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In vitro ketone-supported mitochondrial respiration is minimal when other substrates are readily available in cardiac and skeletal muscle

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Key Points

- Ketone bodies are proposed to represent an alternative fuel source driving energy production, particularly during exercise.
- Biologically, the extent to which mitochondria utilize ketone bodies compared to other substrates remains unknown.
- We demonstrate *in vitro* that maximal mitochondrial respiration supported by ketone bodies is low when compared to carbohydrate-derived substrates in the left ventricle and red gastrocnemius muscle from rodents, and in human skeletal muscle.
- When considering intramuscular concentrations of ketone bodies and the presence of other carbohydrate and lipid substrates, biological rates of mitochondrial respiration supported by ketone bodies are predicted to be minimal.
- At the mitochondrial level, it is therefore unlikely that ketone bodies are an important source for energy production in cardiac and skeletal muscle, particularly when other substrates are readily available.

Wordcount: 125

Abstract

Ketone bodies (KB) have recently gained popularity as an alternative fuel source to support mitochondrial oxidative phosphorylation and enhance exercise performance. However, given the low activity of ketolytic enzymes and potential inhibition from carbohydrate oxidation, it

remains unknown if KBs can contribute to energy production. We therefore determined the ability of KBs (sodium DL-β-hydroxybutyrate, β-HB; lithium acetoacetate, AcAc) to stimulate *in vitro* mitochondrial respiration in the left ventricle (LV) and red gastrocnemius (RG) of rats, and in human *vastus lateralis*. Compared to pyruvate, the ability of KBs to maximally drive respiration was low in isolated mitochondria and permeabilized fibers (PmFb) from the LV (~30-35% of pyruvate), RG (~10-30%), and human *vastus lateralis* (~2-10%). In PmFb, the concentration of KBs required to half-maximally drive respiration (LV: 889 μM β-HB, 801 μM AcAc; RG: 782 μM β-HB, 267 μM AcAc) were greater than KB content representative of the muscle microenvironment (~100 μM). This would predict low rates (~1-4% of pyruvate) of biological KB-supported respiration in the LV (8-14 pmol·sec^{-1}·mg^{-1}) and RG (3-6 pmol·sec^{-1}·mg^{-1}) at rest and following exercise. Moreover, KBs did not increase respiration in the presence of saturating pyruvate, submaximal pyruvate (100 μM) reduced the ability of physiological β-HB to drive respiration, and addition of other intracellular substrates (succinate, palmitoylcarnitine) decreased maximal KB-supported respiration. As a result, product inhibition likely limits KB oxidation. Altogether, the ability of KBs to drive mitochondrial respiration is minimal and they are likely outcompeted by other substrates, compromising their use as an important energy source.

Abstract word count: 249

Keywords: ketone bodies, bioenergetics, metabolism, mitochondria

Abbreviations: AcAc, acetoacetate; Ac-CoA, acetyl-CoA; AcAc-CoA, acetoacetyl-CoA; ACAT, Ac-CoA acetyltransferase; ADP, adenosine diphosphate; ATP, adenosine triphosphate; BDH, β-hydroxybutyrate dehydrogenase; β-HB, β-hydroxybutyrate; ETC, electron transport chain; KB, ketone body; KE, ketone ester; LV, left ventricle; MA, malonic acid; O_2, oxygen; OXCT, succinyl-CoA:3-oxoacid CoA transferase; OXPHOS, oxidative phosphorylation system; PC, palmitoylcarnitine; PDH, pyruvate dehydrogenase; PFK, phosphofructokinase; PK, pyruvate kinase; RCR, respiratory control ratio; RER, respiratory exchange ratio; RG, red gastrocnemius; SDH, succinate dehydrogenase; TCA, tricarboxylic acid cycle; Vmax, maximal respiratory capacity; VO_2max, maximal aerobic capacity; WL, whole muscle lysate.

Introduction

While fat and carbohydrates are classical substrates known to support mitochondrial adenosine triphosphate (ATP) production, ketone bodies (KB) are an alternative fuel source. Produced in the liver during times of fasting, low carbohydrate consumption, or excess lipid

availability, the KBs β-hydroxybutyrate (β-HB) and acetoacetate (AcAc) are capable of driving energy production within the brain, heart, and skeletal muscle (Ruderman & Goodman, 1973; Sokoloff, 1973; Winder *et al.*, 1974; Mikkelsen *et al.*, 2015). Within skeletal muscle, seminal findings based on rates of KB uptake (arteriovenous differences in KB concentrations) have indicated that KBs could represent a priority substrate in this tissue (Ruderman *et al.*, 1971; Ruderman & Goodman, 1973). However, in the absence of measures of direct oxidation rates, the ability to utilize KBs for aerobic energy production within mitochondria remains unknown. Following uptake within extra-hepatic tissues such as skeletal and cardiac muscle, KB utilization occurs as β-HB is re-oxidized to AcAc within the mitochondria through a process producing NADH. Through a series of sequential reactions requiring succinyl-CoA as a CoA donor, AcAc ultimately generates acetyl-CoA (Ac-CoA) and converges with carbohydrate and lipid pathways for oxidative ATP production in the tricarboxylic acid cycle (TCA). Despite lack of physiological significance, *in vitro* combustion of β-HB provides more kJoules of energy per mole carbon (1019 kJ/mol 2C) than the carbohydrate-derived substrate pyruvate (777 kJ/mol C) (Veech, 2004). Additionally, KBs have been shown to increase free energy released from ATP hydrolysis in the heart compared to that of glucose alone (Sato *et al.*, 1995). As a result, it has been suggested that KBs may be a more efficient fuel source to drive ATP production within the oxidative phosphorylation system (OXPHOS).

According to this theory that KBs can substantially support mitochondrial ATP production, considerable interest has been placed on the potential of KBs to spare glycogen, prevent fatigue, and improve exercise performance (Cox *et al.*, 2016; Evans *et al.*, 2017; Pinckaers *et al.*, 2017). In chronic dietary approaches, it has been shown that three weeks of a ketogenic diet (Burke *et al.*, 2017) is capable of elevating plasma ketone levels, decreasing respiratory exchange ratios (RER, suggestive of increased fat oxidation), and decreasing

blood lactate accumulation during exercise. Similar metabolic changes are evident following acute ketone ester (KE) (Leckey *et al.*, 2017) or ketone salt (O'Malley *et al.*, 2017) intake. However, despite alterations in metabolism, these studies have demonstrated impairments in exercise performance compared to individuals consuming a prolonged high carbohydrate diet (Burke *et al.*, 2017; Wroble *et al.*, 2019) or acutely ingesting carbohydrates (Leckey *et al.*, 2017; O'Malley *et al.*, 2017). While other findings suggest ketone ingestion does not alter exercise performance (Shaw *et al.*, 2019*b*; Poffé *et al.*, 2020; Prins *et al.*, 2020), acute consumption of KEs has also been reported to decrease glycolytic intermediates, attenuate glycogen utilization, and improve cycling performance, in which 16-18% of oxygen consumption was estimated to be from β-HB oxidation (Cox *et al.*, 2016). These effects may further be influenced by the structural form of KBs consumed, as ketone salts may be less effective at increasing plasma ketone concentrations compared to ketone monoesters or diesters (Balasse & Ooms, 1968). Clearly, the influence of KBs on exercise performance and skeletal muscle metabolism remains contentious, particularly as the underlying ability of mitochondria to utilize KBs in these situations is largely unknown.

On a cellular level, the activity of enzymes involved in ketolysis (mainly near-equilibrium β-hydroxybutyrate dehydrogenase, BDH; and Ac-CoA acetyltransferase, ACAT) are orders of magnitude lower than glycolytic enzymes mediating carbohydrate breakdown, particularly in skeletal muscle (Baldwin *et al.*, 1973; Winder *et al.*, 1974). This suggests that flux through glycolysis may be dramatically greater than rates of ketone catabolism. In addition, carbohydrate and lipid oxidation could cause product inhibition of KB oxidation, as NADH and Ac-CoA are products of pyruvate dehydrogenase (PDH), β-oxidation, and near-equilibrium ketolysis reactions. This is likely to occur at two sites of regulation from NADH-mediated product inhibition of BDH and Ac-CoA-mediated product inhibition of ACAT. As

a result, KBs may not be important energy sources when carbohydrates (i.e. pyruvate) are readily available, however, this has yet to be directly determined.

Therefore, the current study aimed to examine the *in vitro* ability of β-HB and AcAc to drive mitochondrial respiration compared to carbohydrate-derived substrates in both rodent and human muscle. Furthermore, we estimated biological rates of KB-supported respiration in the presence of other intracellular substrates and concentrations of KBs within the intramuscular environment. Altogether, we establish that the ability of KBs to support mitochondrial respiration is low at rest and is not acutely modulated following exercise. Therefore, KBs are unlikely to contribute substantially to mitochondrial energy production in cardiac and skeletal muscle.

Methods

Ethical approval

All animal experiments were performed in accordance with institutional guidelines approved by the Animal Care Committee at the University of Guelph (AUP#4242) and conform to the ethical principles outlined by *The Journal of Physiology* (Grundy, 2015). Male Sprague Dawley rats were housed with a 12:12 h light-dark cycle and were provided standard chow and water *ad libitum*. For human experiments, the experimental procedures and risks were thoroughly explained, and written informed consent was given by each participant as approved by the Human Research Ethics Board at the University of Guelph (REB17-12-005). The study conformed to the standards set by the Declaration of Helsinki, except for registration in a database.

Rodents

Rats remained sedentary or were subject to an acute bout of treadmill running (Exer-3R treadmill, Columbus Instruments) for 90 min at 18 m/min, 5% grade, following 3 days of acclimation (10 min, 15 m/min, 5% grade). All animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (60 mg/kg; MTC Pharmaceuticals, Cambridge, ON). In exercise experiments, rodents were injected intraperitoneally with sodium pentobarbital (60 mg/kg) and returned to the treadmill until failure to run. Rodents then received isoflurane and oxygen (2%: 98%) to confirm animals remained under the surgical plane while surgeries were rapidly performed to collect red gastrocnemius (RG, within ~1 min of exercise cessation) and left ventricle (LV, within ~2 min of exercise cessation) tissue as previously described (Barbeau *et al.*, 2018). RG and LV tissue was excised for preparation of permeabilized muscle fibers and mitochondrial isolation. All procedures were performed only after assurance of anesthesia depth check by leg retraction after toe pinch, palpebral reflex, and movement of whiskers.

Human participants

Four highly trained, healthy participants (25 ± 1.5 years), three of which were males (58.9 ± 0.8 $mL \cdot kg^{-1} \cdot min^{-1}$ VO_2max, M) and one female (69.1 $mL \cdot kg^{-1} \cdot min^{-1}$ VO_2max, F) were recruited. Participants were concurrently trained cyclists (n=2 M), an ultimate frisbee player (n=1 M) and a former elite distance runner/current competitive cyclist (n=1 F). On average, training time per week performing endurance-based activities was 8.3 ± 4.1 hours (M) and 12 hours (F), over 6.3 ± 1.1 days per week (M) and 7 days per week (F). Years of training experience was 3.7 ± 2.0 years (M) and 10 years (F). Prior to enrollment, participants completed health questionnaires to determine eligibility. Participants were eligible if physically active at least three times per week, free from any injuries, medications, or musculoskeletal disease, and were non-smokers. VO_2max tests were performed on

electronically braked cycle ergometers (Racermate Velotron, Seattle, WA, USA) using breath-by-breath analysis of metabolic parameters (Cosmed Quark CPET, Rome, Italy) as previously described (Coates *et al.*, 2018). On the muscle biopsy day, participants arrived following a 4 hour fast, and having refrained from exercise, alcohol, and caffeine for 12 hours prior. A single resting skeletal muscle biopsy (*vastus lateralis*) was performed using the Bergstrom technique. The sample was immediately placed in ice-cold BIOPS (2.77 mM CaK_2EGTA, 7.23 mM K_2EGTA, 5.77 mM Na_2ATP, 6.56 mM $MgCl_2 \cdot 6H_2O$, 15 mM Na_2PCr, 20 mM imidazole, 0.5 mM dithiothreitol, and 50 mM MES) (Petrick *et al.*, 2019) for preparation of permeabilized muscle fibers and isolation of mitochondria.

Isolated mitochondria

Skeletal muscle (RG and *vastus lateralis)* and LV mitochondria were isolated using differential centrifugation as previously described (Holloway *et al.*, 2009). Tissues were harvested, minced in isolation buffer (100 mM sucrose, 100 mM KCl, 50 mM Tris-HCl, 1 mM KH_2PO_4, 0.1 mM EGTA, 0.2% BSA, and 1 mM ATP; pH 7.4), weighed, and homogenized using a motorized Teflon pestle (800 rpm). Mitochondria were centrifuged at 800 g for 10 min, resuspended in 4 mL of isolation buffer, and treated with 0.025 μg/mg tissue protease (Subtilisin A, Sigma-Aldrich) for 5 min. Thereafter, 10 mL of isolation buffer was added, and the sample immediately spun at 5000 g for 5 min. The pellet was repeatedly resuspended in isolation buffer and pelleted at 10000 g for 10 min. Mitochondria were prepared immediately for respiration experiments. While four human participants were recruited, only two isolated mitochondrial preparations were obtained (one sample from one participant and one sample pooled from three participants) due to tissue limitations. For all experiments subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondrial subpopulations were pooled. In pilot experiments (n=2-3) from rat LV mitochondria, there were no

differences in P/O ratios, RCRs, or the ability of KBs to drive respiration between SS and IMF mitochondria. As a result, we performed all further experiments in pooled SS and IMF isolated mitochondria.

Isolated mitochondria respiration experiments were performed in Oroboros Oxygraph 2K systems (Innsbruck, Austria) at 25˚C. To calculate P/O ratio in the RG and LV, the change in oxygen following the addition of 100 μM adenosine diphosphate (ADP) was used in the presence of 10 mM pyruvate + 2 mM malate; 10 mM sodium DL-β-hydroxybutyrate (β-HB; racemic mixture of D and L isoforms) + 2 mM malate; or 10 mM lithium acetoacetate (AcAc) + 2 mM malate. The interaction between pyruvate and KBs was tested by adding 10 mM KBs (experiments performed each with β-HB and AcAc) following saturating pyruvate (5 mM) + malate (2 mM) + ADP (2 mM). In the reverse experiment, 2 mM malate + 10 mM KBs (β-HB and AcAc in separate experiments) + 2 mM ADP were added to the chamber, followed by 5 mM pyruvate. Excess mitochondria (LV and RG) were frozen in -80˚C for western blot analysis.

Permeabilized muscle fibers

Permeabilized muscle fiber bundles were separated in ice-cold BIOPS using fine-tipped forceps, treated with 40 mg/mL saponin for 30 min at 4°C, and then washed in mitochondrial respiration medium (MiR05) containing 0.5 mM EGTA, 3 mM $MgCl_2 \cdot 6H_2O$, 60 mM potassium lactobionate, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM sucrose, and fatty acid-free bovine serum albumin (BSA; 1 g/l). Mitochondrial respiration experiments were performed in Oroboros Oxygraph 2K systems (Innsbruck, Austria) at 37˚C (rodent RG and human *vastus lateralis*, in the presence of 5 μM blebbistatin) or 25˚C (LV). In human skeletal muscle samples, permeabilized fiber experiments were performed in tissue from all four participants. KB (β-HB and AcAc in separate experiments) titrations (100, 250, 500, 1000,

2000, 4000, 6000, 8000, 10000 μM) were performed in the presence of 1 mM malate + 5 mM ADP; followed by the addition of 5 mM pyruvate, 10 mM glutamate, 10 mM succinate, and 10 μM cytochrome C. Pyruvate titrations (10, 20, 30, 50, 70, 90, 140, 240, 340, 440, 540 μM) were performed in the presence of 1 mM malate + 5 mM ADP; followed by the addition of 10 mM glutamate, 10 mM succinate, and 10 μM cytochrome C. Maximal KB-supported respiration (10 mM β-HB and AcAc in separate experiments) was determined in the presence of 1 mM malate + 5 mM ADP; 1 mM malate + 5 mM ADP + 5 mM pyruvate; 1 mM malate + 5 mM ADP + 100 μM pyruvate; 1 mM malate + 5 mM ADP + 100 μM pyruvate + 1500 μM succinate (LV only); or 1 mM malate + 5 mM ADP + 100 μM pyruvate + 1500 μM succinate + 30 μM palmitoylcarnitine (LV only). Fibers were recovered and freeze-dried to normalize mitochondrial respiration to fiber dry weight.

Western blotting

Western blots were performed on LV and RG isolated mitochondria as previously described (Holloway *et al.*, 2009). Mitochondrial protein samples and a whole muscle lysate control (WL) were equally loaded, separated using SDS/PAGE, and transferred to PVDF membranes. Commercially available antibodies were used to detect α-tubulin (Abcam Ab7291, 1:1000), Caveolin-3 (BD Biosciences 610406, 1:1000), SERCA-2 (Abcam ab2861, 1:1000), VDAC-1 (Abcam ab14734, 1:1000), COX-IV (Invitrogen A21347, 1:1000), and OXPHOS complex ATP synthase (Mitosciences ab110413, 1:1000). Membranes were detected using enhanced chemiluminescence (ChemiGenius2).

Statistical analysis

Results are expressed as bar-and-scatter plots with individual values depicted in grey circles and the mean ± SD superimposed. Michaelis-Menten analysis was performed using

GraphPad Prism 6.0 for pyruvate, β-HB, and AcAc kinetic curves. Groups were compared using a one-way ANOVA followed by a Tukey's multiple comparison post-hoc test, unpaired two-tailed Students *t*-test, or a two-way ANOVA following by Sidak's multiple comparison post-hoc test where appropriate (details and sample sizes listed in figure captions), using GraphPad Prism 6.0 (La Jolla, CA, USA). Statistical significance was assumed to be $p<0.05$.

Results

KB-supported respiration rates are low in isolated mitochondria

We first aimed to test the ability of β-HB and AcAc to drive respiration in isolated mitochondria. The absence of cytosolic, plasma membrane, and endoplasmic reticulum proteins confirmed the purity of left ventricle (LV) *(Figure 1A)* and red gastrocnemius (RG) *(Figure 1B)* mitochondrial preparations. In both the LV and RG, P/O ratios (the amount of O_2 required to use a known concentration of ADP) were not different between pyruvate and KBs *(Figure 1C,D)*. In addition, the ability of mitochondria to respond to saturating concentrations of ADP (respiratory control ratio, RCR) was similar in the presence of KBs or pyruvate in both the LV *(Figure 1E)* and RG *(Figure 1F)*. Although these parameters suggest mitochondrial efficiency was comparable in response to KBs and pyruvate, stark differences existed in the extent to which mitochondria utilized each substrate. In the absence (state 2) or presence (state 3) of ADP, maximal β-HB- and AcAc-supported respiration were ~4-fold lower than pyruvate in the LV (*Figure 1G)* and RG *(Figure 1H)*.

Ketone bodies minimally drive mitochondrial respiration in permeabilized fibers

While KB-supported respiration is minimal in isolated mitochondria, we next examined these parameters in permeabilized fibers which are more representative of the intracellular structure. In the LV, the addition of KBs maximally increased respiration by

~30% of the rate compared to subsequent additions of saturating pyruvate, glutamate (maximal complex I-supported respiration), and succinate (maximal complex I- and II-supported respiration) *(Figure 2A*). The ability of KBs to drive respiration was even lower in the RG *(Figure 2B)* and human skeletal muscle *(Figure 2C)*. Furthermore, these experiments were performed using saturating KB concentrations (10 mM), while biological concentrations are orders of magnitude lower (Balasse & Féry, 1989; Pinckaers *et al.*, 2017). We therefore titrated various submaximal concentrations of β-HB and AcAc in the LV *(Figure 2D)* and RG *(Figure 2E).* This revealed that respiration was half-maximally stimulated (apparent K_m) by 889 μM β-HB and 801 μM AcAc in the LV (*Figure 2D*), while skeletal muscle mitochondrial sensitivity to AcAc (267 μM) was higher than β-HB (782 μM; p=0.0002; *Figure 2E).* However, given the low drive in respiration in human skeletal muscle (~7% and ~2% of pyruvate for β-HB and AcAc, *Figure 2F)*, we did not have the resolution to detect mitochondrial sensitivity to submaximal concentrations of KBs.

KB-supported respiration is not altered following acute exercise

While these data provide evidence that KB utilization is low in two different species and tissues at rest during times of low energy demand, we next examined KB-supported respiration following an acute bout of treadmill running in rodents. However, in permeabilized fibers from both the LV and RG, acute exercise did not alter maximal respiration supported by β-HB *(Figure 3A,B)* or AcAc *(Figure 3C,D);* or mitochondrial sensitivity to these substrates *(Figure 3E,F)*. Therefore, regardless of KB, tissue examined, and energetic status (i.e. rest vs. exercise), it is unlikely that KBs are a substantial source of mitochondrial ATP production.

Estimates of biological interactions between KBs and intracellular substrates

Given our ketone-supported kinetic curves in the LV and RG, we next performed pyruvate titrations in these tissues to compare biological estimates of substrate utilization between KBs and pyruvate. This revealed that maximal and submaximal pyruvate-supported respiration was dramatically greater than β-HB and AcAc in the LV *(Figure 4A)* and RG (*Figure 4B*) at rest. Furthermore, KB concentrations representative of the muscle microenvironment (~100 μM) (Balasse & Féry, 1989; Mikkelsen *et al.*, 2015; Evans *et al.*, 2017) would predict only a ~10-14 pmol·sec^{-1}·mg^{-1} dry wt (~3-4% of maximal pyruvate) drive in mitochondrial respiration in the LV *(Figure 4A),* and rates were even lower in the RG (3-5 pmol·sec^{-1}·mg^{-1} dry wt; ~1-2% of maximal pyruvate) *(Figure 4B).* Similar estimates of mitochondrial respiration are evident in the LV *(Figure 4C)* and RG *(Figure 4D)* following exercise, given the absence of changes in KB-supported respiratory kinetics in these situations. Altogether, this would suggest the ability of KBs to biologically drive respiration is low compared to carbohydrate-derived substrates.

As neither KBs nor pyruvate are present in isolation biologically, we next examined the interaction between these substrates to better model the intracellular environment. In isolated mitochondria, when respiration was supported by pyruvate and ADP, the addition of β-HB (representative O_2 trace, *Figure 5A,B, upper panel)* or AcAc only minimally drove respiration in the LV (*Figure 5C*) and RG *(Figure 5D).* In contrast, the addition of pyruvate following KBs (representative O_2 trace, *Figure 5A,B lower panel*) increased respiration ~3-4-fold *(Figure 1E,F)*. As a result, the ability of KBs to drive O_2 consumption after pyruvate was almost non-detectable, while the addition of pyruvate following KBs dramatically increased respiration *(Figure 1G,H).* In human skeletal muscle isolated mitochondria, (n=2, one sample from one participant in *Figure 6A,B*; and one sample pooled from three participants in *Figure 6C,D*), similar to the RG and LV, the addition of pyruvate stimulated respiration ~10-fold more after saturating β-HB *(Figure 6A)* and AcAc *(Figure 6B)*. In contrast, when added

following pyruvate, β-HB *(Figure 6C)* and AcAc *(Figure 6D)* did not appear to influence respiration. Given our small sample size in isolated mitochondria due to tissue limitations, these data in human skeletal muscle are only observational in nature, yet support our findings in rodent isolated mitochondria (*Figures 1,5*).

In permeabilized muscle fibers prepared from all human skeletal muscle samples (n=4 each PmFb experiment), saturating concentrations of pyruvate (5 mM) blunted the ability of KBs to drive mitochondrial respiration, as pyruvate-supported respiration was not increased following the addition of β-HB or AcAc in the LV *(Figure 7A)* or RG *(Figure 7B)*. Based on these findings, and to model a more biological situation, we used a submaximal concentration of pyruvate (100 μM, ~apparent K_m) followed by either saturating or submaximal KBs to examine the interaction between these substrates. This approach revealed that 100 μM pyruvate did not decrease maximal β-HB- or AcAc-supported respiration in the LV *(Figure 7C,D),* RG *(Figure 7E,F)*, or human skeletal muscle *(Figure 7G,H)*. In contrast, 100 μM pyruvate dramatically impaired the ability of biologically relevant concentrations of β-HB (100 μM) to stimulate respiration in the LV *(Figure 8A,B;* p=0.0058*)*, without affecting the ability of 100 μM AcAc to drive mitochondrial respiration *(Figure 8B,C;* p=0.7472*)*. Combined, this suggests that submaximal pyruvate concentrations (~100 μM) can outcompete, and/or create product inhibition to limit, β-HB- but not AcAc-supported respiration.

While pyruvate drives complex I supported respiration, biologically, carbohydrate substrates also provide reducing equivalents to complex II (i.e. succinate); and lipid substrates (i.e. palmitoylcarnitine, PC) undergo β-oxidation to support both complex I- and II-linked respiration. As a result, we added saturating KBs in the presence of submaximal succinate, pyruvate, and PC to model the intracellular environment where all substrates are available. We performed these experiments in the LV as KB-supported respiration was

highest in this tissue, which would give us the greatest resolution to detect subtle differences in the presence of other substrates. When added following submaximal pyruvate+succinate *(Figure 8D)* and submaximal PC+pyruvate+succinate *(Figure 8E)*, KBs were still capable of increasing absolute mitochondrial respiration. However, while the magnitude of KB-supported respiration was not altered with succinate+pyruvate compared to pyruvate alone (*Figure 8D inset*, p=0.0622 β-HB, p=0.3216 AcAc), the addition of PC (in the presence of submaximal pyruvate and succinate) decreased the ability of both β-HB (p=0.0018) and AcAc (p=0.0224) to drive respiration (*Figure 8E inset)*. As lipids produce both NADH and $FADH_2$, this suggests increasing the $NADH/NAD^+$ ratio from multiple substrates could inhibit KB-supported respiration. Furthermore, the addition of malonic acid (succinate dehydrogenase inhibitor) decreased KB-supported respiration to that of pyruvate *(Figure 8D,E)*, suggesting that the increase in respiration observed with KBs in the presence of pyruvate is largely mediated by complex II, particularly as succinate is a product of AcAc catabolism *(Figure 9*; *left panel)*. While these experiments were performed using saturating KBs to detect subtle differences in respiration in the presence of various substrates, the drive with physiological concentrations of KBs (~100 μM) would be minimal in these situations. Altogether, our data suggest that the ability of KBs to support mitochondrial respiration is low, particularly when other substrates are present and cause product inhibition of KB oxidation.

Discussion

In the current study, we report that while KBs were capable of driving mitochondrial respiration *in vitro*, maximal rates of KB-supported respiration were dramatically lower than that of pyruvate in the LV, RG, and human skeletal muscle. Furthermore, given mitochondrial sensitivity to β-HB and AcAc, and KB content representative of the muscle

microenvironment (~100 μM KB), biological rates of KB-supported respiration are predicted to be very low. Moreover, our data show that the addition of other substrates further limited the ability of KBs to drive mitochondrial respiration. Combined, our data indicate that KBs are not a priority substrate in isolated mitochondria and permeabilized muscle fibers, particularly when other substrates are readily available.

Mitochondrial efficiency

KBs have been considered to be more efficient fuel sources compared to other organic substrates (i.e. glucose, pyruvate) (Sato *et al.*, 1995; Veech, 2004; Murray *et al.*, 2016); however, ATP production from carbohydrate-derived glucose (~38 ATP) or glycogen (~39 ATP) is theoretically greater than that of β-HB (~29 ATP) or AcAc (~26 ATP) because of the additional ATP produced anaerobically within glycolysis. Here, we aimed to test intrinsic mitochondrial fuel efficiency by determining P/O ratios, which refer to the amount of ATP produced per oxygen atom reduced by the respiratory chain (Hinkle, 2005), and RCRs, which represent the ability of mitochondria to respond to exogenous ADP. Within the mitochondria (i.e. excluding glycolysis) prior to A-CoA entry into the TCA and ETC, ketolysis produces two reducing equivalents (one NADH from the oxidation of β-HB to AcAc, and one $FADH_2$ from the conversion of AcAc to AcAc-CoA) (Evans *et al.*, 2017), while pyruvate only produces one reducing equivalent (one NADH from the conversion to Ac-CoA; *Figure 9*). The greater number of reducing equivalents from KB catabolism also alters the $NADH/FADH_2$ ratio compared to that of pyruvate. These changes could in theory alter P/O ratios, as while NADH-derived electrons generate a sufficient electrochemical gradient across the inner mitochondrial membrane to produce a P/O ratio of ~2.5, $FADH_2$-derived electrons are less efficient and generally produce a P/O ratio of ~1.5. However, regardless of these differences in the number and source of reducing equivalents, mitochondrial efficiency

(determined by P/O ratios and RCRs) was not different between KBs and pyruvate. Therefore, our results highlight that mitochondria cannot distinguish the source of reducing equivalents, since all metabolic pathways converge with the production of NADH and $FADH_2$ to drive ATP production within the ETC.

Mitochondrial fuel utilization

Mitochondria receive reducing equivalents from two main macronutrients: carbohydrates (i.e. glucose-derived pyruvate) and fatty acids (activated to fatty acyl-CoA). In the present study, our results highlight that although mitochondria can utilize KBs, in isolation the capacity of KBs to produce ATP is several times lower than other carbohydrate-derived substrates such as pyruvate, glutamate, and succinate. Moreover, our kinetic experiments in permeabilized muscle fibers indicate that concentrations of KBs biologically representative of the muscle microenvironment did not stimulate respiration at a meaningful rate. For instance, after an overnight fast, concentrations of circulating KBs in plasma range from ~0.1-0.5 mM, and can reach ~1-5 mM following a ketogenic diet or acute KE supplementation (Burke *et al.*, 2017; Evans *et al.*, 2017; Leckey *et al.*, 2017). Importantly, at rest, KB uptake into skeletal muscle becomes saturated beyond serum levels of ~0.8 mM β-HB (Mikkelsen *et al.*, 2015). While skeletal muscle can take up ~50% of circulating KBs when plasma values range from ~0.1-0.5 mM, this capacity is reduced to only ~5% when circulating KBs reach ~6 mM (Balasse & Féry, 1989; Pinckaers *et al.*, 2017). During exercise, due to greater energetic demands and increased blood flow to working muscles, KB uptake into skeletal muscle may be up to 5-fold higher than at rest (Wahren *et al.*, 1984; Mikkelsen *et al.*, 2015). This is supported by findings in which KE ingestion increased blood β-HB concentrations nearly 2-fold higher at rest than when performing exercise at 75% Wmax, suggesting greater peripheral uptake of ketones during exercise (Cox *et al.*, 2016). It

would therefore be predicted that KB concentrations within the muscle microenvironment range from ~0.05-0.25 mM (~50-250 μM), which may be capable of reaching higher concentrations during exercise in a ketogenic state. As we report here, respiration was half-maximally stimulated by ~800-900 μM β-HB and AcAc in the LV, and ~300-700 μM in the RG, suggesting that intracellular KB concentrations are several fold lower than the apparent K_m. While mitochondrial sensitivity to AcAc was higher in the RG compared to LV, biological estimates of KB-supported respiration are still dramatically lower in the RG since the ability of KBs to maximally support respiration was ~5-fold lower in this tissue. Therefore, our *in vitro* approaches in combination with previous *in vivo* data of KB concentrations and uptake kinetics predict low oxidation rates of KBs in isolated mitochondria and permeabilized fibers from the LV and RG particularly at rest. While previous historical work (Ruderman *et al.*, 1971) has suggested that KBs may be a priority substrate for skeletal muscle, these estimates are largely based on net KB uptake capacity as opposed to direct assessments of oxidation rates. Given the different plasma membrane transport pathways between various substrates (ketones, glucose, and fatty acids), this could explain why other substrates did not outcompete KB uptake in these situations (Ruderman *et al.*, 1971). While these *in vivo* factors of uptake and blood flow nonetheless need to be considered, our results indicate that on a mitochondrial level, it is unlikely that KBs are a priority substrate for ATP production.

Metabolism is regulated by the interaction between substrate availability, enzyme kinetics, and external/product inhibition; resulting in biological flux rates of enzymatic reactions (Hargreaves & Spriet, 2017). Ketolysis occurs through three key steps which could be subject to regulation *(*summarized in *Figure 9)*, being AcAc production by BDH; AcAc-CoA production by succinyl-CoA:3-oxoacid CoA transferase (OXCT); and Ac-CoA production from ACAT. Importantly, BDH and ACAT are near-equilibrium enzymes, while

OXCT is a non-equilibrium reaction; suggesting BDH and ACAT may be more susceptible to product inhibition. The high activity of glycolytic enzymes and greater capacity for PDH flux compared to ketolytic reactions (Baldwin *et al.*, 1973; Winder *et al.*, 1974) suggest that Ac-CoA production from pyruvate would cause product inhibition of Ac-CoA from ACAT *(Figure 9; left panel)*, therefore limiting KB catabolism (Baldwin *et al.*, 1973; Winder *et al.*, 1974). Furthermore, the increase in NADH/NAD^+ ratio within the mitochondrial matrix due to PDH flux would indirectly inhibit BDH, and as a result, limit AcAc production from β-HB. In support, we determined that submaximal β-HB-supported respiration was blunted in the presence compared to absence of 100 μM pyruvate. Feedback inhibition of BDH would not influence subsequent reactions of AcAc catabolism, which could explain why submaximal AcAc-supported respiration was not changed by the presence of 100 μM pyruvate and would suggest BDH is a primary point of product inhibition.

Since OXCT produces succinate (further generating $FADH_2$; *Figure 9; left panel*), we reasoned that the prior addition of submaximal succinate may cause inhibition of AcAc-supported respiration. However, succinate did not decrease the maximal ability of KBs to drive mitochondrial respiration, which could be a result of the non-equilibrium OXCT reaction. When reducing equivalents were further increased with PC (generating both NADH and $FADH_2$ from β-oxidation), respiration supported by both β-HB and AcAc was blunted. This suggests that a combined increase in reducing equivalents from other substrates feeding the ETC, regardless of source (NADH, $FADH_2$), can decrease KB-supported respiration. The subsequent addition of malonic acid (succinate dehydrogenase complex II inhibitor) in these experiments almost fully inhibited the ability of KBs to drive respiration, suggesting KB-supported respiration in the presence of pyruvate is strongly dependent on $FADH_2$/succinate oxidation with minor NADH-mediated contributions. While the addition of succinate did not decrease KB-supported respiration, this is likely because 1) succinate was added in

submaximal concentrations (1500 μM) which did not saturate electron flow to complex II; and 2) OXCT is a non-equilibrium enzyme which is less susceptible to product inhibition. In addition, the presence of malate prevents the production of succinate from α-ketoglutarate, isolating pyruvate oxidation to complex I, and enabling KB oxidation to stimulate respiration through complex II. Biologically, this would not occur, and therefore the present data indicating low rates of KB utilization, likely overestimates the ability of KB to drive respiration in the presence of pyruvate. These results provide further evidence that all reducing equivalents converge at coenzyme Q within the ETC and suggest that KB oxidation is subject to product inhibition of near equilibrium enzymes (BDH, ACAT) by NADH and Ac-CoA at rest *(Figure 9; left panel)*.

During high intensity exercise, it has been shown that skeletal muscle ketone uptake is increased (Mikkelsen *et al.*, 2015) and whole-body estimates of ketone oxidation are increased particularly when KBs are highly available following supplementation (Cox *et al.*, 2016). However, exercise also upregulates numerous cellular processes globally increasing substrate uptake and oxidation of both glucose and fatty acids to meet the intensity-dependent rise in ATP demand (van Loon *et al.*, 2001). It is therefore likely that product inhibition from increased pyruvate availability, PDH flux, and as a result, increased NADH and Ac-CoA within the mitochondria, limits KB utilization to a greater extent in these situations *(Figure 9; right panel).* This notion is supported by our findings that saturating pyruvate prevented the ability of β-HB and AcAc to increase mitochondrial respiration, suggesting higher levels of NADH and Ac-CoA may overcome oxidation of both KBs by product inhibition of BDH and ACAT. Altogether, this would predict a situation of extremely low KB utilization during or following exercise *(Figure 9*; *right panel).* In addition to changes in substrate concentrations, exercise is also capable of altering the kinetic properties of certain enzymes (Hargreaves & Spriet, 2017; Petrick & Holloway, 2019). However, our data show that mitochondrial

sensitivity to KBs or maximal KB-supported respiration is not altered following acute moderate-intensity exercise. Therefore, although it has been shown that acute exercise might alter KB utilization and metabolism (Evans *et al.*, 2017), our results suggest this does not occur *in vitro* on a mitochondrial level.

Limitations and future perspectives

While our findings provide *in vitro* understanding regarding the ability of KBs to support mitochondrial respiration, we acknowledge that our preparation does not account for many other factors present in the *in vivo* environment. While previous literature has examined plasma membrane KB uptake rates (Mikkelsen *et al.*, 2015), the exact concentrations of KBs within the intramuscular environment, and specifically within the mitochondria, remain to be determined. Based on our data, increases in skeletal muscle KB availability representative of acute/chronic supplementation would not dramatically increase rates of KB-supported respiration. Of course, it remains unknown if enzyme kinetics are altered by short- or long-term nutritional interventions, including ketogenic diets, KE ingestion, or conditions of fasting/starvation when other substrates are not available (Owen, 2005). In addition, prolonged exercise training has been suggested to increase the content and activity of ketolytic enzymes within rodent skeletal muscle (Winder *et al.*, 1975; Askew *et al.*, 1975) supported by the notion that ketolytic enzyme activity is higher in type I oxidative muscle fibers than type II glycolytic muscle fibers (Winder *et al.*, 1974). However, while we do not have direct comparisons in our current study between trained and untrained individuals, or direct quantification of muscle fiber composition, our human participants were highly aerobically trained and rates of KB-supported respiration were nonetheless low. It is therefore possible that the increased ability of skeletal muscle to oxidize KBs following chronic exercise is a result of greater mitochondrial content (increased utilization of all substrates) as

opposed to intrinsic mitochondrial changes promoting KB-specific oxidation. In support, ketolytic enzymes are not increased following training in the LV (Winder *et al.*, 1974; Askew *et al.*, 1975), which aligns with a lack of mitochondrial biogenesis following exercise in this tissue (Winder *et al.*, 1974; Li *et al.*, 2011). While it is unlikely that exercise interventions increase the relative ability of mitochondria to oxidize KBs, future studies could nonetheless investigate if various nutritional interventions can change these parameters. In this respect, it is particularly important to examine direct *in vivo* rates of KB uptake/oxidation, as RER measurements are confounded by oxidation of multiple substrates (carbohydrates, fatty acids, AcAc, β-HB) (Shaw *et al.*, 2019*a*) given their converging metabolic pathways *(Figure 9)*.

In addition, we only performed additions of saturating (10 mM) KBs in human skeletal muscle experiments. Previous findings in human myotubes have indicated that prior (24 hr) incubation with low (0.5 mM), but not moderate or high (1.5 mM, 5 mM) concentrations of β-HB is capable of increasing mitochondrial respiration supported by pyruvate (Mey *et al.*, 2020). However, these data are likely representative of other cellular adaptations (i.e. mRNA transcription, mitochondrial biogenesis) occurring during prolonged KB exposure (Mey *et al.*, 2020), as opposed to our findings examining the substrate nature of KBs to directly support mitochondrial O_2 consumption. Regardless, the possibility of chronic mitochondrial adaptations to a high KB environment warrants further investigation.

Conclusion

We report that while KBs can drive respiration in skeletal and cardiac muscle at rest and following exercise, the biological contribution of KBs to metabolism appears to be low *in vitro*. Furthermore, we demonstrate that competition exists between other substrates and KB oxidation, as the presence of carbohydrate- and lipid-derived substrates are capable of limiting KB-supported mitochondrial respiration. Combined, our research provides important

in vitro insight into the proposed ability of ketone bodies to support mitochondrial respiration, with direct implications for understanding mitochondrial ketone utilization in health and performance settings.

Competing interests

The authors declare that they have no competing interests.

Author contributions

HLP, HSB, EAN, LCJvL, and GPH designed the study. HLP, HSB, CP, JFB, and GPH organized and performed experiments. All authors analyzed and interpreted the data. HLP, HSB, and GPH drafted and edited the manuscript, and all authors approved the final version for publication. HLP and HSB contributed equally as first authors and GPH is the guarantor of the study.

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Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figure Legends

Figure 1: KB-supported respiration is low in isolated mitochondria from the LV and RG of rodents. Purity of mitochondria isolated from the LV (A) and RG (B) was confirmed by the absence of cytosolic and plasma membrane proteins in mitochondrial preparations. In the LV and RG, P/O ratios (C,D) and RCRs (E,F) were similar in response to pyruvate and KBs. However, absolute mitochondrial respiration was ~4-fold higher with pyruvate than KBs, both in the absence (state 2) and presence (state 3) of ADP in the LV (G) and RG (H). White bars depict pyruvate, grey bars depict β-HB, and black bars depict AcAc. Individual values are shown in grey circles (n=3-7 per group). Data expressed as mean±SD. *$p<0.05$ compared to pyruvate. Data analyzed using a one-way ANOVA with Tukey's post-hoc test (C-H). AcAc, acetoacetate; β-HB, β-hydroxybutyrate; Cav, caveolin; JO_2, oxygen consumption; LV, left ventricle; RCR, respiratory control ratio; RG, red gastrocnemius; SERCA, sarcoplasmic-endoplasmic reticulum calcium ATPase; VDAC, voltage dependent anion channel; WL, whole muscle lysate.

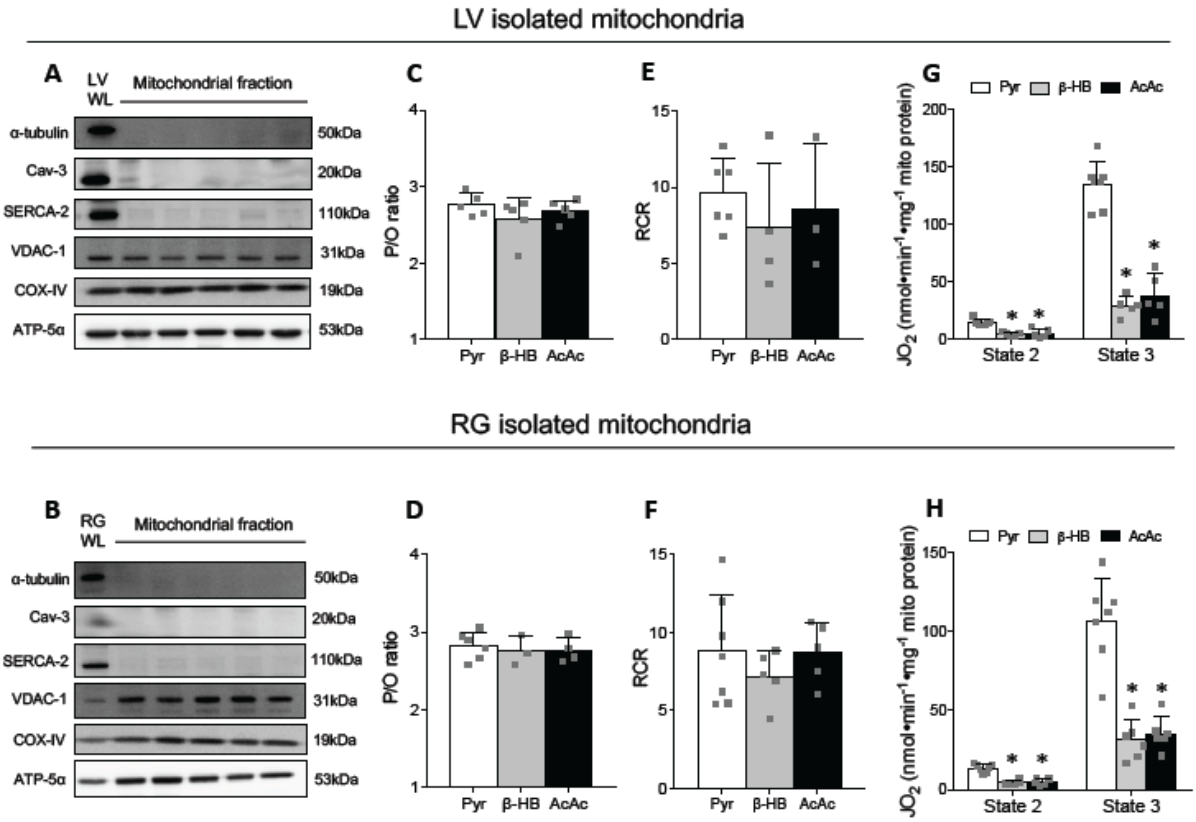


Figure 2: Ketone bodies minimally drive mitochondrial respiration in permeabilized muscle fibers from the LV, RG, and human skeletal muscle. While KBs drove respiration ~30% of pyruvate in the LV (A), KB-supported respiration rates were low in the RG (B) and human skeletal muscle (C). KB titrations were performed to determine mitochondrial sensitivity to β-HB and AcAc in the LV (D) and RG (E), however, due to a minimal drive in respiration with KBs in human skeletal muscle (F), we did not have the resolution to detect KB sensitivity. Grey bars depict β-HB and black bars depict AcAc. Individual values are shown in grey circles (n=4-7 per group). Data expressed as mean±SD. *p<0.05 compared to β-HB. Data analyzed using an unpaired two-tailed Student's *t*-test. AcAc, acetoacetate; β-HB, β-hydroxybutyrate; C, cytochrome C; D, ADP; G, glutamate; JO_2, oxygen consumption; KB, ketone body; LV, left ventricle; M, malate; Pyr, pyruvate; RG, red gastrocnemius; S, succinate; V_{max}, maximal respiratory capacity.

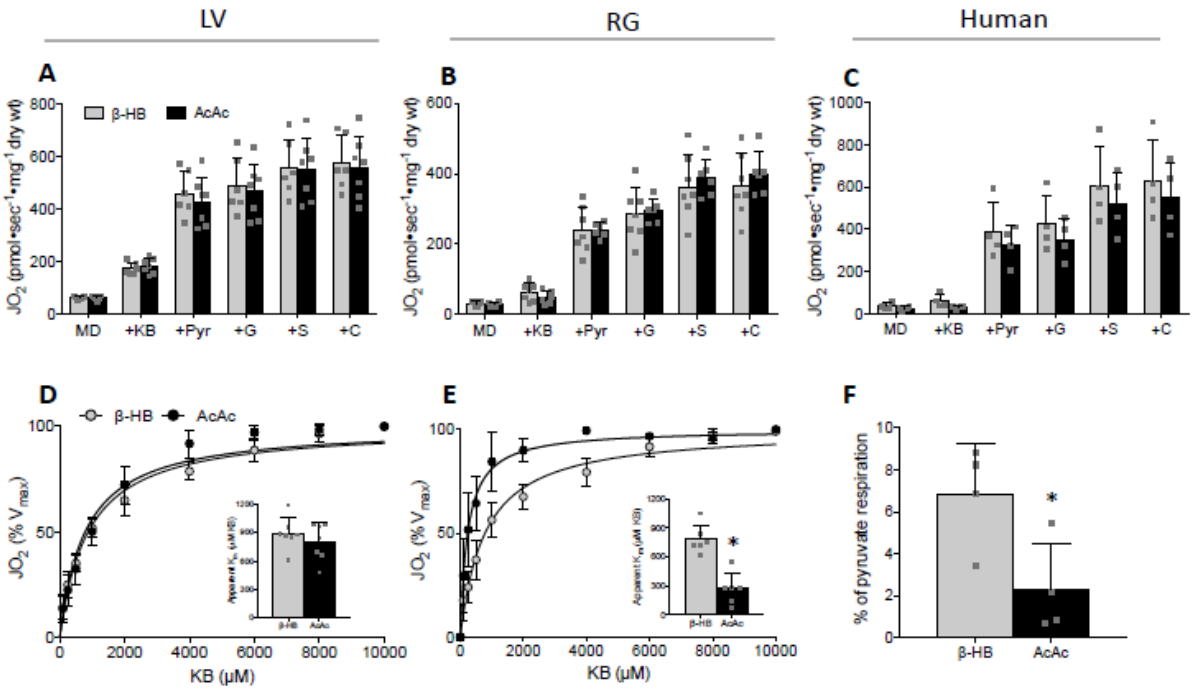


Figure 3: Acute exercise does not alter the ability of KBs to drive mitochondrial respiration. Maximal β-HB- (A,B) and AcAc- (C,D) supported respiration was similar at rest and following an acute bout of treadmill running in both the LV and RG. Furthermore, exercise did not alter mitochondrial sensitivity to KBs (E,F). White bars depict resting states and black bars depict exercise. Individual values are shown in grey circles (n=4-7 per group). Data expressed as mean±SD. Data analyzed using an unpaired two-tailed Student's *t*-test. AcAc, acetoacetate; β-HB, β-hydroxybutyrate; C, cytochrome C; D, ADP; G, glutamate; JO_2, oxygen consumption; KB, ketone body; LV, left ventricle; M, malate; Pyr, pyruvate; RG, red gastrocnemius; S, succinate; V_{max}, maximal respiratory capacity.

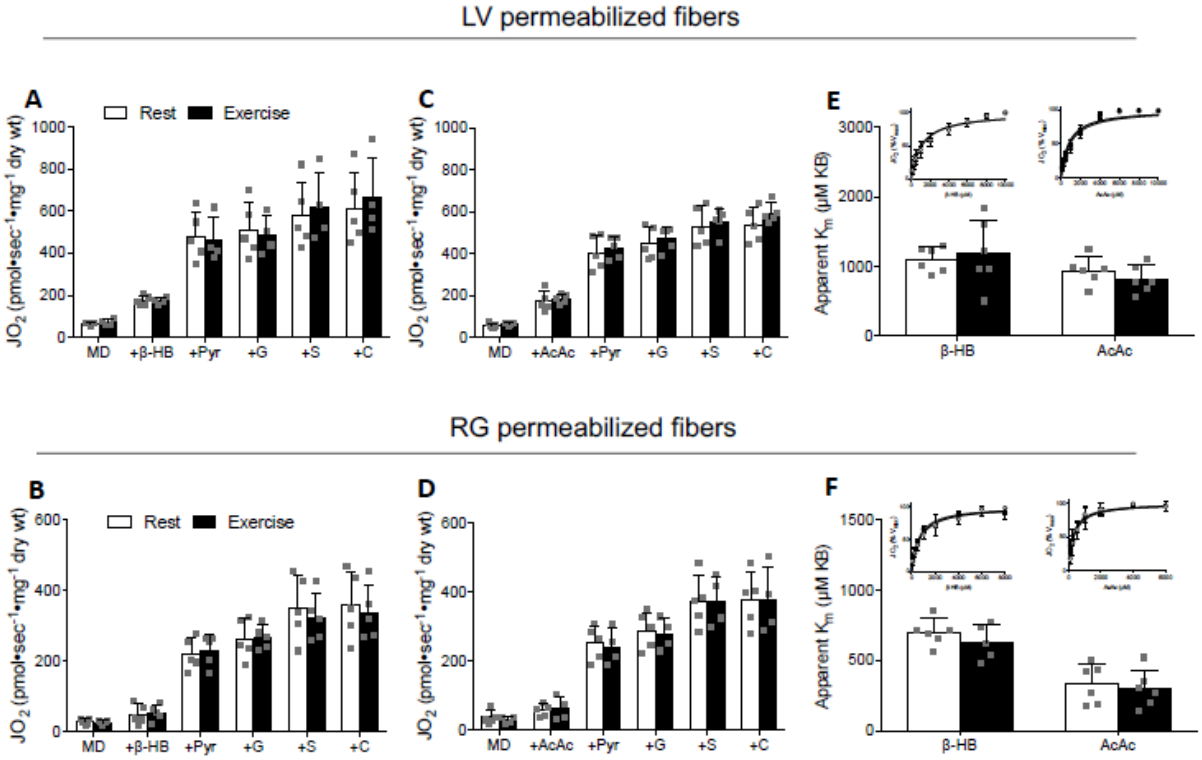


Figure 4: Pyruvate-supported respiration is dramatically higher than that of biological concentrations of KBs in the LV and RG. Based on KB-supported kinetic curves, the biological drive in respiration with β-HB and AcAc (100 μM) would be predicted to be minimal in the LV (A) and RG (B) at rest, particularly when compared to pyruvate. This was also evident following exercise (C,D). White circles depict pyruvate, grey circles depict β-HB, and black circles depict AcAc (n=4-7 per group). Data expressed as mean±SD. AcAc, acetoacetate; β-HB, β-hydroxybutyrate; JO_2, oxygen consumption; KB, ketone body; Pyr, pyruvate.

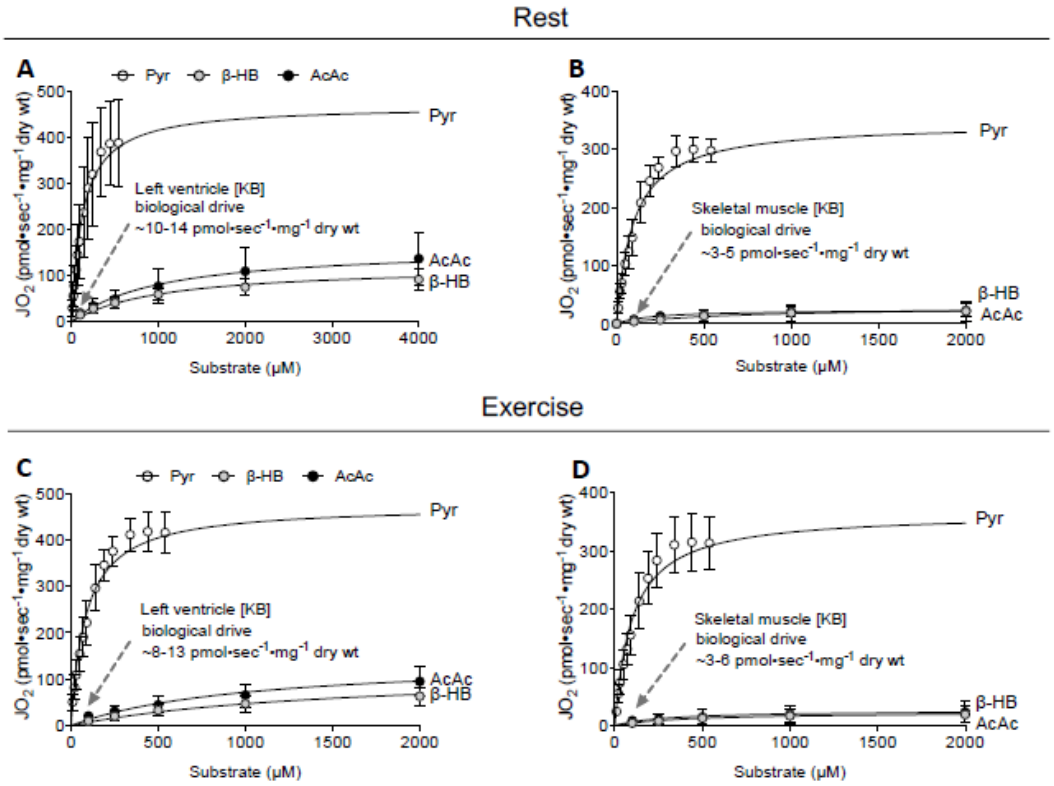


Figure 5: KBs minimally drive respiration following pyruvate, while pyruvate dramatically increased respiration following KBs in mitochondria isolated from the LV and RG. Representative O_2 traces are depicted for the LV (A) and RG (B). When added following pyruvate, β-HB and AcAc minimally drove mitochondrial respiration (C,D), while pyruvate dramatically increased respiration following KBs (E,F). The ability of pyruvate to drive respiration was ~10-fold higher than KBs in the LV (G) and RG (H). Grey bars depict β-HB and black bars depict AcAc. Individual values are shown in grey circles (n=3-6 per group). Slope rates (A,B), depict the absolute change in O_2 consumption over time. Data expressed as mean±SD. Data analyzed using an unpaired two-tailed Student's *t*-test (C-F) or a one-way ANOVA followed by Tukey's post-hoc test (G, H). Main effect of pyruvate ($p<0.05$) compared to KB in the absence of pyruvate (G,H). AcAc, acetoacetate; β-HB, β-hydroxybutyrate; JO_2, oxygen consumption; KB, ketone body; LV, left ventricle; PMD, pyruvate+malate+ADP; Pyr, pyruvate; RG, red gastrocnemius.

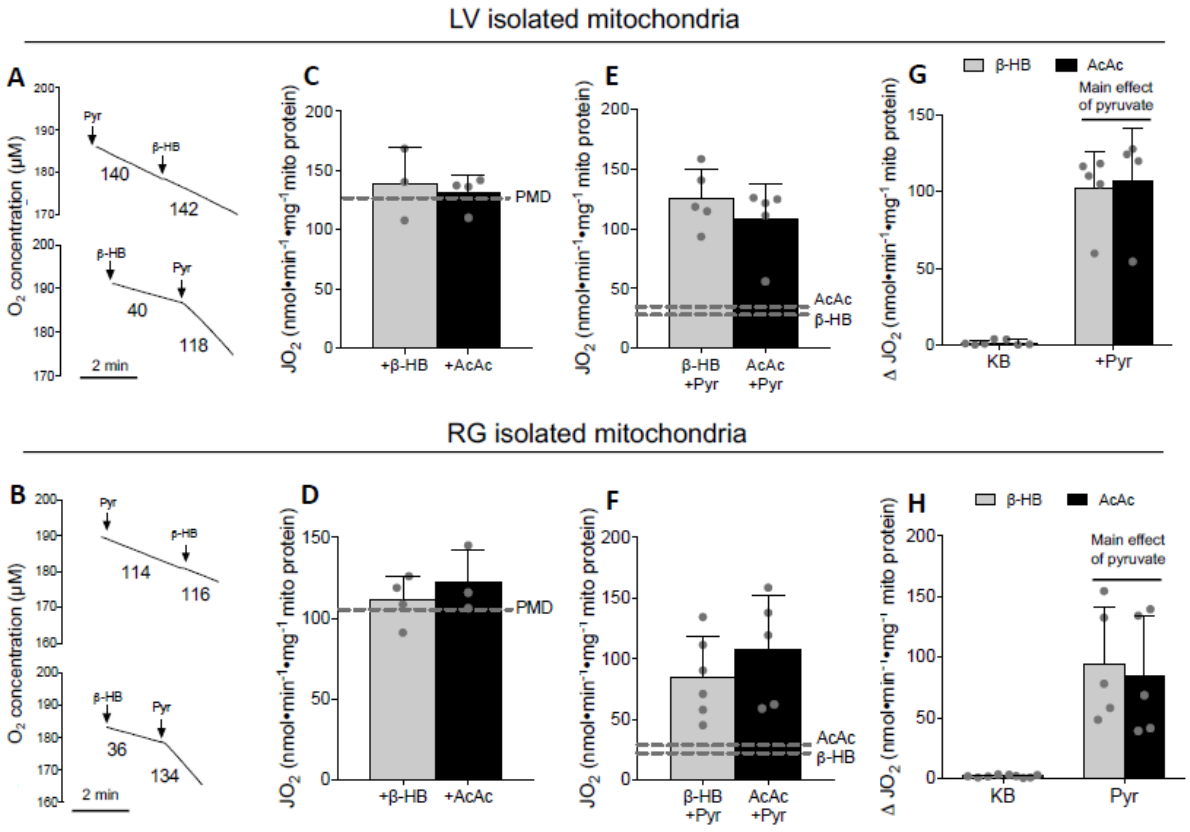


Figure 6: Observational findings in mitochondria isolated from human skeletal muscle. In observational experiments, the addition of pyruvate following β-HB (A) and AcAc (B) dramatically increased respiration, while KBs did not appear to drive respiration following pyruvate (C,D). Insets depict slope rates of the absolute change in O_2 consumption over time after each respective substrate. Grey bars depict β-HB, black bars depict AcAc, and white bars depict respective ketone in the presence of pyruvate. AcAc, acetoacetate; β-HB, β-hydroxybutyrate; M, malate; Pyr, pyruvate; G, glutamate; S, succinate.

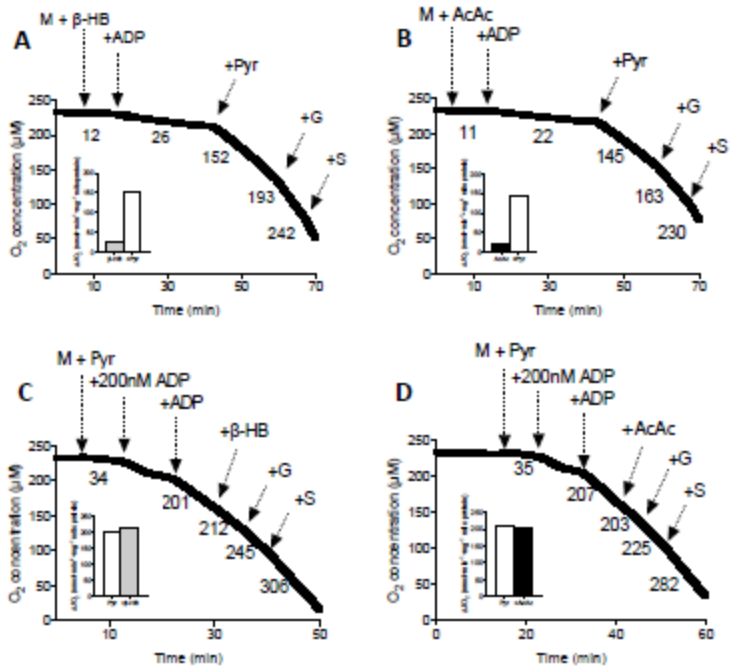


Figure 7: The presence of saturating (5 mM) pyruvate, but not submaximal (100 µM) pyruvate inhibits maximal KB-supported respiration. In permeabilized fibers from the LV (A) and RG (B), KBs were incapable of driving mitochondrial respiration following the addition of saturating pyruvate (5 mM). However, in the presence of submaximal (100 µM) pyruvate, the ability of saturating β-HB or AcAc to support mitochondrial respiration was not altered in LV (C,D), RG (E,F), or human skeletal muscle (G,H). Grey bars depict β-HB, black bars depict AcAc, and white bars depict respective KB in the presence of pyruvate. Individual values are shown in grey circles (n=3-7 per group). Data expressed as mean±SD. *p<0.05 compared to respective KB in the absence of pyruvate (C-H). Data analyzed using a two-way ANOVA followed by Sidak's post-hoc test (A-H). AcAc, acetoacetate; β-HB, β-hydroxybutyrate; JO_2, oxygen consumption; KB, ketone body; LV, left ventricle; Pyr, pyruvate; RG, red gastrocnemius.

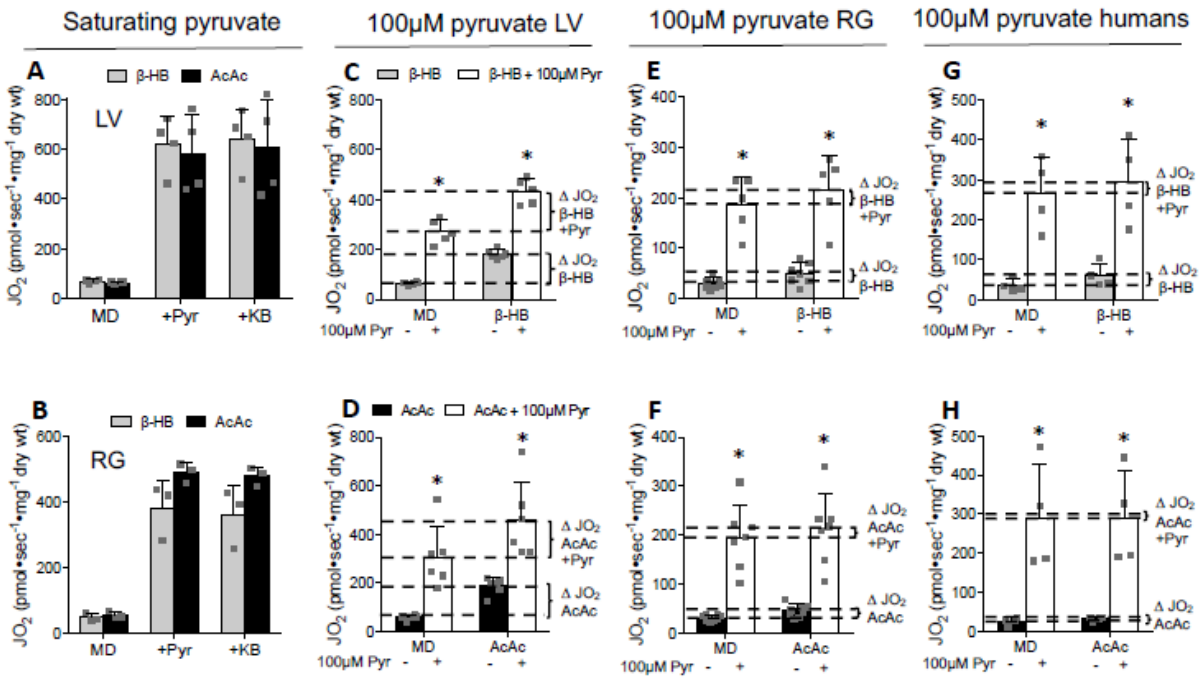


Figure 8: When considering biological concentrations of KBs and the presence of other intracellular substrates in the LV, the increase in respiration with KBs is minimal. The presence of submaximal pyruvate inhibited the ability of 100 μM β-HB (A,B), but not 100 μM AcAc (B,C) to drive mitochondrial respiration in the LV. While submaximal (1500 μM) succinate (in the presence in 100 μM pyruvate) did not inhibit the ability of KBs to maximally drive mitochondrial respiration (D), the addition of 30 μM PC (in the presence of 1500μM succinate + 100 μM pyruvate) decreased KB-supported respiration in the LV (E). The addition of malonic acid almost completely inhibited KB-supported mitochondrial respiration (D,E). Grey bars depict β-HB, black bars depict AcAc, and white bars depict respective KB in the presence of pyruvate. Individual values are shown in grey circles (n=3-7 per group). Data expressed as mean±SD. *$p<0.05$ compared to respective KB in the absence of pyruvate (A,B). *$p<0.05$ with KB compared to previous mixed substrate, #$p<0.05$ with MA compared to previous mixed substrate+KB (D,E). *$p<0.05$ compared to KB in the presence of pyruvate (inset E). Data analyzed using two-way ANOVA followed by Sidak's post-hoc test (A,C); unpaired two-tailed Student's *t*-test (B; inset D,E) or one-way ANOVA followed by Tukey's post-hoc test (D,E). AcAc, acetoacetate; β-HB, β-hydroxybutyrate; D, ADP; JO_2, oxygen consumption; KB, ketone body; LV, left ventricle; M, malate; MA, malonic acid; PC, palmitoylcarnitine; Pyr, pyruvate; RG, red gastrocnemius; S, succinate.

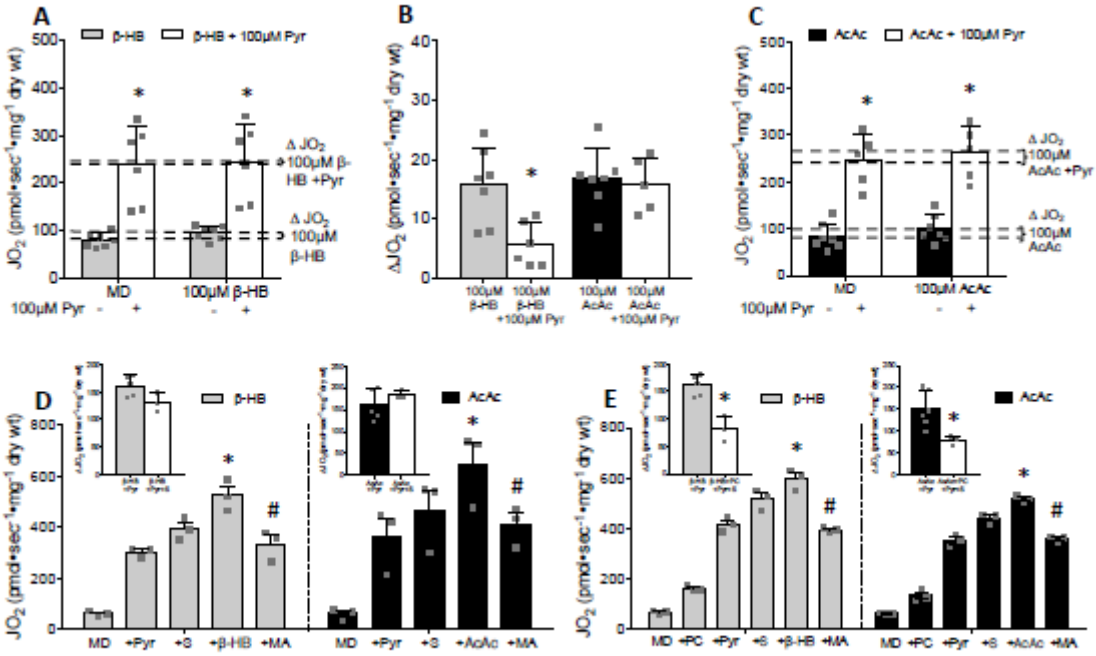
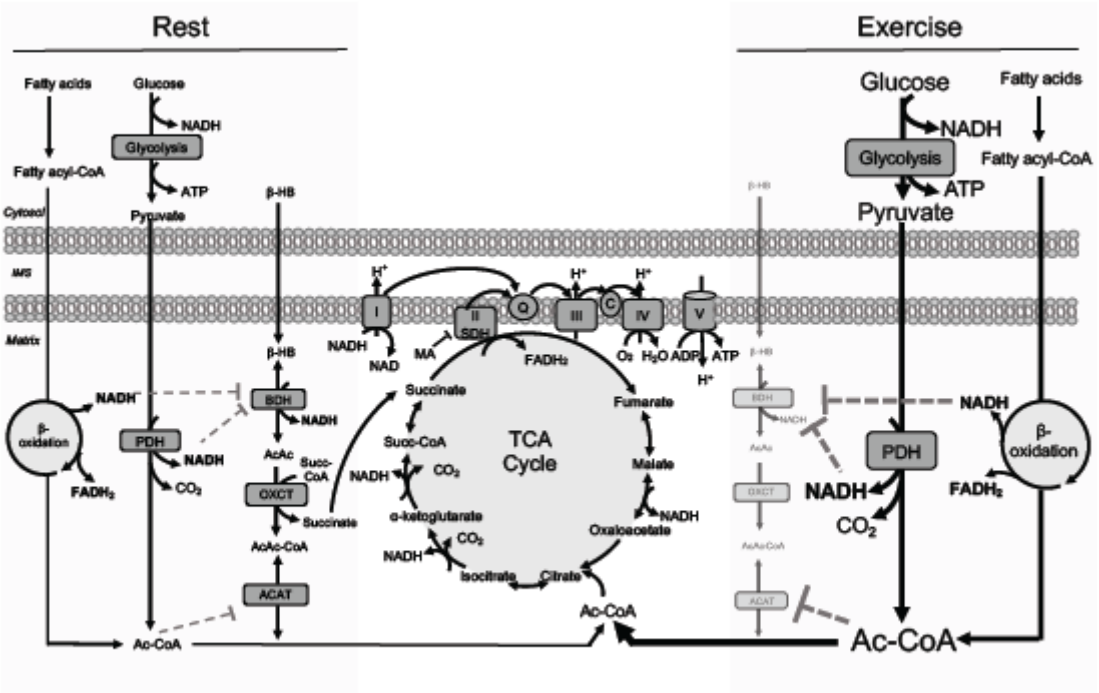


Figure 9: Suggested product inhibition from NADH and Ac-CoA limiting KB oxidation. Pathways involved in KB, glucose, and fatty acid oxidation converge within the mitochondria, suggesting KB oxidation is inhibited at several sites (BDH, ACAT) as other substrates can outcompete KBs. During exercise, when pyruvate is highly available and flux through PDH/ β-oxidation is high, it is likely that KB oxidation is further limited from product inhibition. Dotted lines represent potential product inhibition of KB oxidation. AcAc; acetoacetate; AcAc-CoA; acetoacetyl-CoA; ACAT, Ac-CoA acetyltransferase; Ac-CoA; acetyl-CoA; ADP; adenosine diphosphate; ATP, adenosine triphosphate; BDH, β-hydroxybutyrate dehydrogenase; β-HB, β-hydroxybutyrate; CO_2, carbon dioxide; IMM, inner mitochondrial membrane; IMS, inter-membrane space; O_2, oxygen; OXCT, succinyl-CoA:3-oxoacid CoA transferase; OMM, outer mitochondrial membrane; PDH, pyruvate dehydrogenase; SDH, succinate dehydrogenase; Succ-CoA, succinyl-CoA; TCA, tricarboxylic acid cycle.



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