THE EFFECTS OF ACUTE NUTRITIONAL KETOSIS ON MYOFIBRILLAR PROTEIN SYNTHESIS IN YOUNG MEN

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LIST OF ABBREVIATIONS

4E-BP1 – Eukaryotic translation initiation factor 4E-binding protein 1

AcAc – Acetoacetate

Akt – Protein kinase B

AMPK - AMP-activated protein kinase

ATP – Adenosine triphosphate

AUC – Area under the curve

BiP – Binding immunoglobulin protein

BCAA – Branched chain amino acid

β-OHB – β-hydroxybutyrate

CASTOR1 – Cytosolic arginine sensor for mTORC1 subunit 1

CHO – Carbohydrate

DIAAS – Digestible indispensable amino acid score

EAA – Essential amino acid

eEF2 – Eukaryotic elongation factor 2

eIF4A – Eukaryotic initiation factor 4A

eIF4E – Eukaryotic translation initiation factor 4E

FoxO – Forkhead box class O

FSR – Fractional synthetic rate

GATOR2 – GAP activity towards Rags 2

HMG-CoA – 3-hydroxy-3-methylglutaryl CoA

IGF-1 – Insulin-like growth factor 1

IRE1α –Inositol requiring enzyme 1α

ISWS – Internal standard working solution

KET – Ketone monoester supplement

KET+PRO – Ketone monoester + protein supplement

LC3b – Light chain 3 beta

MAFbx – Muscle atrophy F-box

MPB – Muscle protein breakdown

MPE – Mole percent excess

MPS – Muscle protein synthesis

MyoPS – Myofibrillar protein synthesis

mTOR – Mammalian target of rapamycin

mTORC1 – Mammalian target of rapamycin complex 1

MuRF1 – Muscle RING finger 1

NEAA – Non-essential amino acid

p70S6K – Ribosomal protein S6 kinase beta-1

PDI – Protein disulfide isomerase

PRO – Protein supplement

Rheb – Ras homolog enriched in brain

SOD2 – Superoxide dismutase 2

SUnSET – Surface sensing of translation

TCA – Tricarboxylic acid

TFEB – Transcription factor EB

TSC2 – Tuberous sclerosis complex 2

ULK1 – Unc-51-like kinase 1

UPLC-MS – Ultra-performance liquid chromatography mass spectrometry

V-ATPase – Vacuolar-type ATPase

ABSTRACT

Background: There is some evidence supporting a role for ketone bodies as important regulators of protein synthesis and/or breakdown. To date, no studies have investigated whether orally ingested exogenous ketones can stimulate increased rates of myofibrillar protein synthesis (MyoPS), and whether this effect may be augmented by co-ingestion with dietary protein.

Objectives: To evaluate the effects of elevated β-hydroxybutyrate (β-OHB), induced via the ketone monoester (R)-3-hydroxybutyl (R)-3-hydroxybutyrate, on MyoPS rates when consumed without protein, and when co-ingested with a sub-optimal dose of protein (10 g) in healthy young men.

Methods: In a randomized, double-blind, parallel group design, 36 healthy young men received a primed continuous infusion of L-[ring-$^{2}H_5$]-phenylalanine and were randomly assigned to receive one of three nutritional treatments. A ketone monoester (KET), 10 g whey protein (PRO), or the combination of both (KET+PRO). Blood and muscle biopsy samples were collected during the basal post-absorptive state and during a 300 min postprandial period to assess β-OHB, plasma glucose, insulin, and amino acid concentrations, and MyoPS rates.

Results: Capillary blood β-OHB increased ($P < 0.001$) following ingestion of the ketone monoester and were significantly greater in both KET and KET+PRO than in PRO from 30-180 min in the postprandial period. Incremental area under the curve (iAUC) over the entire 300 min postprandial period for leucine, essential amino acids (EAA), and total amino acids (TAA) was greater ($P < 0.001$) in both KET+PRO and PRO than in KET. iAUC for leucine and EAA was greater ($P < 0.001$) in KET+PRO than in PRO. KET, PRO, and KET+PRO increased MyoPS rates (0-300 min) when compared to basal post-absorptive values (from 0.033 ± 0.011 to 0.053 ± 0.014

%/h, from 0.036 ± 0.011 to 0.049 ± 0.008 %/h, and from 0.029 ± 0.010 to 0.048 ± 0.009 %/h, respectively (Time: $P < 0.001$)), with no differences between treatments (Treatment: $P = 0.38$).

Conclusion: MyoPS rates following the ingestion of a ketone monoester supplement do not differ from rates observed after ingesting a sub-optimal 10 g dose of whey protein or their co-ingestion in moderately active, healthy young men.

RÉSUMÉ

Contexte : Il existe certaines preuves soutenant le rôle des corps cétoniques en tant que régulateurs importants de la synthèse et/ou de la dégradation des protéines. Jusqu'à présent, aucune étude n'a cherché à savoir si des corps cétoniques exogènes ingérés par voie orale peuvent stimuler des taux accrus de synthèse protéique myofibrillaire (MyoPS), et si cet effet peut être augmenté par la co-ingestion de protéines alimentaires.

Objectifs : Évaluer les effets d'un taux élevé de β-hydroxybutyrate (β-OHB), induit par le monoester cétonique (R)-3-hydroxybutyl (R)-3-hydroxybutyrate, sur les taux de MyoPS lorsqu'il est consommé sans protéine, et lorsqu'il est co-ingéré avec une dose sous-optimale de protéine (10 g) chez de jeunes hommes en bonne santé.

Méthodes : Dans une étude randomisée, en double aveugle et en groupes parallèles, 36 jeunes hommes en bonne santé ont reçu une perfusion continue de L-[ring-$^{2}H_{5}$]-phénylalanine et ont été assignés au hasard à l'un des trois traitements nutritionnels. Un monoester cétonique (KET), 10 g de protéines de lactosérum (PRO), ou la combinaison des deux (KET+PRO). Des prélèvements sanguins et des biopsies musculaires ont été effectués pendant l'état basal post-absorptif et pendant une période postprandiale de 300 min pour évaluer les concentrations de β-OHB, de glucose, d'insuline et d'acides aminés plasmatique, ainsi que les taux de MyoPS.

Résultats : Le β-OHB du sang capillaire a augmenté ($P < 0,001$) après l'ingestion du monoester cétonique et était significativement plus élevé chez KET et KET+PRO que chez PRO de 30 à 180 min dans la période postprandiale. L'aire incrémentale sous la courbe (iAUC) sur l'ensemble de la période postprandiale de 300 min pour la leucine, les acides aminés essentiels (EAA) et les acides aminés totaux (TAA) était plus grande ($P < 0,001$) chez KET+PRO et PRO que chez KET. L'iAUC pour la leucine et les EAA était plus grande ($P < 0,001$) chez KET+PRO que chez PRO. KET,

PRO et KET+PRO ont augmenté les taux de MyoPS (0-300 min) par rapport aux valeurs basales post-absorption (de 0,033 ± 0,011 à 0,053 ± 0,014 %/h, de 0,036 ± 0,011 à 0,049 ± 0,008 %/h, et de 0,029 ± 0,010 à 0,048 ± 0,009 %/h, respectivement (Temps : $P < 0,001$)), sans différences entre les traitements (Traitement : $P = 0,38$).

Conclusion : Les taux de MyoPS après l'ingestion d'un supplément de monoester cétonique ne diffèrent pas des taux observés après l'ingestion d'une dose sous-optimale de 10 g de protéines de lactosérum ou de leur co-ingestion chez de jeunes hommes modérément actifs et en bonne santé.

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CONTRIBUTION OF AUTHORS

Jamie Lov (first author): conceived and designed the research, conducted the research, analyzed the data, interpreted the results of the experiments, prepared the figures, drafted the thesis, read and approved the final thesis, and holds primary responsibility for the final content along with the principle investigator (Dr. Tyler A. Churchward-Venne).

Stephanie Hawley: conducted the research

Sarkis Hannaian: conducted the research

Ari Gritsas: conducted the research and analyzed the data

Dr. Tyler A. Churchward-Venne (principle investigator): secured financial support for the research, conceived and designed the research, interpreted the results of the experiments, edited and revised the thesis, read and approved the final thesis, and holds primary responsibility for the final content.

CHAPTER 1: INTRODUCTION

1. INTRODUCTION

Ketone bodies (i.e. β-hydroxybutyrate (β-OHB), acetoacetate (AcAc), and acetone) are lipid-derived molecules normally produced by the liver whose production is amplified in response to starvation or prolonged fasting, a very low carbohydrate (CHO) "ketogenic" diet, and/or prolonged glycogen-depleting exercise (1). β-OHB, the main ketone body in circulation, can act as an alternative energy substrate to CHO and fat via mitochondrial oxidation in metabolically active tissues such as the brain, heart, and skeletal muscles (1). In addition to serving as an alternative energy source under conditions of low CHO availability, ketone bodies are important regulators of CHO, lipid, and protein metabolism (1). Within the context of protein metabolism, there is some evidence supporting a role for ketone bodies as important regulators of protein synthesis (2) and/or breakdown (3). For example, experimentally elevating ketone bodies has been reported to improve nitrogen balance (a proxy measure of protein turnover) under catabolic conditions including post-surgery (4), skeletal trauma (5), and severe burns (6). Additionally, intravenous infusion of sodium β-OHB to ∼2 mM has been reported to reduce leucine oxidation and stimulate increased rates of skeletal muscle protein synthesis (MPS) in humans (2). Recently, ketone bodies have also been demonstrated to enhance the phosphorylation status of key proteins within the mammalian target of rapamycin complex 1 (mTORC1) signaling cascade including p70S6K1 (ribosomal protein S6 kinase beta-1) and 4E-BP1 (eukaryotic translation initiation factor 4E-binding protein 1) (at Thr389 and the percentage of 4E-BP1 in the phosphorylated γ-form, respectively) in human muscle (7). This was in comparison to a placebo condition during the recovery period following intensive glycogen-depleting exercise in which participants also received a high-dose protein-CHO mixture (7). In vitro, ketone bodies also potentiated the increase in mTORC1 activation and protein synthesis in leucine-stimulated myotubes (7).

Most studies to date examining the impact of elevated plasma ketone bodies (i.e., hyperketonemia) on markers of metabolic control have relied on the intravenous infusion of ketone bodies (8–10), or dietary manipulation (e.g., fasting, starvation) (11,12). Recently, orally ingested exogenous ketone supplements have become available (13), permitting a more practical and direct means of testing the metabolic effects of elevated blood ketone bodies without the confounding influence of a ketogenic diet or starvation. For example, the ketone monoester (R)-3-hydroxybutyl (R)-3-hydroxybutyrate has been shown to increase circulating β-OHB concentrations to ~3.0 mM within ~30 min in healthy humans (7,13,14).

Although increases in ketone body availability may enhance mixed skeletal MPS rates (2), it is currently unknown whether orally ingested exogenous ketones can stimulate increased rates of myofibrillar protein synthesis (MyoPS), and whether this effect may be augmented by co-ingestion with dietary protein. Dietary protein represents a source of amino acids (i.e. the "building blocks" of muscle protein), that are well established to stimulate increased MPS rates (15,16). Previous studies have demonstrated a dose-dependent relationship between protein intake and MPS rates in young men (17). For example, the ingestion of 20 g of protein has been reported to stimulate MPS rates greater than that observed following the ingestion of 5 g and 10 g of protein, with no further stimulation with ingestion of 40 g of protein (17). Therefore, per meal ingestion of 20 g protein appears sufficient to maximally stimulate MPS rates (17), with doses below this threshold representing a "sub-optimal" amount of protein to maximally stimulate MPS rates.

1.1. Purpose and Hypothesis

The aim of the present investigation was to evaluate the effects of elevated β-OHB, induced via the ketone monoester (R)-3-hydroxybutyl (R)-3-hydroxybutyrate, on MyoPS rates when consumed without protein (KET), and when co-ingested with a sub-optimal dose (10 g) of protein

(KET+PRO) in healthy young men. It was hypothesized that elevated β-OHB via KET would stimulate increased MyoPS rates compared to basal conditions. Given the dose-response relationship between protein ingestion and MPS rates (17–19), it was also hypothesized that the ingestion of a sub-optimal 10 g dose of whey protein (PRO) would stimulate increased MyoPS rates; however, it was hypothesized that MyoPS rates would be further increased when the ketone monoester (R)-3-hydroxybutyl (R)-3-hydroxybutyrate was co-ingested with 10 g of whey protein (KET+PRO).

CHAPTER 2: LITERATURE REVIEW

2. LITERATURE REVIEW

2.1. Introduction

The goal of this literature review is to present the pertinent research surrounding ketone bodies and their effect on protein/amino acid metabolism in order to provide a framework for the objectives and hypothesis of the current study. The literature review begins with a physiological overview of ketone bodies, with emphasis placed on ketosis induced via exogenous ketone supplements. The role of dietary protein in regulating protein metabolism is then discussed, followed by an overview of the role of ketone bodies in the regulation of whole-body and skeletal muscle protein turnover. The literature review finishes with an overview of contemporary methodology to measure *in-vivo* rates of human skeletal MPS based on the precursor-product principle in order to provide a rationale for its application in the current study.

2.2. Overview of ketone bodies

Ketone bodies are lipid-derived molecules normally produced by hepatic mitochondria under conditions of starvation or prolonged fasting, a very low CHO "ketogenic" diet, and in response to prolonged glycogen-depleting exercise (1). As a result of these conditions, the body enters a state known as "ketosis". A blood ketone body concentration > 0.5 mM is often defined as the threshold for ketosis or hyperketonaemia (20), although concentrations > 0.2 mM have also been proposed (21). The production of endogenous ketone bodies and the resulting state of endogenous ketosis is driven by the combination of low CHO availability in the body and low blood insulin concentration (22). With low blood insulin concentrations, the balance between triacylglycerol formation and lipolysis favours the latter, causing adipocytes to release non-esterified fatty acids into the bloodstream. Circulating fatty acids are taken up by the liver (i.e., by hepatocytes), which is where ketogenesis primarily occurs (23). As a result of beta-oxidation, the

excess fatty acids are converted into acetyl-CoA (22). However, during conditions of low CHO availability, there is insufficient production of oxaloacetate, a tricarboxylic acid (TCA) cycle intermediate, to sustain the complete entry of all the newly formed fatty acid-derived acetyl-CoA into the TCA cycle (22). Instead of entering the TCA cycle in the hepatic mitochondria, acetyl-CoA can undergo a series of reactions to eventually produce AcAc (22). This series of reactions include the formation of acetoacetyl CoA and 3-hydroxy-3-methylglutaryl CoA (HMG CoA) as intermediary steps, catalyzed by *thiolase* and *HMG CoA synthase*, respectfully. The AcAc can then directly enter the bloodstream, be further reduced to β-OHB, via the enzyme *3-hydroxybutyrate dehydrogenase*, before entering the bloodstream, or spontaneously decarboxylate to form CO_2 and acetone (both of which exit the body via ventilation) (22,24). Once in the bloodstream, AcAc and β-OHB can be transported into the mitochondrial matrix of extrahepatic cells (24). In extrahepatic tissues (e.g., brain, heart, muscle), β-OHB is oxidized back to AcAc which is ultimately used to generate acetyl-CoA. The latter reaction includes the formation of acetoacetyl CoA from AcAc, catalyzed by *succinyl CoA oxoacid tranferase*, leading to the eventual cleavage of an acetyl group and formation of acetyl-CoA, catalyzed by *methylacetoacetyl CoA thiolase* (22). Ketone body-derived acetyl-CoA can then enter the TCA cycle and serve as an energy source.

2.2.1. The ketogenic diet

Up until recently, the only way of achieving a state of ketosis via dietary changes (independent of prolonged fasting or starvation) was through the adoption of a traditional ketogenic diet. The initial focus of the ketogenic diet was its application as a therapeutic approach for pathological conditions such as those involving insulin resistance or for the treatment of certain diseases like epilepsy (25). Characterized by the adherence to a dietary intake pattern high in lipids

and low in CHO, this diet relies on the production of endogenous ketone bodies through hepatic ketogenesis in order induce ketosis. The most common design for the ketogenic diet is structured in a 4:1 fat to non-fat ratio with the former constituting 75-80% of daily energy intake while protein and CHO comprise 15-20% and <5%, respectively (26,27). The ketogenic diet induces a metabolic state that closely resembles that of starvation whereby the deprivation of glucose results in the subsequent production of ketone bodies in order to provide an alternative energy substrate that bodily tissues can utilize (28). It is important to highlight that ketone bodies can account for nearly 2/3 of the brain's energy needs during periods of starvation due to their permeability across the blood-brain barrier (22). Nutritional ketosis can be achieved after ~4 days on a ketogenic diet, with ketone body concentrations varying depending on the extent of CHO restriction (1). There is however a period of time that may be required before body organs and systems begin to acclimate/adapt to using ketone bodies as a primary fuel source, a process termed "keto-adaptation" (29,30). Keto-adaptation is thought to require adherence to a ketogenic diet for at least 3-4 weeks along with sodium and potassium supplementation (11). Before full adaptation, symptoms of fatigue, a general loss of well-being, and an increase in the perception of effort during exercise can occur as the body adapts to this shift in fuel reliance (27). A major metabolic change that accompanies keto-adaptation is the increased capability to oxidise and utilize fat for energy (29). Following adaptation, rates of maximal fat oxidation during exercise can represent 200-250% of values obtained during a high CHO diet (31,32). The keto-adaptation process also leads to a decrease in CHO oxidation during exercise (31–33). This has been attributed to the corresponding scarcity of CHO that results from dietary restriction as well as an impairment in the ability to oxidise stored glycogen (27). With the sustained restriction of dietary CHO during a ketogenic diet, blood β-OHB levels can reach ~7-8 mmol/L without a change in blood pH (26). However, it

can be understood that the adoption of a ketogenic diet can be quite a lengthy process and problems with sustained compliance due to the dietary restrictions can arise. Due to the emphasis on fat as the main dietary macronutrient, the ketogenic diet may pose an increased risk of elevating serum cholesterol and triglyceride levels (34). There could also be the additional challenge of experiencing adverse symptoms that have been associated with the ketogenic diet, termed the "keto-flu" (35). The dietary restrictions and the time required to achieve keto-adaptation represent major hurdles with regards to the feasibility and practicality of implementing a ketogenic diet (36).

2.2.2. Exogenous ketone supplements

Recently, orally ingested exogenous ketone supplements have become commercially available that can induce varying levels of nutritional ketosis (i.e., increase blood ketone body concentration) within several minutes without the need for dietary CHO restriction (1). The two main forms of exogenous dietary ketone supplements that are currently available are ketone salts and ketone esters (36), with the latter being the more potent and reliable option for inducing nutritional ketosis (36). Ketone salt supplements (e.g., sodium, potassium and/or calcium β-OHB) may result in excessive mineral intake and often provide a racemic mixture of the two optical isoforms of β-OHB, namely D-β-OHB, and L-β-OHB (36,37). Only D-β-OHB is bioactive, while L-β-OHB is not normally found in blood. Furthermore, the concentration of blood β-OHB achieved with exogenous ketone salt supplements is typically <1 mM (38). Alternatively, ketone ester supplements may include ketone monoester (37,38) and diester compounds (38). The ketone monoester (R)-3-hydroxybutyl (R)-3-hydroxybutyrate (**Figure 1**) is a non-racemic ketone supplement (37) that can rapidly (within 30 minutes) raise blood β-OHB concentration to 3–5 mM (13), concentrations similar to that seen after several days of fasting (13,39). After ingestion of the ketone monoester (R)-3-hydroxybutyl (R)-3-hydroxybutyrate (**Figure 2**), the monoester bond is

cleaved by esterase enzymes in the gut, yielding both β-OHB and butanediol (37). These molecules are absorbed into the portal circulation where butanediol is then taken up by the liver and converted into β-OHB by the enzyme *alcohol dehydrogenase* (37). The β-OHB then leaves the liver via monocarboxylate transporters and enters systemic circulation (37). Exogenous ketone supplements can come with their own set of adverse effects, the main reported symptom being gastrointestinal discomfort following ingestion (37). However, the frequency and severity of this can be influenced by factors including the dosage and type (i.e., ketone salt or ester) of exogenous ketone consumed (40). In fact, the ingestion of the ketone monoester (R)-3-hydroxybutyl (R)-3-hydroxybutyrate, three times daily has been shown to be safe and tolerable in healthy adults over a duration of 28 days (41).

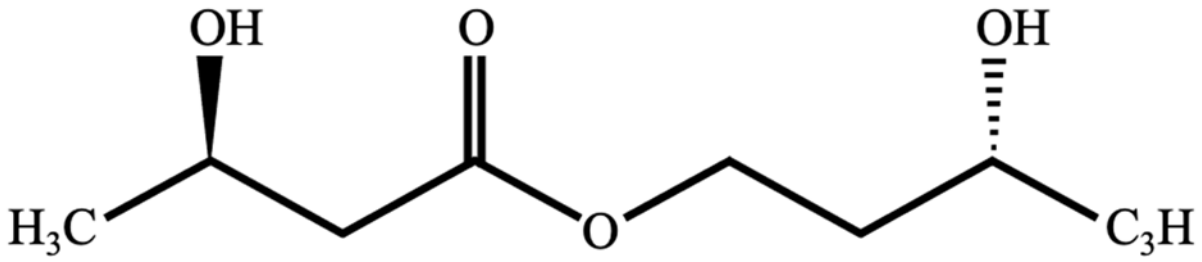

Figure 1. The chemical structure of (R)-3-hydroxybutyl (R)-3-hydroxybutyrate. Adapted from (42).

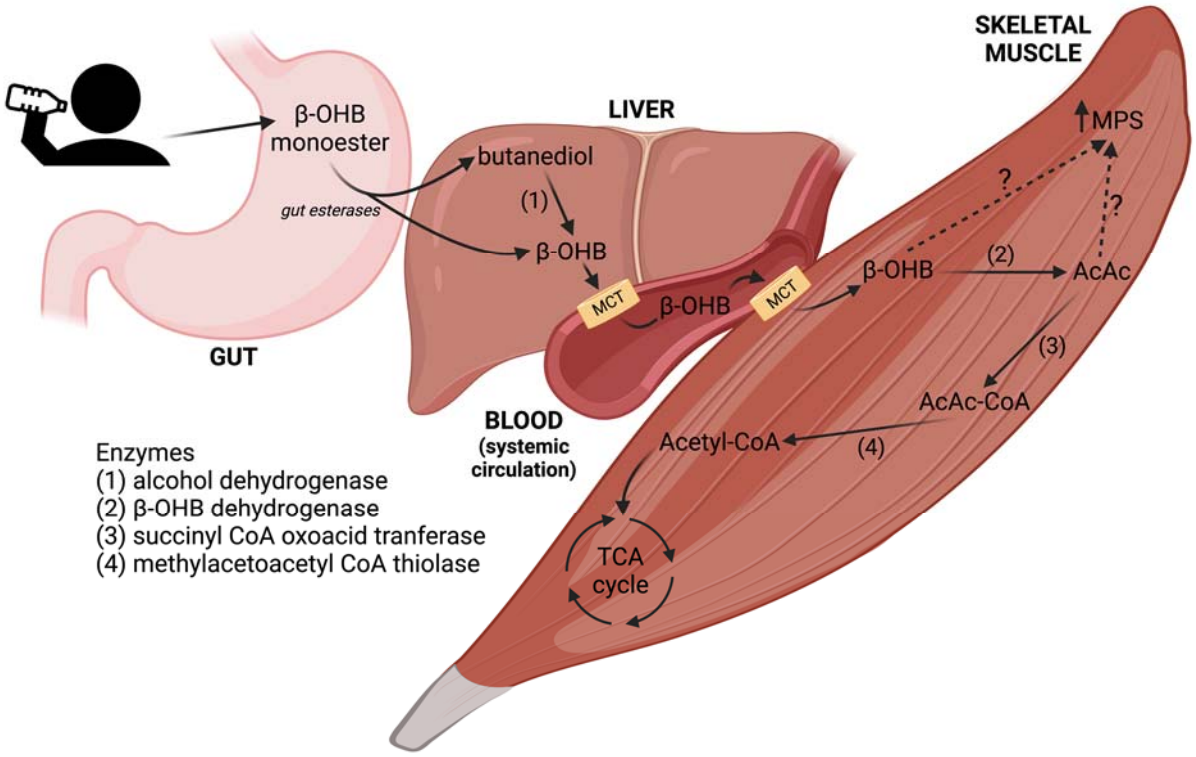

Figure 2. The metabolism of the β-OHB monoester. Following ingestion, the monoester bond is cleaved by gut esterases. The resulting β-OHB and butanediol are then taken up by the liver with the latter further converted into β-OHB by the enzyme alcohol dehydrogenase. β-OHB enters and leaves systemic circulation via monocarboxylate transporters (MCT) to reach peripheral tissues such as muscle. Within the muscle, β-OHB is oxidized back to AcAc, catalyzed by β-OHB dehydrogenase. Acetoacetyl CoA is generated from AcAc, catalyzed by succinyl CoA oxoacid tranferase. An acetyl group is cleaved from acetoacetyl CoA leading to the formation of acetyl-CoA, catalyzed by methylacetoacetyl CoA thiolase. The ketone body-derived acetyl-CoA can subsequently enter the TCA cycle. Within the muscle, β-OHB and AcAc may also have roles in stimulating an increase in MPS. Adapted from Soto-Mota and colleagues (37).

Despite the effective induction of nutritional ketosis with exogenous ketone supplements, the metabolic state that follows is not identical to that which occurs in response to a ketogenic diet or starvation (36,43). The latter conditions rely on the scarce availability of CHO to drive endogenous ketone production and requires more time to adapt the body's fuel utilization preferences (29). Without the necessity of dietary adjustments, the state of ketosis induced by exogenous sources can coexist with regular CHO intake and low free fatty acid concentrations (38). Despite this, reductions in blood glucose concentrations can still be observed under exogenous ketosis due to an inhibition of gluconeogenesis and an increase in peripheral glucose uptake (38,44). Thus, in contrast to the ketogenic diet, exogenous ketosis can improve blood glucose control without the added risk of high fat intake (38). Although indirect *in-vivo* evidence for increased insulin secretion has been previously presented (via the increase in levels of plasma C-peptide) (9), it's relationship with exogenous ketone sources remains unclear (38). Notwithstanding, a benefit of exogenous ketone supplements is that they permit testing of the direct effects of ketone bodies themselves (36), without the potential confounding influence of dietary CHO restriction or starvation. However, given the acute nature of exogenous ketone supplements (i.e., they induce ketosis for only a few hours), whether there is an adaptation period similar to that seen with the ketogenic diet remains to be elucidated (36).

2.2.3. Impact of nutritional ketosis

The earliest application of nutritional ketosis was the adoption of the ketogenic diet for its demonstrated therapeutic potential in diseases characterized by metabolic abnormalities and certain neurodegenerative diseases (for a comprehensive review, see (25)). More recently, there has been growing interest in nutritional ketosis, particularly via exogenous ketone supplements, to enhance sport and/or exercise performance (26). Indeed, β-OHB has been suggested to represent

an efficient alternative fuel source, modulate the availability and catabolism of other energy substrates, and act as a powerful signalling molecule (1,45). As mentioned earlier in section 2.2.1., the demonstrated ability of ketone bodies to reduce CHO oxidation has been a key point of interest (1). In addition to their potential ability to spare CHO utilization, ketone bodies may also be energetically advantageous when compared to the oxidation of CHO or fat. Compared to the 10 adenosine triphosphate (ATP) produced through the combustion of glycolytic-derived pyruvate, the end-result of D-β-OHB combustion is 13 ATP (46). This is a consequence of ketone bodies being more reduced, containing a higher H-C ratio (25). As such, ketone bodies may produce more ATP per mole of substrate and represent the more efficient fuel source (37). In support of this notion, infusing ketones and glucose versus glucose alone resulted in a ~30% greater hydraulic efficiency (J/mol of O_2 consumed) of the working perfused rat heart, suggesting that the oxidation of ketones may increase muscular output for a given oxygen requirement (47). Even with this demonstrated superior efficiency over CHO-derived substrates, the body's selectivity for utilizing ketone bodies as an energy source remains a topic of debate (45,48). Indeed, a recent series of *in-vitro* experiments on rodent and human muscle showed that ketone bodies contribute little to mitochondrial respiration, especially in the presence of pyruvate where product inhibition can arise (48).

At the level of the brain, ketone bodies can also act as a source of fuel, as with glucose, due to their ability to cross the blood-brain barrier (49). The brain normally depends on CHO as a substrate, being unable to metabolize fat. However, during starvation and CHO scarcity, lipids are used to form ketone bodies via hepatic ketogenesis, and via ketone bodies, lipids sustain the brain. It has been previously shown that ketone bodies can supply over 60% of the energy needs associated with brain metabolism (50). From the perspective of survival during periods of

starvation or metabolic crisis, ketone bodies not only serve as alternative fuel source for the brain, heart, and skeletal muscle but also exert anti-catabolic effects including inhibition of glycolysis and adipose tissue lipolysis (14). These life-saving features have created great interest in the potential application of ketosis, via both the ketogenic diet and exogenous ketone supplements, within the context of endurance exercise where finite muscle and liver glycogen stores can limit performance (51,52).

High-fat ketogenic diets have produced mixed and controversial results in terms of their effects on exercise performance (27,53). In a 12-week intervention on endurance-trained athletes, those who were assigned to a ketogenic diet (% CHO:protein:fat = 65:14:20) saw a respective 0.8 and 1.4 watt/kg increase in peak power during a six second sprint and critical power test performed on a cycle ergometer (33). The improvement in performance in this intervention was partially attributed to the 12-week duration which allowed the athletes to be properly keto-adapted (33). Another study showed performance decrements in a 30-s Wingate and a 45-min cycling bout following a 6 week high-fat/moderate-protein diet (fat = 61%) in non-highly trained men (54). The overall consensus with this strategy of adopting a ketogenic diet emphasizes the consideration of event characteristics and personal experience due to the risk of performance decrements (27). The main concern being that the absence of a sufficient amount of CHO can hinder performance due to the inability to properly fuel high-intensity tasks (55). Additionally, although many timelines have been proposed (11,27,33), the guidelines for the adoption of the ketogenic diet with the goal of improving performance remain unclear along with the potential undesirable effects of the keto-adaptation process (27). Although further clarity is needed, a general recommendation for athletes, apart from an introspective audit of preferences and needs (27), is to not undertake a ketogenic diet within 4-6 weeks prior to an event (33).

In terms of exercise performance, exogenous ketone supplements may hold more promise than ketogenic diets as their use does not necessitate dietary CHO restriction to induce ketosis. With the ingestion of a ketone ester, plasma lactate concentrations were shown to be significantly reduced compared to a CHO condition following a 1-hour session of constant load cycling at 75% of maximum workload (14). It was also demonstrated that the co-ingestion of a ketone ester with CHO could significantly reduce muscle glycolysis even during exercise conditions that are normally highly glycolytic (14). However, a recent study reported that ketone ester ingestion led to impaired 30-min cycling time-trial performance (56). There seems to be a general consensus that the use of exogenous ketone supplements may not be beneficial for exercise that is of high-intensity (i.e. where CHO dependence >80%) (1,55,56). Under these conditions, glycolysis may be impaired such that the ensuing glycolytic flux is unable to support the required workload (1,55,56). Overall, past research on the effects of exogenous ketone supplements on exercise performance have been equivocal (57). A recent meta-analysis provided evidence that there is no effect, positive or negative, of exogenous ketone supplements on exercise performance (58).

The role of ketosis and exogenous ketone supplements on human exercise performance remains unclear and warrants more research in order to fully explore and understand their potential as an ergogenic aid (1). In addition to exploring the direct effects of nutritional ketosis on exercise performance, there is also emerging interest in their application within the context of post-exercise recovery (7,39). Ketone bodies represent unique signalling molecules (45), and have been shown to augment post-exercise glycogen re-synthesis (59), mTORC1 pathway activation/phosphorylation (7), and sustainable exercise training load during 3-weeks of overreaching (60). There is also evidence that ketone bodies are important regulators of MPS (2,7) and breakdown (3,61); key processes involved in skeletal muscle remodelling and adaptation.

2.3. Muscle protein turnover

Skeletal muscle typically exists in a state of dynamic equilibrium, whereby daily rates of MPS are normally equal and opposite to rates of muscle protein breakdown (MPB), resulting in a neutral net protein balance (MPS = MPB) and the maintenance of muscle mass. In healthy and recreationally active adults, the rate of skeletal muscle protein turnover is ~1.2% day^{-1} (62). For example, an individual with 35 kg of total body skeletal muscle mass will synthesize and degrade ~420 g muscle protein per day. In the overnight post-absorptive state, rates of MPB exceed those of MPS resulting in a negative net protein balance (MPS < MPB) (62). Amino acids liberated from the breakdown of muscle protein may be reutilized for protein synthesis in muscle or other tissues, oxidized as a fuel source, and/or support gluconeogenesis in the liver (62). Ingestion of a protein-containing meal (e.g., bacon and egg breakfast) represents a key stimulus supporting increased MPS rates in humans (62). Protein ingestion stimulates increased MPS rates, suppresses MPB rates (an effect largely attributed to increases in postprandial insulin concentration), and results in a positive net protein balance (MPS > MPB) (62). Therefore, fed-state gains in muscle protein are necessary to replace fasted-state losses in muscle protein and maintain muscle mass. Proteins are comprised of one or more polypeptide chains which in itself is composed of a combination of amino acids. It is the sequence of these amino acids that ultimately dictate the exact biological structure of a protein, and thus its function within the body. The liver is able to control plasma amino acid levels following nutrient intake as it metabolizes ~60% of all absorbed amino acids during a single pass process before they enter systemic circulation and become available to other organs such as skeletal muscle (63). The branched chain amino acids (BCAA) leucine, isoleucine, and valine are unique in that they are not heavily metabolized in the liver (63), and therefore are highly available within systemic circulation following a protein-containing meal. The effect of

feeding on MPS is transient, lasting only a few hours. Even during a situation of continuous amino acid availability, rates of MPS will return to baseline levels after 2-3 h of a constant amino acid infusion (64). A similar transient MPS response has been observed following the ingestion of an oral bolus of protein (65). Two of the most important factors regulating the magnitude and duration of fed-state increases in MPS include the source/type (e.g., animal-derived vs. plant-derived protein) and the per meal dose/amount of ingested protein.

2.3.1. Dietary protein source/type

The source, and by extension quality, of dietary protein is an important factor to consider with regards to the regulation of MPS. Sources of dietary protein differ in their amino acid composition and digestibility, and therefore may have different effects on postprandial MPS rates. The 20 amino acids that represent the building blocks of muscle protein can be categorized based on their dispensability within the human body. Dispensable or non-essential amino acids (NEAA) comprise amino acids that can be adequately synthesized *de novo* by the body to meet requirements and are thus not critically required through the diet (66). Indispensable or essential amino acids (EAA) comprise amino acids that cannot be synthesized *de novo* by the body or are synthesized in insufficient amounts to meet the body's requirements (66). This group of amino acids must therefore be consumed through dietary intake (66). Protein sources are usually considered complete if they provide all the required EAA. Typically, animal-sourced proteins such as beef, chicken, and dairy are considered the most complete proteins based on their amino acid profiles (67). On the other hand, plant-based proteins often lack one or more of the EAA and are therefore deemed incomplete proteins (67). The ability of the protein source to be efficiently digested and absorbed by the small intestine is also an important factor to consider when determining protein quality. The digestible indispensable amino acid score (DIAAS) represents the currently

recommended method to assess dietary protein quality (68). It is based on the relative digestible content of the indispensable amino acids within a protein and the amino acid requirement pattern (for review see (68)). Animal-based proteins such as whey protein isolate and chicken breast have a DIAAS of 1.09 and 1.08, respectively, whereas vegetable-based protein sources such as pea protein concentrate have a DIAAS of 0.82 (69). Whey protein in particular is considered one of the highest quality protein sources because of its high bioavailability and high content of EAA (and especially leucine) (70). Whey is one of the end products of milk coagulation and can be further purified into whey protein isolate (67,70). The latter represents the purest form of whey, with protein concentrations that can exceed 90% while containing minimal fat and CHO (67,70). This form of whey also has the added benefit of being low in the disaccharide lactose which usually allows it to be consumed by those with lactose intolerance (67). Compared to its dairy protein counterpart micellar casein, the rate of digestion for whey is much faster resulting in a more rapid rise in postprandial plasma amino acid concentration and can result in a robust stimulation of MPS rates (71).

2.3.2. Protein dose response

In addition to the quality of a protein source, the quantity ingested also plays a key role with regards to regulating the postprandial MPS response. It has previously been shown in healthy young men that postprandial MPS rates after an acute bout of resistance exercise are maximized following the consumption of 20 g of egg protein (~8.6 g EAAs) (17). In a now seminal study, Moore and colleagues (17) had young healthy men perform an acute bout of resistance exercise and ingest 0, 5, 10, 20, and 40 g of dietary protein. Mixed MPS and whole-body leucine oxidation were measured over 4 h post-exercise recovery via a primed constant infusion of [1-^{13}C] leucine. MPS displayed a dose response relationship (**Figure 3**) to dietary protein ingestion and was

maximally stimulated with 20 g ingested protein (17). Doses larger than 20 g (i.e. 40 g) showed no additional stimulatory effect on MPS rates but increased irreversible amino acid oxidation (17). Alternatively, protein doses below 20 g represent 'sub-optimal' doses of ingested protein that do not saturate the MPS response to ingested protein. However, Churchward-Venne and colleagues (72) reported that a low dose of ingested protein (6.25 g) was as effective as a high dose (25 g) of ingested protein at stimulating increased MPS rates when supplemented with 5.0 g leucine. Therefore, it may be possible to enhance the MPS response to sub-optimal doses of ingested protein via co-ingestion with specific nutrients that display anabolic properties.

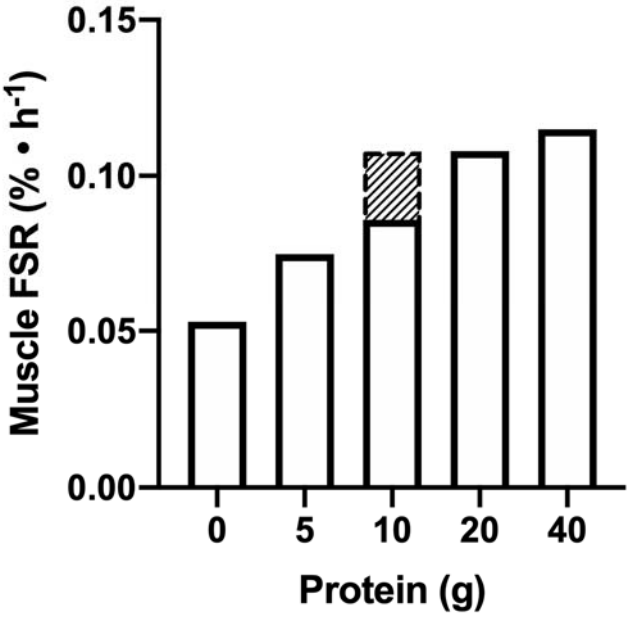

Figure 3. Dose-response relationship between MPS and ingestion of dietary protein following a bout of resistance exercise. MPS was found to be maximally stimulated with a 20 g dose of protein with no additional stimulatory effect observed with higher doses (i.e. 40 g). The shaded area represents a hypothetical enhancement of MPS when a sub-optimal dose of protein is co-ingested with nutrients displaying anabolic properties. Adapted from Moore and colleagues (17).

2.3.3. Molecular regulation of protein synthesis

Synthesizing new proteins involves the transcription of DNA into mRNA and the subsequent process of mRNA translation into polypeptide chains. In muscle, transcription and translation have been shown to be influenced by nutrition, exercise, and hormones (73). Both insulin and insulin-like growth factor 1 (IGF-1) are hormones that can modulate protein metabolism (74). Increases in insulin have been shown to inhibit MPB, while also acting to regulate amino acid uptake into the muscle (62,75). About half of the anti-proteolytic effect observed in response to feeding can be attributed to insulin (62,76). That is to say that even in the presence of a high concentration of amino acids, this effect cannot be replicated without insulin, as insulin is the main driver of MPB suppression during feeding (62,76). Although insulin plays a key role in regulating MPB, it does not appear to directly stimulate increased MPS rates, as MPS can be stimulated despite low levels of the peptide hormone (76). Both insulin and IGF-1 can stimulate the signalling cascade associated with the mammalian target of rapamycin (mTOR) (74,77). mTOR is an evolutionary conserved Ser/Thr protein kinase that integrates signals from growth factors and nutrients in order to regulate cell growth and cell cycle progression (78). Special attention is given to the activation of mTORC1 which is largely responsible for the stimulation of MPS that results from feeding through the promotion of mRNA translation (74). The activation of mTORC1 can also lead to a reduction in autophagy which facilitates a positive net protein balance by not having two competing systems active at the same time (79). IGF-1 can bind to receptor tyrosine kinases on the periphery of the cell to activate the IGF-1/Akt (protein kinase B) /mTOR pathway (77). Once bound, the downstream activation of Akt promotes MPS by stimulating mTORC1 activation in addition to exhibiting effects on other pathways (80). Most notably, the activation of Akt also inhibits atrophy-associated FoxO (forkhead box class O) proteins, leading

to a reduction in MPB (80). The mTOR pathway is strongly activated in the presence of amino acids, with increased sensitization in the presence of growth factors (81). The BCAAs are mentioned as a modulator of mTOR activation although there is some debate as to whether mTORC1 actually distinguishes different amino acids or if it instead senses the intracellular pool of amino acids as a whole (81,82). Some amino acids have been found to interact with amino acid sensors upstream of mTORC1 around the lysosomal surface (83). The amino acid leucine and arginine have been found to inhibit the proteins Sestrin2 and CASTOR1 (cytosolic arginine sensor for mTORC1 subunit 1), respectively, which ultimately leads to the activation of Rag GTPases (83). Rag GTPases have been found to be necessary for mTORC1 activation in response to amino acid availability (84,85). During periods of amino acid deprivation, mTORC1 is dispersed within the cell (79). In the presence of amino acids, the Rag GTPases interact with mTORC1 by initiating the intracellular localization of the latter to the lysosomal surface which also contains the Ras homolog enriched in brain (Rheb) GTPase (77,85). Thus, the main role of amino acids in the signalling cascade appears to be the translocation of mTORC1 to its activator, Rheb (86).

Evidence has been presented regarding the vacuolar H^+-ATPase (v-ATPase) as a potential sensor of amino acids within the lysosomal lumen (87). During periods of amino acid availability, v-ATPase interacts with Ragulator, a scaffolding protein that anchors Rag GTPases to the lysosomal surface (87). Thus, if amino acids are being sensed from within the lysosome, mTORC1 activation may actually be an "inside-out mechanism" (86,87). However, more research is needed in order to fully elucidate the mechanisms behind how the v-ATPase senses amino acids during the fed state.

mTORC1 relies on downstream effectors to coordinate the activation of the necessary programs to achieve a positive net protein balance via stimulation of MPS (79). Once activated by

Rheb, mTORC1 activates p70S6K1 through phosphorylation which leads to the downstream activation of both eEF2 (eukaryotic elongation factor 2) and eIF4A (eukaryotic initiation factor 4A), which are important for translation, and ultimately for the synthesis of proteins to occur (77). Activation of p70S6K1 can also directly activate the ribosomal protein s6 (rpS6) through phosphorylation (77). mTORC1 also inhibits 4E-BP1 which leads to the downstream activation of eIF4E (eukaryotic translation initiation factor 4E) to promote translation (77). As mentioned earlier, mTORC1 can regulate autophagy and thus reduce protein degradation (77,88). It does this by inhibiting ULK1 (Unc-51-like kinase 1) and TFEB (transcription factor EB) which are responsible for the induction of autophagy (86,88). It was also mentioned that growth factors could stimulate the mTOR pathway and ultimately promote protein synthesis (74). This effect is seen upstream of mTORC1 activation, running parallel to the amino acid activation of Rag GTPases (77). Growth factors activate Protein kinase B, or Akt, which ultimately leads to the activation of the Rheb GTPase which can interact with mTORC1 and lead to activation of the aforementioned downstream pathways that promote protein synthesis (77). More specifically, Akt inhibits the tuberous sclerosis complex 2 (TSC2) which ensures that Rheb is in its active GTP-bound state for it to successfully activate mTORC1 (86).

It is important to note that during periods of fasting there is reduced activation of the mTOR pathway which contributes to net muscle protein catabolism (89). With net catabolism, nitrogen balance (a proxy for net protein balance) is normally negative during the post-absorptive state (80). During a period of fasting, reductions in the level of circulating nutrients and growth factors make it necessary for the body to prioritize the maintenance of essential functions (89). With MPB, proteins are degraded to liberate amino acids which can be used for other processes during periods of stress (80). Also, with the inactivation of mTORC1, ULK1 and TFEB are no longer inhibited

and can induce autophagy as a result (88). Periods of fasting represent a time of energetic stress within the cell which may also lead to the activation of AMP-activated protein kinase (AMPK) (90). AMPK is known for its role in regulating energy homeostasis by prioritizing cellular processes based on the energy status of the cell (90). As such, AMPK can act to inhibit mTORC1 activity and the energetically expensive process of protein synthesis in order to prioritize processes that support energy availability (90).

A number of studies have highlighted the importance of EAA for stimulating mTOR-mediated MPS with no apparent need of NEAA (91–93). This is in contrast to earlier work which suggested that the complete deprivation of any individual amino acid would effect mTORC1 activation, with leucine and arginine being the most impactful (94). It has been hypothesized that NEAA may serve a more critical role for MPS during conditions where there is greater anabolic drive such as that following resistance exercise (72). Other studies have emphasized the importance of BCAAs by their dual role as substrates for MPS but also signalling molecules that can act independently of growth factors to activate mTORC1 (82). Nevertheless, this highlights the importance of having a high-quality protein source like whey, as it offers a complete amino acid profile, while other sources that lack certain EAA may limit the MPS response (95). In addition, the consumption of whey can also ensure that the NEAA will not eventually become rate-limiting during the process of MPS (95).

The EAA leucine has been identified as a key regulator at a number of sites upstream of mTORC1 (96,97). A number of past studies have identified the protein Sestrin2 to be a cytosolic leucine sensor (83,98). Upon binding to Sestrin2, leucine can disrupt the protein's inhibition of the GATOR2 (GAP activity towards Rags 2) complex which supports the downstream activation of mTORC1 (98). With increased concentrations of leucine, there is some demonstrated improvement

in components of mTOR signalling with some indications of reduced autophagy (99). However, even when leucine is available at a higher concentration within an EAA solution, no enhancement of the rate of MPS could be observed (99). Similarly, no differences were found between leucine and an EAA (minus leucine) mixture on MPS rates when co-ingested with a sub-optimal dose (6.25g) of whey protein (72). Both of the aforementioned treatments were found to have similar effects on MPS as a 25 g dose of whey protein which suggests that the impact of leucine on mTOR signalling may have its limits (72). Despite prior studies (16) having demonstrated enhanced signalling of downstream effectors of mTORC1 with increased leucine concentrations, increased signalling activity does not necessarily translate into a greater increase in MPS rates (76,99). Rather than focusing solely on leucine, intracellular availability of amino acids may be a more important factor for increasing MPS rates, as greater leucinaemia does not necessarily translate into increased transport into the muscle (99,100). Leucine may be important for priming the muscle tissue for the ensuing influx of amino acids following the intake of a dietary protein, but cannot independently provide an optimal anabolic response (95).

Overall, it is still unclear as to whether it is in fact intracellular (100) or extracellular (15) increases in amino acid availability that are sensed in order to regulate MPS rates. Certain models have proposed that the aggregate of all amino acid inputs via sensing signals may be converging at the level of the Rag GTPases in order to activate mTORC1 (101). Whether one specific amino acid, all the amino acids, or a by-product of an amino acid (e.g., amino acid-derived metabolite) is being sensed to activate the mTOR signalling cascade needs more clarification (79). Furthermore, the role of other non-amino acid based dietary molecules with the capacity to stimulate MPS via mTORC1 activation is an area of current research interest since such compounds may have the potential to protect and restore muscle mass in clinical conditions characterized by muscle loss.

2.4. Role of ketone bodies in the regulation amino acid and protein metabolism

The protein anabolic and anti-catabolic potential of ketone bodies at the whole-body and skeletal muscle level in response to ketosis has been studied since the 1970's (3). During short-term fasting, skeletal muscle proteins are mobilized and utilized in order to supply amino acid precursors required for hepatic gluconeogenesis (12). The glucose produced is a critical fuel source for the brain until hepatic ketogenesis and plasma ketone body concentration is sufficient to serve as an alternative energy substrate. Indeed, over 60% of the substrate requirement for the brain can be met via ketone bodies during prolonged fasting (50). The availability of ketone bodies as an oxidizable energy substrate for the brain may reduce the requirement for glucose, which during fasting primarily occurs using amino acids via gluconeogenesis. It has been hypothesized (102) that ketone bodies possess anti-catabolic properties in muscle by sparing muscle protein normally used to supply amino acid precursors for glucose production to fuel the brain (and possibly other cells such as erythrocytes). However, the role of ketone bodies in the regulation of MPS has been less explored. The goal of the following section is to provide an overview regarding current knowledge on the role of ketone bodies in the regulation of amino acid and protein metabolism.

2.4.1. Ketone bodies and muscle protein breakdown

As indicated above, the potential anti-catabolic effect of ketone bodies has mainly centred around their role as an alternative fuel substrate to glucose. With an increased reliance on ketone bodies as source of energy during periods of starvation, the need to breakdown body protein for the purpose of gluconeogenesis may be reduced (3). The effect of ketone bodies on the regulation of MPB is still not fully understood, as differences can arise depending on the approach implemented to achieve nutritional ketosis (36). It was previously highlighted that although ketosis can be achieved with the ketogenic diet or use of exogenous ketone supplements, the ketosis occurs

under vastly different metabolic states (36,103) and may therefore have different effects on protein metabolism.

The initial days of CHO restriction following the onset of a ketogenic diet are inevitably catabolic until the availability of ketone bodies are sufficient to spare tissue amino acids (26). Most human studies have reported that the adherence to a ketogenic diet usually has positive effects on fat mass loss (104,105), but either has no effect or results in a loss of lean body mass compared to a non-ketogenic control group (104–106). Arguably more important for the preservation of muscle mass is that the adopted ketogenic diet contain an adequate amount of dietary protein (80,104,107). It has been previously shown that although initial mean nitrogen balance may be negative during the start of a ketogenic diet, there is a net positive balance after 4 weeks of adherence in lean men (31). The results from this study demonstrate that lean body mass can be maintained while following a ketogenic diet provided that that there is a moderate level of dietary protein intake (in this case, 1.75 g/kg bodyweight/day) (31). As nitrogen is a key structural component of amino acids, nitrogen balance represents a proxy measure of amino acid /protein metabolism (108). As nitrogen cannot be synthesized within the body, the intake of nitrogen through dietary protein and its excretion from the body can serve as a proxy measure of the dynamic nature of protein metabolism (108). With the ketogenic diet, assessment of protein turnover responses and/or associated molecular signaling in humans are still limited and most of the available data has been obtained from rodent models (80). With mice, a 7-day ketogenic diet was found to mimic a state of starvation, whereby ketones displace glucose as the primary fuel source, while also upregulating genes (MAFbx (muscle atrophy F-box), MuRF1 (muscle RING finger 1), and Lc3b (light chain 3 beta)) associated with skeletal muscle atrophy and autophagy (109). In comparison to mice fed a normal diet, those in the ketogenic diet group experienced a reduction in muscle mass along with

decreased plasma glucose, insulin, IGF-1, and albumin; an index of nutritional status (109). However, longer-term adherence to the ketogenic diet has been reported to slightly attenuate protein degradation after 26 months of dietary adherence in mice (110). This attenuation was attributed to reductions in oxidative (increase in BiP (binding immunoglobulin protein), IRE1α (inositol requiring enzyme 1α), and PDI (protein disulfide isomerase) protein levels) and endoplasmic reticulum (increase in SOD2 (superoxide dismutase 2) and catalase protein levels) stress that was seen within the ketogenic diet mice after 26 months (110).

Alternatively, use of exogenous ketones has provided more consistent findings with regards to the attenuation of MPB (2,3,61). In men, the induction of hyperketonemia through the infusion of sodium DL-β-OHB has been shown to result in plasma hypoalaninemia which may occur due to a reduction in alanine release from the muscle (3). A reduction in muscle alanine efflux may also reflect attenuation of BCAA oxidation as BCAAs can be used to synthesize alanine for protein-derived gluconeogenesis (3). Similar observations have been made *in-vitro*, with reductions in alanine release being observed following the addition of either sodium DL-β-OHB or sodium AcAc to the medium (111). In these isolated rat diaphragms, the presence of glucose and BCAA during the addition of ketone bodies still resulted in decreased production of both alanine and glutamine with the inhibition of glycolysis (111).

Evidence for ketone-mediated anti-catabolic effects is also supported by the observation of decreased nitrogen excretion following the infusion of sodium DL-β-OHB in men (3). With a reduction in nitrogen excretion compared to pre-ketone infusion levels, it has been suggested that exogenous ketones play a potential role in decreasing protein catabolism (3). A decrease in nitrogen excretion in response to hyperketonemia has also been observed in humans during normally catabolic events such as the post-operative state (112,113). Another notable finding is

that plasma concentrations of leucine do not appear to diminish during the course of ketone body infusion (3). This is important to consider since a reduction in leucine oxidation (and oxidative amino acid losses) would theoretically increase the availability of this amino acid for protein synthesis. Indeed, another study utilizing a sodium DL-β-OHB infusion in humans reported a 30% reduction in whole-body leucine oxidation in fasted subjects compared to a control saline infusion (2). The amino acid phenylalanine has also been used as a metabolic tracer to estimate the effect of ketosis on protein dynamics (61). Thomsen and colleagues (61) reported a ~70% reduction in net forearm phenylalanine release in response to the infusion of sodium DL-β-OHB to 3.5 mM during acute lipopolysaccharide-induced inflammation in young males; however, there was no change in the phosphorylation status of molecular regulators of protein breakdown (e.g., FOXO3A$^{Ser318/321}$, MURF1, LC3II:LC3I, and ULK1^{Ser757}). There was also an decrease in phenylalanine to tyrosine conversion (i.e., phenylalanine hydroxylation) during the infusion which supports the overall finding of a net anabolic response with β-OHB (61). Overall, these results support the notion that ketone bodies possess anti-catabolic properties in muscle.

2.4.2. Ketone bodies and muscle protein synthesis

Compared to protein breakdown, the effect of ketone bodies on MPS is less understood as pertinent information regarding this relationship remains unavailable. As with breakdown, the effect of ketosis on MPS and indicators thereof may be influenced by the method of keto-induction. Although a ketogenic diet may still provide an adequate amount of dietary protein to support resistance exercise-induced muscle hypertrophy, the fasting-like metabolic state that is created may not support such activities (26,105). A ketogenic diet (for 7 days) has been reported to decrease MPS rates in mice based on surface sensing of translation (SUnSET) analysis using puromycin and decrease muscle weight, muscle fiber area, and muscle strength (109).

Alternatively, Wallace and colleagues (110) reported that 12-month old mice assigned to a ketogenic diet until 16 or 26 months of age had greater muscle mass at 26-months than mice on an energy-matched control diet. The ketogenic diet was also associated with a shift in fiber type from type IIb to IIa fibers, and a decrease in protein synthesis and proteasome activity (110). The ketogenic diet has also been associated with the activation of AMPK (80). Within mice fed a ketogenic diet, AMPK activity within the soleus muscle increased threefold compared to a control diet (114). The phosphorylation of AMPK, also induced by fasting, can act to inhibit mTORC1 (77). Another rodent study found increased levels of phosphorylated 4E-BP1 in the skeletal muscle of mice after 1 month on the ketogenic diet, however this result did not differ from those fed with a mixed macronutrient Western diet (107). As with protein breakdown, the distinct physiological state induced with a ketogenic diet makes it difficult to isolate the effects of ketone bodies per se on muscle anabolic responses.

In contrast to studies based on the ketogenic diet, several studies utilizing exogenous ketone bodies have provided evidence for an anabolic effect (2,7,115). In an early animal study, Umpleby and colleagues (115) reported an increase in the rate of [1-^{14}C]-leucine incorporation into protein with the infusion of sodium DL-β-OHB into dogs. An increased rate of leucine appearance has also previously been shown to occur in response the infusion of sodium DL-β-OHB in the both the fasted and postoperative state in humans (116). The increase in the rate of leucine appearance suggests a possible rise in protein breakdown. Given that previous studies have demonstrated that ketone bodies reduce amino acid oxidation (2,3), the authors suggested that hyperketonemia may have enhanced protein synthesis through the inhibition of leucine oxidation (116). In support of this notion, Nair and colleagues (2) reported that sodium DL-β-OHB infusion to induce ketosis (~2.0 mM) led to a ~30% reduction in leucine oxidation and a ~10% increase in

the rate of mixed MPS. The authors noted that the fractional mixed MPS rates were positively correlated with the increase (~11.5%) in non-leucine flux during the ketone infusion. (2).

Recently, the ketone monoester (R)-3-hydroxybutyl (R)-3-hydroxybutyrate has been used to investigate the anabolic potential of ketone bodies in healthy young men (7). It was shown that ingestion of a ketone monoester during the recovery period from glycogen-depleting exercise increased the phosphorylation status of p70S6K1 (at Thr^{389}) and 4E-BP1 (the percentage of 4E-BP1 in the phosphorylated γ-form, 4E-BP1%γ) within the mTORC1 signaling cascade after 5 hours (7). The phosphorylation of AMPK (at Thr^{172}) was also reduced following the ingestion of the ketone monoester which may have facilitated the phosphorylation (and presumably activation) of the mTORC1 pathway (7). Additional experiments were also carried out in an *in-vitro* experiment in C_2C_{12} myoblasts that further demonstrated the anabolic capacity of ketone bodies (7). Although neither β-OHB or AcAc were successful in increasing the phosphorylation status of either p70S6K (at Thr^{389}) or 4E-BP1%γ alone, their combination alongside leucine significantly increased the phosphorylation status of both mTORC1 downstream effectors compared to leucine alone (7). Furthermore, the combined incorporation of β-OHB, AcAc, and leucine into the medium resulted in levels beyond any level reached by either of the substrates alone (7). It should also be noted that the addition of either β-OHB or AcAc with leucine also resulted in significant increases in phosphorylation status, albeit inferior to the combination of all three (7). The *in-vitro* experiments also demonstrated a two-fold increase in the rate of protein synthesis when β-OHB and AcAc (4 mM and 1.4 mM, respectively) were added to 1.5 mM of leucine compared to the latter alone (7). It is also interesting to note that the rate achieved with this combination was similar to that observed with a supraphysiological concentration of leucine (5 mM) (7). Further research is needed to determine the effect of orally ingested ketone bodies on MPS rates *in vivo* in humans,

and whether their effect is augmented when co-ingested with protein to provide the amino acid building blocks ultimately required for polypeptide synthesis.

2.5. The primed continuous intravenous infusion of stable isotope-labelled amino acids to assess skeletal muscle protein synthesis rates in humans *in-vivo*

With the use of a stable isotope-labeled amino acid (e.g., L-[ring-$^{2}H_{5}$]-phenylalanine), the synthesis rate of skeletal muscle protein can be determined by measuring the change in protein-bound enrichment in muscle samples collected via needle biopsies, along with measurement of precursor pool enrichment and the tracer incorporation period (117). More specifically, the fractional synthetic rate (FSR) of a muscle protein can be calculated based on changes in protein-bound enrichment in collected muscle tissue samples, assessment of plasma or muscle precursor pool enrichment, and accurate recording of the tracer incorporation period (118). Isotopes are molecules that are chemically and functionally identical to their natural form with the exception of their mass due to a differing number of neutrons present in the nuclei, which renders it either radioactive, or stable and nonradioactive (119). Because of the potential dangers of introducing radioactive molecules into the human body, stable isotopes are the preferred choice in human research as they are naturally occurring and do not spontaneously decay (119). Common stable and nonradioactive tracers used in the study of protein metabolism include L-[1,2–$^{13}C_{2}$]-leucine and L-[ring-$^{2}H_{5}$]-phenylalanine (120). By infusing a known concentration of a stable isotope *in-vivo*, the molecule can subsequently be traced during analysis to provide more insight into metabolic processes (121). This method can especially prove useful when looking at the efficacy of a nutritional intervention on MPS rates. However, it is important to note that feeding studies incorporating the ingestion of a protein bolus should consider adding a small quantity of the selected tracer to the nutritional treatment (122). This would serve the purpose of minimizing

disturbances in the steady-state precursor conditions created by the intravenous infusion of the tracer (122).

Although they are important to the study of *in-vivo* protein metabolism and its molecular regulation, skeletal muscle biopsies are invasive. A sequential biopsy approach is typically utilized during tracer studies whereby two muscle biopsies are performed during the primed, continuous intravenous infusion of the tracer in order to calculate FSR (123). However, the ability to reduce the required number biopsies would be quite advantageous from a methodological and/or ethical standpoint (123). Indeed, it has been previously shown that a single biopsy approach can serve as a valid alternative to sequential biopsies (122). The approach determines FSR by relying on plasma proteins for baseline enrichments along with the analysis of the obtained biopsies, eliminating the need to perform another biopsy (122). With this approach, it is recommended to have a long period (3 h) of tracer incorporation prior to the biopsy in order to offset potential inter-subject variability with muscle enrichments (122). It is also important to highlight that the single biopsy approach is only reliable if the participant has never previously been subject to a tracer infusion as residual amounts of tracer may remain for an uncertain period of time (122). The development of these validated and reliable methods has greatly facilitated the ability to investigate the dynamics of protein turnover in response to a variety of conditions including exercise, aging, disease, and nutrition.

CHAPTER 3: MANUSCRIPT

3. MANUSCRIPT

3.1. Introduction

Ketone bodies (i.e., β-OHB, AcAc, and acetone) are lipid-derived molecules that are normally endogenously produced via the process of ketogenesis in the liver in response to starvation or prolonged fasting, a very low CHO "ketogenic" diet, and prolonged glycogen-depleting exercise (1). Under these conditions, ketone bodies circulate via the bloodstream and are taken up by metabolically active tissues such as the brain, heart, and skeletal muscles where they serve as an alternative energy source (1). In addition to serving as an alternative fuel source, β-OHB has been recognized as a signaling metabolite regulating oxidative stress, inflammation, and gene expression (124). Recently, orally ingested ketone supplements have been developed that can induce hyperketonemia within minutes to concentrations observed with prolonged fasting (~4-5 mM) without the need for dietary restriction (13,39). Therefore, exogenous ketone supplements permit direct testing of the metabolic effects of hyperketonemia without the confounding influence of dietary manipulation due to a ketogenic diet or prolonged fasting.

A number of studies to date have evaluated the effects of ketone bodies on anabolic and/or anticatabolic processes in skeletal muscle (102). Early *in-vitro* preparation studies reported that protein synthesis rates were decreased (125,126) or unchanged (127) in incubated animal muscles via the addition of ketone bodies. Alternatively, Umpleby and colleagues (115) reported that sodium β-OHB infusion into fasted dogs stimulated an increase in protein synthesis based on an increase in [1-^{14}C]-leucine incorporation into protein. In humans, experimentally elevating ketone bodies has been reported to improve nitrogen balance (a surrogate measure of protein turnover) in traumatized man (5) post-operative patients (4), and in response to fasting (3). Additionally, Nair and colleagues (2) reported that intravenous infusion of sodium β-OHB to ~2 mM in healthy men,

reduced leucine oxidation by ~30% and stimulated increased rates of mixed MPS by ~10% using a L-[1-^{13}C]-leucine tracer. More recently, Vandoorne and colleagues (7) reported that ingestion of a ketone ester supplement along with a standard protein-containing post-exercise recovery beverage enhanced the post-exercise activation of components of the mTORC1 pathway in young men. Further, ketone bodies were shown to potentiate the increase in mTORC1 activation and protein synthesis in leucine-stimulated myotubes *in vitro* (7).

It is well established that protein ingestion stimulates increased skeletal MPS rates (17,108). There is a dose-dependent relationship between protein (17) and essential amino acid (128) intake and skeletal MPS rates. Moore and colleagues (17) reported that ~20 g of protein stimulated MPS after exercise above that observed in response to both 5 g and 10 g of protein but was not further stimulated with ingestion of 40 g of protein. Therefore, ~20 g of high-quality protein (17) or ~10 g of essential amino acids (128) appear saturating for MPS rates in young adults.

Currently, there is limited and conflicting information available on the effects of elevated ketone body availability (i.e., hyperketonemia) on rates of skeletal MPS in humans (2,129). Furthermore, studies to date examining the effects of ketone bodies on protein turnover responses in humans have primarily induced hyperketonemia via intravenous infusion of ketone bodies; no information is available on the effects of orally ingested ketone supplements on MyoPS rates. Given what we know about the ingested protein dose-response of MPS rates, the aim of the present study was to evaluate the effects of elevated β-OHB, induced via oral ingestion of the ketone monoester (R)-3-hydroxybutyl (R)-3-hydroxybutyrate when consumed without protein (KET), and when co-ingested with a "sub-optimal" dose (10 g) of protein (KET+PRO) on MyoPS rates compared to isolated protein ingestion (PRO). It was hypothesized that elevated β-OHB via KET

would stimulate increased MyoPS rates compared to basal post-absorptive conditions and the response would be equivalent to that observed following ingestion of a sub-optimal 10 g dose of protein (PRO). It was further hypothesized that co-ingesting the ketone monoester (R)-3-hydroxybutyl (R)-3-hydroxybutyrate with 10 g protein (KET+PRO) would stimulate greater MyoPS rates than both KET and PRO.

3.2. Methods

3.2.1. Participants

Thirty-six healthy recreationally active young men volunteered to participate in this randomized, double-blind, parallel group study. In order to be eligible to participate, participants had to have a BMI >18.5 and < 30 kg/m^2 and be healthy and moderately active based on responses to a routine screening questionnaire. Exclusion criteria included: the presence of any identified metabolic or intestinal disorders, use of tobacco products, allergies to milk proteins, lactose intolerance, phenylketonuria, a history of neuromuscular problems, previous participation in a stable-isotope tracer study, adherence to a strict vegetarian or vegan diet, current use of ketone supplements or adherence to a ketogenic diet, use of medications known to affect protein metabolism (i.e. corticosteroids, anti-inflammatories, prescription strength acne medications), diagnosis of diabetes, and engagement in sports or physical exercise for 5 or more days per week. Participants were recruited through advertisements on dedicated bulletin boards within McGill University as well as posts on social media (e.g. Facebook). All participants were informed about the purpose of the study, the experimental procedures, and possible risks prior to providing informed written consent to participate. This informed consent was obtained before commencing any form of assessment. The study was conducted in accordance with the ethical standards of the Faculty of Medicine Institutional Review Board at McGill University on human experimentation

and in accordance with the Helsinki Declaration of 1975 as revised in October 2013. The study was prospectively registered on ClinicalTrials.gov (NCT04565444) prior to the enrollment of the first participant.

3.2.2. Research ethics approval

The study was approved by the Faculty of Medicine Institutional Review Board at McGill University on January 6, 2020 (IRB Study Number: A11-M51-19A). All participants provided written informed consent prior to study participation.

3.2.3. Preliminary testing

Participants 18-35 years of age with a BMI >18.5 and <30.0 underwent an initial screening visit to assess height, weight, blood pressure, and body composition (by dual-energy X-ray absorptiometry; GE Healthcare; WI, USA). Participants were deemed healthy based on their responses to a medical questionnaire and screening results. Questions regarding physical activity and exercise preferences were also asked in order to determine daily habitual activity status. Participants with an exercise frequency between 3-4 times per week were considered recreationally active and were included in the study.

3.2.4. Diet and physical activity

Study participants were asked to refrain from strenuous physical activity and alcohol consumption for 2 days immediately prior to the experimental trial. In addition, all participants filled out food intake and physical activity logs for 2 days immediately prior to the experimental trial. Dietary intake was analyzed using commercially available software (Food Processor version 11.9; ESHA Research; OR, USA). On the evening before the experimental trial, all participants consumed a standardized meal purchased from a local grocery store (Michelina's Beef and Macaroni; Bellisio Foods, Inc.; MN, USA) which provided 2134 kJ of energy and consisted of

52% carbohydrates, 31% fat, and 17% protein. This standardized meal was provided to the participants following their initial screening visit and they were directed to store it frozen until preparation for consumption. The standardized meal also constituted the only component of the participants' dinner (other than water) and represented the last meal that was consumed prior to the experimental visit. The participants were instructed to stop consuming food or beverages (except water) by 21:00 h, after which they remained fasted until testing the following morning.

3.2.5. Study design

The present study employed a randomized, double-blind, parallel group design in which research participants reported to the laboratory for a single visit (not including the visit for preliminary testing). Participants were randomly assigned to one of three nutritional beverage treatment groups, each consisting of 12 participants. The randomization procedure to allocate treatment group order was executed via a random-number generator (www.randomization.com). An investigator not directly affiliated with the study was responsible for the randomization. Beverage specifications are outlined below in **Table 1**, section **3.2.6. Nutritional treatments**. Beverages were iso-energetic, matched for volume, and prepared in non-transparent plastic containers. To limit diurnal and intrasubject variation, all measures were carried out according to a standardized time schedule at the same time of day.

3.2.6. Nutritional treatments

An investigator not directly affiliated with the study was responsible for preparation of the study beverages on the morning of the experimental visit. KET and KET+PRO treatment groups ingested the ketone monoester (R)-3-hydroxybutyl (R)-3-hydroxybutyrate (DeltaG®, TDeltaS® Ltd, UK; Oxford, UK; used under exclusive license by HVMN Inc.; CA, USA) at a dose of 0.36 g kg^{-1} body weight per serving. Previous studies have shown a single 0.36 g kg^{-1} body weight dose

of (R)-3-hydroxybutyl (R)-3-hydroxybutyrate to be effective in raising plasma levels of β-OHB to ~3 mmol/L all the while being safe and tolerable by the research participants (13,39). PRO and KET+PRO consumed protein beverages consisting of 10 g of whey protein powder (Isagenix, AZ, USA). The protein beverage was enriched to 4% with tracer according to a phenylalanine content of 3.14% in the whey protein (i.e. 0.04 x [0.0314 x 10 g] = 12.56 mg). This was done in order to minimize disturbances to the blood enrichments and better maintain steady-state conditions in the plasma precursor pool (122). In order to energy- and volume- match for KET+PRO, carbohydrates were added to the KET and PRO treatments. This consisted of a combination of Glacier Cherry Gatorade (G2 - Gatorade Company, Inc.; IL, USA), dextrose powder (NOW foods, Inc.; IL, USA), stevia (Stevia Select Inc, USA), and vanilla flavouring (McCormick & Company, Inc, ON, Canada). The stevia and vanilla flavouring were added to the nutritional treatments in order to mask differences in taste. Following their consumption, the bottle containing the nutritional treatment was rinsed with 40 ml of water in order to dislodge any remnants and ensure that all the content in the bottle was consumed. The participants had 5 minutes to ingest the nutritional treatment assigned to them. Details regarding the composition of the nutritional treatments are presented in **Table 1**

Table 1. Amino acid, ketone monoester, carbohydrate, fat, and protein content of nutritional treatments

	Nutritional treatment group		
	KET	PRO	KET+PRO
Amino acid content			
Alanine, g	—	0.49	0.49
Arginine, g	—	0.25	0.25
Aspartic acid, g	—	1.03	1.03
Cysteine, g	—	0.26	0.26
Glutamic acid, g	—	1.67	1.67
Glycine, g	—	0.19	0.19
Histidine, g	—	0.18	0.18
Isoleucine, g	—	0.64	0.64
Leucine, g	—	1.04	1.04
Lysine, g	—	0.84	0.84
Methionine, g	—	0.23	0.23
Phenylalanine, g	—	0.32	0.32
Proline, g	—	0.59	0.59
Serine, g	—	0.49	0.49
Threonine, g	—	0.68	0.68
Tryptophan, g	—	0.22	0.22
Tyrosine, g	—	0.32	0.32
Valine, g	—	0.58	0.58
Σ NEAA, g	—	4.73	4.73
Σ EAA, g	—	5.27	5.27
Beverage Totals			
Ketone Monoester, g	23.98 – 29.66	—	18.32 – 29.74
Carbohydrate, g	12.16	24.36 – 36.58	1.11
Fat, g	—	0.56	0.56
Protein, g	—	10.00	10.00
Energy, kJ	586 – 815	627 – 844	601 – 830

KET, ketone monoester; PRO, 10 g protein; KET+PRO, ketone monoester co-ingested with 10 g protein; *Σ NEAA, sum total nonessential amino acids; Σ EAA, sum total essential amino acids.*

3.2.7. Experimental protocol

An overview of the experimental protocol is shown in **Figure 4**. Study participants were asked to come to the laboratory at approximately 7:30 am, following a ~10-hour overnight fast. Upon arrival, participants rested comfortably on a bed in the laboratory for ~10 minutes while health status and compliance to the pre-experimental visit guidelines were confirmed. Participants

then had a Teflon catheter inserted into an antecubital vein for the intravenous infusion of L-[ring-$^{2}H_5$]-phenylalanine. The stable isotope tracer L-[ring-$^{2}H_5$]-phenylalanine (ACP Chemicals, QC, Canada) was dissolved in 0.9% saline for intravenous infusion. The tracer solution was prepared at a pharmacy (Gentès & Bolduc, Pharmaciens, Inc.; QC, Canada) and subsequently tested for sterility. Following the administration of a priming dose (2.2 μmol · kg^{-1}) of the L-[ring-$^{2}H_5$]-phenylalanine tracer, a calibrated syringe pump (Harvard Apparatus; MA, USA) was used to deliver a continuous infusion of the tracer during the entire experimental trial (8 h, 0.05 μmol · kg^{-1} · min^{-1}).

A second Teflon catheter was inserted into the dorsal hand vein or antecubital vein of the opposite arm for arterialized venous blood sampling. Once inserted, the Teflon catheters were connected to a 3-way stop-cock and placed under a heated (60°C) blanket for 10 minutes prior to obtaining arterialized venous blood samples. The baseline blood sample was obtained from the antecubital vein of the arm destined for tracer infusion prior to commencement of the infusion protocol. Subsequent blood samples were obtained from the dorsal hand vein or antecubital vein of the opposite arm. A saline drip was connected to the stop-cock to keep the catheter patent for repeated blood sampling. Arterialized venous blood samples were drawn into a prechilled 10-mL blood collection tube (BD Vacutainer®; NJ, USA) coated with K_2EDTA. All tubes were inverted 10 times and centrifuged at 3,000 x g for 15 min at 4°C. After centrifugation, the plasma samples were aliquoted out into microtubes. All plasma samples were frozen in liquid nitrogen and transferred into a -80 °C freezer until further analysis.

Following basal blood collection (8 mL; t = -180 min), the plasma L-[ring-$^{2}H_5$]-phenylalanine pool was primed with a single intravenous dose (2.2 μmol · kg^{-1}) of tracer and a continuous tracer infusion (0.05 μmol · kg^{-1} · min^{-1}) commenced. Another arterialized venous

blood sample (8 mL) was drawn at t= -60 min. Prior to the nutritional treatment, a muscle biopsy was collected along with another arterialized venous blood sample (t= 0 min). Three muscle biopsies were taken through three separate incisions during the trial. The muscle biopsies (0, 120, and 300 min following beverage intake) permitted the measurement of the temporal response of MyoPS between the different nutritional treatments. These biopsies were necessary in order to be able to measure the fractional synthesis rate of myofibrillar proteins in the postabsorptive state prior to treatment administration, and in the postprandial state following treatment administration. Biopsies were obtained from the middle region of the *vastus lateralis* muscle (15 cm above the patella) and approximately 2 cm below entry through the fascia by the percutaneous needle biopsy technique (130). In short, following sterilization of the site, the skin and muscle fascia were locally anesthetized using 2% lidocaine. A small incision was then made in the skin and fascia after which the sterilized biopsy needle (Millennium Surgical; PA, USA) was introduced in the muscle. Vacuum was applied to the needle and a small sample of muscle tissue was obtained (~150 mg). Following the muscle biopsy, the skin was closed using a Steristrip® and covered with a pressure bandage. Muscle samples were separated from any visible non-muscle material, immediately frozen in liquid nitrogen and transferred into a -80 °C freezer until further analysis.

Immediately after the skeletal muscle biopsy and arterialized venous blood sample at t=0 min, participants ingested one of the three beverage treatments based on group randomization (i.e. PRO, PRO+KET, or KET). Arterialized venous blood samples (8 ml each) were subsequently collected at t= 15, 30, 60, 90, 120, 150, 180, 240, and 300 min during the postprandial period following beverage intake (12 x 8 mL samples = 96 mL of venous blood). Capillary blood samples also were collected at t= 0, 30, 60, 120, 180, 240, and 300 min to measure β-OHB concentration using a handheld blood ketone monitor (FreeStyle Precision Neo, Abbott Laboratories, UK). The

capillary samples were collected using a lancet following cleaning with alcohol and allowing the area to air dry. The first blood droplet sample was discarded with a cotton swab and the subsequent droplet samples were used for analysis. At 120 min and 300 min in the postprandial period, the second and third skeletal muscle biopsy samples were obtained from the same leg as the first biopsy but from a different incision site.

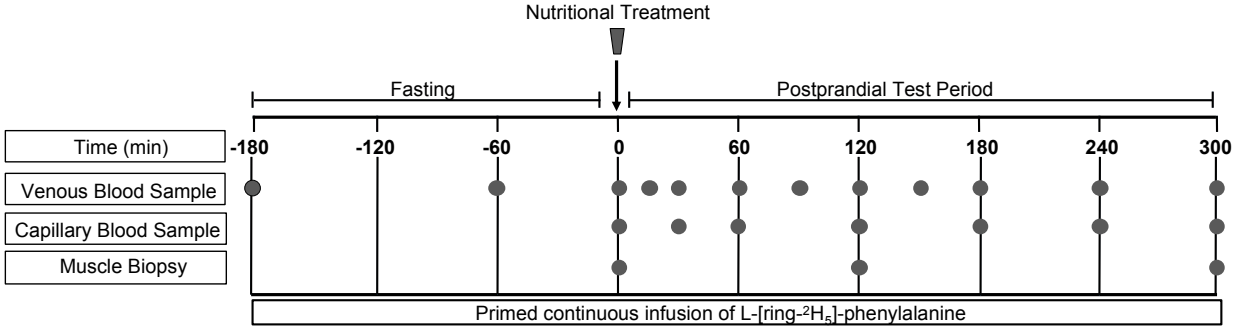

Figure 4. Overview of experimental protocol.

3.2.8. Blood glucose, insulin, and amino acid analyses

Plasma glucose and insulin concentration were measured by the Clinical Biochemistry Laboratory of McGill University Health Centre (Montreal, QC). Plasma glucose concentration was measured using a chemistry analyzer (Beckman Coulter AU5800), while insulin was measured via immunoenzymatic assay (Beckman DxI800). According to data provided by the laboratory, the coefficient of variation (CV) was 2.5% for glucose and 5% for insulin.

Plasma amino acid concentrations were assessed in collaboration with the Proteomics and Clinical Mass Spectrometry platform at the Research Institute of the McGill University Health Centre (Montreal, QC). Amino acids were extracted from plasma using protein precipitation and derivatized with 6- aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC; Toronto Research Chemicals; ON, Canada) for analysis using reversed phase ultra-performance liquid

chromatography mass spectrometry (UPLC-MS). Plasma samples were extracted alongside a calibration curve of amino acids in 0.1N HCl with norvaline as an internal standard (all amino acids and norvaline purchased from Sigma-Aldrich; MO, USA). A calibration curve of 5 to 1000 μM was used for all amino acids except cysteine (2.5 to 500 μM). An internal standard working solution (ISWS) containing 50 μM norvaline in 5% 5-sulfosalicylic acid was used to extract plasma and calibration samples. ISWS aliquots (25 μL) were added to sample aliquots (25 μL) in microcentrifuge tubes, vortexed and centrifuged at 10,000 x g at 10°C for 10 mins. Supernatant aliquots (10 μL) were transferred into glass tubes containing 70 μL buffer solution (0.2M sodium borate pH 8.8) along with 20 μL derivatization solution (10 mM AQC in acetonitrile), mixed and incubated for 10 min at 55°C. After cooling to room temperature, aliquots (10 μL) were transferred to autosampler vials containing 990 μL Type-1 water for UPLC-MS analysis.

Extracts were analyzed by UPLC-MS using an Agilent 6460 triple quadrupole mass spectrometer coupled with an Agilent 1290 UPLC system (Agilent; CA, USA). Extracts (5 μL) were injected onto an Agilent Eclipse Plus C18 100 x 2.1 mm (1.8 μm) column and chromatographed with a reverse phase gradient at 0.200 mL/min using 0.1% formic acid in water and 0.1% formic acid in acetonitrile. The derivatized amino acids were detected using electrospray positive mode ionization followed by MS/MS fragmentation. Data acquisition was performed using Agilent MassHunter Data Acquisition (version B.04.01) software. Peak area measurements from selected product ions, calibration curve regression analysis and resulting sample quantification were performed using Agilent MassHunter Quantitative Analysis (version B.05.00) software.

3.2.9. Blood and muscle phenylalanine enrichment analysis

Enrichments of L-[ring-$^{2}H_5$]-phenylalanine were assessed in free and protein bound plasma and protein bound muscle using separate sample preparation methods but with the same UPLC-MS method.

Free plasma enrichments of L-[ring-$^{2}H_5$]-phenylalanine were determined using protein precipitation. Aliquots (25 μL) of an ISWS containing 50 μM methyl-DL-phenylalanine in 5% 5-sulfosalicylic acid were added to sample aliquots (25 μL) in microcentrifuge tubes, vortexed, and centrifuged at 10,000 x g at 4°C for 5 min. Supernatant aliquots (25 μL) were transferred to 75 μL Type-1 water in another set of microcentrifuge tubes and vortexed. Aliquots (10 μL) were transferred to autosampler vials containing 490 μL Type-1 water for UPLC-MS analysis.

Bound plasma enrichments of L-[ring-$^{2}H_5$]-phenylalanine were determined using protein isolation followed by acid hydrolysis and solid phase extraction. Plasma sample aliquots (250 μL) were added to 10% trichloroacetic acid in water aliquots (250 μL) in microcentrifuge tubes, vortexed, and centrifuged at 1,000 x g at 4°C for 10 min. The supernatants were discarded and 850 μL aliquots of 10% trichloroacetic acid in water were added. The pellets were resuspended with vigorous mixing and centrifuged again at 1,000 x g at 4°C for 10 min. The supernatant removal with pellet resuspension and centrifugation were repeated twice more. All possible supernatant was removed from the final pellets, and they were resuspended in 800 μL 6N HCl for acid hydrolysis. The resuspended pellets were transferred to screw cap glass tubes. The microcentrifuge tubes were rinsed twice with 800 μL 6N HCl and the solutions transferred to their corresponding glass tubes for a final hydrolysis solution volume of 2.4 mL. The samples were heated in a dry bath at 105°C for 16.5 hours. The hydrolysates were cooled to room temperature and transferred to glass tubes. Aliquots (1 mL) of hydrolysates were added to pre-conditioned (1 mL methanol

then 1 mL 0.1N HCl) 30 mg Strata-X-C cartridges (Phenomenex; CA, USA). The cartridges were washed then twice with 1 mL water. After the analytes were eluted with two 600 µL aliquots of 5% ammonium hydroxide in water. The eluted samples were evaporated to dryness under nitrogen at 37°C. The samples were reconstituted in 1mL water.

To determine muscle (myofibrillar) protein-bound enrichments, a piece (~30 mg) of muscle was homogenized on ice using a standing tissue homogenizer (Kinematica Inc.; USA) in 2mL Optima water (Fisher Scientific; ON, Canada). The homogenate was transferred to screw top conical tubes, vortexed, and centrifuged at 1,500 x g at 4°C for 10 mins to separate the myofibrillar and collagen proteins. The myofibrillar pellet was broken up and washed with 2mL Optima water. The sample was then vortexed and centrifuged at 1,500 x g at 4°C for 10 mins after which the supernatant was aspirated and the washing process was repeated twice more using the same steps. After the washes, the myofibrillar pellet was resolubilized in 1.5ml 0.3M NaOH (Fisher Scientific; ON, Canada), vortexed, and centrifuged at 1,500 x g at 4°C for 10 mins. The resulting supernatant was hydrolyzed with 1.5ml 6M HCL (Fisher Scientific; ON, Canada) inside a reaction vial. The reaction vial was heated to 110°C for 16 h. Subsequently, aliquots (0.4ml) were transferred to glass culture tubes and evaporated under nitrogen gas at 37°C until completely dry and then stored at -20°C. The dried myofibrillar fractions were reconstituted with 1mL Type-1 water. Aliquots (50 µL) were transferred to injection vials containing 950 µL internal standard solution (10 nM methyl-DL-phenylalanine in water) for UPLC-MS analysis.

Enrichment samples were analyzed by UPLC-MS using the same Agilent 6460 triple quadrupole mass spectrometer coupled with an Agilent 1290 UPLC system with the same Agilent MassHunter Data Acquisition and Quantitative Analysis software. Extracts (5 µL) were injected onto an Agilent Eclipse Plus C18 50 x 2.1 mm (1.8 µm) column and chromatographed with a

reverse phase gradient at 0.300 mL/min using 0.1% formic acid in water and 0.1% formic acid in acetonitrile. L-phenylalanine, L-[ring-$^{2}H_5$]-phenylalanine and methyl-DL-phenylalanine were detected using electrospray positive mode ionization followed by MS/MS fragmentation. L-phenylalanine was monitored using three transitions at *m/z* 166-120 (primary isotope), *m/z* 168-120 (+2 isotope) and *m/z* 169-120 (+3 isotope). L-[ring-$^{2}H_5$]-phenylalanine was monitored at *m/z* 171-125 (primary isotope). Methyl-DL-phenylalanine (internal standard) was monitored at *m/z* 180-134 (primary isotope). The internal standard was added to monitor system stability and was not used in any enrichment calculations. Additional isotopes for L-phenylalanine were monitored to verify potential signal saturation in samples with high L-phenylalanine amounts.

Tracer-to-tracee calculations for free plasma samples were made by dividing L-[ring-$^{2}H_5$]-phenylalanine peak area by L-phenylalanine peak area and multiplying by a response factor. The response factor was used to correct for the difference in UPLC-MS response between labelled and unlabelled phenylalanine. A TTR calibration curve was deemed unnecessary to use for free plasma as the L-[ring-$^{2}H_5$]-phenylalanine amounts were large in the samples and well above the limit of detection.

Low L-[ring-$^{2}H_5$]-phenylalanine signals were expected so tracer-to-tracee calculations for bound muscle samples were made by using a TTR solution calibration curve which was injected along with the samples. The curve consisted of standards with different amounts of L-[ring-$^{2}H_5$]-phenylalanine (0.5-10 nM) with a constant amount of phenylalanine (5000 nM) which resulted in a TTR curve range from 0.0001 to 0.0020 (0.01% to 0.20%). No significant differences were observed in determined TTR values using L-phenylalanine concetrations from 1000 to 5000 nM which encompassed the range observed in the sample extracts.

3.2.10. Calculations

The fractional synthesis rate of myofibrillar proteins was assessed using the standard precursor-product equation:

$$FSR\ (\%\cdot h^{-1}) = \Delta E_p / [E_{pl} \times t] \times 100\%$$

where ΔE_p is the change in protein-bound L-[ring-$^2H_5$]-phenylalanine enrichment between two muscle biopsies, E_{pl} is the weighted mean plasma L-[ring-$^2H_5$]-phenylalanine precursor enrichment (mole percent excess) across the two biopsy samples, and t is the tracer incorporation time in hours. Weighted mean plasma enrichments were calculated by taking the measured enrichment between consecutive time points and correcting for the time between these sampling time points. The use of tracer-naïve participants in this study allowed the use of the pre-infusion blood sample (t= -180) (i.e., a mixed plasma protein fraction) as the baseline enrichment for the calculation of basal (post-absorptive) FSR. This approach has been previously validated by past research groups (122,131,132).

3.2.11. Statistical analysis

Sample size was determined by completing a power analysis based on differences in postprandial muscle protein FSR from previous published data (133,134). This determined a sample size of 10 in each group would provide statistical power at 80%, with an alpha level of 0.05. However, to preserve power and account for dropouts, we recruited n=12 per group. With three groups, the total number of participants to be recruited was 36.

Participant characteristics (height, weight, blood pressure, HR, % fat, bone- and fat-free mass) were assessed using a 1-factor (treatment) ANOVA. The plasma glucose, insulin, amino acid, and β-OHB data were assessed using a 2-factor (treatment x time) repeated measures ANOVA and 1 factor (treatment) ANOVA (incremental AUC). Plasma tracer enrichments (L-

[ring-$^{2}H_5$]-phenylalanine) were assessed using a 2-factor (treatment x time) repeated measures ANOVA. Skeletal muscle MyoPS rates (i.e. FSR) were assessed using a 2-factor (treatment x time) repeated measures ANOVA. The postprandial time-course (i.e. 0-120 min and 120-300 min) and aggregate (i.e. 0-300 min) FSR were analysed separately and were compared to the basal postabsorptive (i.e. -180-0 min) FSR.

Bonferroni-corrected post hoc comparisons were performed when significant main effects and interactions were observed in the time course data. Assumptions of the statistical methods were assessed using Levene's test (for 1-factor ANOVA), Mauchley's test and the D'Agostino–Pearson omnibus normality test at a significance of $P < 0.05$. If a significant Mauchley's test was determined, the Greenhouse-Geisser correction factor was used to adjust the degrees of freedom accordingly. For data that did not pass normality, values were transformed with the ln of the value. The statistical analysis was performed on transformed data, but non-transformed data are presented in graphic or tabular form for clarity. If a significant Levene's test was determined in the 1-factor ANOVA, Welch's ANOVA and the Dunnett T3 post hoc comparison were used accordingly to test for group mean differences. Statistical analysis was performed with use of the Statistical Package for the Social Sciences (SPSS, Version 26. IBM Corp.; NY, USA). In all statistical analysis, statistical significance was set at $p<0.05$. Values are expressed as mean ± SD.

3.3 Results

3.3.1. Participants' characteristics

No differences were present between KET, PRO, and KET+PRO groups for any of the participants' characteristics (Table 2).

Table 2. Characteristics of study participants who ingested nutritional treatments consisting of ketone monoester only, protein only, or ketone monoester co-ingested with protein

	Nutritional treatment group			
Characteristic	KET	PRO	KET+PRO	*P*
Age (y)	23.3 ± 3.6	24.0 ± 4.7	25.4 ± 3.9	0.43
Height (m)	1.75 ± 0.05	1.78 ± 0.07	1.73 ± 0.08	0.17
Weight (kg)	74.5 ± 5.2	71.9 ± 8.6	70.0 ± 10.5	0.42
BMI (kg/m^2)	24.3 ± 1.6	22.6 ± 1.7	23.4 ± 2.3	0.11
Systolic BP (mmHg)	121 ± 13	112 ± 10	118 ± 12	0.16
Diastolic BP (mmHg)	77 ± 11	72 ± 10	77 ± 8	0.43
Resting heart rate (bpm)	78 ± 13	77 ± 13	73 ± 13	0.60
Body fat (%)	22.1 ± 6.5	21.2 ± 4.8	19.6 ± 6.2	0.57
Bone- and fat-free mass (kg)	55.5 ± 5.0	53.8 ± 6.6	53.5 ± 8.2	0.75

Values represent mean ± SD, n = 12 per group. Data were analyzed using a 1-factor ANOVA. bpm, beats per minute; KET, ketone monoester; PRO, 10 g protein; KET+PRO, ketone monoester co-ingested with 10 g protein.

3.3.2. Dietary intake

Dietary intake data prior to the experimental visit is shown in **Table 3**. No differences were present between KET, PRO, and KET+PRO groups for average total energy, carbohydrate, fat, protein, relative protein intake per kg body weight, as well as relative carbohydrate, fat, and protein intake.

Table 3. Average 2-d dietary intake of study participants who ingested nutritional treatments consisting of ketone monoester only, protein only, or ketone monoester co-ingested with protein

	Nutritional treatment group			
	KET	PRO	KET+PRO	*P*
Energy, kJ · d^{-1}	9511 ± 2213	7741 ± 2145	8992 ± 2642	0.18
Carbohydrate, g	294 ± 73	224 ± 67	238 ± 89	0.08
Fat, g	81 ± 22	74 ± 35	86 ± 37	0.66
Protein, g	99 ± 38	80 ± 25	108 ± 60	0.54
Protein, g · kg^{-1} · d^{-1}	1.3 ± 0.5	1.1 ± 0.3	1.5 ± 0.8	0.43
Carbohydrate %, total energy	52 ± 7	49 ± 11	46 ± 15	0.47
Fat %, total energy	32 ± 7	35 ± 10	35 ± 12	0.76
Protein %, total energy	17 ± 4	18 ± 4	19 ± 8	0.78

Values represent mean ± SD, n = 12 per group. Data were analyzed using a 1-factor ANOVA; KET, ketone monoester; PRO, 10 g protein; KET+PRO, ketone monoester co-ingested with 10 g protein.

3.3.3. Capillary blood β-OHB concentrations

Capillary blood β-OHB (Figure 5, Panel A) concentration (mM) increased (Interaction, $P < 0.001$) following ingestion of the ketone monoester and were significantly greater in both KET & KET+PRO than in PRO from 30-180 min in the postprandial period. At 240 min, capillary blood β-OHB concentrations were significantly greater in KET ($P = 0.039$) than in PRO. The iAUC for capillary blood β-OHB (Figure 5, Panel B) over the 300 min postprandial period was greater ($P < 0.001$) in both KET and KET+PRO than in PRO but were not different from each other ($P = 0.613$).

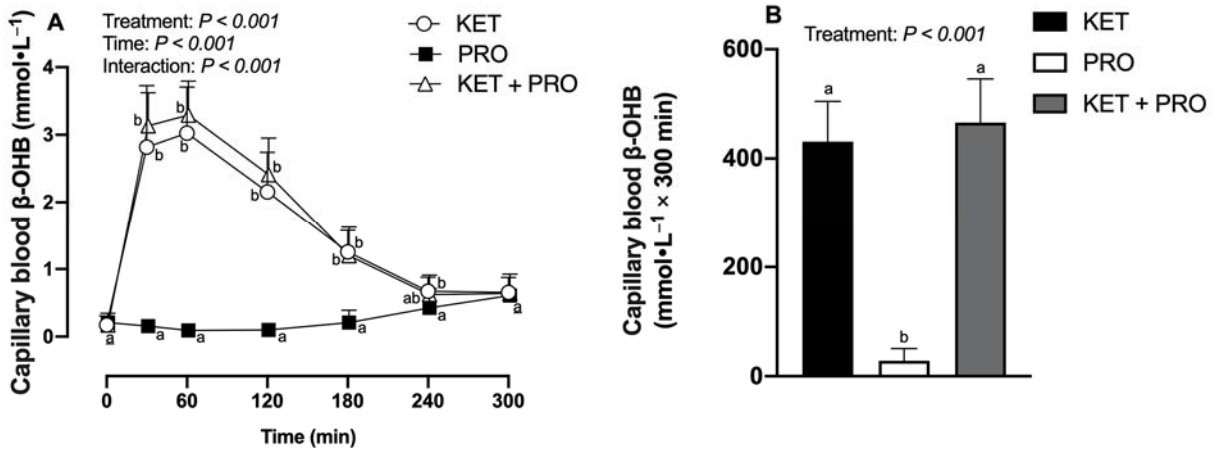

Figure 5. Capillary blood β-OHB (A) concentrations (mmol·L^{-1}) during postabsorptive conditions (t = 0 min) and during postprandial conditions (t = 15-300 min), and corresponding incremental area-under-the curve (B) for β-OHB after beverage intake in young men. Values represent means ± SD, n = 12 per group. In the time-course data, labeled means without a common letter differ within each time point, $P < 0.05$. In the iAUC data, labeled means without a common letter differ, $P < 0.05$. Time course data were analyzed with a two-factor repeated measures ANOVA. Bonferroni post hoc tests were performed to determine the difference between means within each time-point. Incremental area-under-the curve data were analyzed with a one-factor ANOVA. Bonferroni post hoc tests testing was used to detect differences between groups. If a significant Levene test was determined, Welch's ANOVA and the Dunnett T3 post hoc comparison were used accordingly to detect differences between groups. iAUC, incremental area under the curve; KET, ketone monoester; PRO, 10 g protein; KET+PRO, ketone monoester co-ingested with 10 g protein.

3.3.4. Plasma glucose, insulin, and amino acid concentrations

Plasma glucose (Figure 6, Panel A) concentrations (mmol·L^{-1}) increased (Interaction, $P <$ 0.001) and were greater in PRO than in both KET and KET+PRO from 15-120 min in the postprandial period. At 30 min, plasma glucose concentrations were greater in KET (P = 0.007)

than in KET+PRO. Plasma insulin (Figure 6, Panel C) concentrations ($pmol \cdot L^{-1}$) increased (Interaction, $P < 0.001$) and were greater in PRO than in KET+PRO from 15-30 min in the postprandial period and were also greater than in KET at 15 min. The iAUC for glucose (Figure 6, Panel B) over the 300 min postprandial period was greater ($P < 0.001$) in PRO than in both KET and KET+PRO. The iAUC for glucose was also greater ($P < 0.001$) in KET than in KET+PRO. The iAUC for insulin (Figure 6, Panel D) over the 300 min postprandial period was greater ($P = 0.002$) in PRO than in KET+PRO, but both were not different from KET ($P = 0.068$ and $P = 0.543$, respectively).

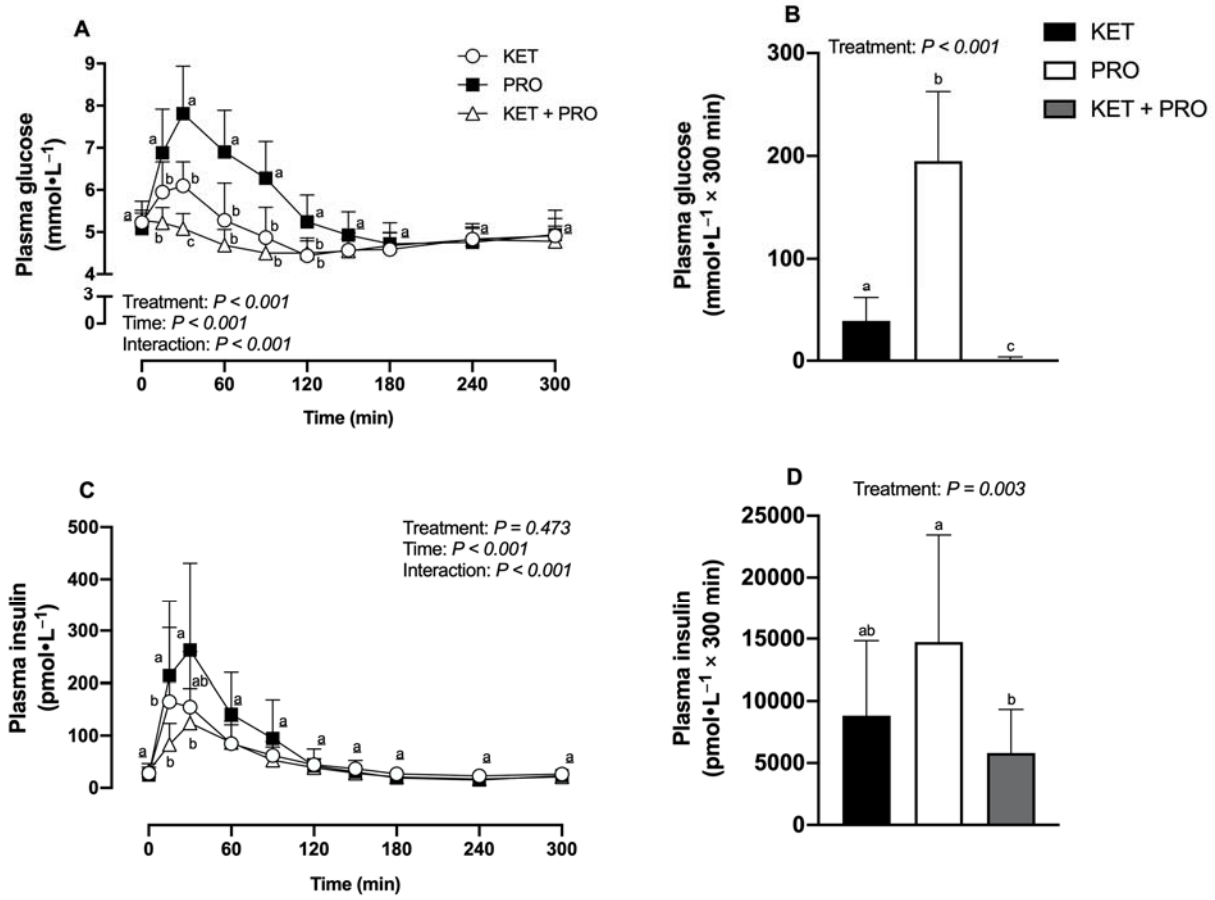

Figure 6. Plasma glucose (A) and insulin (C) concentrations during postabsorptive conditions (t = 0 min) and during postprandial conditions (t = 15-300 min), and corresponding incremental area-under-the curve for glucose (B), and insulin (D) after beverage intake in young men. Values represent means ± SD, n = 12 per group. In the time-course data, labeled means without a common letter differ within each time point, $P < 0.05$. In the iAUC data, labeled means without a common letter differ, $P < 0.05$. Time course data were analyzed with a two-factor repeated measures ANOVA. Bonferroni post hoc tests were performed to determine the difference between means within each time-point. Incremental area-under-the curve data were analyzed with a one-factor ANOVA. Bonferroni post hoc tests testing was used to detect differences between groups. iAUC,

incremental area under the curve; KET, ketone monoester; PRO, 10 g protein; KET+PRO, ketone monoester co-ingested with 10 g protein.

Postprandial plasma leucine (Figure 7, Panel A) concentrations ($mmol \cdot L^{-1}$) increased (Interaction, $P < 0.001$) and were greater in KET+PRO than in KET from 15-300min and were also greater than in PRO from 60-240 min. Postprandial plasma leucine concentrations were greater in PRO than in KET from 15-90 min. The iAUC for leucine (Figure 7, Panel B) over the 300 min postprandial period was greater ($P < 0.001$) in both PRO and KET+PRO than in KET. The iAUC for leucine was also greater ($P < 0.001$) in KET+PRO than in PRO. Postprandial plasma EAA (Figure 7, Panel C) concentrations ($mmol \cdot L^{-1}$) increased (Interaction, $P < 0.001$) and were greater in KET+PRO than in KET from 15-180 min and were also greater than in PRO from 90-180 min. Postprandial plasma EAA concentrations were greater in PRO than in KET from 15-120 min. iAUC for EAA (Figure 7, Panel D) over the 300 min postprandial period was greater ($P < 0.001$) in both PRO and KET+PRO than in KET. The iAUC for EAA was also greater ($P = 0.016$) in KET+PRO than in PRO. Postprandial plasma TAA (Figure 7, Panel E) concentrations ($mmol \cdot L^{-1}$) increased (Interaction, $P < 0.001$) and were greater in KET+PRO than in KET from 15-180 min and were also greater than in PRO from 120-180 min. Postprandial plasma TAA concentrations were greater in PRO than in KET from 15-90 min. The iAUC for TAA (Figure 7, Panel F) over the 300 min postprandial period was greater ($P < 0.001$) in both PRO and KET+PRO than in KET but were not different from each other ($P = 0.056$).

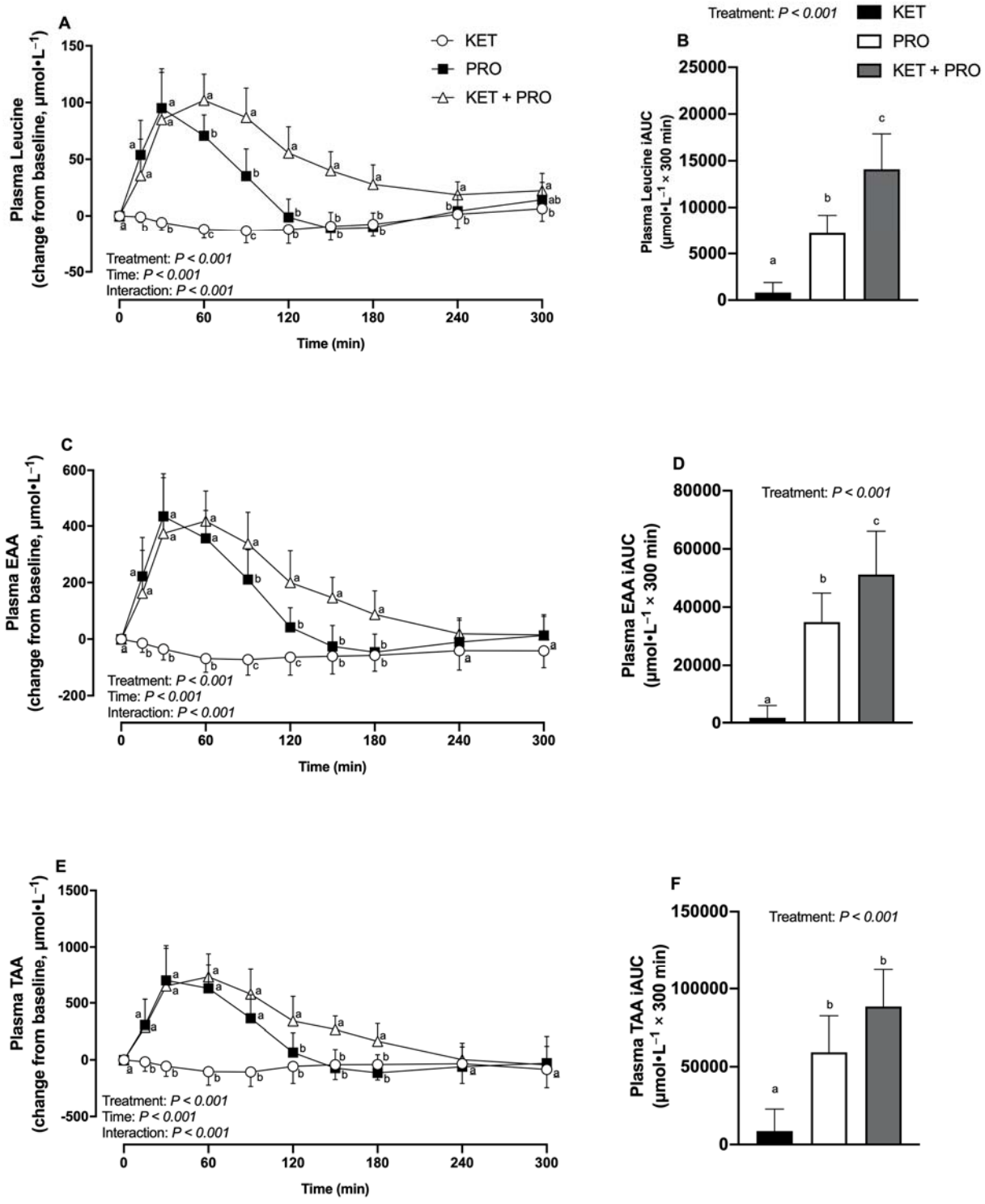
A
Plasma Leucine (change from baseline, µmol•L⁻¹)
KET
PRO
KET + PRO
Treatment: P < 0.001
Time: P < 0.001
Interaction: P < 0.001
Time (min)
B
Treatment: P < 0.001
Plasma Leucine iAUC (µmol•L⁻¹ × 300 min)
C
Plasma EAA (change from baseline, µmol•L⁻¹)
Treatment: P < 0.001
Time: P < 0.001
Interaction: P < 0.001
Time (min)
D
Treatment: P < 0.001
Plasma EAA iAUC (µmol•L⁻¹ × 300 min)
E
Plasma TAA (change from baseline, µmol•L⁻¹)
Treatment: P < 0.001
Time: P < 0.001
Interaction: P < 0.001
Time (min)
F
Treatment: P < 0.001
Plasma TAA iAUC (µmol•L⁻¹ × 300 min)

Figure 7. Plasma leucine (A), essential amino acid (C) and total amino acid (E) concentrations during postabsorptive conditions (t = 0 min) and during postprandial conditions (t = 15-300 min), and corresponding incremental area-under-the curve for leucine (B), essential amino acid (D) and total amino acid (F) after beverage intake in young men. Values represent means ± SD, n = 12 per group. In the time-course data, labeled means without a common letter differ within each time point, $P < 0.05$. In the iAUC data, labeled means without a common letter differ, $P < 0.05$. Time course data were analyzed with a two-factor repeated measures ANOVA. Bonferroni post hoc tests were performed to determine the difference between means within each time-point. Incremental area-under-the curve data were analyzed with a one-factor ANOVA. Bonferroni post hoc tests testing was used to detect differences between groups. If a significant Levene test was determined, Welch's ANOVA and the Dunnett T3 post hoc comparison were used accordingly to detect differences between groups. EAA, essential amino acids; TAA, total amino acids; iAUC, incremental area under the curve; KET, ketone monoester; PRO, 10 g protein; KET+PRO, ketone monoester co-ingested with 10 g protein.

3.3.5. Plasma L-[ring-$^{2}H_{5}$]-phenylalanine enrichment

Plasma L-[ring-$^{2}H_{5}$]-phenylalanine (Figure 8) enrichment (MPE) increased (Interaction, $P < 0.001$) and were greater in PRO than in KET from 150-180 min in the postprandial period. Plasma L-[ring-$^{2}H_{5}$]-phenylalanine enrichment was also greater ($P = 0.002$) in PRO than in KET+PRO at 150 min.

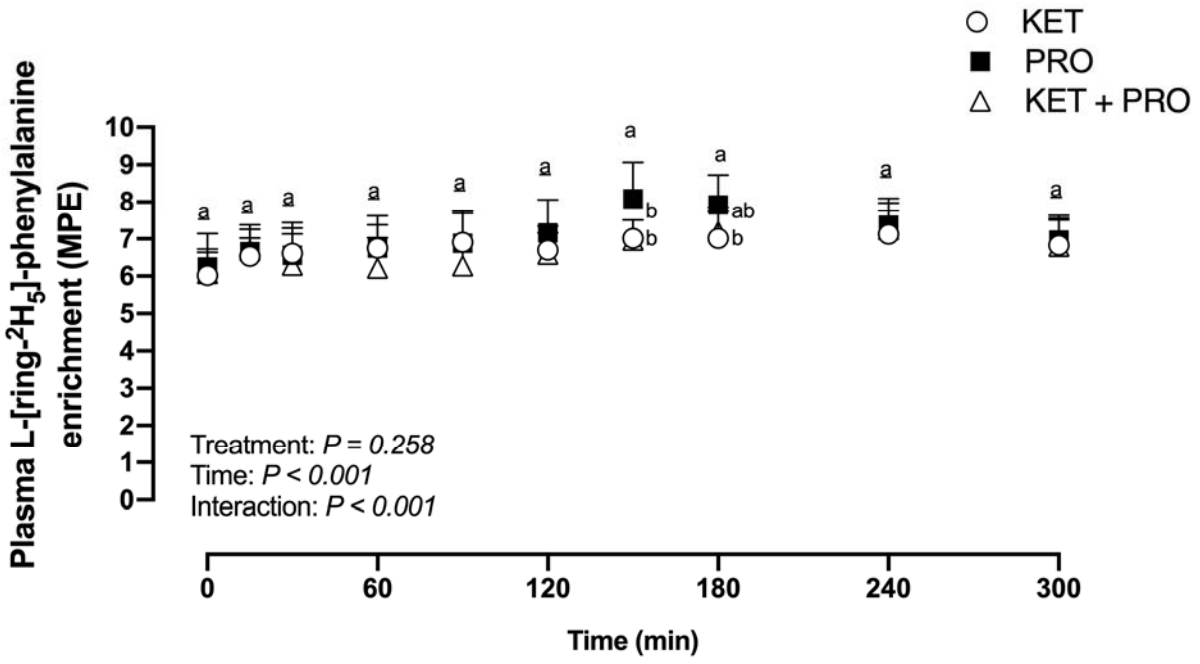

Figure 8. Plasma L-[ring-$^{2}H_{5}$]-phenylalanine enrichment. Values represent means ± SD, n = 12 per group. Labeled means without a common letter differ within each time-point, $P < 0.05$. Data were analyzed with a two-factor repeated measures ANOVA. Bonferroni post hoc tests were performed to determine the difference between means within each time-point. MPE, mole percent excess; KET, ketone monoester; PRO, 10 g protein; KET+PRO, ketone monoester co-ingested with 10 g protein.

3.3.6. Myofibrillar FSR

L-[ring-$^{2}H_{5}$]-phenylalanine-determined myofibrillar FSR, assessed during the early (0–120 min) and late (120–300 min) phase of the postprandial period (Figure 9, Panel A) were significantly higher (Time: $P = 0.001$) compared to basal postabsorptive conditions (-180–0 min), with no differences between treatment groups (Treatment: $P = 0.301$). Similarly, myofibrillar FSR assessed during the aggregate (0–300 min) postprandial period (Figure 9, Panel B) was significantly higher (Time: $P < 0.001$) compared to basal postabsorptive conditions, with no

differences between treatment groups (Treatment: $P = 0.383$). Basal L-[ring-$^2H_5$]-phenylalanine MyoPS rates averaged 0.033 ± 0.011 %/h in KET, 0.036 ± 0.011 %/h in PRO, 0.029 ± 0.010 %/h in KET+PRO over -180–0 min in the basal post-absorptive period. Postprandial L-[ring-$^2H_5$]-phenylalanine MyoPS rates averaged 0.051 ± 0.012 %/h in KET, 0.049 ± 0.012 %/h in PRO, 0.048 ± 0.018 %/h in KET+PRO over 0–120 min, and 0.053 ± 0.022 %/h in KET, 0.046 ± 0.014 %/h in PRO, 0.046 ± 0.016 %/h in KET+PRO over 120–300 min after beverage intake. Postprandial L-[ring-$^2H_5$]-phenylalanine MyoPS rates 0.053 ± 0.014 %/h in KET, 0.049 ± 0.008 %/h in PRO, 0.048 ± 0.009 %/h in KET+PRO over 0–300 min after beverage intake.

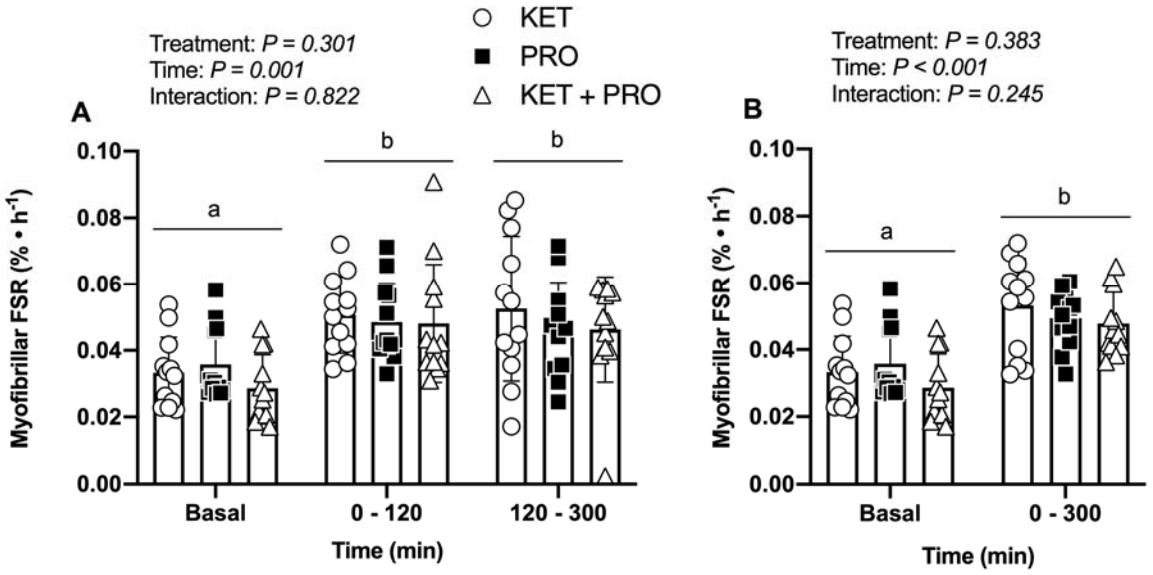

Figure 9. Basal, early (0-120 min) and late (120-300 min) (A), and basal and aggregate (0-300 min) myofibrillar FSR (B). Values represent means ± SD along with individual participant data, $n = 12$ per group. Labeled means without a common letter differ, $P < 0.05$. Data were analyzed with a two-factor repeated measures ANOVA. FSR, fractional synthetic rate; KET, ketone monoester; PRO, 10 g protein; KET+PRO, ketone monoester co-ingested with 10 g protein.

3.4 Discussion

In the present study, we evaluated postprandial changes in capillary blood β-OHB concentration, plasma glucose, insulin, and amino acid (leucine, EAA, and TAA) concentrations, and MyoPS rates in response to the ingestion of a ketone monoester (KET), 10 g whey protein (PRO), or their co-ingestion (KET+PRO) in healthy, moderately active, young men. To better match the energy content of the nutritional treatments, both KET and PRO were co-ingested with carbohydrate (Table 1). Both KET and KET+PRO resulted in a substantial elevation in capillary blood β-OHB concentrations compared to PRO from 30-180 min in the postprandial period. PRO increased plasma glucose concentration to a greater extent than both KET and KET+PRO from 15-120 min in the postprandial period. Plasma insulin concentration was also greater in PRO than both KET and KET+PRO at 15 min in the postprandial period. Both PRO and KET+PRO led to an increase in postprandial plasma amino acid concentration; however, both the temporal and iAUC response differed between treatments. Leucine, EAA and TAA iAUC over the entire 300 min postprandial period was greater in both PRO and KET+PRO compared to KET. Additionally, leucine and EAA iAUC over the entire 300 min postprandial period was greater in KET+PRO than PRO. Treatment ingestion increased postprandial MyoPS rates compared to basal post-absorptive rates, with no difference in the time-course (i.e., 0-120 min and 120-300 min; $P = 0.301$) or aggregate (i.e., 0-300 min; $P = 0.383$) response between treatments.

Consistent with previous work from our lab group (39), and others (7,13), oral ingestion of the ketone monoester (R)-3-hydroxybutyl (R)-3-hydroxybutyrate resulted in a pronounced (β-OHB $C_{max} = 3.3 \pm 0.5$), rapid (within 30 min), but transient (~180 min) nutritional ketosis (Figure 5A). However, it should be noted that in the present study, participants co-ingested the ketone monoester alongside other macronutrients (i.e., carbohydrate or 10 g whey protein). Stubbs and

colleagues (38) reported that meal intake before ingestion of a ketone monoester drink reduced blood D-β-OHB C_{max} by 33% compared to ingestion of the same ketone monoester drink on an empty stomach (i.e., under fasted conditions). Nonetheless, the pronounced, rapid, but transient rise in blood β-OHB concentration observed in the present study is consistent with findings from other studies (7,13,38,39) and confirms that exogenous ketone supplements consumed as a ketone monoester drink represents an efficacious way to induce ketosis.

Exogenous ketone supplements decrease blood glucose concentration in humans (135), with significantly greater glucose-lowering effects reported with ketone monoester supplements compared with ketone salt supplements (135). The mechanisms underlying the glucose-lowering effect of ketone monoester intake are not entirely clear but is likely a direct effect of β-OHB since infusion of β-OHB lowers circulating glucose concentrations (8,9). Exogenous ketosis appears to lower blood glucose concentration via limiting gluconeogenesis in the liver and enhancing peripheral glucose uptake (44). In the present study, treatment groups that ingested the ketone monoester (i.e., KET and KET+PRO) demonstrated a minimal rise in plasma glucose concentration compared to PRO (Figure 6A). Therefore, it is possible that the postprandial glucose response was altered following ketone monoester intake. However, it should be noted that the PRO treatment contained the greatest amount of carbohydrate amongst the three treatments, which likely explains the greater increase in plasma glucose iAUC seen in PRO (Figure 6B).

Intravenous ketone body infusions have been reported to stimulate pancreatic β-cell insulin secretion (136–138). Similarly, previous studies using exogenous ketone monoester drinks have reported an increase in blood insulin concentration during a post-exercise hyperglycaemic clamp (59) and in the fasted state (38,139). The greater increase in plasma insulin concentration in PRO vs. KET and KET+PRO at 15 min in the postprandial period is likely a result of the greater amount

of total carbohydrate ingested (Table 1). Insulin is a key peptide hormone involved in the regulation of muscle protein turnover, where its primary role is to suppress MPB rather than stimulate MPS (140). Given that insulin is permissive, rather than stimulatory for MPS, it is perhaps unsurprising that the greater insulin concentration observed in PRO compared to KET and KET+PRO was not associated with a greater increase in postprandial MyoPS rates (Figure 9, Panel A-B).

The postprandial increase in circulating amino acids, and leucine in particular, after protein ingestion, is thought to be a key regulator of MPS rates (141). Specifically, the anabolic effects of protein ingestion are primarily driven via the transfer and incorporation of amino acids from ingested dietary protein, into skeletal muscle proteins (62). While previous studies have examined the effects of carbohydrate- (142) and fat- (143) co-ingestion with protein on postprandial plasma aminoacidemia, the present study is the first to our knowledge to investigate the effects of ketone monoester co-ingestion with protein on postprandial plasma amino acid concentrations in healthy young men. As expected, there was an increase in postprandial plasma leucine concentration (Figure 7A) after consumption of the nutritional treatments containing 10 g whey protein (i.e., PRO and KET+PRO). However, both the time-course (Figure 7A) and iAUC (Figure 7B) for postprandial plasma leucine concentration differed between PRO and KET+PRO treatments. Specifically, KET+PRO was associated with a more protracted increase in postprandial plasma leucine concentration that resulted in an overall greater plasma leucine iAUC compared to PRO. Similar findings were also observed for postprandial plasma essential amino acid (Figure 7C and 7D) and total amino acid (Figure 7E and 7F) concentration. The reason(s) for differences in postprandial plasma amino acid concentrations between PRO and KET+PRO despite the ingestion of an identical amount of protein (10 g) are unclear but may relate to the greater postprandial iAUC

for plasma insulin concentration in PRO vs. KET+PRO (Figure 6D). Previous studies (144,145) have reported that increases in plasma insulin concentration can reduce plasma amino acid concentrations. The stimulation of endogenous postprandial insulin release represents a key factor facilitating increases in microvascular perfusion (146), supporting amino acid delivery/uptake into skeletal muscle (147). Therefore, the shorter rise in plasma amino acid concentration that coincided with a greater increase in circulating insulin concentration may suggest that disappearance of amino acids from the circulation into skeletal muscle was enhanced in PRO compared to KET+PRO. Alternatively, it is possible that co-ingestion of the ketone monoester with protein (KET+PRO) decreased amino acid retention in splanchnic tissues compared to co-ingestion of protein with carbohydrate (PRO), allowing more of the ingested amino acids to become available in the circulation. Future studies utilizing intrinsically-labelled dietary protein (148) to accurately assess protein digestion/absorption kinetics could address this question.

The role of ketone bodies and hyperketonemia in the regulation of nitrogen, amino acid, and protein metabolism has been of interest since the 1970's (100). Ketone bodies have long been hypothesized to conserve body protein during fasting and therefore possess anti-catabolic effects (12,50). During prolonged fasting, ketone bodies replace glucose as the primary fuel for the brain; therefore, the requirement for amino acid precursors for gluconeogenesis decreases and protein catabolism decreases (3,12,50). Support for this hypothesis comes from human studies in which experimentally elevating ketone bodies has been reported to improve nitrogen balance in traumatized man (5) post-operative patients (4), and in response to fasting (3). Since these initial studies, recent research applying contemporary isotope tracer methods to study protein metabolism has provided evidence that β-OHB has potent anti-proteolytic effects in muscle during acute inflammatory stress (61) and situations characterized by combined systemic inflammation, fasting,

and immobilization (129). In addition to possessing anti-catabolic effects in muscle, there is some evidence that elevated β-OHB/hyperketonemia may stimulate increases in MPS rates (2,7). Using the "gold-standard" tracer incorporation approach (i.e., precursor-product method) to measure MPS, Nair and colleagues (2) reported that intravenous infusion of sodium β-OHB to ~2 mM in healthy men, reduced leucine oxidation by ~30% and stimulated increased rates of mixed MPS by ~10%. In the present study, we applied a recently developed orally ingested ketone monoester supplement, co-ingested with (KET+PRO) and without (KET) 10 g of whey protein, to evaluate the effects of hyperketonemia and combined hyperketonemia/hyperaminoacidemia on postprandial MyoPS rates in young men. Given the well-established dose-response relationship between protein/EAA ingestion and MPS rates, whereby 20 g high-quality protein (17) or 10 g EAA (128) saturates the MPS response, we compared the ketone treatments to a 'sub-optimal' 10 g dose of whey protein (PRO). Despite large differences in postprandial plasma amino acid concentrations between treatments (Figure 7A-F), we observed a robust increase in postprandial MyoPS rates compared to basal conditions (Figure 9A-B) that did not differ between treatments.

It is well-established that dietary protein intake stimulates increased MPS rates (17,108). There is a dose-dependent relationship between protein (17) and EAA (128) intake and MPS rates. Specifically, Moore and colleagues (17) reported ~20 g protein stimulated MPS rates after resistance exercise above that observed with both 5 g and 10 g of protein but was not further stimulated with ingestion of 40 g of protein, indicating that ingestion of 20 g of protein is saturating for MPS in young men. As such, we hypothesized that co-ingesting a 'sub-optimal' amount (10 g) of protein with the ketone monoester supplement (KET+PRO) would further increase MyoPS rates when compared to ingesting a 'sub-optimal' amount (10 g) of protein (PRO) and exogenous ketones without additional protein/amino acids (KET). In support of this hypothesis, Vandoorne

and colleagues (7) reported in an *in vitro* model that the addition of AcAc and β-OHB to a low-dose of leucine (1.5 mM) stimulated a ~2-fold increase in protein synthesis (as measured by puromycin incorporation into nascent peptide chains) in C_2C_{12} cells that was comparable to that achieved with a high-dose of leucine (5.0 mM). However, in the present study there was no additional increase in postprandial MyoPS rates with KET+PRO compared to both KET and PRO. Recently, Mose and colleagues (129) reported that co-ingestion of β-lactoglobulin with exogenous ketones reduced protein synthesis (i.e., reduced the rate of phenylalanine disappearance based on arterio-venous tracer exchange across the forearm) compared to β-lactoglobulin alone under catabolic conditions of combined inflammation, fasting, and bed rest. That is, combined β-lactoglobulin with exogenous ketones eliminated the usual stimulatory effect of β-lactoglobulin (a leucine-rich protein) on MPS. Differences between the present study and that of Mose and colleagues (129) are numerous but include background metabolic state (overnight postabsorptive vs. combined inflammation, fasting, and bed rest), and methods used to measure MPS (precursor-product method vs. arterio-venous tracer exchange).

In conclusion, ketone monoester ingestion, with or without dietary protein co-ingestion, results in elevated β-OHB/hyperketonemia. Treatment ingestion increased postprandial MyoPS rates over the early (0-120 min), late (120-300 min), and aggregate (0-300 min) postprandial period compared to basal postabsorptive conditions. The postprandial MyoPS response following ketone monoester intake, and combined ketone monoester-protein intake (10 g) does not differ from the response observed following protein ingestion (10 g) in healthy young men.

CHAPTER 4: OVERALL CONCLUSION AND SUMMARY

4. CONCLUSION AND SUMMARY

There is currently (re)emerging interest into the metabolic, therapeutic, and performance effects of ketone bodies in humans, largely driven by the development and availability of orally-ingested exogenous ketone supplements. Commercially available ketone supplements such as the ketone monoester (R)-3-hydroxybutyl (R)-3-hydroxybutyrate can induce a transient (~3 hours) hyperketonemia (i.e., elevated β-OHB) within minutes, without starvation or dietary carbohydrate restriction (13,39). Ketone bodies have long been hypothesized to spare body protein, since elevating ketone bodies can improve nitrogen balance under catabolic conditions in traumatized man (5) post-operative patients (4), and in response to fasting (3). Indeed, several studies provide support for the notion that ketone bodies largely support protein anabolism via inhibition of MPB (61,102,126,129). However, the effects of ketone bodies on MPS rates are less clear, with some studies reporting that ketone bodies decrease protein synthesis (102,126,129), and others reporting that ketone bodies increase protein synthesis (2,7). Nonetheless, using the gold-standard precursor-product method to measure fractional synthesis rates of skeletal muscle proteins via L-[1-^{13}C]leucine, Nair and colleagues (2) reported that intravenous infusion of sodium β-OHB to ~2 mM attenuated leucine oxidation by ~30% and increased mixed MPS by ~10% compared to a saline infusion in healthy young men. This study provided initial evidence that ketone bodies stimulate MPS in humans.

The present thesis aimed to explore the effects of acute nutritional ketosis, induced via orally-ingested ketone supplements, on MyoPS rates in healthy young men. Specifically, we evaluated the effects of the ketone monoester (R)-3-hydroxybutyl (R)-3-hydroxybutyrate, co-ingested with (KET+PRO) and without (KET) dietary protein (10 g whey). We compared these ketone-based treatments to protein (10 g whey) alone (PRO), since dietary protein unequivocally

stimulates MyoPS rates (62,108,141). We found that ketone monoester ingestion, with (KET+PRO) or without (KET) dietary protein co-ingestion, resulted in elevated β-OHB. Interestingly, treatment ingestion increased postprandial MyoPS rates above basal postabsorptive conditions across all time-periods (i.e., 0-120, 120-300, and 0-300 min), with no time × treatments interaction. Therefore, the increase in postprandial MyoPS rates does not differ following ketone monoester intake (KET), 10 g protein intake (PRO), and combined ketone monoester-protein intake (KET+PRO) *in vivo* in humans.

The results generated from this study suggest that ketone bodies (i.e., elevated β-OHB) possess anabolic effects in human skeletal muscle. Despite no exogenous source of amino acids, the postprandial FSR that resulted following the ingestion of the ketone monoester supplement (KET) was similar to that observed with the consumption of 10 g whey protein (PRO), and a combination of the two (KET+PRO). Further studies are warranted in order to fully explore the molecular mechanisms behind ketone body-mediated anabolism after ketone monoester intake, in addition to their potential interaction with ingested protein/amino acids. The present study represented an acute and tightly controlled investigation during which participants remained rested during the duration of the experimental trial. Given that exercise can modulate ketone body production and stimulate MPS, future studies may wish to explore the effects of ketone supplements on MyoPS rates during post-exercise recovery conditions. In addition, future studies may want to explore their potential use in counteracting and mitigating muscle atrophy. The present study was also conducted in young men who were healthy and moderately active. Future studies may also look to explore whether similar results could be extended to other types of research populations such as women, the elderly, obese individuals, or those living with metabolic disorders.

In conclusion, ketone monoester ingestion, with or without dietary protein co-ingestion, results in elevated β-OHB/hyperketonemia. Treatment ingestion increased postprandial MyoPS rates over the early (0-120 min), late (120-300 min), and aggregate (0-300 min) postprandial period compared to basal postabsorptive conditions. The postprandial MyoPS response following ketone monoester intake, and combined ketone monoester-protein intake (10 g) does not differ from the response observed following protein ingestion (10 g) in healthy young men.

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