) was from Sigma Aldrich (St. Louis, MO, USA). Protease (Complete Ultra Tablets) and phosphatase (PhosStop) inhibitors were from Roche Diagnostics GmbH (Mannheim, Germany). 2-deoxy-D-[H^3] glucose (0.5 μCi/ml) was purchased from Perkin Elmer (Seer Green, United Kingdom). Cytochalasin B. D-[U-^{14}C] glucose and [1-^{14}C] palmitic acid were from GE Healthcare Radiochemicals (Quebec City, Quebec, Canada); and human insulin (Humulin) was from Eli Lilly (Indianapolis, Indiana, USA). Phosphate buffered saline (PBS) tablets were bought from Bioshop (Burlington, Ontario, Canada). Glycogen carrier. The lactate colorimetric assay kit was obtained from BioVision (San Francisco, CA, USA). Cell viability CellTiter-Glo® Luminescent Cell Viability Assay was purchased form Promega (Madison, WI, USA).

Cell culture

Stock rat L6 skeletal muscle cell cultures were obtained from the American Type Culture Collection and kept in a humidified atmosphere of 95% O_2 and 5% CO_2 at 37°C. Cells were grown in minimum essential medium (α-MEM) containing 10% (v/v) Fetal Bovine Serum (FBS) and 1% (v/v) antibiotic antimycotic referred to as growth medium. Cells were kept in 2 T75 standard flasks which were split at 70%-80% confluence. Each of the assays involved either a time course or a dose response treatment. Treatment began with cells being trypsinized and reseeded for the assay being run. Cells were given 24 hours to allow for surface attachment before changing the media to

remove any DMSO and beginning treatment with DL-β-hydroxybutyric acid sodium salt obtained from Sigma Aldrich. A 20mM stock solution of βHB was used to achieve the desired concentrations of 0.0 to 2.5mM (0, 0.5, 1.0, 1.5, 2.0, or 2.5mM) for the dose response treatment. This range was chosen because serum βHB concentrations typically range from approximately 100uM to 1-3mM while on a sustained ketogenic diet[7,112]. An assay done with the dose response treatment would indicate which βHB concentrations are effective in eliciting some sort of functional change. Based on this, a single βHB concentration would be chosen for the time course treatment to determine the duration of time needed to elicit the response seen in previous assays. Cells will be treated for up to 5 days for the time course treatment, and exactly 5 days with the does response treatment. Growth medium was changed and treatment was readministered every other day.

Glucose Uptake

Cells were seeded into 24-well plates and divided between basal, insulin stimulated, and non-specific uptake conditions. On the day of the assay, cells were serum starved (α-MEM with antibiotic antimycotic only) for 4 hours. Wells assigned to the insulin condition were treated with insulin (100nM) for 30 minutes. Cells were washed twice with Hepes-buffered saline (HBS) solution (140mM NaCl, 20mM Hepes-Na, 2.5mM $MgSO_4$, 1mM $CaCl^{2+}$, 5mM KCl, pH 7.4) at room temperature and aspirated dry. A specific glucose uptake solution of 10μM 2-deoxy-D-glucose and labelled 2-deoxy-D-[H^3] glucose (0.5μCi/mL) made in HBS solution was administered to all basal and insulin stimulated wells. Wells designated for non-specific uptake were prepared by using an uptake solution containing cytochalasin B (10μM). Cells were allowed to incubate for 5 minutes. The plates were then put on ice and washed twice with ice cold 0.9% saline solution in order to stop the reaction. Wells were aspirated to dryness. 250uL of 0.05N

NaOH solution was added to each well and put on to a shaker for 15 minutes to aid in cell lysis. 200uL of the resulting lysate was put into scintillation vials filled with 2mL of scintillation fluid, for counting. The remaining 50uL of lysate was used for protein quantification via Bradford assay.

Glycogen Synthesis

After completing the time course treatment, on the day of the assay, cells were serum starved for 3 hours in starve media (FBS-free α-MEM containing antibiotic and antimycotic only). The starve media was aspirated and the control and insulin groups were treated with 500uL of test media containing non-labeled glucose (5.5mM) and [U-^{14}C] d-glucose (0.2μCi/mL) made in starve media without and with insulin (100nM) respectively. The cells were allowed to incubate for another 2 hours after which 100uL of media was collected and stored in the -20^{o}C for future experiments. The glycogen synthesis reaction was stopped by aspirating the test media, putting the plates on ice, and washing with ice cold PBS. Wells were treated with KOH (1M) and placed on a shaker for 30 minutes to aid in cell lysis. Lysates were transferred to eppendorf tubes and heated for 30 minutes at 65^{o}C. 50μL of each sample was taken for protein measurement via Bradford assay. 100uL of glycogen carrier was added to each Eppendorf. Afterwards, 80uL of Saturated Na_2SO_4 and 1.2mL of cold ethanol was added to each. Subsequently, the tubes were vortexed and stored at -20^{o}C overnight. Samples were then be centrifuged for 20 minutes at 5000-10000 rpm at room temperature after which a pellet was formed. The supernatant was discarded and the remaining pellet was dissolved in distilled water where 400uL was transferred to scintillation vials filled with 2mL of scintillation fluid for radioactivity counting[113].

Palmitate Oxidation

Cells were seeded into 35x10mm tissue culture dishes and subjected to a time course treatment. On the day of the assay, cells were serum starved with starve media (α-MEM with antibiotic antimycotic only) for 4 hours. The βHB treatments was added to each dish as the starve media did not contain βHB. Starve media was then aspirated and 500uL of a solution containing non-labeled palmitate (20μM) and labeled (0.2 μCi/ml [1-^{14}C] palmitic acid) in αMEM was added to each dish. Dishes were sealed using parafilm with a piece Whatman paper attached using tape, to the side of the film facing the inside of the dish. Cells were allowed to incubate for 1 hour. After 1 hour of incubation, a syringe was used to pierce the parafilm and saturate the Whatman paper with 100μL of phenylethylamine-methanol (1:1) to capture the CO_2 produced during the incubation period. Then using another syringe, 200μL of H_2SO_4 (4M) was added to the media. The holes created by the syringes were covered with pieces of tape to ensure there was no leakage of gasses from the dishes. One additional hour of incubation was given for $^{14}CO_2$ collection. After incubation, the pieces of Whatman paper were separated from the parafilm in a way that ensures the paper does not touch the media. The piece of Whatman paper were then be placed in scintillation vials for radioactivity counting. Vials were filled with 5mL of scintillation fluid to ensure the piece of whatman paper were fully submerged.

Glucose Oxidation

Cells were seeded into 35x10mm tissue culture dishes and subjected to a time course treatment. On the day of the assay, cells were serum starved with starve media (αMEM with antibiotic antimycotic only) for 4 hours. The βHB treatments was added to each dish as the starve media did not contain βHB. Starve media was aspirated and 750uL of a solution containing labeled (0.1 μCi/mL [1-^{14}C] glucose) in αMEM which on its own contains glucose at a concentration of

1ug/uL, was added to each dish. Dishes were sealed using parafilm with a piece Whatman paper attached using tape, to the side of the film facing the inside of the dish. Cells were allowed to incubate for 1 hour. After 1 hour of incubation, a syringe was used to pierce the parafilm and saturate the Whatman paper with 100µL of phenylethylamine-methanol (1:1) to capture the CO_2 produced during the incubation period. Then using another syringe, 200µL of H_2SO_4 (4M) was added to the media. The holes created by the syringes were covered with pieces of tape to ensure there was no leakage of gasses from the dishes. One additional hour of incubation was given for $^{14}CO_2$ collection. After incubation, the pieces of Whatman paper were separated from the parafilm in a way that ensures the paper does not touch the media. The piece of Whatman paper were then be placed in scintillation vials for radioactivity counting. Vials were filled with 5mL of scintillation fluid to ensure the piece of whatman paper were fully submerged.

Lactate Production

Cells were seeded into 6-well plates and were subjected to a time course treatment. On the fourth day of the treatment, when cells designated for 1 day of treatment are given βHB, media in all the wells was changed. Instead of the usual growth being used, a version using a form of αMEM without phenol red was used. This created a colorless media which would not interfere with later steps in the Lactate colorimetric assay kit. Cells were treated with a 20mM βHB stock solution created using colorless growth medium as well. After the 5 days of the time course treatment was completed, 500uL sample was taken from each well, flash frozen and stored in a -80^{o}C freezer until the next step of the assay. Once ready to conduct the assay, samples were deproteinized first. Samples were loaded into tubes containing removable 10 kda filters and were then centrifuged at 13 000 rpm for 15 minutes at 4^{o}C. The remaining sample at the bottom of the filter tubes were put into Eppendorf tubes. An initial assay was done to create a standard curve as well as determine the

optimal amount of sample needed for the assay, which was found to be 2.5uL. To prepare the standard curve, the lactate standard in the kit was diluted to 1nM/uL by adding 100uL of the 100nM/uL lactate standard to 990uL of lactate assay buffer. After mixing well, 0, 2, 4, 6, 8, and 10uL of the diluted lactate standard was added to a series of wells in duplicates in the same 96-well plate. The volumes of the standards were adjusted to 50uL using the lactate assay buffer to generate 0, 2, 4, 6, 8, and 10nM/well concentrations of the lactate standard. 50uL of a reaction mix containing 46uL of lactate assay buffer, 2uL of lactate enzyme mix, and 2uL of probe, was added to all sample and standard wells. After adding the reaction mix, the plate was allowed to incubate at room temperature for 30 minutes in the dark. Absorbance of the plate was measured at 570 nm in a microplate reader. It was determined that 2.5uL was the optimal amount of sample to use for the assay. For the actual assay, 2.5uL of each sample was loaded into wells on a 96-well plate in duplicates. Volumes were adjusted to 50uL with lactate assay buffer and 50uL of reaction mix was added. the plate was allowed to incubate at room temperature for 30 minutes in the dark. Absorbance of the plate was measured at 570nm in a microplate reader.

Cell Viability

Cells were seeded into a 96-well, opaque walled, clear bottom plate and subjected to a time course treatment. Cell viability was measured using the CellTiter-Glo® Luminescent Cell Viability Assay from Promega. This assay determines the number of viable cells in culture by lysing the cells to expose their contents to the assay substrate. Prior to the start of the assay, the assay buffer and assay substrate were thawed and allowed to equilibrate to room temperature. After equilibration, 10mL of the buffer was added to the substrate to fully reconstitute all the lyophilized enzyme/substrate mixture thereby creating the reaction mixture. The reaction mixture was gently mixed by vortexing and inverting to ensure a homogenous solution. At the start of the assay

standards were created by dissolving powdered ATP in growth medium to create a 100mM stock solution. From this stock solution, dilutions were created in order to obtain solutions with the concentrations 1, 0.5, 0.25, 0.1, and 0.01uM which would be used to create the standard curve. 100uL of each standard solution was loaded into the same 96-well plate in duplicates. All sample wells in the 96-well plate were then aspirated. 50uL of the reaction mixture was added to all wells, standard and experimental. Blanks were prepared as well by adding the reaction mixture to empty wells to obtain a value for background luminescence. The plate was allowed to equilibrate at room temperature while on a shaker to induce cell lysis, for approximately 30 minutes. The plate was then taken off the shaker and allowed to sit for 10 minutes so that the luminescent signal may stabilize. The luminescence was then recorded on a microplate reader.

Analysis of Proteins in Related Signaling Pathways using Western Blotting

Cells were seeded into 6-well plates and underwent a dose response or time course treatment. Once treatment was completed, cells were washed twice with PBS to ensure the removal of all growth medium. Growth medium contains proteins and so and amount left in the wells could alter protein measurements. Cell lysis was induced using lysis buffer containing protease (Complete Ultra Tablets) and phosphatase (PhosStop) inhibitors. Once washing with PBS was complete and wells were aspirated dry, 100uL of the lysis buffer was added to each well. Lysate was scraped from the wells using a cell scraper and further broken down mechanically by using the syringe to repeatedly take up and then dispense the lysate. Sample lysates were collected in eppendorfs and stored in a -80^{o}C freezer until next use. Once collected, an aliquot of the samples was used to measure protein by the Bradford method. Samples were diluted 1:1 (v/v) with 2X Laemmli sample buffer, heated to 95°C for 5 minutes, subjected to SDS-PAGE and transferred to PVDF membrane. Membranes were subsequently probed with primary antibodies (1:1,000

dilution), followed by horseradish peroxidase-conjugated anti-rabbit secondary antibody (dilution of 1:2,000). β-actin was used as a loading control. Blots were visualized using chemiluminescence (Luminata forte, Millipore, Billerica, MA) and scanned directly into an image quantification program.

Statistical Analysis

Results are expressed as means ± SEM and statistical significance was established at $P < 0.05$. Statistical analysis was carried out using Graphpad Prism 8 software. For experiments where basal and insulin-stimulated conditions were examined, two-way ANOVA was performed. For experiments involving only basal conditions, one-way ANOVA was performed. Additional post hoc Tukey corrections for multiple comparisons were carried out where appropriate.

Results

Glucose uptake assay following 5-day dose response treatment with βHB – Glucose uptake overall increased in both basal and insulin-stimulated conditions, reaching significance with treatment concentrations of 2.0 and 2.5mM βHB. Under basal conditions, treatment with 2.0 and 2.5mM βHB resulted in an increase of 44% and 55%, respectively, compared to control, while under insulin stimulated conditions, treatment resulted in an increase of 26% and 42%, respectively (Fig. 1). An increase of 22% and 25% in basal and insulin-stimulated conditions was seen with 0.5mM, although it was not significant due to the presence of many other concentrations in the statistical analysis. It's been established that individuals on a KD can achieve serum βHB levels of 1-2.5mM and that the threshold for the effects of βHB on glucose and lipid metabolism appears to be at least 1mM[111]. In this context, a concentration of 1mM, representative of a less carbohydrate-restricted KD was examined in subsequent experiments.

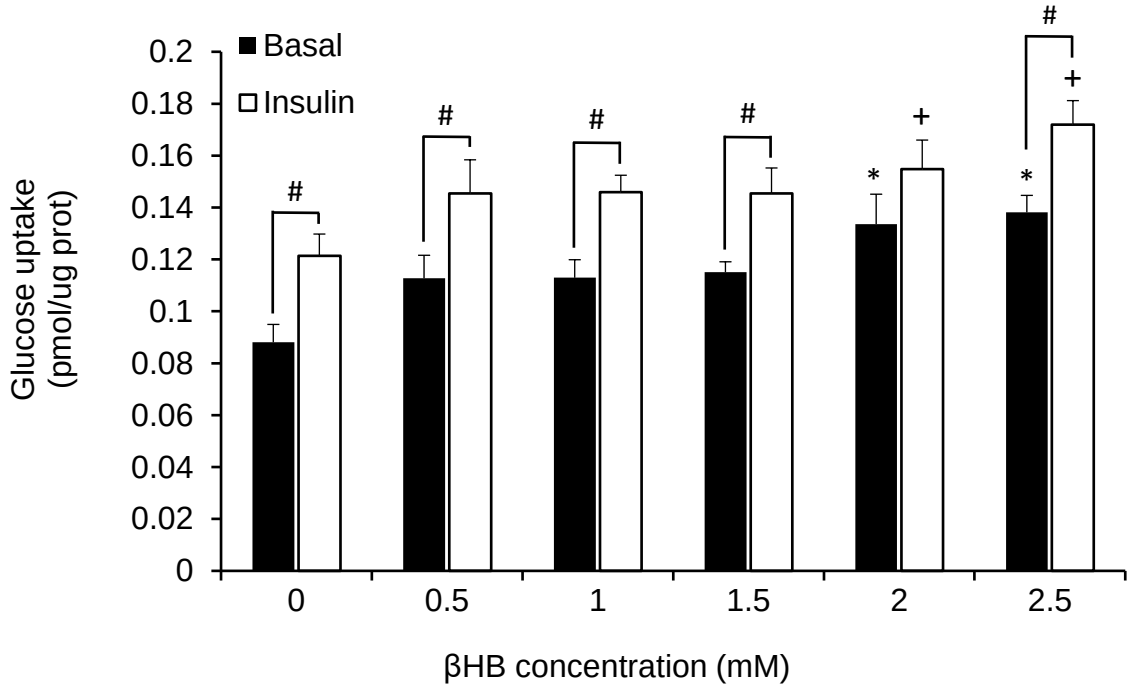

Figure 1: Effects of different concentrations of βHB ranging from 0 to 2.5mM on glucose uptake of L6 muscle cells after 5 days of treatment. Two-way ANOVA, n = 8-9. * $P < 0.05$ vs. 0mM Basal. + $P < 0.05$ vs. 0mM Insulin. # $P < 0.05$.

Glucose uptake assay following 1mM time course treatment with βHB – Under basal conditions, glucose uptake increased significantly by 40% after a single day of treatment with βHB, compared to the control. After 5 days, there was a 60% increase in basal glucose uptake (Fig. 2). Under insulin stimulation, glucose uptake also increased after a single day of treatment with βHB, however the effect did reach significance until 5 days of treatment with a 29% increase compared to control. Interestingly, the results showed a greater increase in glucose uptake under basal conditions compared to the insulin condition following treatment with βHB. This is shown by comparing the difference between basal and insulin-stimulated glucose uptake in controls and cells treated for 5 days. Under control conditions (0mM βHB), there is a 44% increase in glucose uptake with insulin stimulation, whereas after 5 days of treatment, there is only a 15% increase in glucose uptake following insulin stimulation. This suggests that βHB supplementation may have a greater effect on basal glucose compared to insulin stimulated glucose uptake.

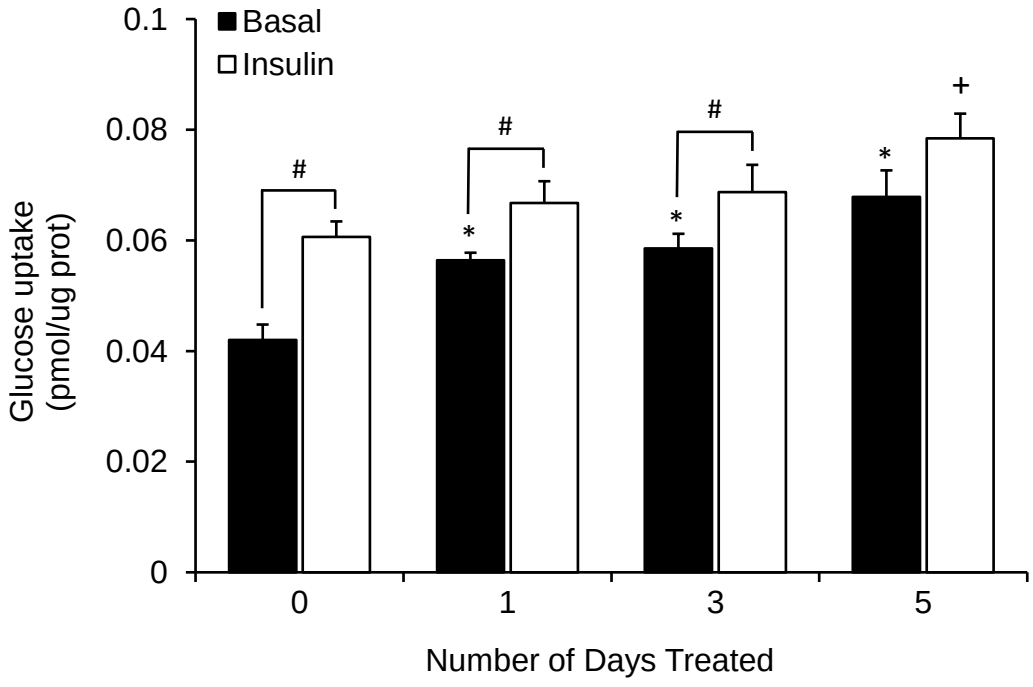

Figure 2: Glucose uptake of L6 muscle cells after 5-day time course treatment at 1mM of βHB. Two-way ANOVA, n = 8-9. * *P* < 0.05 vs. 0 days treated Basal. + *P* < 0.05 vs. 0 days treated Insulin. # P < 0.05.

Glucose oxidation assay following 1mM time course treatment with βHB – 1mM βHB treatment was shown to increase basal and insulin stimulated glucose uptake (Fig. 3). Glucose oxidation was then analyzed to determine if βHB had any effects and if the increased glucose being taken up was being directed towards oxidation. As expected, glucose oxidation in L6 cells increased after a single day of treatment with 1mM βHB, reaching significance after 5 days with a 47% increase compared to control. These results support the idea that glucose oxidation is one of the pathways by which the increased glucose is being directed.

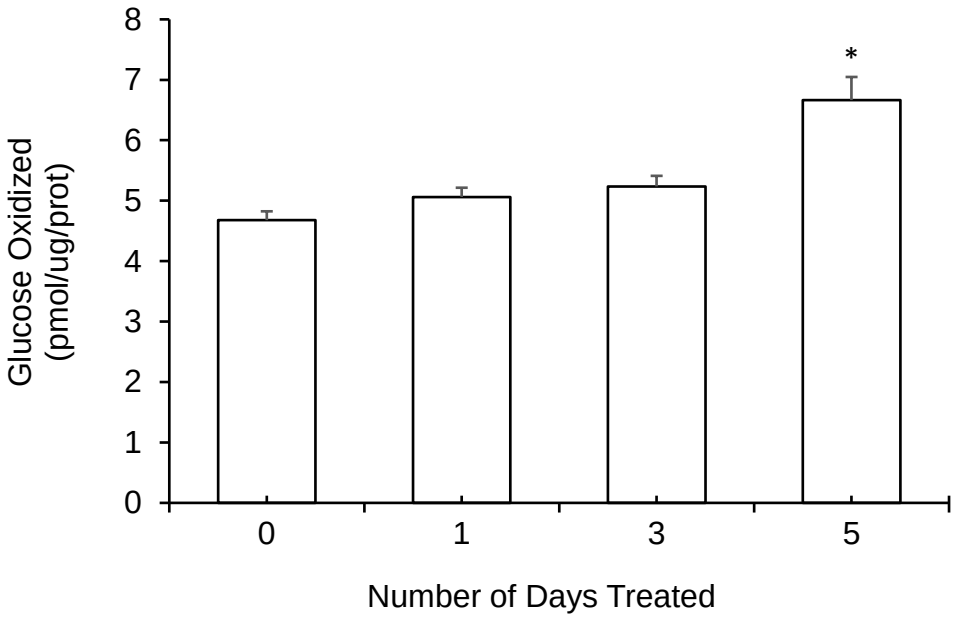

Figure 3: Glucose oxidation in L6 muscle cells after 5-day time course treatment with 1mM βHB. One-way ANOVA, n = 15-16. * $P < 0.05$ vs. 0 days.

Glycogen Synthesis following 1mM time course treatment with βHB – Basal glycogen synthesis increased after 1 day of treatment, reaching significance with a 33% increase after 5 days of treatment with βHB compared to control (Fig. 4). Insulin stimulated glycogen synthesis increased by 11% and 19% after 1 and 5 days of treatment compared to untreated, insulin-stimulated cells. This suggests that in addition to the increase in glucose oxidation, increased glucose taken up by cells following treatment with 1mM βHB may also be directed towards glycogen synthesis.

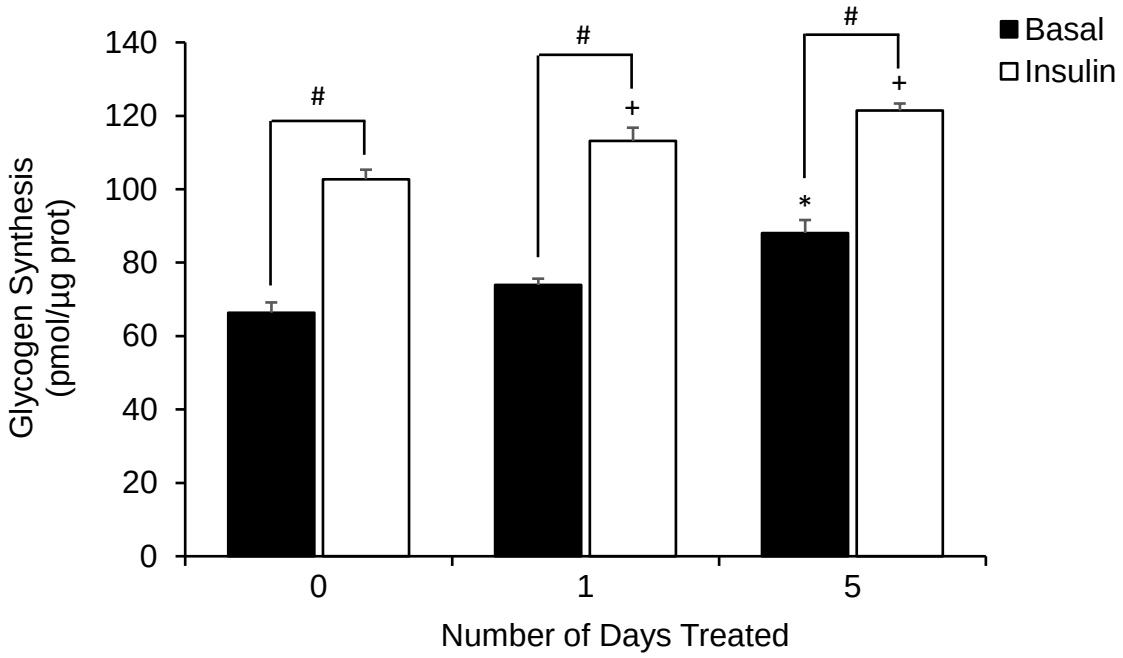

Figure 4: Glycogen synthesis in L6 muscle cells after 5-day time course treatment with 1mM βHB. Two-way ANOVA, n = 8. * *P* < 0.05 vs. 0 Days Basal. + *P* < 0.05 vs. 0 Days Insulin. # P < 0.05.

Lactate production following 1mM time course treatment with βHB – Treatment with 1mM βHB resulted in a 13%, 24% and 28% increase in lactate production following 1, 3 and 5 days of treatment, respectively (Fig. 5). Along with the increased glucose oxidation and glycogen synthesis, these results indicate that lactate production is another metabolic pathway that consumes the increased glucose being taken up by the cells following βHB treatment.

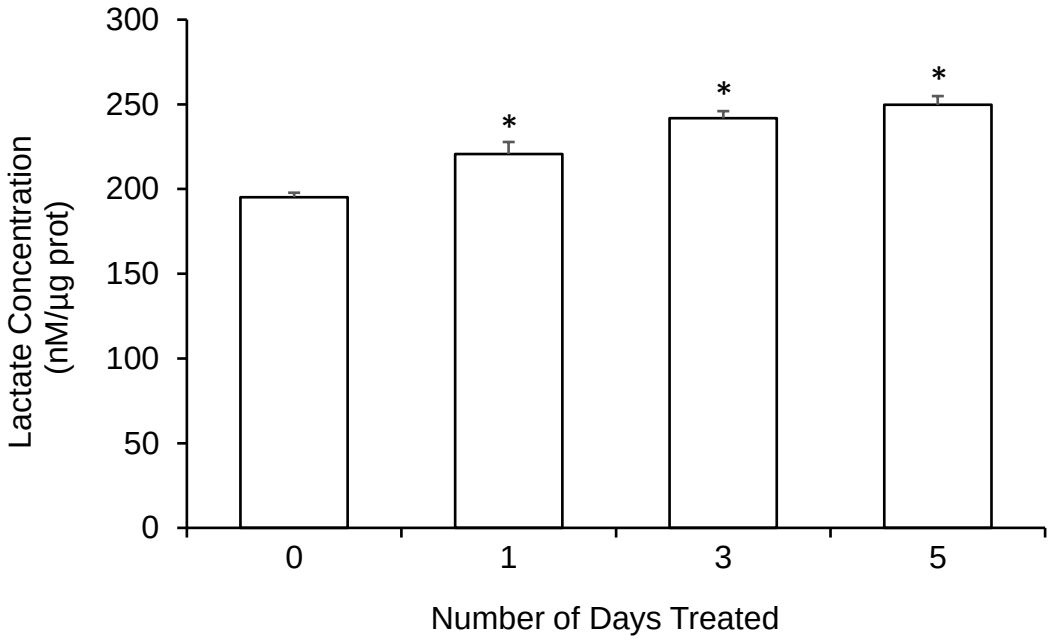

Figure 5: Lactate production of L6 muscle cells after 5-day time course treatment with 1mM βHB. One-way ANOVA, n = 18. * $P < 0.05$ vs. 0 Days.

Palmitate oxidation following 1mM dose response treatment with βHB – L6 muscle cells were subjected to a 5-day dose response treatment with a range of βHB concentrations from 0.0 to 2.0mM (Fig. 6). Given that literature has shown that fatty acid oxidation is enhanced when on a KD, it was expected that palmitate oxidation in L6 muscle cells would increase with βHB treatment. Contrary to what was expected, no significant change in palmitate oxidation was seen at any βHB concentration. These results indicate that the increased fatty acid oxidation seen when on a KD may be a product of the increased fat intake of the diet, and not an effect of βHB acting on muscle cell metabolism.

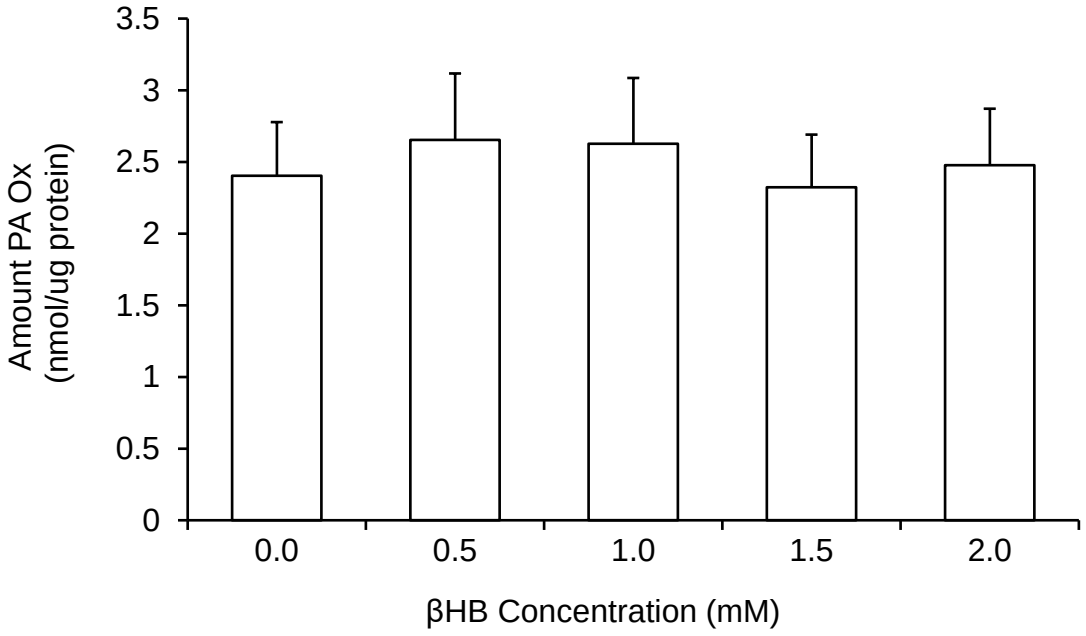

Figure 6: Palmitate oxidation of L6 muscle cells after a dose response treatment at 1mM βHB. No significant differences were found between the various treatment conditions. One-way ANOVA, n = 19.

ATP cell viability assay following a time course treatment with 1mM βHB – The 1mM βHB treatment showed no signs of toxicity or negative effects on cell viability for any treatment duration when compared to the control (Fig. 7a). Unexpectedly, cell viability significantly increased following 1,3, and 5 days of treatment as shown by an increase in ATP concentration. Bradford assay indicated similar protein concentrations for all four conditions, which suggest that the βHB treatment may have increased cell turn over. Figure 7b shows the average protein content of the wells in each of the treatment durations. As can be seen, there is no significant difference between any of the treatment durations indicating that the βHB treatment did not alter protein content.

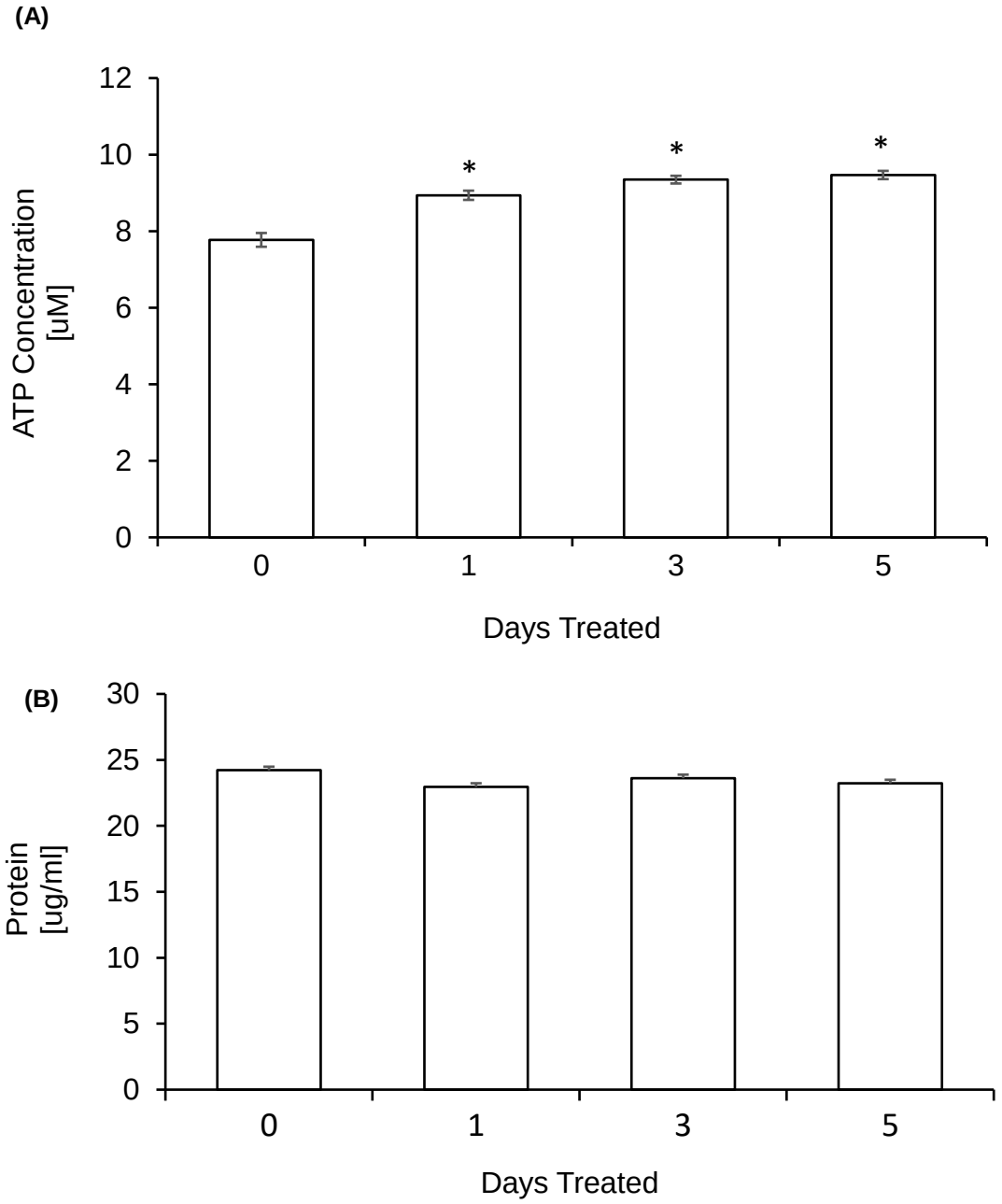

Figure 7a: Cell viability of L6 muscle cells after a time course treatment with 1mM βHB. One-way ANOVA, n = 18. * $P < 0.05$ vs. 0 Days. (B) **7b:** Measure of total protein concentration in samples used for determination of ATP concentration. One-way ANOVA, n = 18.

Content and Phosphorylation of AMPK and AKT in L6 muscle cells following a time course treatment with 1mM βHB – Phosphorylation of AKT was significantly reduced by 1.4-fold, 1.6-fold, and 1.7-fold following 1,3, and 5 days of treatment, respectively (Fig. 8b). This contradicts the findings in figure 2 that showed an increase in glucose uptake as the phosphorylation of AKT is required for the translocation of GLUT4 to the plasma membrane in order for glucose uptake to occur under insulin-stimulated conditions. However, there are alternative, insulin-independent pathways that may be activated following βHB. The phosphorylation of AMPK significantly increased by 2-fold, 2.4-fold, and 2.8-fold following 1, 3, and 5 days of βHB treatment respectively (Fig. 8a). This increase in the phosphorylation of AMPK indicates that the increase in glucose uptake may be occurring through insulin-independent pathways, which could explain the increase in glucose uptake in figure 2.

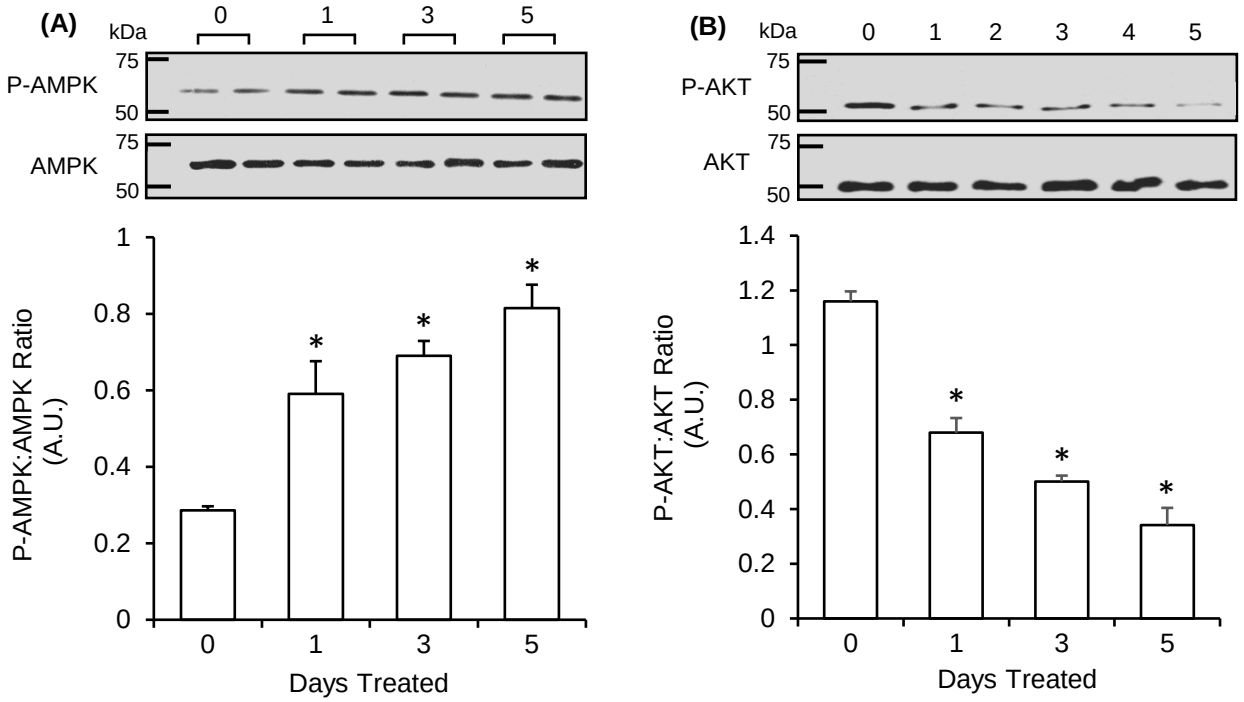

Figure 8a, b: Western blot analysis of the AMPK and AKT and their phosphorylation. One-way ANOVA, n = 6-8. * *P* < 0.05 vs. 0 Days.

Content and phosphorylation of GSK3 and GS in L6 muscle cells following a time course treatment with 1mM βHB -Phosphorylation of GSK3 was enhanced significantly 2.2-fold, and 2.3-fold following 3 and 5 days of βHB treatment respectively (Fig. 9a). Based on this result, it was expected that the phosphorylation of GS would be reduced with βHB treatment. This is due to the phosphorylation of GSK3 leading to its inactivation making it unable to phosphorylate GS. This would allow GS to remain active and catalyze glycogen synthesis. However, there was no significant change in the phosphorylation of GS with any of the treatment durations (Fig. 9b). An increase in the phosphorylation of PGSK3 suggests that less GSK3 is active and available to phosphorylate and inactivate GS. These results may support the significant increase in glycogen synthesis shown in figure 4.

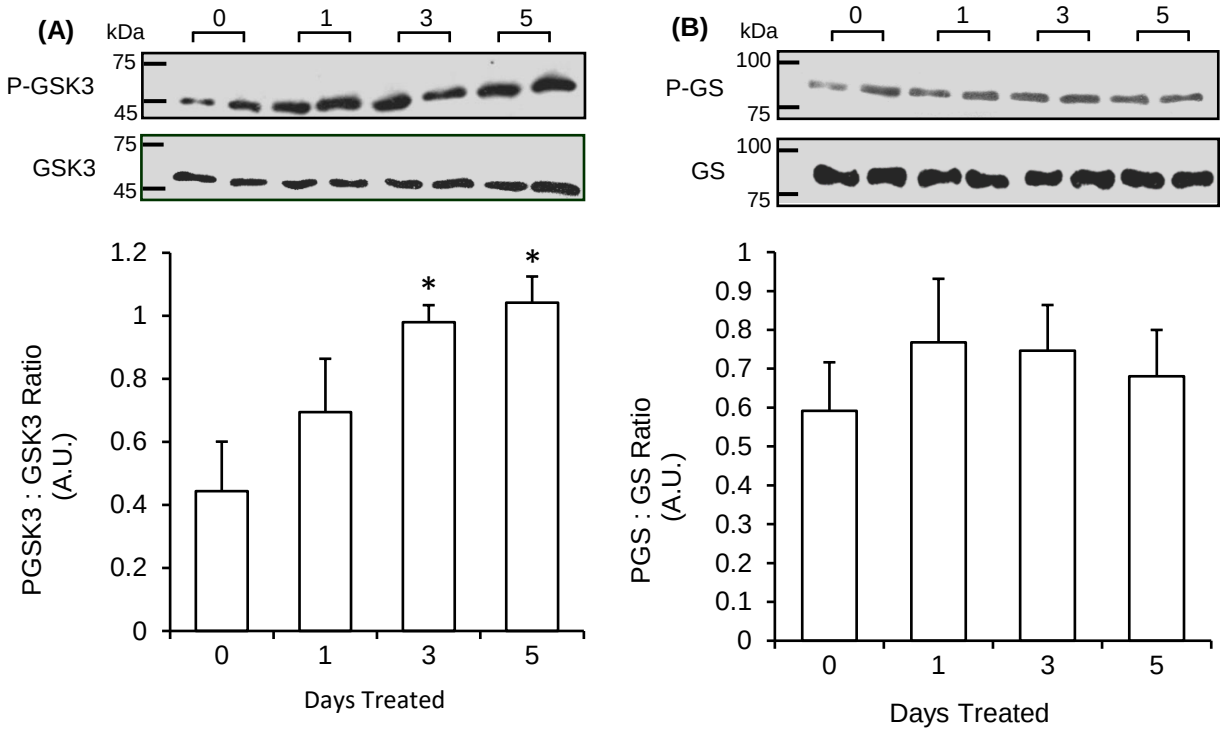

Figure 9: Western blot analysis of GSK3 and GS and their phosphorylation. One-way ANOVA, n = 6-8. * $P < 0.05$ vs. 0 Days.

Content of CPT1B in L6 muscle cells following a time course treatment with 1mM βHB – Based on the results of figure 6 which showed that there was no change in palmitate oxidation with βHB treatment, it was expected that CPT1B content would remain unchanged as well. However, CPT1B content significantly increased 4.2-fold, 4.8-fold, and 6.6-fold after 1, 3, and 5 days of βHB treatment.

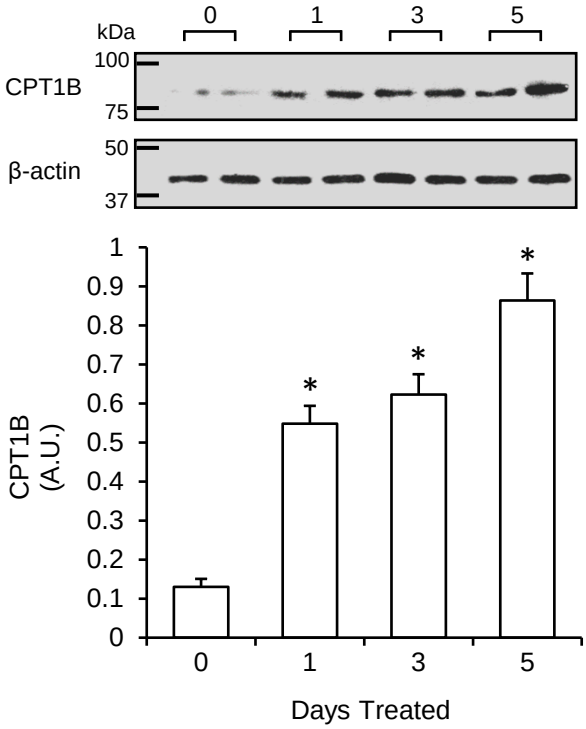

Figure 10: Western blot analysis of CPT1B. One-way ANOVA, n = 6-8. * *P* < 0.05 vs. 0 Days.

Discussion

Herein, we provide evidence that βHB supplementation can alter the glucose and fat metabolism of L6 skeletal muscle cells. The glucose uptake assay was used initially to evaluate various concentrations of βHB representing ketogenic diets with varying levels of carbohydrate restriction. A concentration of 1mM representative of a less carbohydrate-restricted KD was examined in subsequent experiments. This is in agreement with previous work that found that the threshold for the effects of βHB on glucose and lipids appears to be at least 1mM[111]. On a 1mM βHB time course, basal and insulin-stimulated glucose uptake in L6 muscle cells increased significantly (Fig, 2).

Though there is a lack of literature examining the metabolic effects of βHB supplementation on cell metabolism using in vitro models, our results are in agreement with results from studies using in-vivo models. One such study compared the effects of a ketogenic diet (4% of calories from carbohydrates) to a control diet (35% of calories from carbohydrates) on various physiological variables in obese men over a 4-week period[114]. Among the various improvements in health experienced by the KD-fed participants including a decrease in caloric intake, reduced hunger, and increased fat loss, including a decrease in blood glucose and insulin levels, and an overall improvement in the HOMA-IR by approximately 50%[114]. Another study by Mikkelsen et al., examined the effects of βHB infusion on skeletal muscle βHB utilization and metabolism in healthy human adult males[115]. Participants were subjected to low βHB infusion (4.7μM /kg/min) for 1 hour, medium infusion (9.4μM/kg/min) for the next hour, and high infusion (18.8μM/kg/min) for the final hour[115]. Samples of blood metabolites were taken before and during each of the infusion periods. Results showed that blood glucose and endogenous glucose production decreased as βHB infusion increase under basal and insulin-stimulated conditions[115]. Therefore, both our

research using an in vitro model and research using in vivo models shows an enhancement of glucose metabolism following treatment with βHB. Most human in-vivo studies propose various explanations for the observed decrease in basal blood glucose levels. This includes; the signaling actions of ketones (primarily βHB) in key tissues such as skeletal muscle, as well as an improvement in whole body glucose control/clearance with skeletal muscle playing a key[114,115]. Specifically, βHB treatment may altering skeletal muscle metabolism in a way that increases basal glucose uptake. These results were preserved with the stimulus of a meal which would be similar to our insulin stimulated conditions[114,115]. Another interesting finding was that the difference between basal and insulin mediated glucose uptake decreased with treatment. The 44% difference found in the controls diminished to only 15% after 5 days of treatment. It is clear that βHB is acting on signaling pathways involved in glucose uptake which are the PI3K-AKT and AMPK pathways, representing the insulin dependent and independent pathways respectively. However, βHB seems to be increasing insulin independent glucose uptake more than insulin dependent glucose uptake, as our results showed an enhanced phosphorylation of AMPK but not AKT with βHB treatment. As has been confirmed in other research, βHB treatment is not enhancing insulin-stimulated glucose uptake, but appears to be acting in an insulin-independent manner to increase glucose uptake[111].

To begin to understand the changes in the metabolic pathways responsible for the functional changes we observed, the content and phosphorylation of AKT and AMPK were measured. After a 1mM βHB time course treatment, L6 muscle cells displayed decreased phosphorylation of AKT, which is a part of the PI3K-AKT pathway (Fig. 8b). The mechanism behind this decrease has yet to be determined. This significant decrease in AKT phosphorylation would normally lead to a decrease in GLUT4 translocation to the plasma membrane and a

subsequent decrease in glucose uptake. This decrease however, seems to be compensated for by increase in the phosphorylation of AMPK, which plays a role in insulin independent glucose metabolism (Fig. 8a). In its active (phosphorylated) form, AMPK phosphorylates AS160, thereby deactivating it and allowing Rab to stay in its active GTP bound form. This allows GLUT4 translocation to the plasma membrane and an increase in glucose uptake. Therefore, this significant increase in AMPK phosphorylation may explain the increase in glucose uptake we found (Fig. 2).

Once it was established that glucose uptake increased significantly with βHB treatment, we wanted to determine what was the fate of the increased glucose within the cells. It is understood that glucose can be directed towards three main glucose utilization pathways; glucose oxidation, glycogen synthesis, and lactate production. We observed significant increases in glucose oxidation with βHB treatment suggesting that this is one of the metabolic pathways that glucose is being directed towards (Fig. 3). Although literature measuring glucose oxidation in similar experimental conditions could not be found, Cox et al., investigated metabolic changes in human male athletes pre, during, and post-exercise as a result of ketone supplementation[110]. While there was a decrease in plasma glucose concentrations and an increase in intramuscular glucose pre-exercise, muscle concentrations of the glycolytic intermediates, glyceraldehyde-3-phosphate, 2&3-phosphoglycerate, and pyruvate, were significantly lower following KE consumption compared with high carbohydrate, and high fat diets[110]. This decrease was proportional to increases in intramuscular βHB concentrations[110]. These results along with the fact that participants ingested their beverage only 15 minutes before testing, after an overnight (12h) fast, suggests that ketone supplementation suppled skeletal muscle glycolysis[110]. This study highlights the fact that observed effects of ketone (βHB) supplementation is heavily dependent on carbohydrate availability, as well as duration of treatment with ketones. In this context, it is possible that participants that have

achieved ketosis for at least 24 hours, while on a normal diet may also experience increases in glucose oxidation similar to what we have shown.

Glycogen synthesis was also examined as a potential fate of glucose, and we observed a significant increase in glycogen synthesis with 1mM βHB treatment under both basal and insulin-stimulated conditions (Fig. 4). These results are in agreement with a study that determined whether a dietary ketone ester (βHB), combined with plentiful glucose, could increase post-exercise glycogen synthesis in human skeletal muscle[116]. Subjects given a ketone ester drink experienced 50% greater muscle glycogen synthesis after 2h hyperglycemic clamp compared to controls on the clamp[116]. This difference was even greater when compared to controls given saline[116]. This study was very similar to our work considering that our cells also had a constant supply of glucose while supplemented with βHB. It is important to note that the difference between basal and insulin stimulated glycogen synthesis decreased from 54% to 33% with treatment, similar to what was seen in glucose uptake. This indicates that βHB signaling may play part in manipulating the signaling pathways involved in glycogen synthesis, and that it is also one of the pathways glucose may be directed towards.

To further investigate the mechanism behind the increase in glycogen synthesis, the content and phosphorylation of GSK3 and GS were measured. Cells treated with 1mM βHB displayed an enhancement in the phosphorylation of GSK3 (Fig. 9). The phosphorylation of GSK3 inactivates it, leaving it unable to phosphorylate and inactivate GS. Based on this, it was expected that PGS content would decrease. However, our results show that the phosphorylation of GS was unaffected by 1mM βHB treatment. While unphosphorylated, GS is in its active form and catalyzes glycogen synthesis. Together, an increase in the phosphorylation of GSK3 with no change in GS phosphorylation don't explain the significant increase in glycogen synthesis we found earlier. It is

possible that glycogen synthesis may have increased simply due to an increase in available substrate/glucose as a result of the increase in glucose uptake.

Finally, lactate production was measured and similarly to the previous glucose utilization pathways, it increased significantly in L6 muscle cells treated with 1mM βHB (Fig. 5). Although there is a lack of literature examining the effects of βHB on lactate concentrations using in vitro models, there is work that has examined the effects of a KD on aerobic performance and exercise metabolism in off-road cyclists[117]. However, results showed a decrease in plasma lactate concentrations in KD-fed participants at rest and during moderate intensity endurance exercise (between 50% and 70% VO2max)[117]. This is comparison to the same participants on a high carbohydrate diet (70% of calories from carbohydrates)[117]. A possible explanation is that the ability of the body to clear lactate from circulation through the Cori cycle may have increased[117,118]. This increase in efficiency may be due to the low-carbohydrate aspect of the KD making glucose cycling much more important. The Cori cycle refers to the metabolic pathway by which lactate is produced by anaerobic glycolysis in the muscles. Lactate leaves the muscle and is taken up by liver where it is converted back to glucose for further use by muscle[117,118]. In this context, the lactate produced by the L6 muscle cells would remain in the growth media after production as there would be no clearance mechanism. This is important because the assay used to analyze lactate production did so by measuring the lactate concentration in the growth media.

In addition to changes in glucose metabolism, we also examined fatty acid metabolism following 1mM βHB treatment. Significant changes to fatty acid metabolism were expected with βHB treatment given previous research that has shown this. We began by measuring the effects of various concentrations of βHB on palmitate oxidation in a dose response treatment. Concentrations ranging from 0.5mM to 2.5mM, representing ketogenic diets with varying levels of carbohydrate

restriction, were chosen. We found that there was no significant change in palmitate oxidation at any βHB concentration (Fig. 6). Although an increase was expected, a possible reason for this result was that the high circulating concentrations of FFAs seen in humans and rodents on KDs, was not present in the cell culture media. There would also need to be a reduction in available glucose, which would help transition skeletal muscle from using glucose as a fuel, to relying on FFAs. Therefore, there wasn't enough substrate available for β-oxidation to increase even if βHB was capable of altering fat metabolism. These concepts are explored by O'Malley et al., which examined the effects of βHB salt supplementation on cycling performance in healthy individuals[11]. They found that ketone salts were capable of altering metabolic response during exercise[11]. Specifically, the respiratory exchange ratio decreased at 30%, and 60% ventilatory threshold (VT) indicating a shift towards fatty acid oxidation during exercise[11]. They also found that total fat oxidized increased by 23% while total carbohydrates oxidized decreased by 8% after the 3 5-minute cycling stages and time trial stage[11]. It is proposed that there would be a degree of glucose depletion as the subjects completed the cycling stages in a fasted state[11]. Therefore, it is possible that glucose depletion along with βHB signaling played a role in promoting lipid utilization during aerobic exercise. Our experimental design differs as the growth media for our L6 muscle cells had a physiological concentration of glucose, but a relatively limited amount of FFAs. Our results may have differed had we also experimented with varying levels of substrate provided to our cells.

To confirm whether βHB treatment may have altered the content of proteins involved in fatty acid metabolism, we began by measuring CPT1B content, which is involved in fatty acid oxidation. CPT1B was chosen because it is responsible for catalyzing the rate limiting step in long chain fatty acid transport into the mitochondrial matrix for β-oxidation. Although there was no change in palmitate oxidation, we expected βHB to alter CPT1B content in a way that would

accommodate a large influx of FFAs, which would be normally present in individuals on a KD. We found that CPT1B content significantly increased in L6 muscle cells treated with 1mM βHB (Fig. 10). An increase in CPT1B indicates that βHB may preparing the cell for a larger influx of FFAs. The importance of CPT1B is highlighted by Wicks et al., which created CPT1B knockout mice. Skeletal muscle fatty acid oxidation in these mice was 50% lower than controls, and similar to control mice treated with etomoxir, a nonselective whole-body CPT1 inhibitor[119]. Interestingly, the knockout mice displayed enhanced carbohydrate utilization signaled by lowered blood glucose levels, increased PDH activity and pyruvate oxidation[119]. The enhancement of CPT1B activity, although in the context of possible effects on skeletal muscle physiology, has been explored[120]. Henique et al., were able to express a mutant version of CPT1B, which was insensitive to malonyl-CoA, its physiological inhibitor, in the tibialis anterior of mice. Results showed that fatty acid oxidation increased 40%, which mimic our idea that the increase in CPT1B we saw can lead to significant increases in fatty acid oxidation, given enough substrate[120].

To ensure that the 1mM βHB treatment was not having a negative effect on the viability of the L6 muscle cells, an ATP assay was used. The assay measured intracellular ATP concentrations as an indicator of cell health/viability (Fig. 7). Our results showed that there was no negative effect on cell viability. Interestingly however, there was a significant increase in ATP concentration with no difference in protein content between treatment conditions (Fig. 7). These results suggest that the βHB treatment may be increasing cell turnover, which would explain the increased ATP production. The exact mechanism behind these results is unclear, however it is possible that the increase in glucose oxidation we saw may play a role.

Conclusion

The overall objective of this study was to determine if βHB supplementation can mimic the effects of a KD on skeletal muscle glucose and fat metabolism. Based on our finding it is clear that βHB does in fact alter glucose metabolism. We have shown that 1mM of βHB, which represents a less carbohydrate restrictive diet, significantly increased glucose uptake, as well as the utilization of glucose through glucose oxidation, glycogen synthesis, and lactate production. Interestingly, βHB treatment had no effect on palmitate oxidation, despite increasing the content of CPT1B. This suggests to us that given enough substrate, our L6 muscle cells may have displayed an increase in palmitate oxidation. Analysis of a few key proteins involved in glucose metabolism; AKT, AMPK, GSK3, and GS supported the outcomes of our functional assays. Our results suggest that the enhance glucose uptake is due to an enhancement of the insulin-independent glucose uptake pathway.

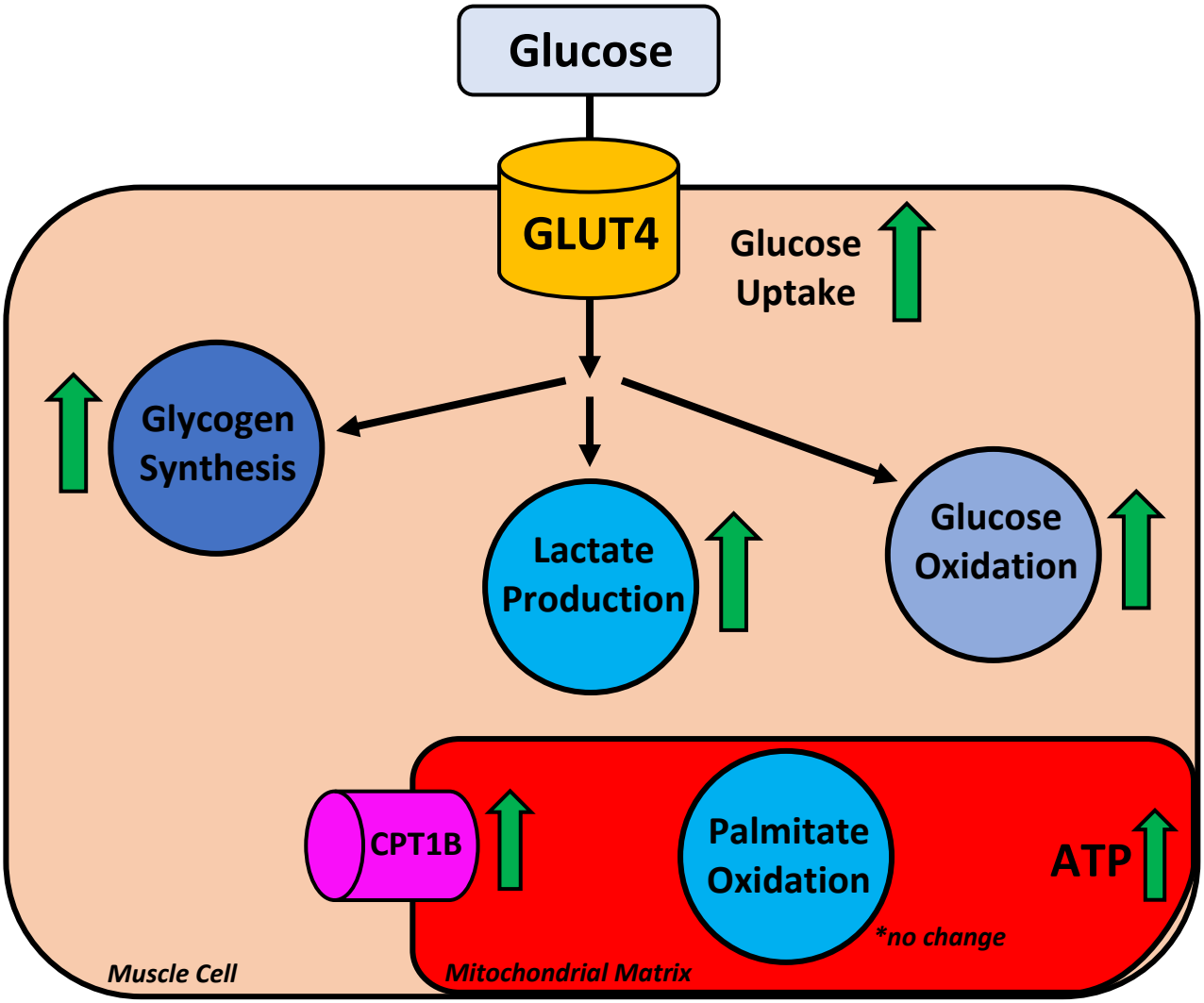

Figure 11: Outline of the effects of BHB supplementation on L6 muscle cell metabolism

Our study showed that supplementing L6 muscle cells with 1mM βHB resulting in changes in metabolism. Specifically, we showed that glucose uptake increased significantly after a single day of treatment. To determine the fate of the increased glucose within the cells, assays to measure the three main pathways that glucose can enter; glycogen synthesis, lactate production, and glucose oxidation, were done. We found that each of these aspects of glucose metabolism increased significantly. This suggested that the increased glucose within the cell is directed towards all three pathways. Palmitate oxidation was measured to find any potential effects on fat metabolism. No significant change was found. The content of the protein CPT1B, responsible for the transport of long chain fatty acids into the mitochondria matrix, increased significantly. This suggested a possible priming effect that may be preparing the cell for large influx of free fatty acids. A cell viability assay was run to determine if βHB supplementation may be having negative effects on cell health. This was done by measuring ATP within cells which indicates viable cells. We found that ATP concentration increased with treatment possibly indicating increased cell turnover.

Future Directions

In our study we observed that 1mM βHB is capable of altering skeletal muscle glucose metabolism, while changes in fat metabolism require further study. Although there was no change in palmitate oxidation with the dose response treatment, there was an increase in CPT1B content. CPT1B is the first component of the carnitine palmitoyl transferase system and is responsible for catalyzing the rate limiting step. It is also important to note that the cell culture growth media possess a fraction of the FFAs normally present in physiological conditions. In this context, the results suggest that the L6 muscle cells are capable of increased fatty acid oxidation if given adequate amounts of substrate. To confirm this, experiments in which L6 muscle cells are subjected to a dose response treatment using growth media with additional FFAs should be carried out. These experiments should be done with varying concentrations of FFAs that are representative of a range of physiological conditions. It is also important that the content of key proteins involved in the process of fatty acid oxidation also be measured. These include proteins in the carnitine palmitoyl transferase system as well as those responsible for transport into the cell such as fatty acid transport protein (FATP) 1, and 4, and CD36. These proteins are especially worthy of investigation as their behavior resembles that of GLUT4[121,122]. Both CD36 and GLUT4 are recruited from intracellular pools by the insulin-stimulated PI3K-AKT pathway[121,122]. Similarly, muscle contraction, and AMPK activation induce translocation of GLUT4, CD36, as well as FATP1 and 4[121,122]. These experiments would help to clarify if βHB concentrations representing KDs with varying levels of carbohydrate restriction can alter palmitate oxidation.

When considering the possible mechanisms behind the βHB induced changes in the molecular pathways that caused the changes in glucose and fat metabolism we observed, the answer remains unclear. It has also been established that βHB is a HDAC inhibitor and is capable

of regulating post translational protein modifications such as acetylation, succinylation, and β-hydroxybutyrylation. Through these interactions, βHB can alter the expression of various genes including those involved in glucose and fat metabolism. For example, PPARα, FGF21, PGC1α, and some FOXO genes such as FOXO3 are known to be regulated by βHB indirectly through HDACs 1 and 2. Experiments that measure the gene expression and content of proteins that may be subjected to these βHB interactions, using cells grown in media with and without additional FFAs, should be carried out.

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APPENDICES

Appendix A - Detailed Experimental Methods

Lysis Buffer for Homogenization

Reagent	Concentration/MW
NaCl	135mmol/L (MW=58.44)
$MgCl_2$	1mmol/L (MW=203.3)
KCl	2.7mmol/L (MW=74.55)
Tris (pH 8)	20mmol/L (MW=121.14)
Triton 1%	
Glycerol 10%	

Prepare lysis buffer stock and store at -20°C. Aliquot desired volumes and add protease (cOmplete ULTRA Tablets) and phosphatase (PhoStop) inhibitors immediately prior to use.

Laemmli Sample Buffer (2x) - (Bio-Rad, Cat#161-0737)
Per 1mL: 950µl of 2x Laemmli sample buffer
50Ul β-Mercaptoethanol

Store at room temperature. Dilute the sample (1 in 2) with sample buffer and heat for 5min at 95°C.

Preparation of Tissue Lysates

1. Upon extraction, immediately snap freeze samples and store at -80°C.
2. Weigh sample (~ 20mg for liver) and add to 350µl of lysis buffer containing both protease & phosphatase inhibitors. (Always keep samples on ice and avoid thawing while weighing tissue)
3. Thoroughly homogenize the sample.
4. Centrifuge the tissue lysate for 10min @ 12,000rpm (4°C).
5. Extract the supernatant and transfer to a fresh micro tube.
6. Take one aliquot for protein determination by Bradford method.
7. Aliquot remaining lysate and store at -80°C.
8. For western blot purposes, dilute lysates with 2x lammeli buffer (1 to 1 v/v) as needed and heat for 5 min at 95°C prior to use.

Western Blotting Buffers

10x Electrophoresis/Running Buffer (pH - 8.3)
30.34g Tris base
144g Glycine
10g SDS
Dissolve contents in 1L of ddH20 and store at room temperature.

1x Running Buffer (pH- 8.3)
10% 10x Running buffer
90% ddH20
Mix solutions and store at room temperature.

10x Transfer Buffer (pH- 8.3)
30.3g Tris base
144g Glycine
Dissolve contents in 1L of ddH20 and store at room temperature.

1x Transfer Buffer (pH- 8.3)
10% 10x Transfer buffer
20% Methanol
70% ddH20
Mix solutions and store at -20°C prior to use.

10x Wash Buffer
60.57g Tris base
87.66g Sodium Chloride (NaCl)
Dissolve contents in 1L of ddH20, store at room temperature.

1x Wash Buffer
10% 10x Wash buffer
90% ddH20
Add 500Ul/L of Tween-20 and NP-40. Mix solutions and store at room temperature.

Blocking Buffer
3% BSA (w/v: 1.5g/50mL)
Dissolve in 1x Wash buffer, store at 4°C.

Antibody (Ab) Buffer
1° Ab – 1 part blocking buffer + 2 parts wash buffer + 0.02% NaAzide (stock in ddH20)
2°Ab – 1 part blocking buffer + 2 parts wash buffer (NO NaAzide).
Typically 1:1000-1:2000 dilution is appropriate for an Ab. This may vary depending on how good the signal is.

Resolving gel Tris Buffer (1.5M) (pH-8.8)
90.86g/500mL of ddH20

Stacking gel Tris Buffer (0.5M) (pH-6.8)
30.3g/500mL of ddH20

10% APS Solution
10% (w/v) Ammoniumperoxide Sulfate in ddH20.
Use 0.1g/mL Store at -20°C.

10% SDS Solution
10% (w/v) Sodium dodecylsulfate in ddH20
Use 1g/10mL Store at room temperature.

Western Blotting

1. Take samples out of -80° freezer and place on ice.
2. Place gels into cassettes, and add the cassette to tank ensuring red and black terminals of cassette correspond with red and black markings of tank respectively.
3. Add 1x running buffer to fill the tank.
4. Once samples have thawed, spin in centrifuge for a few seconds.
5. Take out combs from gel and pipette 7ul Bio-Rad protein ladder.
6. Add samples into each well according to Bradford values.
7. Top up with running buffer to make sure tank is full.
8. Ensure positive and negative electrodes are matched (black to black, red to red)
9. Turn on the voltage for 60V for 30min, and then turn it up to 110V for ~1.5hrs until dye runs off the gel.
10. While gel is running, can prepare 1x transfer buffer. Once transfer buffer is well mixed, cover with parafilm and place in the -20°C freezer until ready for transfer.

Transferring the Gel onto a membrane

1. Fill Pyrex dish with cold transfer buffer.
2. Cut out equal sized membranes and dip in methanol to activate (2min). Also cut out equal sized filter papers and prepare the appropriate number of foam pads.
3. Place membranes in transfer buffer after activation.
4. Once dye has run off the gel, remove the gels from tank and soak in transfer buffer. Carefully remove glass plates. Cut off and discard combs of the gel. Loosen gel from the glass plate with scraper while keeping it emerged in the buffer.
5. In the pyrex dish, place the black side of the cassette on the bottom, and place two foam pads followed by 3 filter papers on top. Ensure there are no bubbles.
6. Carefully place gel on top of filter paper and use the roller to get any air bubbles out. Make sure gel is in the correct orientation so that the ladder will appear on the left side of the membrane when removed. Note: transfer runs from negative (black) to positive (red). Always ensure proteins will run from gel to the membrane.
7. Carefully place the membrane on top of the gel and roll out any bubbles.
8. Place 3 more filter papers on top and roll out any bubbles.
9. Add one foam pad and roll out any bubbles.
10. Carefully close sandwich and place into transfer tank. Make sure black matches black and red matches red.
11. Place ice pack in tank to keep buffer cold. Close the lid ensuring positive and negative electrodes are matched (black to black, red to red)
12. Surround transfer tank with ice to keep cold. 12. Turn on transfer at 120V for 2.5 hours or at 60V overnight.
13. Look for bubbles on ends of cassette to indicate successful transferring.
14. Check on temperature throughout transfer time to ensure no overheating.

Probing the membrane

1. Prepare containers to hold blocking buffer for each membrane, approx. 10mL per container.
2. Once transfer has finished, open cassettes and quickly place membranes in containers with blocking buffer.
3. Allow the membranes to surf in the blocking buffer for 1hr at room temperature on the orbital shaker.
4. Pour out blocking buffer and add 1°Ab.
5. Incubate overnight on shaker at 4°C. Ensure containers are fully sealed to avoid evaporation.
6. The next day, remove 1°Ab and wash membranes 5x at 10min intervals with 10mL of 1x wash buffer to rid the membrane of any unbound Ab.
7. Add 2° Ab and allow membranes to surf on orbital shaker for 1hr at room temperature.
8. Remove 2°Ab and wash membranes 5x at 10min intervals with 10mL of 1x wash buffer to rid the membrane of any unbound 2°Ab.
9. Membranes are ready for developing

Developing the membrane

1. For each membrane, use 3mL chemiluminescence (Millipore Immobilon Western Chemiluminescent HRP substrate) per membrane and incubate for 3 minutes.
2. Dip membranes into ddH20 to rinse and place in radiography cassette.
3. In the darkroom, expose film for desired time.
4. Place film in developer for a few seconds until signal appears. Dip into water to stop the reaction, and place in fixer solution. Ensure ample fixing time.
5. Rinse with water and allow drying.