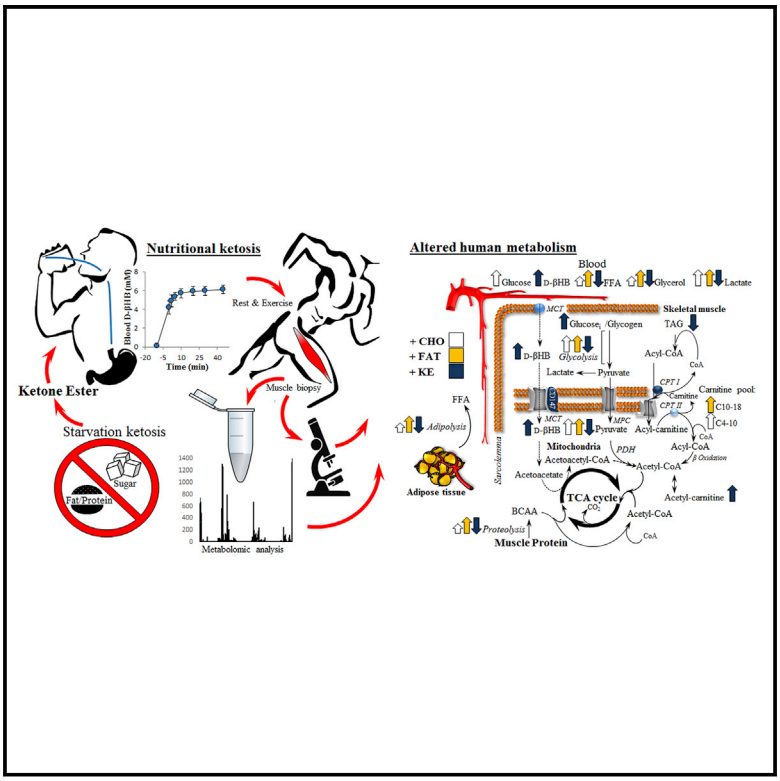
Clinical and Translational Report

Cell Metabolism

Nutritional Ketosis Alters Fuel Preference and Thereby Endurance Performance in Athletes

Graphical Abstract



Highlights

- Nutritional ketone bodies can promote the advantageous aspects to starvation ketosis
- Nutritional ketosis alters the hierarchy of substrate competition for respiration in exercise
- Ketosis increases metabolic flexibility during exercise, reducing glycolysis and increasing muscle fat oxidation
- Improved performance during cycling time trial suggests ketosis during exercise may be beneficial for some athletes

Authors

Pete J. Cox, Tom Kirk, Tom Ashmore, ..., Richard L. Veech, Julian L. Griffin, Kieran Clarke

Correspondence

petejcox456@gmail.com

In Brief

Cox et al. show the metabolic benefit of ketone metabolism through the administration of a ketone ester-based drink to athletes during exercise. The physiological alterations achieved by acute nutritional ketosis may improve human physical performance in some athletes as indicated by initial endurance test results.

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Nutritional Ketosis Alters Fuel Preference and Thereby Endurance Performance in Athletes

Pete J. Cox,[1,2,*] Tom Kirk,[1] Tom Ashmore,[3] Kristof Willerton,[1] Rhys Evans,[1] Alan Smith,[4] Andrew J. Murray,[5] Brianna Stubbs,[1] James West,[3] Stewart W. McLure,[1] M. Todd King,[6] Michael S. Dodd,[1] Cameron Holloway,[1,2] Stefan Neubauer,[2] Scott Drawer,[4] Richard L. Veech,[6] Julian L. Griffin,[3] and Kieran Clarke[1]
[1]Department of Physiology, Anatomy and Genetics, University of Oxford, Oxford OX1 3PT, UK
[2]Department of Cardiovascular Medicine, University of Oxford, Oxford OX3 9DU, UK
[3]Department of Biochemistry & Cambridge Systems Biology Centre, University of Cambridge & MRC Human Nutrition Research, Cambridge CB1 9NL, UK
[4]UK Sport, 40 Bernard Street, London WC1N 1ST, UK
[5]Department of Physiology, Development and Neuroscience, University of Cambridge, Cambridge CB2 3EG, UK
[6]Laboratory of Metabolic Control, NIAAA/NIH, Rockville, MD 20852, USA
*Correspondence: petejcox456@gmail.com
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SUMMARY

Ketosis, the metabolic response to energy crisis, is a mechanism to sustain life by altering oxidative fuel selection. Often overlooked for its metabolic potential, ketosis is poorly understood outside of starvation or diabetic crisis. Thus, we studied the biochemical advantages of ketosis in humans using a ketone ester-based form of nutrition without the unwanted milieu of endogenous ketone body production by caloric or carbohydrate restriction. In five separate studies of 39 high-performance athletes, we show how this unique metabolic state improves physical endurance by altering fuel competition for oxidative respiration. Ketosis decreased muscle glycolysis and plasma lactate concentrations, while providing an alternative substrate for oxidative phosphorylation. Ketosis increased intramuscular triacylglycerol oxidation during exercise, even in the presence of normal muscle glycogen, co-ingested carbohydrate and elevated insulin. These findings may hold clues to greater human potential and a better understanding of fuel metabolism in health and disease.

INTRODUCTION

Ketone body metabolism is a survival trait conserved in higher organisms to prolong life during an energy deficit or metabolic crisis. The advantages of ketone body metabolism during starvation are clear; providing an oxidizable carbon source to conserve precious glucose/gluconeogenic reserves while simultaneously satisfying the specific fuel demands of the brain. Ketone bodies, when present, act not only as respiratory fuels to power oxidative phosphorylation but as signals regulating the preferential oxidation and mobilization of fuel substrates (Robinson and Williamson, 1980). The conservation of CHO reserves in the form of glycogen and gluconeogenic skeletal muscle protein is a hallmark of starvation induced ketosis (Cahill, 1970), dramatically increasing survival duration (Cahill and Owen, 1968; Felig et al., 1969). Ketosis may also provide thermodynamic advantages over other carbon substrates by increasing the free energy conserved in ATP (ΔG_{ATP}) by the oxidation of ketones during mitochondrial oxidative phosphorylation (Sato et al., 1995). The combination of improved energetic efficiency and fuel sparing is vitally important not only during famine, but could also provide clues to new methods of sustaining human performance, or restoring dysregulated substrate metabolism.

Produced continuously under normal physiological conditions, a significant increase in the ketone bodies, D-β-hydroxybutyrate (D-βHB) and acetoacetate (AcAc), rarely manifests in concentrations above 1 mM (Robinson and Williamson 1980). However, the production of ketone bodies increases rapidly in response to calorie deprivation or energy deficit such as starvation, prolonged exercise, and as part of the clinical manifestations of diseases, such as uncontrolled diabetes (Robinson and Williamson, 1980).

As a fuel source, ketone bodies are readily oxidized by most body tissues (Robinson and Williamson, 1980), the major exception being the liver due to its lack of the enzyme succinyl-CoA:3-ketoacid CoA transferase, which permits oxidative disposal of ketones in the TCA cycle. The favorable thermodynamic characteristics of ketone body oxidation and their regulatory role controlling the preferential use and release of other substrates, such as fat (FAT) and glucose, may also have therapeutic utility for the treatment of disease (Veech, 2004; Keene, 2006).

Achieving ketosis by feeding D-βHB in an acid or salt form is not advisable due to the accompanying acid/salt load. To circumvent this, and the unwanted dietary restriction of adhering to a ketogenic diet, we generated an edible form of a ketone body by transesterifying ethyl *(R)*-3-hydroxybutyrate with *(R)*-1,3-butanediol using lipase (Figure 1; Table S1). Previously we have shown the nutritional ingestion of this *(R)*-3-hydroxybutyl *(R)*-3-hydroxybutyrate ketone ester (KE) is a safe and effective way of elevating blood ketone levels (Clarke et al., 2012; Shivva et al., 2016) and provides a means of investigating human ketone metabolism independent of caloric or CHO deficit.

In some ways, the metabolic demands of prolonged exercise parallel (albeit on a more rapid scale) the metabolic conditions important to survival in starvation; it being well known that

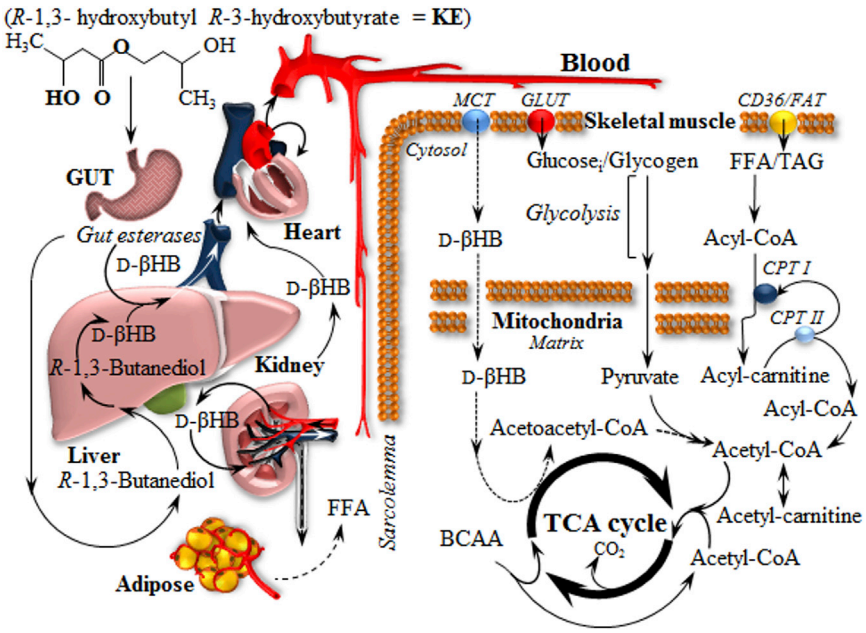

Figure 1. Proposed Ketone Ester Metabolism

Ingested KE is hydrolysed in the small intestine by nonspecific gut esterases (Van Gelder et al., 2000), which cleave it into D-β-hydroxybutyrate (D-βHB) and (*R*)-1,3-butanediol. Both metabolites are absorbed into the portal circulation, with butanediol undergoing first-pass metabolism in the liver to form D-βHB (Desrochers et al., 1992). D-βHB is subsequently released into the circulating blood, to be transported into muscle cytosol and mitochondria via the monocarboxylate transporters (MCTs) (Halestrap and Meredith 2004). Glucose is transported across the sarcolemma by GLUTs, and FFAs are transported by the FAT/CD36 transporters. Once inside the mitochondrial matrix, all substrates are metabolized to acetyl-CoA and oxidized in the TCA cycle.

skeletal muscle fuel selection shifts as exercise intensity rises, placing a premium on CHO reserves, resulting in an almost exclusive reliance on glycogen and blood glucose for its energy requirements (Romijn et al., 1993; van Loon et al., 2001). We reasoned that the combination of improved energetic efficiency and fuel sparing induced by ketosis is vitally important not just in famine, and that harnessing the metabolic actions of ketosis in nutritional form may provide a method of sustaining human physical performance (Cox and Clarke 2014).

Therefore, we sought to determine the mechanisms governing skeletal muscle substrate metabolism during acute nutritional ketosis in exercising humans, as well as their effects on endurance performance in this unique metabolic state.

RESULTS

Exercise Intensity Alters the Metabolism of Nutritional Ketosis (Study 1)

To determine whether exercise intensity altered the metabolism of diet-derived ketones, we examined the effects of steady-state exercise on the clearance of blood and urinary D-βHB in six male endurance athletes (Table S2A). An identical amount of KE was consumed by athletes at rest, and during 45 min of cycling exercise (40% and 75% of W_{Max}) in a randomized crossover design (Figure 2A). Ingestion of a drink containing 573 mg/kg body weight of KE resulted in a rapid rise in circulating D-βHB from overnight fasted levels (0.1 mM) to ~3 mM after 10 min of rest. After the onset of exercise, D-βHB concentrations were divergent, reaching new steady-state concentrations after approximately 10 min, with high-intensity (75% W_{Max}) exercise reducing D-βHB concentrations by 1.05 ± 0.2 mM compared to workloads of 40% W_{Max}, and by 3.1 ± 0.4 mM compared with resting conditions (Figure 2B). D-βHB area under curve (AUC) during 45 min of rest or exercise was significantly decreased with increasing exercise intensity (Figure 2C) and correlated closely with increasing oxygen consumption (Figure 2D). Indirect calorimetry equations were adjusted for ketone oxidation (Frayn, 1983) (Supplemental Experimental Procedures) and used to calculate relative contributions of each substrate to total oxygen consumption during exercise at 40% and 75% W_{Max} (Figure 2E). D-βHB oxidation was estimated to account for 16%–18% of total oxygen consumption during exercise.

Estimated D-βHB oxidation during steady state exercise increased from 0.35 g/min at 40% W_{Max} to ~0.5 g/min at 75% intensity (Figure 2F). Urinary elimination of D-βHB during exercise was negligible, ranging from 0.05 to 0.3 g (~0.2% of total ingested KE) over the entirety of the protocol, although it correlated positively with D-βHB AUC (Figure 2G).

The Metabolic Effects of Nutritional Substrate Alteration during Exercise (Study 2)

Each athlete (n = 10, Table S2B) completed three experimental trials consisting of 1 hr of constant load cycling at 75% of W_{Max} in a randomized, single-blind, cross-over design (Figure 3A). Isocaloric drinks contained a minimum of 96% of their calories from the one substrate (Figure 3A; Supplemental information). Subjects ingested 573 mg/kg BW of KE, isocaloric CHO, or FAT 15 min prior to the start of exercise, and 191 mg/kg BW KE 45 min into each 1 hr trial. Resting blood ketone body kinetic profiles, using an identical protocol, were determined on a separate (non-exercising) study day.

Ingestion of a drink containing 573 mg/kg body weight of KE resulted in a rapid rise in circulating D-βHB from overnight fasted levels of 0.13 ± 0.1 mM to 3.5 ± 0.3 mM during 10 min of rest, where they remained throughout 1 hr of exercise (Figure 3B). When no exercise was performed, plasma D-βHB concentrations increased to >5 mM.

Lactate concentrations were the same at baseline for all conditions (Figure 3C). However, after the onset of exercise, blood lactate concentrations were significantly lower on KE, resulting in average exercise lactate concentrations ~2–3 mM (~50%) lower than CHO, and lower than FAT at 30 and 45 min.

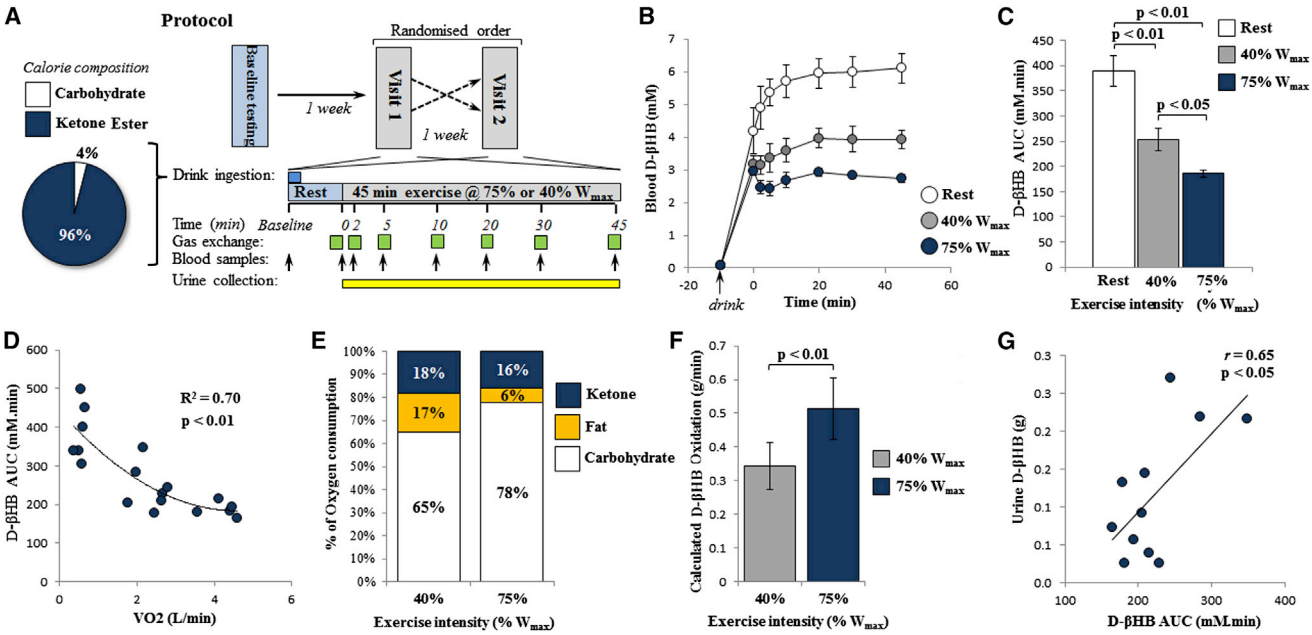

Figure 2. Effects of Exercise on D-βHB Metabolism (Study 1)

Incremental exercise intensity increases the clearance of blood ketosis (lowering blood ketone concentrations) following the consumption of identical quantities of KE.

(A) Study protocol and interventions.

(B) D-βHB concentrations at rest and exercise at 40% and 75% W_{Max}.

(C) D-βHB AUC during rest and exercise at 40% and 75% W_{Max}.

(D) D-βHB AUC versus oxygen consumption.

(E) Calculated contribution of D-βHB oxidation to total O_2 consumption in exercise.

(F) Calculated oxidation of D-βHB (g/min) at 40% and 75% W_{Max}.

(G) D-βHB AUC versus urinary elimination during exercise.

All data are means ± SEM.

FFA concentrations were significantly higher at baseline on FAT after 24 hr of high-FAT low-CHO meals (Figure 3D), remaining elevated throughout exercise compared with CHO or KE, reaching 0.85 mM at the end of exercise. FFA concentrations were lower than FAT at baseline before and fell after CHO or KE ingestion. Ketosis suppressed the rise in FFA seen after 25 min of exercise compared with FAT and, to a lesser extent, CHO. Exercise caused significant increases in plasma glycerol following both CHO and FAT ingestion (Figure 3E), but not after KE.

Plasma glucose concentrations were similar for all athletes at baseline but increased significantly after consuming CHO (Figure 3F). Glucose fell during the first 10 min of exercise after CHO or KE and was significantly lower after KE than either FAT or CHO intake within 5 min of exercise, remaining lower than FAT for much of the exercise protocol.

Plasma insulin concentrations were significantly elevated following CHO compared with FAT and KE (Figure 3G). Insulin concentrations peaked 10 min after the CHO drink and fell to baseline levels after 25 min of exercise. There were no significant differences in insulin after FAT and KE intake. Gas exchange (RER) was higher on CHO, with values consistently close to unity on all arms (Table S3). There were no significant differences between FAT and KE.

Ketosis Altered Skeletal Muscle Metabolism at Rest and during Exercise

D-βHB and other metabolites were measured in skeletal muscle biopsies before and after bicycle ergometer exercise (Supplemental Information). At rest, after KE intake, intramuscular concentrations of D-βHB were ~3-fold higher than after the ingestion of CHO or FAT (Figure 4A) and remained double the concentrations following either FAT or CHO after 1 hr of exercise. Intramuscular glucose was increased pre-exercise following CHO versus FAT and KE, but was significantly greater at the end of exercise on KE (Figure 4B). Pre-exercise muscle concentrations of the glycolytic intermediates, glyceraldehyde-3-phosphate, 2&3-phosphoglycerate, and pyruvate, were significantly lower following KE consumption compared with CHO and FAT. Fructose-1,6-bisphosphate and 1,3-bisphosphoglycerate were similar at rest in all subjects (Figures 4C–4G and S1).

Following exercise, concentrations of all measured muscle glycolytic intermediates were significantly lower after KE versus CHO and FAT. The sum of glycolytic intermediates also decreased proportionately with increased intramuscular D-βHB concentration (Figure 4H). Taken together, these findings suggest that ketosis suppressed skeletal muscle glycolysis, explaining the lower blood lactate concentration described previously. Glycolytic intermediates were not different following FAT and CHO.

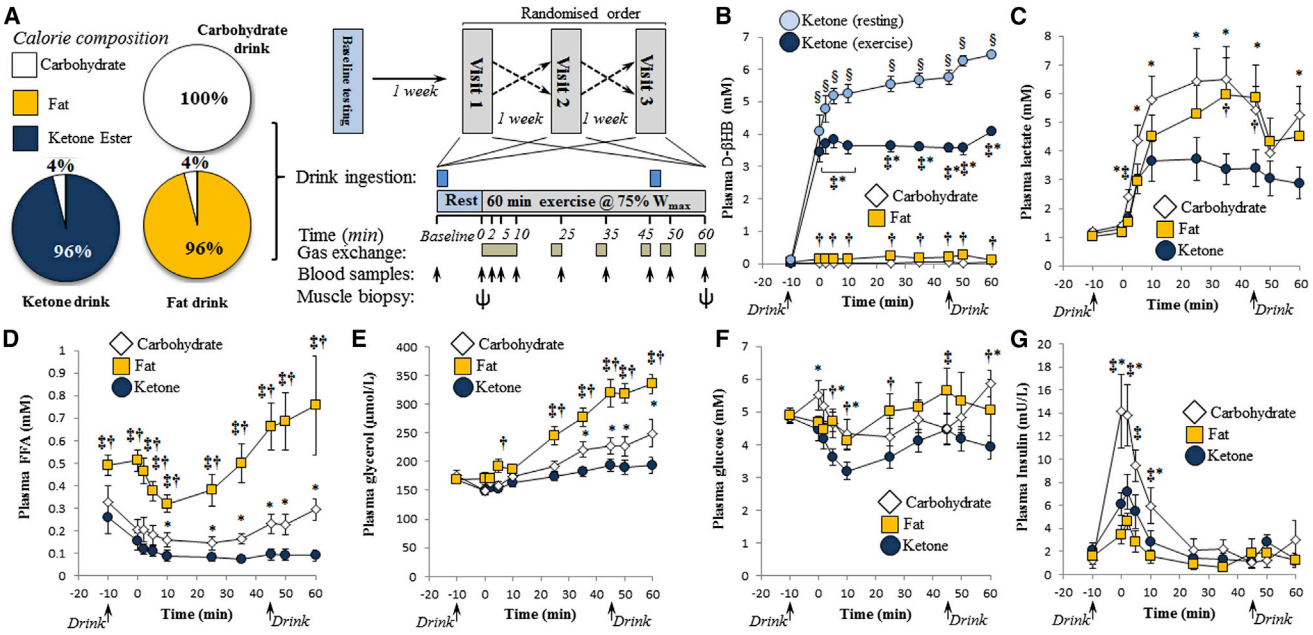

Figure 3. Effects of Dietary Substrates on Plasma Metabolites during Exercise (Study 2)

Acute nutritional substrate provision before exercise resulted in significant alterations in circulating metabolite concentrations, with KE ingestion increasing blood ketone levels, while reducing blood lactate, and circulating fats.

(A) Study protocol and interventions.

(B) D-βHB concentrations.

(C) Plasma lactate concentrations.

(D) Plasma FFA concentrations.

(E) Plasma glycerol concentrations.

(F) Plasma glucose concentrations.

(G) Plasma insulin concentrations.

All data are means ± SEM. $^{\S}p < 0.05$ exercise versus resting, †$p < 0.05$ KE versus FAT, *$p < 0.05$ KE versus CHO, ‡$p < 0.05$ CHO versus FAT.

Drink ingestion did not change free carnitine and acyl-carnitine concentrations before exercise, but after 1 hr of high-intensity exercise, free carnitine was lower and acetyl- and short-chain C3-carnitines were higher following KE versus CHO and FAT (Figures 4I and S1), with a positive relationship between acetyl-carnitine/free carnitine ratio and D-βHB (Figure 4J). C8 and C10 carnitine derivatives were higher following the CHO drinks, whereas C16 and C18 longer chain acyl-carnitines were increased following FAT intake (Figures 4K, 4L, and S1). The pool of TCA intermediates remained largely unaffected by substrate provision, both at rest and following exercise, albeit expanding ~2-fold with exercise. With the exception of increased oxaloacetate concentrations following FAT, and lower malate concentrations after CHO (Figures 4M and 4N), TCA metabolites were unchanged by the type of nutritional substrate (Figure S1).

Branched-chain amino acids (BCAAs), leucine, isoleucine, and valine, are mobilized during exercise as muscle energetic and anaplerotic demands increase (van Hall et al., 1995). At rest, skeletal muscle BCAAs were significantly higher after FAT than CHO or KE (Figure S2A). During exercise, leucine + isoleucine increased, but were 50% lower following the ketone drink than CHO or FAT. The exercise-induced demand for anaplerotic substrates was reflected in the strong positive relationship between muscle leucine + isoleucine and muscle pyruvate (Figure S2B). Reducing glycolytic demand during exercise by increasing intramuscular D-βHB proportionately decreased leucine + isoleucine, and pyruvate (Figures S2C and S2D).

The Effects of Synergistic CHO and Ketone Delivery on Human Substrate Metabolism (Study 3)

The provision of CHO with high ketone levels would never usually co-exist with an intact insulin axis and is unique to this form of ketosis. In order to determine the metabolic effects of synergistic nutritional provision of KE and CHO during exercise, each athlete (n = 8, Table S2C) completed three experimental trials consisting of 1 hr of constant load cycling at 75% W_{Max} in a randomized, single-blind, cross-over design (Figure S3A). Alterations in plasma metabolites were highly reproducible, with ingestion of KE increasing D-βHB levels versus CHO and B3 (Figure S3B) similar to Studies 1 and 2. To mimic the effects of ketone agonism of the nicotinic acid receptor (Taggart et al., 2005), but without the oxidizable carbon source, nicotinic acid (B3) was ingested as a control. Blood lactate concentrations were significantly decreased during exercise after KE+CHO versus CHO and B3 (Figure S3C), with no differences observed between the latter. Plasma FFA concentration fell on all arms after administration of study drinks or B3. During exercise on CHO, FFA concentration rose in identical fashion to Study 2, significantly higher than KE+CHO or B3 (Figure S3D) after ~30 min, as would be expected.

Plasma glucose remained virtually unaltered by vitamin B3 consumption; however, CHO and KE+CHO conditions resulted in transient decreases in plasma glucose on initiation of exercise, which returned to pre-exercising concentrations after 35–45 min (Figure S3E). Alterations in plasma glucose can be explained by the increases in plasma insulin following CHO-containing drinks on KE+CHO and CHO (Figure S3F). No changes in plasma insulin were observed after B3 ingestion, which remained low throughout exercise. No differences in plasma insulin concentration were observed between KE+CHO and CHO conditions. Gas exchange (RER) was similar between all three arms, with values consistently around unity (Table S4).

Synergistic Substrate Delivery Alters Human Skeletal Muscle Metabolism

At rest, following KE+CHO ingestion, intramuscular concentrations of D-βHB were ~7-fold higher than after the ingestion of CHO or vitamin B3 (Figure 5A), and >5-fold at the end of exercise. Consumption of drinks containing CHO resulted in significant increases in intramuscular total hexose (CHO) concentration at rest. However, following 1 hr of exercise at 75% W_{Max}, hexose concentrations were significantly higher on KE+CHO versus CHO or B3 reflecting preserved intramuscular CHO stores (Figure 5B). Average plasma lactate concentration during exercise negatively correlated with end exercise intramuscular hexose (Figure 5C), while intramuscular hexose concentrations at the end of exercise correlated positively with free carnitine (Figure 5D). Intramuscular glutamine concentrations were increased on KE+CHO versus B3 and CHO (Figure 5E). No correlation was found between blood D-βHB and intramuscular D-βHB (Figure S4A), in keeping with selective trans-sarcolemmal transport by monocarboxylate transporters (MCT) (Halestrap and Meredith, 2004). A strongly positive correlation ($r = 0.72$, $p < 0.05$) was found between intramuscular D-βHB concentration and intramuscular hexose at the end of exercise on KE+CHO (Figure S4B).

Alterations in Carnitine Metabolism

Free carnitine concentrations were elevated on KE+CHO versus B3 at rest and significantly greater than both CHO and B3 after exercise at 75% W_{Max} for 60 min (Figure 5F). Acetyl-carnitine/free carnitine ratio was elevated on KE+CHO versus CHO or B3 at rest, likely reflecting alterations in acetyl-CoA/CoA ratio. After exercise, however, the reverse was observed with a pronounced increase in ratio on CHO and B3, but not on KE+CHO, where a decrease occurred (Figure 5G). Commensurate with these changes, an increase in acetyl-carnitine was observed on KE+CHO at rest versus CHO and B3. However, after exercise no differences in acetyl-carnitine were observed between nutritional conditions (Figure 5H). Considerable increases in C_4-OH carnitine ("keto-carnitine") levels were observed following KE+CHO both at rest and after exercise (Figure 5I), likely reflecting buffered intra-mitochondrial ketone, and a strongly positive relationship ($r = 0.93$, $p < 0.01$) was observed between C_4-OH-carnitine concentration and acetyl-carnitine on KE+CHO (Figure 5J).

The Effect of Nutritional Ketosis on Intramuscular Fat and Glycogen Stores in Prolonged Exercise (Study 4)

Having demonstrated the actions of acute nutritional ketosis on skeletal muscle energy metabolism, we sought to determine whether these changes resulted in altered intramuscular stores of FAT and glycogen during prolonged (2 hr) exercise (Figure 6A).

Alterations in plasma metabolites were highly reproducible between study participants (n = 7, Table S2D) and comparable with those in Studies 1–3. Ingestion of ketone ester increased D-βHB levels from 0.1 mM after an overnight fast to 2.2 mM ($p < 0.01$) following KE+CHO ingestion (Figure 6B). Blood D-βHB concentration continued to slowly increase throughout exercise with regular ingestion of drinks, reaching 3.2 ± 0.2 mM after 2 hr of exercise. Similar profiles in blood AcAc were observed (Figure S5A). D-βHB concentration remained unchanged on CHO throughout exercise (0.1 ± 0.05 mM, $p < 0.01$ versus KE+CHO). Blood lactate concentrations were significantly decreased during exercise on KE+CHO versus CHO (Figure 6C). Plasma glucose concentrations were, on average ~1–2 mM higher on CHO following ingestion of high-CHO-containing drinks (Figure 6D). Plasma FFA concentration fell progressively on KE+CHO over the course of the study. Similar alterations were observed on CHO at rest. However, during exercise, FFA concentration was significantly higher than KE+CHO after 2 hr. (Figure 6E). No significant differences were observed in plasma insulin or cortisol (Figures S5B and S5C). In contrast to the previous studies involving shorter and higher intensity exercise, respiratory exchange ratios were consistently lower for much of the 2 hr exercise duration on KE+CHO studies versus CHO (Table S5) suggesting greater lipid oxidation.

Intramuscular triacylglycerol (IMTG) content was not significantly different between nutritional conditions at baseline. However, after 2 hr of exercise at 70% $VO_{2\ Max}$, intramuscular lipids fell by 24% during KE+CHO, but only 1% on CHO ($p < 0.01$) (Figure 6F). Intramuscular glycogen content was not significantly different between nutritional conditions, with all athletes demonstrating a high level (dark staining) of intramuscular glycogen before exercise (Figure 6G). As expected after 2 hr of exercise, glycogen concentrations fell on both arms with reductions in dark PAS staining and proportionate increases in moderate and light staining intensities. The degree of change was most marked on CHO, where significantly more glycogen deposits appeared moderate or light, or were no longer visible versus KE+CHO ($p < 0.05$).

The Effect of Nutritional Ketosis on Endurance Exercise Performance (Study 5)

Finally, to determine whether exercise performance could be altered by the metabolic changes arising from nutritional provision of CHO and KE, we examined the effects of steady-state exercise and time trial performance in (n = 8) highly trained endurance athletes (Table S2E). Following an overnight fast, study participants completed two blinded bicycle exercise trials consisting of 1 hr steady-state workload at 75% W_{Max} followed by a blinded 30 min time trial (TT) for maximum distance (Figure 7A; Supplemental Information). Ingestion of a drink containing 573 mg/kg body weight of KE resulted in a rapid rise in circulating D-βHB from overnight fasted levels to ~2 mM after 20 min. Ketone concentrations remained elevated throughout subsequent exercise with a fall in concentration on initiation of exercise at 75% W_{Max} workload, after which blood concentration rose reaching a new approximate steady state after 30 min, where they remained for the rest of the protocol.

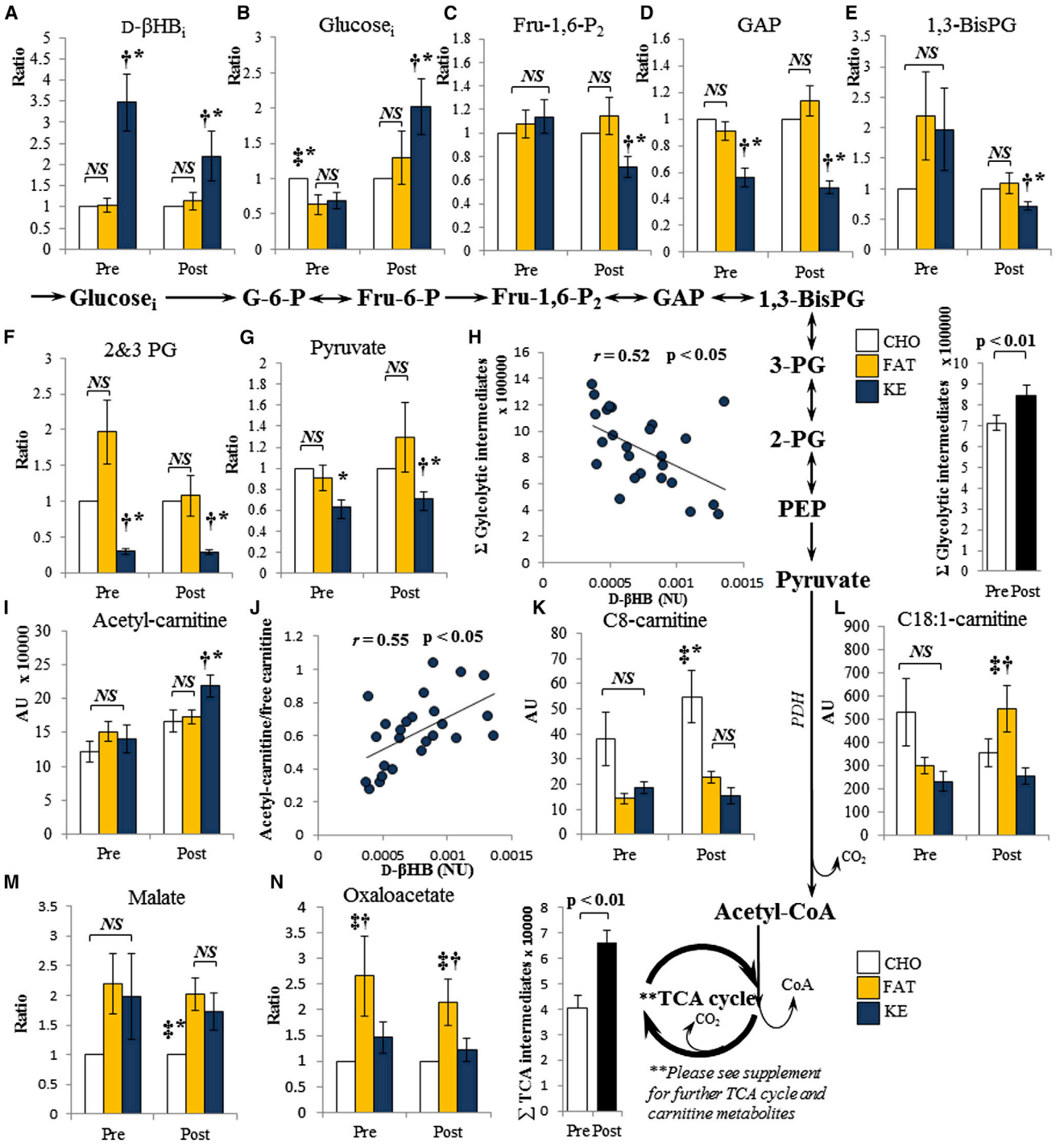

Figure 4. Metabolic Effects of Dietary Substrates on Human Skeletal Muscle Metabolism before and after Exercise (Study 2)

The effects of CHO, FAT, and KE ingestion on skeletal muscle metabolism pre- (Pre) and post (Post)-cycling exercise for 1 hr at 75% W_{Max}. Glycolytic and TCA cycle intermediates are expressed relative to CHO.

(A) D-βHB concentrations.

(B) Intramuscular glucose concentrations.

(C) Fru-1,6-P2: fructose-1,6-bisphophosphate concentrations.

(D) GAP: glyceraldehyde-3-phosphate concentrations.

(E) 1,3-BisPG: 1,3-bisphosphoglycerate concentrations.

(F) 2&3-PG: 2- and 3-phosphoglycerate concentrations.

(G) Pyruvate concentrations.

(legend continued on next page)

In almost identical fashion to studies 3 and 4, blood lactate concentrations increased during exercise, but were ~1.5–2 mM lower on KE+CHO versus CHO (Figure 7C). Blood glucose was raised following ingestion of both drinks at rest, but fell during the first 10 min of exercise (Figure 7D). Glucose concentrations were lower on KE+CHO versus CHO during the first 25 min but were similar by 1 hr. KE+CHO significantly suppressed the exercise induced rise in FFA seen after 25 min of exercise versus CHO (Figure 7E). No significant differences in gas exchange parameters were detected during the 1 hr constant load exercise (Table S6).

Time trial performance following 1 hr of high-intensity exercise was significantly improved in KE+CHO versus CHO conditions. Athletes cycled on average 411 ± 162 m further ($p < 0.05$) over 30 min on KE+CHO versus CHO equating to a mean performance improvement of 2%. Pooled and individual TT performances are shown in Figures 7F and 7G. The metabolic changes arising from altered nutritional substrate provision during exercise are summarized in Figure 7H.

DISCUSSION

In common with many disease conditions, the possible range of oxidizable carbon sources to power exercise becomes highly selective, favoring glucose as energetic demands increase (Romijn et al., 1993; van Loon et al., 2001). Here we show how a nutritional source of ketone bodies alters conventional muscle fuel metabolism and physical performance, alone and in combination with nutritional CHOs. This physiological state operates in contrast to that of endogenous ketosis, where replete glucose reserves, an intact insulin axis, and elevated ketone bodies would never usually coexist.

Ketosis Alters the Hierarchy of Skeletal Muscle Substrate Metabolism

Substrate metabolism in the normal human body is flexible: our bodies having evolved to utilize different fuel sources depending on their availability (Randle et al., 1963). During exercise, energy expenditure increases dramatically above resting levels, with rapid turnover of mobilized fuels required to keep pace with ATP demand (Spriet and Peters, 1998). Usually, as exercise intensity increases, mitochondrial oxidation of fatty acids reaches a ceiling, shifting the burden of energy provision to CHO so that glycolytic supply of pyruvate is the major carbon source for oxidation during heavy exercise (Romijn et al., 1993; van Loon et al., 2001). Despite the stimulation of sustained exercise here, the elevated circulating ketone concentrations significantly decreased human skeletal muscle glycolytic intermediates, including pyruvate. Remarkably, this suppression of glycolysis occurred despite physical workloads that would normally be highly glycolytic (~75% W_{Max}).

Conversely, the same exercise overrode inhibition of glycolysis by fatty acids, in agreement with evidence suggesting that the glucose-FFA cycle (Randle et al., 1963) does not operate during intense exercise. Rather, we suggest that ketone metabolism may hold hierarchical preference over CHO and FAT metabolism, even during conditions that strongly favor CHO oxidation, such as heavy exercise. In essence, ketosis allows substrate competition for respiration during exercise that is not observed in their absence. In support of this theory, intramuscular D-βHB and acetyl-carnitine levels were raised ~3- to 7-fold by KE ingestion, while glycolytic intermediates were decreased without altering the pool of TCA cycle metabolites during exercise. While direct calculation of metabolic flux is not possible from single time point measurements, these data suggest that ketones and FATs were oxidized as an alternative to pyruvate, easing the reliance on glycolysis to provide acetyl-CoA to the TCA cycle. Furthermore, ketosis reduced intramuscular BCAA concentrations, supporting previous evidence that ketosis tightly regulates glycolysis (and therefore pyruvate), ultimately reducing the requirement for BCAA deamination (Thompson and Wu, 1991). Such metabolic effects may have sound survival advantages, limiting the catabolism of CHOs and skeletal muscle protein for gluconeogenesis in starvation.

Taken together, these findings support a mechanism whereby ketosis alters substrate signaling, oxidation, and energy transduction in working muscle, free of the confounding effects of elevated FFA, and reduced CHO reserves that occur with endogenous ketosis (Phinney et al., 1983a, 1983b).

Ketosis in a Glycogen-Replete State

We have shown here how nutritional ketosis enables comparable physiological function to that of glucose, but via very different metabolic actions. Preservation of physiological function is very much in keeping with survival metabolism, where maintenance of homeostasis during conditions of altered fuel availability is vital (Cahill and Owen, 1968). Ample evidence during starvation (Hagenfeldt and Wahren, 1971; Féry and Balasse, 1983), and during high-FAT diets (Phinney et al., 1983b), suggests that ketone oxidation by skeletal muscle is minimal following the transition from fed to starvation states (Féry and Balasse, 1983)—conditions where glycogen is exhausted and FFA oxidation predominates. The observations that "starved" skeletal muscle does not utilize significant quantities of ketone bodies are in contrast to our findings in this post-absorptive (glycogen replete) state that ketone body oxidation may account for ~10%–18% of the total oxygen consumption during exercise; values in close agreement with radio-isotope studies of

(H) Σ Glycolytic intermediates versus D-βHB concentrations.
(I) Acetyl carnitine concentrations.
(J) Acetyl carnitine/free carnitine ratio versus D-βHB concentrations.
(K) C8-canitine concentrations.
(L) C18:1 Carnitine concentrations.
(M) Malate concentrations.
(N) Oxaloacetate concentrations.
†$p < 0.05$ KE versus FAT. *$p < 0.05$ KE versus CHO, ‡ $p < 0.05$ FAT versus CHO. All data are means ± SEM.
G-6-P: glucose-6-phosphate; Fru-6-P: fructose-6-phosphate; PDH: Pyruvate dehydrogenase; PEP: phosphoenolpyruvate. Arbitrary units: AU; normalized units, NU.

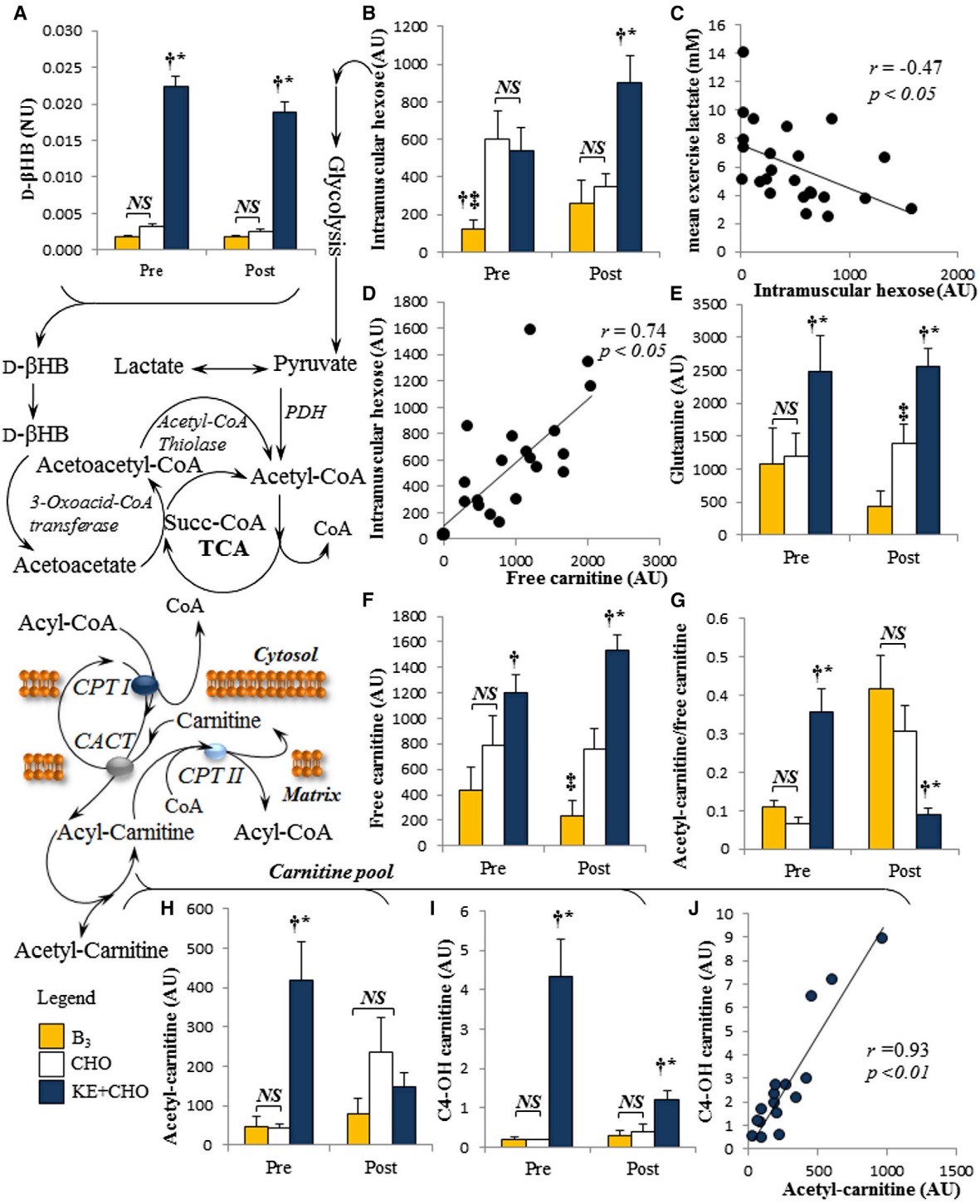

Figure 5. Metabolic Effects of Dietary Substrates on Human Skeletal Muscle Metabolism before and after Exercise (Study 3)

Nutritional substrate provision significantly altered the major pathways of muscular energy transduction, with KE ingestion increasing total CHO levels, and shifting the carnitine axis.

(A) Intramuscular D-βHB concentrations.

(B) Intramuscular Hexose concentrations.

(C) End exercise intramuscular hexose versus mean plasma lactate during exercise.

(D) End exercise intramuscular hexose versus free carnitine.

(E) Intramuscular glutamine concentrations.

(F) Intramuscular free carnitine concentrations.

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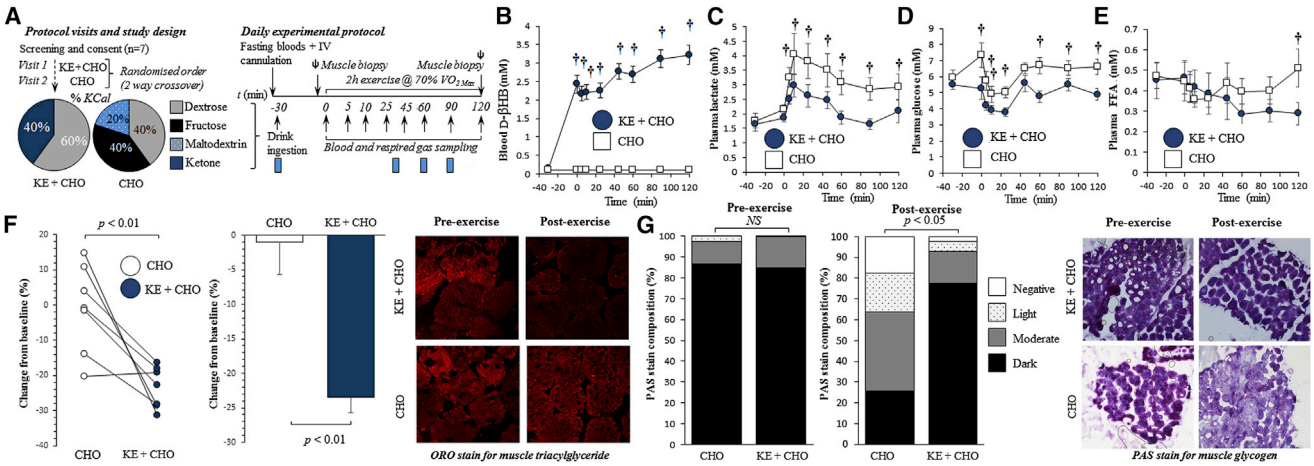

Figure 6. The Effects of Ketosis on Intramuscular Fat and CHO Fuel Reserves during Prolonged Exercise (Study 4)

Combined provision of nutritional ketosis with CHO ingestion increased intramuscular triacylglycerol breakdown while preserving muscle glycogen during sustained endurance exercise.

(A) Study protocol, and interventions.

(B) Plasma D-βHB concentrations.

(C) Plasma lactate concentrations.

(D) Plasma glucose concentrations.

(E) Plasma FFA concentrations.

(F) Intramuscular triacylglycerol (IMTAG) levels (expressed as a % change during exercise).

(G) Intramuscular glycogen (PAS stain intensity).

All data are means ± SEM. †p < 0.05 KE+CHO versus CHO.

exercising man (Balasse et al., 1978). Furthermore, the permissive link between the supply of CHO to sustain anaplerosis, and thus TCA flux, during rat heart perfusion with acetoacetate is well known (Russell and Taegtmeyer 1991a, 1991b). As such, it seems that ketone bodies may "burn in the flame of CHOs," whereupon the ensuing cataplerosis (and exhaustion of muscle glycogen) in 'starved' muscle may limit ketone body oxidation to preserve a circulating substrate for the brain.

It should also be emphasized that the coincident oxidation of ketone bodies as fuel substrates alongside FAT and glucose confounds the conventional interpretation of RER to determine fuel oxidation by indirect calorimetry (Frayn, 1983). The stoichiometry of ketone body oxidation yields RQ values of 1.0 and 0.89 for acetoacetate and βHB, respectively, making isolated inferences of FAT and CHO metabolism based on RER inaccurate during ketosis (Frayn, 1983).

Substrate Competition for Respiratory Oxidation

We have demonstrated how ketosis alters the hierarchy of fuel selection, restoring substrate competition for respiration where fatty acid oxidation cannot conventionally keep pace with TCA flux. In support of previous work in rodents (Sato et al., 1995; Kashiwaya et al., 1997; Ruderman et al., 1999), we have provided evidence of a combined action between CHOs and ketone bodies, accentuating vital elements of the major fuel pathways known to influence muscular energy transduction. In comparison to CHO consumption alone (CHO), nutritional ketosis from KE+CHO consumption significantly increased human skeletal muscle IMTAG oxidation. Remarkably, this occurred despite highly glycolytic workloads and with increased concentrations of glucose and insulin, as both drinks contained significant quantities of CHO. Conversely, feeding isocaloric CHOs during the same exercise demonstrated no appreciable change in IMTAG levels after 2 hr of cycling. Inhibition of lipolysis via nicotinic acid receptor agonism (Taggart et al., 2005) could conceivably reduce circulating FFA availability, thus increasing IMTAG oxidation. However, this seems unlikely, as providing nicotinic acid, with no oxidizable carbon source, only increased the reliance on glycolysis for energy provision in Study 3, similar to the findings of Bergström et al. (1969). Furthermore, FFA levels following CHO and KE ingestion are suppressed, and any small (<0.1 mM) differences in circulating FFA cannot account for the magnitude of change in the intramuscular lipids observed. It is tempting to suggest that a greater capacity to oxidize fatty-acid-derived carbon moieties during ketosis could power improvements in exercise capacity (as shown here) where exhaustion of glycogen

(G) Intramuscular acetyl-carnitine/free carnitine ratio.

(H) Intramuscular acetyl-carnitine concentrations.

(I) Intramuscular C_4-OH carnitine concentrations.

(J) Intramuscular C_4-OH carnitine versus acetyl-carnitine.

All data are means ± SEM. †p < 0.05 KET versus B3, *p < 0.05 KET versus CHO, ‡ p < 0.05 B3 versus CHO. TCA: tri-carboxylic acid cycle; PDH: pyruvate dehydrogenase; Succ-CoA: succinyl-CoA; CACT: carnitine-acylcarnitine translocase; CPT I and II: carnitine palmitoyltransferase; normalized units, NU; arbitrary units, AU.

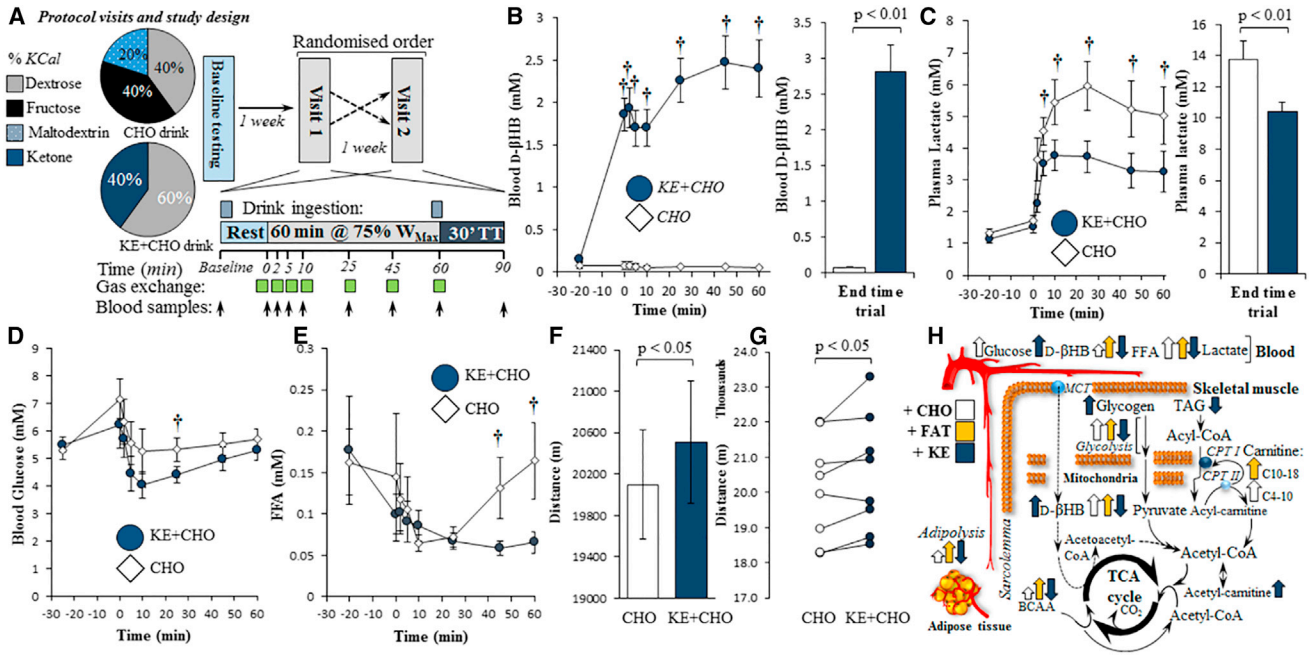

Figure 7. The Effects of Altered Fuel Metabolism on Human Physical Performance

Combined provision of nutritional ketosis with CHO ingestion to fuel exercise altered fuel metabolism, and increased bicycle time-trial performance after 1 hr of fatiguing exercise.

(A) Study protocol, and interventions.

(B) Plasma D-βHB concentrations.

(C) Plasma lactate concentrations.

(D) Plasma glucose concentrations.

(E) Plasma FFA concentrations.

(F) Time trial (TT) performance (expressed as % versus CHO).

(G) Bland Altman plot of TT performance.

(H) Summary of the changes in the major fuel pathways involved in skeletal muscle energy transduction during exercise following nutritional ingestion of FAT, CHO, and KE.

All data are means ± SEM. †$p < 0.05$ KE+CHO versus CHO.

reserves limit physical endurance. However, further work is required to confirm this. Skeletal muscle adaptions to exercise training may have influenced the changes in substrate preference observed here, and it remains to be seen whether similar changes occur in untrained individuals.

Randle Cycle Revisited?

The promotion of intramuscular lipid oxidation during ketosis is in effect signaling an "energetic crisis" in the organism (Robinson and Williamson, 1980; Newman and Verdin, 2014), conserving glucose by forcing skeletal muscle to shift substrate oxidation to more ample fat reserves. Unlike glucose or FAT, acetyl group production from ketone bodies is independent of both PDH and CPT transporters (Halestrap and Meredith, 2004), with the increased acetyl-carnitine concentrations observed during ketosis representing an increase in acetyl-CoA production from ketones or FAT, rather than glycolysis (Sato et al., 1995, Kashiwaya et al., 1997). As was proposed by Randle (Randle et al., 1963; Randle, 1998), feedback inhibition of glycolysis by a high acetyl-CoA/CoA ratio or NADH/NAD^+ ratio during ketosis could account for the observed decrease in glycolytic intermediates and preserved intramuscular CHO stores, as has been reported in rodent muscle (Maizels et al., 1977) and heart (Williamson and Krebs, 1961; Sato et al., 1995). Ketones may have improved the efficiency of either the carnitine transport of acyl-CoA or β-oxidation, resulting in greater acyl-group oxidation. KE ingestion resulted in profound differences in carnitine species, increasing free carnitine during exercise when fed with CHO. As suggested by Wall et al. (Stephens et al., 2007; Wall et al., 2011) (who observed an improvement in physical performance with greater free carnitine availability), the matching of TCA flux with acetyl-CoA supply may have been improved, rendering oxidative ATP production more efficient. Ketosis may also augment (or mimic) the physiological actions of CHO and insulin (Kashiwaya et al., 1997), increasing ketone body disposal in preference to glucose or FAT. Such metabolic actions suggest a plausible mechanism to allow the rapid clearance of ketone bodies on re-feeding following starvation, thus restoring conventional fuel metabolism. Similar findings have been shown during hyperinsulinemic clamp and ketone salt infusions in man (Keller et al., 1988). However, the exact mechanism of how ketones promoted skeletal muscle fatty acid oxidation during conditions in which glucose is conventionally preferred, and in the presence of an intact insulin axis, is unknown.

Altered Athletic Performance

In some ways, the demands of endurance exercise parallel (albeit more rapidly) the metabolic constraints pertinent to survival in starvation, placing a premium on glucose reserves and effective oxidative respiration. We have shown here the benefit of inducing ketosis and how the combination of metabolic alterations achieved by nutritional ketosis may create a potentially advantageous physiological state, distinctly different from that of endogenous ketosis (Cahill, 1970). Athletic adaptions to harness greater circulating fuels for combustion (including ketones) are well known (Johnson and Walton, 1972; Winder et al., 1974), making athletes ideally placed to capitalize on altered substrate provision. However, it remains unclear whether similar changes to those shown here can occur in untrained individuals.

In study 5, bicycle ergometer time trial performance was ~2% greater following KE+CHO versus CHO, representing a modest increase in physical capacity in these highly trained athletes, despite significant changes in muscular metabolism. These findings suggest that the ceiling for human performance is not purely constrained by muscular energetics (Noakes, 2011). However, ketosis may not be advantageous in physiological conditions that rely almost solely on anaerobic glycolysis, or extremely high glycolytic flux for ATP production, such as sprint or short-duration exercise. Furthermore, highly glycolytic exercise may even be impaired if ketone body oxidation restricts glycolysis by negative feedback, either by an increase in NADH/NAD^+ or acetyl-CoA/CoA ratio. Therefore, the utility of nutritional ketosis appears more suited to metabolic conditions were dysregulated substrate selection exists, and where incremental improvements in energy transduction, or CHO preservation, may translate to significant increases in muscular endurance. Further work to determine the factors influencing human performance in ketosis, including the role of cerebral metabolism, is already underway.

CONCLUSION

We have demonstrated the metabolic effects of elevated circulating ketone bodies as a fuel and biological signal to create a unique physiological condition. Ketosis may alter substrate competition for respiration, while improving oxidative energy transduction under certain conditions, such as endurance exercise. Consequently, nutritional ketosis may help to unlock greater human metabolic potential.

EXPERIMENTAL PROCEDURES

Subjects and Screening

Endurance athletes (n = 39) participated in a series of studies investigating the effects of nutritional ketosis on resting and exercise metabolism (see Figure S6 for diagrammatic overview). Ethics approvals for all human studies were granted in accordance with Oxfordshire Regional Ethics Committee (OXREC) and NHS national research ethics service (NRES) requirements. The trials were deemed not to constitute clinical trials and were not registered as such. Written informed consent was obtained from all participants following an explanation of the risks associated with participation, and all testing conformed to the standards of ethical practice as outlined in the declaration of Helsinki. Prior to their inclusion, athletes completed a confidential medical questionnaire and received a resting ECG before exercise testing. Participants were asked not to perform strenuous exercise within 48 hr of each test, to refrain from alcohol and caffeine for 24 hr, and to consume an identical pre-testing meal the night before every test. Water intake was provided ad libitum to each participant. In all studies comparing the effects of nutritional substrates, drink allocation was concealed and the trials were conducted in a randomized, single-blind, crossover fashion. A double randomization method was used; the order of drink allocation was determined using a random number generator, and the order of participation was determined by participant enrolment.

General Study Design (Study 1)

To determine whether exercise intensity altered the metabolism of diet-derived ketosis, we examined the effects of steady-state exercise on the clearance of blood and urinary D-βHB in six male endurance athletes (Table S2A). An identical amount of KE (573 mg/kg BW) was consumed by athletes at rest, and during 45 min of cycling exercise 40% and 75% of W_{Max} in a randomized crossover designed trial (Figure 2A) with 1 week between trials.

General Study Design (Study 2)

In order to compare the metabolic alterations arising from the provision of ketones as an alternative fuel during the same physical workload, male athletes (n = 10) (Table S2B) undertook a three-way crossover study of fixed intensity cycling at 75% W_{Max} for 1 hr. Before each test, athletes consumed a taste-matched, isocaloric flavored beverage containing ≥96% of calories from CHO (dextrose = CHO), KE (573 mg/kg BW), or FAT. Blood and respiratory gas samples were collected at regular intervals throughout exercise (Figure 3A). Muscle biopsy was performed before and after exercise on all participants.

General Study Design (Study 3)

In order to investigate the metabolic alterations arising from the synergistic combination of fuel substrates at rest and during the same physical workload, male athletes (n = 8) (Table S2C) undertook a three-way crossover study of fixed intensity cycling at 75% W_{Max} for 1 hr. Before each test, athletes consumed a taste-matched, isocaloric flavored beverage containing 60% of calories from CHO (dextrose), and KE (573 mg/kg BW) = KE+CHO, or a mixture of carbohydrates (CHO). On the third arm, no calories were provided in the beverage, and vitamin B3 (1,000 mg = B3) was ingested as a control to mimic the effects of ketone agonism of the nicotinic acid receptor, but without the oxidizable carbon source. Blood and respiratory gas samples were collected at regular intervals throughout exercise (Figure S3A). Muscle biopsy was performed before and after exercise on all participants.

General Study Design (Study 4)

To investigate the effects of ketosis on intramuscular fuel reserves during prolonged exercise a further study of n = 7 male athletes (Table S2D) undertook a two-way crossover study of fixed intensity bicycle ergometry at 70% $VO_{2\ Max}$ for 2 hr. All participants consumed a taste-matched, isocaloric flavored beverage containing 60% of calories from CHO (dextrose) and 40% from KE = KE+CHO, or a mixture of CHOs . 50% of the total KE (573 mg/kg BW) was ingested at baseline, with the remaining 50% ingested as equal aliquots at 30 min, 1 hr, and 90 min during exercise. Blood and respiratory gas samples were collected at regular intervals throughout exercise (Figure 6A). Muscle biopsy was performed before and after exercise on all participants.

General Study Design (Study 5)

To determine the effect of altered substrate metabolism on human physical performance, study participants (n = 6 male, n = 2 female) (Table S2E) completed two blinded exercise trials following an overnight fast, consisting of 1 hr steady-state workload at 75% W_{Max} followed by a blinded 30 min time trial for maximum distance. Before each test, athletes consumed a drink containing either ketone and dextrose, or CHOs alone, in randomized order (Figure 7). Athletes completed all trials on identical bike set up dimensions (SRM training systems, Germany), with no external stimuli. Blood and pulmonary gas measurements were collected during the first 1 hr fixed workload period; however, athletes were left free of distractions throughout the time trial, with a blood sample obtained immediately after the completion of the time trial. Athletes were blinded to work output, heart rate, and cadence during the 30 min time trial, and only elapsed time was visible to athletes.

Baseline Testing and Workload Prescription

All participants undertook a stepped (25 W/3 min) incremental exercise test to exhaustion on an electronically braked bicycle ergometer (Ergoline, Germany) for the determination of $VO_{2\ Max}$ (Cortex Biophysik, Germany) and W_{Max} at least 1 week prior to the start of each trial (Supplemental Information). The same ergometer was used for subsequent exercise tests.

Substrate Drinks

In Studies 1 and 2, participants ingested drinks containing >96% of total calories from a single dietary fuel substrate as CHO, KE, or long-chain FAT (Supplemental Information). In Studies 3 and 4, participants' ingested drinks containing isocaloric quantities of CHO+KE, or 1:1:2 mixtures of dextrose, fructose, and maltodextrin. In both latter studies, a minimum of 1.2 g/min of CHO supply was ensured during exercise trials to allow comparisons according to evidence based "optimal CHO feeding strategy" (Jeukendrup and Jentjens, 2000; Jentjens et al., 2004). In Studies 3–5, drinks were prepared that contained KE as 40% of calories, with the remainder made up from CHO (dextrose). The dose response, determined previously (Clarke et al., 2012; Shivva et al., 2016), showed that 500 mg of KE/kg body weight produced blood D-βHB concentrations of ~3 mM after 30–60 min. All drinks were taste, color, and volume matched (Supplemental Information).

Pulmonary Gas Exchange and Blood Sampling

Respiratory gas collections (Cortex Biophysik, Germany) were obtained at identical times during exercise as blood was sampled (Supplemental Information). Blood samples (2 ml) were obtained via a venous catheter inserted percutaneously into an antecubital vein (Supplemental Information). Samples were immediately stored on ice, centrifuged (3,600 rpm for 10 min), and stored at −80°C until further analysis. Glucose, FFA, triglycerides, D-βHB, and lactate were assayed using a commercial automated bench-top analyzer (ABX Pentra, France). Glycerol and insulin assays were performed using ELISA kits (Mercodia, Sweden). Acetoacetate was assayed using enzymatic methods (Bergmeyer and Gawehn, 1974).

Muscle Biopsy

Muscle tissue was collected using percutaneous needle biopsies from the lower third of the vastus lateralis muscle (Bard Monopty, USA). Samples were obtained from new incisions at rest and immediately following exercise. Tissue was frozen immediately in liquid nitrogen and stored at −80°C until further analysis.

Metabolite Extraction from Skeletal Muscle

Metabolites were extracted from approximately 100 mg tissue using a modified Folch method (Le Belle et al., 2002). The aqueous and organic fractions were separated and split into two identical volumes to allow multiple analyses (Supplemental Information). Histological analyses were performed using staining and confocal microscopy methods described previously (Gollnick et al., 1973; Halkjaer-Kristensen and Ingemann-Hansen, 1979; Koopman et al., 2001) (Supplemental Information).

^{1}H-NMR Analysis of Aqueous Metabolites

Half of the aqueous fraction (~25 mg wet weight tissue) was dried under nitrogen and resuspended in 600 μl D_2O containing 0.09% w/v NaCl (Sigma), 0.01% w/v NaN_3 (Sigma), and 0.25 mM deuterated sodium-3-trimethylsilylpropionate (NaTMSP-2,2,3,3-D4, Cambridge Isotope Laboratories, USA) as a chemical shift reference. Samples were analyzed on a Bruker NMR spectrometer interfaced with an 11.8 Tesla superconducting magnet at 310K using a ^{1}H-NOESY 1D pulse sequence with 128 scans. Data were integrated using fixed integral sizes of 0.02 ppm within 1D Spec Manager (v12, Advanced Chemistry Development, Canada).

Carnitine Analysis

Half the aqueous fractions were combined with half the organic fraction, and 200 μl acyl-carnitine standard containing eight deuterated species was added (Cambridge Isotope Laboratories, Inc.). Samples were dried under nitrogen and butylated with 3 M butanolic-HCl (Sigma). Samples were resuspended in 200 μl of 4:1 acetonitrile:water containing 0.1% v/v formic acid (Sigma) and analyzed using multiple reaction monitoring on a Waters Quattro Premiere XE triple quadrupolar mass spectrometer. Chromatograms were integrated using QuanLynx v4.1 (Waters Ltd, UK).

Statistics

Results are expressed as means ± SEM and significance was established a priori at $p < 0.05$. All clinical and laboratory data were analyzed for all subjects (Supplemental Information). Statistical analysis was performed using SPSS (V21, USA). For the human trials containing paired data with three arms, repeated-measures ANOVA was performed following initial tests to ensure sphericity assumptions were not violated, and then corrected with additional post hoc Tukey corrections for multiple comparisons where appropriate (Supplemental Information). Cycling performance results were paired comparisons containing two arms, with comparisons performed using a two tailed paired t test. Correlations were tested using a two-tailed Pearson's test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, ten tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2016.07.010.

AUTHOR CONTRIBUTIONS

Study design: P.J.C., T.K., K.C. Conducting studies: P.J.C., T.K., A.S., S.W.M., B.S., S.D., C.H., S.N., R.L.V., M.T.K. Analysis: P.J.C., T.A., T.K., C.H., J.W., B.S., J.L.G., A.J.M., M.S.D., S.W.M., R.E. Manuscript preparation: P.J.C., K.C. Manuscript editing: All authors.

CONFLICTS OF INTEREST

The intellectual property and patents covering the uses of ketone bodies and esters are owned by BTG Ltd, The University of Oxford, the NIH and TdeltaS Ltd. Should royalties ever accrue from these patents, R.L.V., K.C., A.J.M., M.T.K., and P.J.C. as named inventors may receive a share of royalties as determined by the terms of the respective institutions. K.C. is director of TdeltaS, a spin out company of the University of Oxford, to develop and commercialize products based on the ketone ester. B.S., T.K., and S.W.M. are employees of TdeltaS Ltd.

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Cell Metabolism, Volume *24*

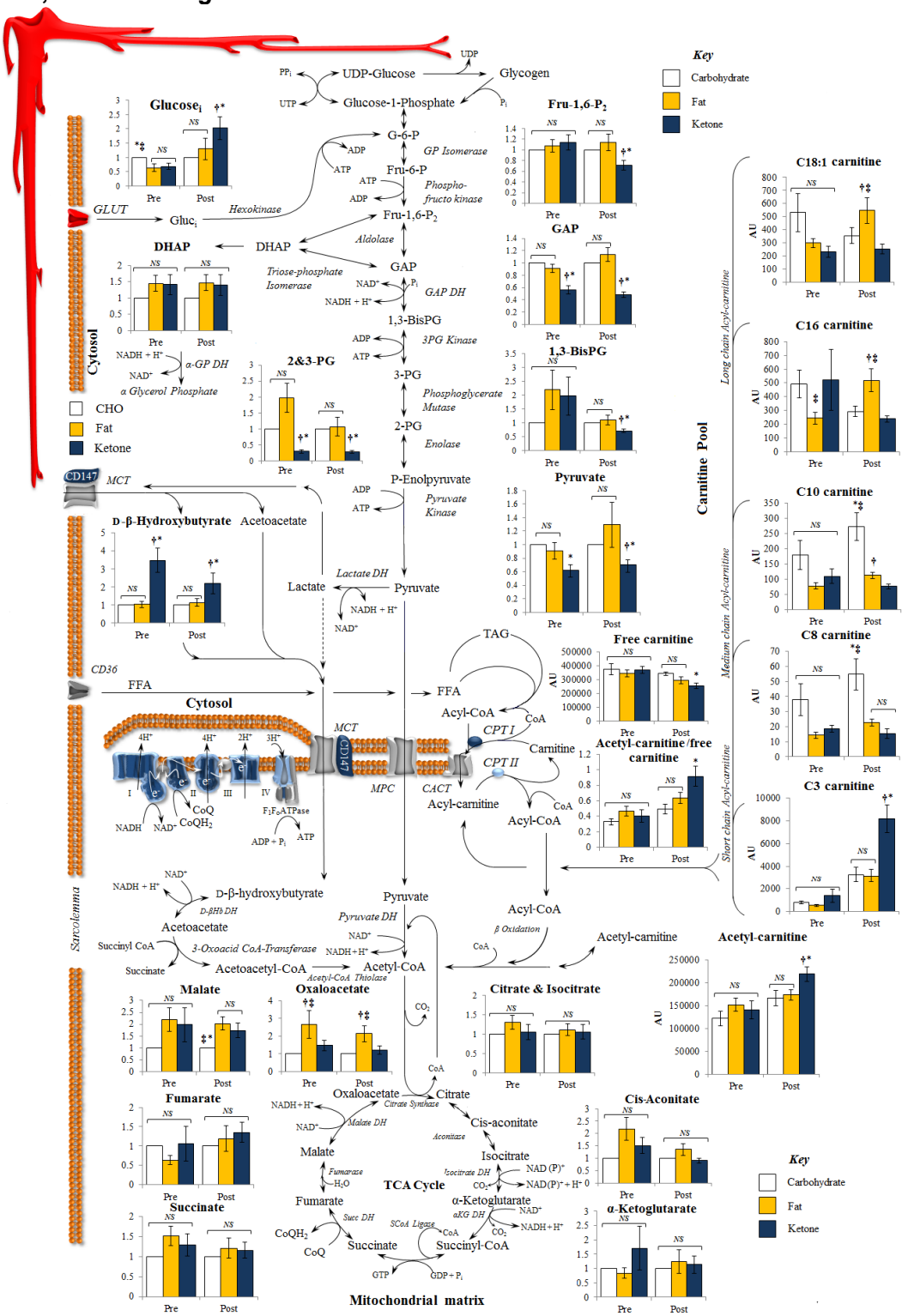
Supplemental Information

Nutritional Ketosis Alters Fuel Preference and Thereby Endurance Performance in Athletes

Pete J. Cox, Tom Kirk, Tom Ashmore, Kristof Willerton, Rhys Evans, Alan Smith, Andrew J. Murray, Brianna Stubbs, James West, Stewart W. McLure, M. Todd King, Michael S. Dodd, Cameron Holloway, Stefan Neubauer, Scott Drawer, Richard L. Veech, Julian L. Griffin, and Kieran Clarke

Supplemental figures:

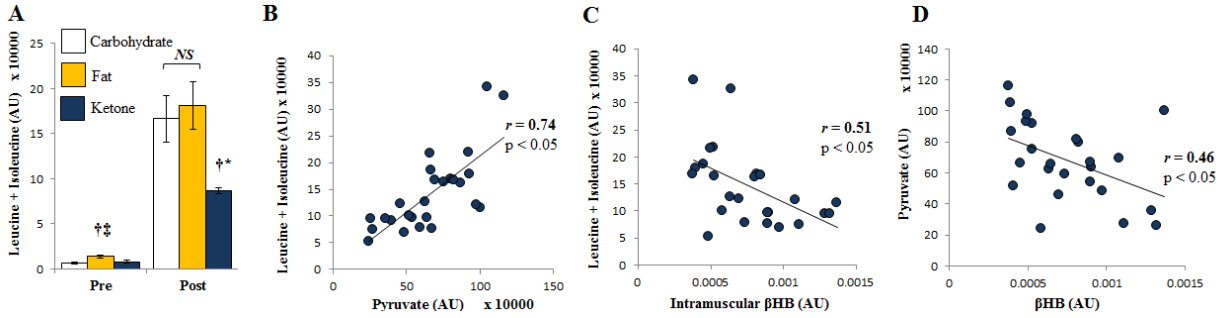
Figure S1, *related to Figure 4.*



Nutritional ketosis alters fuel preference and thereby endurance performance in athletes.

Supplemental information *Cell metabolism*

3 **Figure S2, *related to Figures 4 & S1.***



4 **Figure S3, *related to Figure 5.***

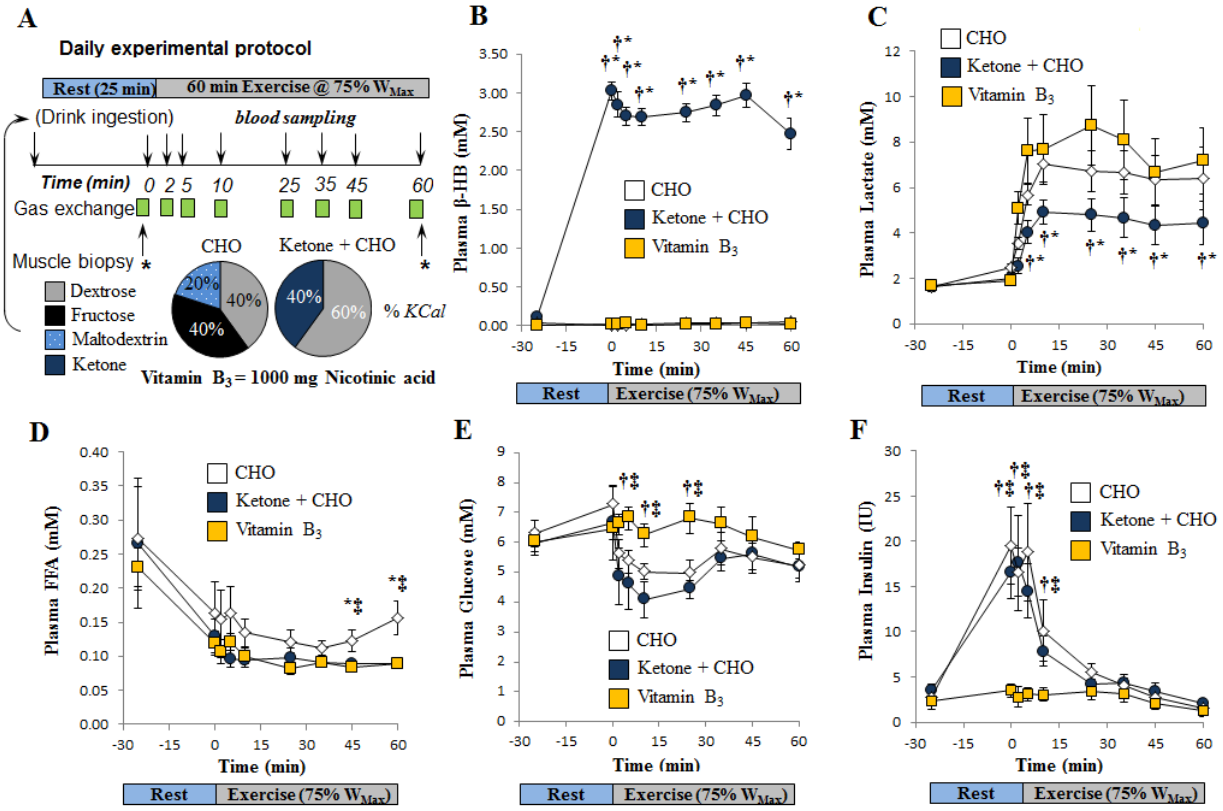
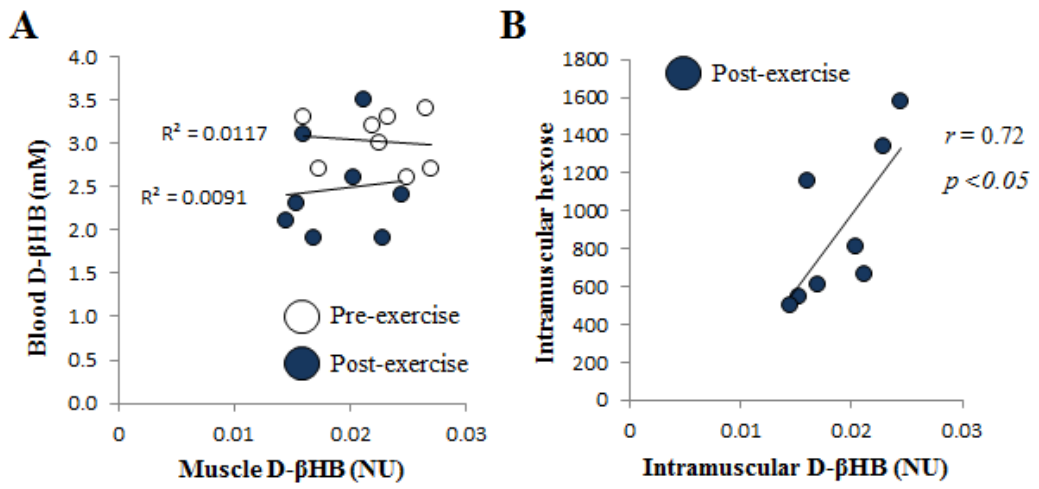
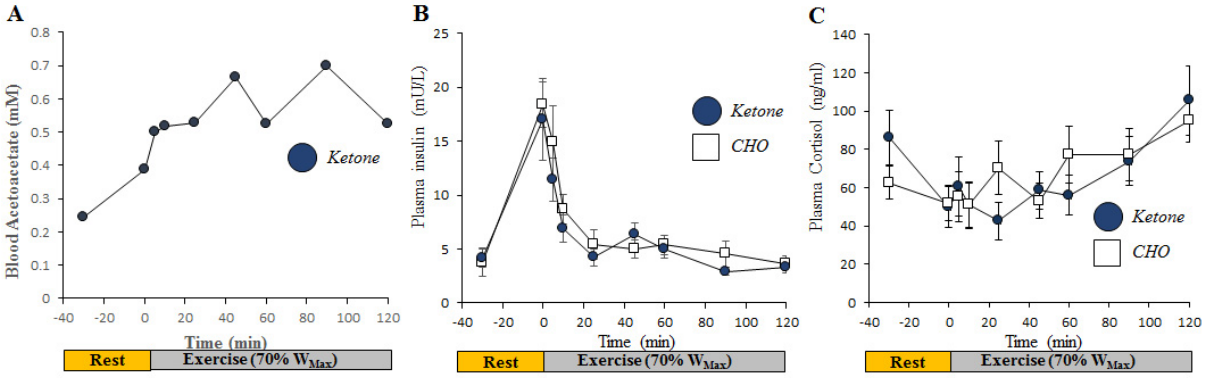


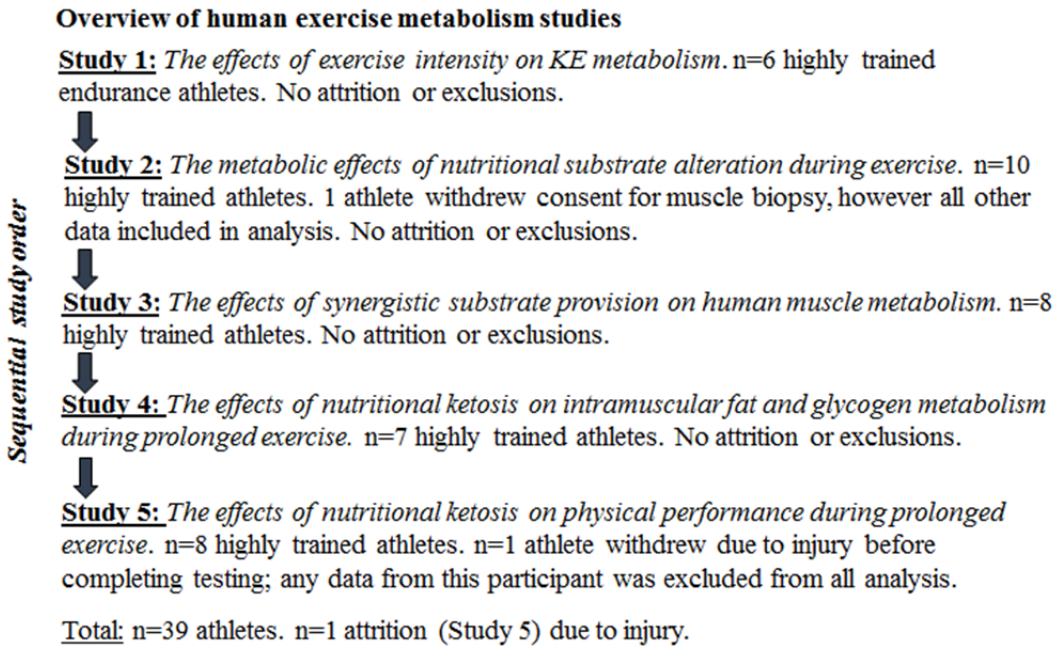
Figure S4, *related to Figure 5.*



6 **Figure S5, *related to Figure 6.***



7 **Figure S6, *relates to experimental procedures.***



Supplemental tables:

Table S1, *relates to Figure 1*. Physical properties of KE

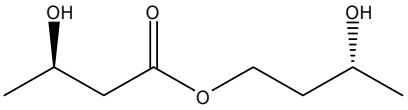
Monoester	Physical property
Chemical name	D-β-hydroxybutyrate-R 1,3-Butanediol Monoester
Molecular formula	$C_8H_{16}O_4$
Molecular weight	176
Physical structure	
Appearance	Colourless, Viscous Oil
Taste	Extremely bitter
Boiling point	145°C at 1.8 Torr
State at room temperature	Liquid
Density	1.0731 g/mL at 22°C

Table S2, *relates to Figures 2-7*.

Anthropometric characteristics of study participants.

Study (n)	**n=6** (*M*)	**n=10** (*M*)	**n=8** (*M*)	**n=7** (*M*)	**n=6** (*M*) **n=2** (*F*)
	***(A)** Study 1*	***(B)** Study 2*	***(C)** Study 3*	***(D)** Study 4*	***(E)** Study 5*
	Mean *(±SE)*	Mean *(±SE)*	Mean *(±SE)*	Mean *(±SE)*	Mean *(±SE)*
Age (yr)	28.8 (± 0.3)	27.6 (± 1.6)	27.4 (±1.0)	29.4 (± 1.0)	29.4 (± 1.0)
Height (cm)	189 (± 6)	191 (± 4)	185 (± 4)	185 (± 4)	187 (± 4)
Weight (kg)	89.7 (± 8.0)	91.3 (± 5.2)	79.8 (± 4.7)	79.8 (± 4.7)	84.9 (± 5.2)
VO_{2Max} (L/min) *male*	5.35 (± 0.3)	5.35 (± 0.2)	5.15 (± 0.2)	4.85 (± 0.2)	5.37 (± 0.3)
VO_{2Max} (L/min) *female*					3.30 (± 0.1)
W_{Max} (W) *male*	386 (± 19)	392 (± 12)	387 (± 19)	387 (± 19)	404 (± 12)
W_{Max} (W) *female*					282 (± 14)
BMI (kg/m^2)	24.6 (± 0.6)	24.8 (± 0.7)	23.1 (± 0.6)	23.1 (± 0.6)	24.3 (± 0.9)

M = male, *F*= female

Table S3, *related to Figures 3 & 4*.

Gas exchange obtained during rest and exercise in Study 2

Time	VO_2 (L/min)			RER		
	KE	*CHO*	*FAT*	*KE*	*CHO*	*FAT*
Rest	0.53 ± 0.12	0.54 ± 0.14	0.51 ± 0.14	0.98 ± 0.05	1.02 ± 0.04	0.94 ± 0.06
2min	3.39 ± 0.11	3.35 ± 0.12	3.17 ± 0.15	0.88 ± 0.03	0.96 ± 0.03‡*	0.85 ± 0.03
5 min	3.72 ± 0.08	3.77 ± 0.12	3.67 ± 0.15	0.99 ± 0.02	1.03 ± 0.02‡*	0.98 ± 0.02
10 min	3.82 ± 0.09	3.88 ± 0.12	3.75 ± 0.16	0.97 ± 0.01	0.99 ± 0.01	0.96 ± 0.02
25 min	3.81 ± 0.11	3.83 ± 0.12	3.72 ± 0.13	0.96 ± 0.02	0.98 ± 0.02‡	0.94 ± 0.02
35 min	3.75 ± 0.11	3.87 ± 0.11	3.70 ± 0.16	0.95 ± 0.01	0.97 ± 0.02	0.93 ± 0.02
45 min	3.88 ± 0.13	3.95 ± 0.13	3.61 ±0.17	0.94 ± 0.01	0.95 ± 0.01	0.93 ± 0.02
50 min	3.80 ± 0.12	3.82 ± 0.13	3.77 ± 0.16	0.93 ± 0.01	0.95 ± 0.01‡	0.90 ± 0.02
60 min	3.87 ± 0.14	4.05 ± 0.14	3.85 ± 0.12	0.95 ± 0.02	0.95 ± 0.01	0.91 ± 0.02

Data are expressed as mean ± SEM. ‡ $p<0.05$ fat (FAT) vs. carbohydrate (CHO) and * $p < 0.05$ KE vs. carbohydrate.

Table S4, *related to Figure 5*.

Gas exchange data obtained during rest and exercise in Study 3

Time	VO_2 (L/min)			RER		
	KE+CHO	*CHO*	*B3*	*KE+CHO*	*CHO*	*B3*
Rest	0.59 ± 0.06	0.56 ± 0.05	0.61 ± 0.06	1.00 ± 0.03	1.04 ± 0.04	0.93 ± 0.06
2min	3.89 ± 0.16	3.89 ± 0.15	3.76 ± 0.18	0.99 ± 0.03	1.04 ± 0.03*	1.09 ± 0.04†
5 min	4.07 ± 0.14	4.04 ± 0.16	4.06 ± 0.19	0.99 ± 0.02	1.01 ± 0.02	1.03 ± 0.02
10 min	4.10 ± 0.16	4.06 ± 0.13	4.06 ± 0.16	0.96 ± 0.01	0.99 ± 0.01	1.01 ± 0.02
25 min	4.05 ± 0.13	4.19 ± 0.16	4.00 ± 0.15	0.97 ± 0.02	0.99 ± 0.02	1.02 ± 0.02
35 min	4.10 ± 0.13	4.18 ± 0.16	4.05 ± 0.17	0.96 ± 0.02	0.97 ± 0.02	1.01 ± 0.03
45 min	4.18 ± 0.13	4.20 ± 0.16	4.08 ± 0.17	0.96 ± 0.02	0.96 ± 0.01	1.00 ± 0.04
60 min	4.21 ± 0.12	4.22 ± 0.16	4.20 ± 0.20	0.96 ± 0.02	0.97 ± 0.02	1.00 ± 0.03

Data expressed as mean ± SEM. † $p < 0.05$ between KE+CHO and B3.* $p < 0.05$ between KE+CHO and CHO.

19 **Table S5, *related to Figure 6*.** Gas exchange data obtained during exercise in Study 4.

Time	VO_2 (L/min)		RER	
	KE+CHO	*CHO*	*KE+CHO*	*CHO*
Rest	0.56 ± 0.08	0.57 ± 0.07	0.98 ± 0.03	0.98 ± 0.05
5min	3.49 ± 0.17	3.34 ± 0.14	0.94 ± 0.03	0.99 ± 0.02
10 min	3.38 ± 0.17	3.32 ± 0.18	0.94 ± 0.01†	0.99 ± 0.02
25 min	3.45 ± 0.17	3.28 ± 0.19	0.93 ± 0.01†	0.98 ± 0.01
45 min	3.49 ± 0.19	3.35 ± 0.19	0.91 ± 0.01†	0.96 ± 0.01
60 min	3.51 ± 0.13	3.35 ± 0.20	0.91 ± 0.01†	0.98± 0.01
90 min	3.54 ± 0.19	3.46 ± 0.20	0.92 ± 0.01†	0.98 ± 0.01
120 min	3.63 ± 0.19	3.49 ± 0.20	0.92 ± 0.01	0.96 ± 0.01

20 Data expressed as mean ± SEM. († $p < 0.05$).

Table S6, ***related to Figure 7.*** Gas exchange data obtained during exercise in Study 5.

Time	VO_2 (L/min)		RER	
	KE+CHO	*CHO*	*KE+CHO*	*CHO*
Rest	0.52 ± 0.05	0.58 ± 0.07	1.00 ± 0.04	1.02 ± 0.06
2min	3.63 ± 0.27	3.54 ± 0.24	1.05 ± 0.03	1.05 ± 0.02
5 min	3.76 ± 0.27	3.93 ± 0.28	1.02 ± 0.01	1.03 ± 0.02
10 min	3.91 ± 0.27	3.98 ± 0.29	1.02 ± 0.01	1.00 ± 0.01
25 min	3.99 ± 0.29	3.95 ± 0.29	1.00 ± 0.01	1.01 ± 0.01
45 min	4.11 ± 0.33	4.04 ± 0.27	0.98 ± 0.01	0.99 ± 0.01
60 min	4.08 ± 0.29	4.09 ± 0.27	0.98 ± 0.01	0.98 ± 0.01

Data expressed as mean +/- SEM.

Supplemental figure legends:

Figure S1: The effects of dietary substrate alteration on skeletal muscle fuel metabolism (Study 2)

The effects of carbohydrate (CHO), fat (FAT) and ketone drinks on skeletal muscle metabolism pre (Pre) and post (Post) cycling exercise for 1 h at 75% W_{Max}. Glycolytic and TCA cycle intermediates are expressed relative to carbohydrate (CHO), except carnitine values which are relative to each other (AU). All data are means ± SEM. †$p<0.05$ KE vs. FAT. *$p<0.05$ KE vs. CHO, ‡ $p<0.05$ FAT vs. CHO.

$Gluc_i$: intracellular glucose, G-6-P: glucose-6-phosphate, Fru-6-P: fructose-6-phosphate, Fru-1,6-P2: fructose-1,6-bisphophosphate, DHAP: dihydroxyacetone phosphate, GAP: glyceraldehyde-3-phosphate, 1,3-BisPG: 1,3-bisphosphoglycerate, 2&3-PG: 2- and 3-phosphoglycerate, P-Enolpyruvate: phosphoenolpyruvate, GP-Isomerase: glucose phosphate isomerase, LDH: lactate dehydrogenase, MCT : monocarboxylate transporter, CPT: carnitine palmitoyltransferase, βHBDH: D-β-hydroxybutyrate dehydrogenase, αKG-DH: α-ketoglutarate dehydrogenase. Arbitrary units; AU.

Figure S2: The effects of dietary fuels on skeletal muscle BCAA and relationships to intramuscular ketone bodies (Study 2)

A. Intramuscular isoleucine + leucine before (Pre), and after exercise (Post)

B. Intramuscular isoleucine + leucine vs. intramuscular pyruvate

C. Intramuscular isoleucine + leucine vs. intramuscular D-βHB concentrations

D. Intramuscular pyruvate vs. D-βHB concentrations

All data are means ± SEM. †$p<0.05$ KE vs. FAT, *$p<0.05$ CHO vs. KE, ‡ $p<0.05$ CHO vs. FAT

AU (Arbitrary units).

Figure S3: Effects of dietary substrates on plasma metabolites during exercise (Study 3)

E. Study protocol, showing baseline testing, cross-over design, cycling exercise with study interventions, and substrate drink calorie composition (% Kcal)

F. D-βHB concentrations

G. Plasma lactate concentrations

H. Plasma FFA concentrations

I. Plasma glucose concentrations

J. Plasma Insulin concentrations

All data are means ± SEM. †$p<0.05$ KE+CHO vs. B3, *$p<0.05$ KE+CHO vs. CHO, ‡ $p<0.05$ B3 vs. CHO.

Figure S4: Skeletal muscle relationships to intramuscular and circulating ketone bodies (Study 3)

A. Blood D-βHB vs. intramuscular D-βHB concentrations

B. Intramuscular hexose vs. intramuscular D-βHB concentrations.

NU (Normalised units).

Figure S5: Plasma metabolites (Study 4)

A. Plasma acetoacetate concentrations

B. Plasma insulin concentrations

C. Plasma cortisol concentrations

61 ***Figure S6: Study overview diagram***

Supplemental experimental procedures:

Effect of exercise intensity on the metabolism of D-βHB (Study 1):

Six high performance athletes from endurance sports were recruited to take part in this study (anthropometric characteristics are shown in Table S2A). All participants undertook a stepped (25 W/3 min) incremental exercise test to exhaustion on an electronically braked bicycle ergometer (Ergoline, Germany) for the determination of $VO_{2\ Max}$ and W_{Max} at least 1 week prior to the first trial. The same ergometer was used for subsequent exercise tests, which were completed by all athletes.

Drink preparation:

All athletes consumed a drink containing 96% of ketone ester as total calories before each trial, and dosed at 573mg/kg body weight for each athlete.

Urine collection:

Athletes were asked to completely empty their bladder immediately prior to the ingestion of KE, and once again immediately after the completion of the 45 min exercise period. Urine was collected into 3 L containers (Simport Plastics Ltd, Beloeil, Canada). Total urine volume was measured and 2 ml aliquots frozen at -80ºC until further analysis. Urinary D-βHB was assayed using a commercial automated bench-top analyzer (ABX Pentra, Montpellier, France). Total D-βHB elimination was calculated as n (mol) = C x V where C represents mol. concentration of D-βHB, and V the total volume of urine collected.

Indirect calorimetry and corrections for ketone oxidation:

Total substrate oxidation equations derived from indirect calorimetry do not account for the oxidation of ketone bodies under conventional conditions. Therefore corrections to the calculated total substrate oxidation rates during ketosis were undertaken according to the methods described by Frayn (Frayn 1983). Briefly, differences in blood D-βHB AUC calculated at rest, and during exercise conditions were used to determine the oxidation (mol) of circulating D-βHB, assuming a volume of distribution of 0.2 L/kg body weight (Beylot, Beaufrere et al. 1986). Total oxygen consumption, and carbon dioxide production resulting from ketone oxidation were therefore adjusted assuming 1 mol of gas occupies a volume of 22.4 L, and the stoichiometry of D-βHB oxidation requires 4.5 mol of oxygen per mol utilised as fuel (Frayn 1983). Conventional substrate oxidation equations (Frayn 1983, Peronnet and Massicotte 1991) were subsequently used to determine relative contributions of fat and carbohydrate to total oxygen consumption.

The metabolic effects of nutritional substrate alteration during exercise (Study 2):

Ten male high performance athletes from endurance sports were recruited to take part in this study (anthropomorphic characteristics in Table S2B). One athlete withdrew consent for muscle biopsy, but all remaining collected data were included in the analyses.

Drink preparation:

Drinks were isocaloric in energy (mean calorific value 337 ± 15 Kcal) and taste matched using sweeteners (Neotame™, NutraSweet, USA) or bitter additive (Symrise, product number 648352, UK) to ensure blinding. Substrate calories were bodyweight-adjusted, and dosed to ensure a minimum carbohydrate delivery of 1.2 g/min of exercise (Jeukendrup 2004, Jeukendrup 2008) on the carbohydrate arm. Drinks were made up from commercially available sports water (Glaceau, UK), and matched for tonicity (13% solutions for all arms). All drinks contained a minimum of 96% of their energy from a sole substrate, as carbohydrate (maltodextrin:fructose, 5:1, Gu Gels, Berkeley, USA), long chain triglyceride (Calogen™, Zoetermeer, Netherlands), or KE. Subjects ingested 75% of each drink (total volume 393 ± 23 ml) 15 min prior to the start of exercise, over a 5 min time interval. At 45 min, athletes paused for 1 min to ingest the remaining 25% of the drink as a 'top up' for the final 15 min of exercise. During the study, subjects were allowed water *ad libitum.*

Standardised diets:

All athletes completed a 3 day diet diary in a representative training week to determine their individual average calorie intake. This calorie requirement was then used to determine meal plans overseen by a study dietician, with athletes instructed to adhere to the same diet/calorie intake for 24 hours before each study visit. Prior to the FAT trial, athletes consumed an isocaloric high fat low carbohydrate diet for 24 hours instead of their habitual diet in order to raise circulating FFA. Athletes received individual meal plans, and consumed diets consisting of

approximately (in %kcals) 70% fat, 25% protein, and ~5% carbohydrate similar to previous work from our laboratory (Edwards, Murray et al. 2011).

The effects of synergistic carbohydrate and ketone delivery on human substrate metabolism (Study 3):

In order to investigate the metabolic alterations arising from the synergistic combination of fuel substrates at rest and during the same physical workload, male athletes (n = 8) (Table S2C) undertook a 3 way cross-over study of fixed intensity cycling at 75% W_{Max} for 1 h.
Dink preparation: In all studies a minimum of 1.2 g/min of carbohydrate supply was ensured so as not to feed isocaloric carbohydrates at a 'disadvantage' according to evidence based nutritional guidance for exercise performance. Drinks were prepared that contained *(R)*-3-hydroxybutyl *(R)*-3-hydroxybutyrate as 40% of calories, with the remainder made up from carbohydrate (dextrose). Additional matched calories from either ketone, or 'multiple transportable carbohydrates' were provided to allow comparisons according to evidence based 'optimal feeding strategy'. In order to mimic the physiological action of ketone bodies on the nicotinic acid receptor, but not to provide an oxidisable fuel source for muscle, participants ingested 1000 mg of nicotinic acid (vitamin B3) as a control. Calorie, tonicity and taste matching was performed as previously described in Study 2.

The effect of nutritional ketosis on intramuscular fat and glycogen oxidation in prolonged exercise (Study 4)

To investigate the effects of ketosis on intramuscular fuel reserves during prolonged exercise a further study of n = 7 male athletes (Table S2D) undertook a 2-way cross over study of fixed intensity at 70% $VO_{2\ Max}$ for 2 h.
Dink preparation: Identical composition drinks to study 3 were ingested by participants, with drinks were prepared that contained KE (*(R)*-3-hydroxybutyl *(R)*-3-hydroxybutyrate) as 40% of calories, with the remainder made up from carbohydrate (dextrose). On the CHO arm, calories derived from 'multiple transportable carbohydrates' were provided as 1:1:2 mixtures of dextrose, fructose, and maltodextrin. 50% of the total drink (573 mg/kg BW) was ingested at baseline, with the remaining 50% ingested as equal aliquots at 30 min, 60 min and 90 min during the exercise trial. This method of administering nutritional calories was chosen to maximize the contributions of ingested carbohydrate to energy production.
Calorie, tonicity and taste matching was performed as previously described in Study 2.

The effect of nutritional ketosis on human physical performance during prolonged exercise (Study 5).

To determine the effect of altered nutritional substrate metabolism on human physical performance, study participants (n=6 male, n=2 female)) (Table S2E) completed two blinded exercise trials following an overnight fast, consisting of 60 min steady state workload at 75% W_{Max} followed by a blinded 30 min time trial (TT) for maximum distance. Before each test, athletes consumed a drink containing either ketone and dextrose, or carbohydrates alone, in randomised order. Athletes completed all trials on identical bike set up dimensions (SRM training systems, Germany), with no external stimuli. Blood and pulmonary gas measurements were collected during the first 60 min fixed workload period; however athletes were left free of distractions throughout the time trial, with a blood sample obtained immediately after the completion of the time trial. Athletes were blinded to work output, heart rate and cadence during the 30 min time trial, and only elapsed time was visible to athletes. No muscle biopsy was performed in this study.
Dink preparation: Identical composition drinks to study 3 and 4 were ingested by participants. Drinks were prepared that contained KE (*(R)*-3-hydroxybutyl *(R)*-3-hydroxybutyrate) as 40% of calories, with the remainder made up from carbohydrate (dextrose). On the CHO arm, calories derived from 'multiple transportable carbohydrates' were provided as 1:1:2 mixtures of dextrose, fructose, and maltodextrin. 50% of the total drink (573 mg/kg BW) was ingested at baseline, with the remaining 50% ingested as equal aliquots at 30 min, and before the TT at 60 min.
Calorie, tonicity and taste matching was performed as previously described in Study 2.

Participants and eligibility

Target populations for all studies were highly trained elite and sub-elite athletes currently participating in endurance sports requiring a large aerobic training base. All athletes were recruited from professional/semi-professional

sporting clubs in the UK. Only athletes who were at a steady state phase of training were eligible for inclusion, so as not to be influenced by early or late season shifts in specific conditioning. All study participants were tested within the same macrocycle of training and at the same time within a training week. Participants presented following an overnight fast and testing was at the same time of day (starting at 8 am) to reduce the effect of diurnal patterns on subsequent measurements. All participants were healthy non-smokers, not on medication and with no history of major illness. Testing was conducted at the John Radcliffe hospital, Oxford, UK.

Eligibility criteria

- Age 18-40 years
- Currently undertaking aerobic endurance training > 12 h per week for the past 3 months
- Physical injury and illness free
- Elite or sub elite performers in the endurance sports of rowing, cycling, or triathlon currently resident in the UK.

Exclusion criteria

- Any previous history of cardiovascular, neuromuscular, endocrine or neurological illness, or any other medical condition requiring long-term medication.
- Smoking (of any description)
- Pregnancy or current breastfeeding, or female athletes not taking the oral contraceptive pill (due to the variability in hormonal patterns and substrate levels with different parts of the menstrual cycle)
- Inability or loss of ability to give informed consent
- Alcohol in the 24 hours prior to any study visit

Body composition analyses were performed using a bio-impedance Bodystat® 1500 Body Composition Analyzer (Douglas, Isle of Man). Anthropometric characteristics for each study (Table S2) are shown.

Determination of maximal workload on bicycle ergometer

Baseline testing to determine workloads for subsequent prescriptive steady state exercise, and to characterise study populations was performed on a stationary bicycle ergometer (Ergoline, Germany) following personalised saddle, and handlebar adjustments. Subjects commenced all tests in the post-absorptive state following an overnight fast of 8-12 hours, and were allowed water *ad libitum* prior to the start of the test to ensure adequate hydration. No standardised warm up was undertaken; instead workloads were initiated at 100 watts (low intensity) to allow a controlled progressive lead in to the maximal test.

Wattage was fixed in the rpm independent mode of the ergometer for all testing to ensure constant workloads, and increased by 25-35 watts every 3 min until volitional fatigue, with maximal workload (W_{Max}) calculated according to the formula:

$$W_{Max} = \text{Watts}_{\text{Step N-1}} + (\text{time elapsed}/180\text{ s} \times \text{Wattage Increment})$$

Maximal oxygen uptake was defined as the average value of oxygen consumed at peak effort over 20 seconds. Furthermore, the attainment of two of the following criteria was required:

1. VO_2 did not increase with greater intensity, resulting in a plateau showing less than a 0.2 L/min increase in oxygen uptake.
2. Heart rate within 10 beats per min of age predicted maximum (220- age)
3. Respiratory exchange ratio greater than 1.10
4. Physical exhaustion

$VO_{2\ Max}$ was in all cases expressed as volume in L/min.

Pulmonary gas exchange and blood sampling:

Pulmonary gas exchange analysis was used to quantify volumes of oxygen and carbon dioxide via indirect calorimetry. All data were collected in real time, and displayed online (Metasoft®, V7.9.1, Germany) on a dedicated

study computer connected to the indirect calorimetry system (Metalyzer 3BR2, Cortex, Germany). Breath by breath data were subsequently time weighted into 10 second averages, and pooled to calculate a grand average over this interval.

Calibrations and apparatus

Prior to every use, thorough calibration of the indirect calorimetry system was performed. Two point calibrations for gas concentration (ambient air vs. standardised calibrant gas, 17% O_2 /5% CO_2) were performed before every test and adjusted for barometric pressure. A known 3L volume syringe (Hans Rudolph, Germany) was used to calibrate gas flow through the pneumotachograph turbine. Real-time changes in temperature were adjusted automatically by a housed temperature sensor within the pneumotachograph casing. The pneumotachograph and gas sampling assembly was attached to snug fitting face masks (Hans Rudolph V2®, Cranlea, UK) sized appropriately for each participant.

Respiratory gas collections (Metalyzer 3BR2, Cortex Biophysik), obtained at the same times during exercise as blood was sampled, were performed continuously for 3 min prior to the onset of exercise. Thereafter respired gases were collected for 2 min intervals at identical time points to blood sampling.

Participants had a single 22 G (BD Venflon™, UK) venous catheter inserted percutaneously into their non-dominant forearm under aseptic conditions. Following insertion of the catheter, a small (2 ml) resting baseline sample of blood was taken in all trials, and a 3 way tap connecter (BD Connecta™, UK) was placed on the catheter hub to allow repeated blood draws.

During exercise, blood samples were obtained via the 3 way tap (2 ml) and the catheter flushed with a small, 1-2 ml, prime of sterile isotonic (0.9%) saline (BD, Plymouth, UK) to prevent coagulation. Blood samples obtained during exercise were immediately transferred into cooled 4 ml blood tubes containing ethylenediaminetetraacetic acid (EDTA) (BD Vacutainer, UK), and immersed in an ice bath at + 4ºC until the end of the test.

Ketone ester

This ketone ester has been designated as GRAS by the FDA allowing it to be used as a foodstuff in the USA. The chemical and physical properties of the KE are shown in Table S1.The safety and toxicology of this ester has been published previously (Clarke, Tchabanenko et al. 2012, Clarke, Tchabanenko et al. 2012). Prior to all studies involving competitive athletes, approval was obtained from the world anti-doping agency (WADA) who confirmed this nutritional ketone ester did not constitute a banned substance for use by athletes. Ketone ester was produced via the trans-esterification of the two major reagents R-1,3-butanediol (R-1,3-hydroxybutyl) and ethyl-β-hydroxybutyrate using enzymatic catalysts in a patented process to produce a > 99.8% purity of raw (D-β-hydroxybutyrate-R 1,3-Butanediol) monoester. Each batch of raw monoester was quality tested for impurities before use according to strict food grade standards as set out by the FDA. Smaller aliquots of raw ester were individually tested for microbial contamination (IFN, Reading, UK). All ketone ester was stored at or below room temperature in air tight containers to minimize contamination or exposure to environmental esterases.

Skeletal muscle biopsy:

Muscle tissue was collected via percutaneous needle biopsy technique, modified for use with a biopsy gun (Bard Monopty™, Bard biopsy systems, USA), from the lower third of the Vastus Lateralis muscle. All samples were obtained under aseptic conditions following skin cleaning with 0.5% chlorhexidine spray (Hydrex®, Ecolab Ltd, UK). Following this, 1-2 ml of local anaesthetic (1% Lidocaine hydrochloride without adrenaline, Hameln Pharmaceuticals, Gloucester, UK) was infiltrated into the subcutaneous layer, then a small incision was made in both the dermis and deep fascia to allow percutaneous passing of the biopsy device. Biopsy samples were obtained from new incisions at each time point with the direction of the needle angulated away from the previous pass to ensure undisturbed fibres were sampled on each occasion. Four passes were made on each sampling time point, with approximately 20-30 mg of muscle tissue obtained on each pass, stored separately in labelled eppendorf tubes. Once removed, tissue was immediately frozen in liquid nitrogen and stored at -80º C until further processing.

Metabolite extraction from skeletal muscle

Metabolites were double-extracted from approximately 100 mg tissue using a modified Folch method (Le Belle 2002). The aqueous and organic fractions were separated and further split into 2 identical volumes to allow multiple analyses.

^{1}H-NMR analysis of aqueous metabolites

Half of the aqueous fraction (~25 mg wet weight tissue) was dried under nitrogen, and resuspended in 600 µL D_2O containing 0.09% w/v NaCl (Sigma), 0.01% w/v NaN_3 (Sigma) and 0.25 mM deuterated sodium-3-trimethylsilylproprionate (NaTMSP-2,2,3,3-D4, Cambridge Isotope Laboratories, Inc.) as a chemical shift reference. Samples were analysed on a Bruker NMR spectrometer interfaced with an 11.8 Tesla superconducting magnet at 310K using a ^{1}H-NOESY 1D pulse sequence with 128 scans. Data were integrated using fixed integral sizes of 0.02 ppm within 1D Spec Manager (v12, Advanced Chemistry Development, Inc.Canada).

Carnitine analysis

Half the aqueous fraction was combined with half the organic fraction, and 200 µL acyl-carnitine standard containing eight deuterated species was added (Cambridge Isotope Laboratories, Inc. USA). Samples were dried under nitrogen and butylated with 3 M butanolic-HCl (Sigma). Samples were dried once more before resuspension in 200 µL acetonitrile containing 0.1% v/v formic acid (Sigma). Samples were analysed using multiple reaction monitoring on a Waters Quattro Premiere XE triple quadrupolar mass spectrometer and chromatograms integrated using QuanLynx v4.1 (Waters Ltd, Hertfordshire, UK).

TCA cycle and glycolytic intermediate analysis

Tissue extracts from the carnitine analysis were recovered, dried and resuspended in 50:50 water:acetonitrile containing universally-labelled glutamate as internal standard (Cambridge Isotope Laboratories Inc.). Metabolites were identified using a mass scan from *m/z* 50-1200 on a Waters Xevo G2 quadrupolar time-of-flight (qTOF) mass spectrometer. Tricarboxylic acid cycle intermediates and pyruvate were detected in butylated form and identified using fragmentation data from the high-energy function. Remaining glycolytic intermediates were detected underivatised and identified using fragmentation data. Chromatograms were integrated using QuanLynx v4.1 (Waters).

Lipid staining and quantification:

Histological staining techniques for intra-myocellular triglyceride (IMTG) obtained from muscle biopsy specimens were performed using the modified methods of Koopman, et al. (Koopman, Schaart et al. 2001). Muscle tissue (~30 mg) was embedded into Tissue-Tek™ (Sakura Finetek Europe, Zoeterwoude, the Netherlands) on dry ice, before sectioning with a Cryostat Microtome (Leica CM3050 S) into serial sections of 4 µm. Samples were chemically fixed for 1 hour using 3.7% formaldehyde solution (diluted in deionised water). Excess formaldehyde was removed by rinsing three times for 30 seconds in deionised water. Sections were then immersed in a working solution of ORO for 30 min. Samples were further rinsed and covered with a coverslip using 10% glycerol in phosphate-buffered saline (PBS). Image acquisition of samples was carried out immediately. Image analysis was performed using an Olympus Fluoview FV1200 microscope. ORO stained sections were examined using a Texas red excitation filter (540–580 nm). Images were analysed with ImageJ™ software to quantify the size and density of the lipid droplets giving results as the mean grey value.

PAS staining for muscle glycogen:

All steps were carried out at room temperature, using a protocol developed from the Periodic acid-Schiff (PAS) staining system kit (Sigma-Aldrich) and (Halkjaer-Kristensen and Ingemann-Hansen 1979). Samples were fixed for 5 minutes in Formalin-Ethanol Fixative Solution, and then subsequently rinsed under running tap water for 1 min. Slides were immersed in Periodic acid solution for 5 minutes, and repeatedly rinsed in distilled water. Schiff's reagent was then applied for 15 minutes before washing under running tap water for 5 minutes. Slides were counterstained in hematoxylin solution for 90 seconds and then rinsed under running tap water for 15–30 seconds. Sections acting as a negative control were pre-incubated with porcine pancreas α-amylase (Sigma-Aldrich) for 30 minutes. Images were obtained with a DSS1 Nikon Slide Scanner (x10magnification). Blinded, randomized,

analysis of n=200 individual muscle fibers was performed on each muscle sample, and graded according to their PAS staining intensity according to the methods of Gollnick and Saltin et al (Gollnick, Armstrong et al. 1973). All slides were reviewed by an experienced histopathologist.

Statistics:

Results are expressed as means ± SEM and significance was taken at $p < 0.05$. One subject declined to have skeletal muscle biopsies in study 2, otherwise all clinical and laboratory data were analysed for all subjects (no attrition or exclusions) with the exception of *^{1}H-NMR* muscle biopsy data from one athlete on the post exercise fat arm (Study 2) excluded as an outlier (> 4 SD from the group mean). Statistical analysis was performed using SPSS (V21, Chicago, USA). For the human trials containing paired data with 3 arms, repeated measures ANOVA was performed following initial tests to ensure sphericity assumptions were not violated, and then corrected with additional post-hoc Tukey corrections for multiple comparisons where appropriate. Paired data were compared using two tailed student-t tests. Correlations were tested using a two-tailed Pearson's test.

Sample size estimates:
Study 1:
No *a-priori* data on the influence of exercise on the kinetics of oral KE was available prior to this study. Previous data obtained from resting studies demonstrated a blood D-βHB concentration of 3 +/- 0.2 mM is reliably obtained following the ingestion of 500 mg/kg KE (Clarke, Tchabanenko et al. 2012). Furthermore previous infusions of radiolabeled ketone salts during moderate exercise demonstrated a 1-2 mM fall in blood concentration, and that 10-18% of VCO_2 production was attributable to ketone oxidation (Fery and Balasse 1986). Therefore to detect a 1 mM fall in circulating blood ketosis from a mean pre-exercise value of 3 mM, with SD of 0.2 mM with 80% power (α = 0.05) would require n=6 participants to reject the null hypothesis in a 2 tailed 3-way cross over design.

Study 2.
No *a-priori* data on the influence of ketosis on human skeletal muscle metabolism was available prior to this study. Therefore we estimated sample size based on the previous work conducted by Sato et al who provided glucose and a mixture of glucose and ketones in the working perfused rodent heart (to determine the energetic implications of altered substrate metabolism) (Sato, Kashiwaya et al. 1995). In this work it was shown the addition of ketones to working muscle reduced glycolytic intermediates by 2-3 fold whilst sustaining contractile function (Sato, Kashiwaya et al. 1995). Therefore we proposed that to detect a (conservative estimate) of 20% change in human skeletal muscle pyruvate (as a surrogate marker of glycolysis) with a SD of 12.5% with 80% power (α = 0.05) would require n=10 participants to reject the null hypothesis in a 2 tailed 3-way cross over design.

Study 3.
Data obtained in study 2 demonstrated a ~40 % higher intramuscular glycose concentration after KE (450 +/- 115) vs. CHO (275 +/-112) in n=10 athletes at the end of 1 h of exercise consistent with the downstream reductions in glycolytic intermediates observed. We reasoned that the addition of 60% of calories to the KE drink would reduce this difference if supplementary oral glucose could restore conventional muscle fuel preference for glucose. Therefore we estimated that to observe a 40% change in intramuscular carbohydrate content after 1 h of exercise between KE+CHO vs. carbohydrate with 80% power (α = 0.05) would require a sample size of n=8 athletes to reject the null hypothesis in a 2 tailed 3-way cross over design.

Study 4.
In study 2 and 3 we demonstrated a significant reduction in intramuscular glucose metabolism during exercise following KE ingestion vs. carbohydrate. We reasoned that this cumulative reduction in muscle glucose use would result in a sparing of total muscle glycogen content and a decrease in total intramuscular triacylglycerol levels. We validated the microscopy methods previously described to determine intra-muscular ORO signal intensity in needle biopsy specimens of 25-35 mg with mean values of 375 +/- 50 (AU) (n=8). We estimated that to detect a 24% change in triacylglycerol content published by (Wendling, Peters et al. 1996) to represent a 'meaningful' change in lipid content during exercise, with a SD of 13%, and 80% power (α = 0.05) would require a sample size of n=7 athletes in a 2-tailed t-test to reject the null hypothesis.

Study 5.

No *a-priori* data on the effect of nutritional ketosis on cycling performance were known before this study; however the high reliability (CV ~1-1.5%) of repeated bicycle ergometer trials in well trained athletes has been published previously (Currell and Jeukendrup 2008). Therefore to detect a 2% difference in performance with 80% power (α = 0.05) would require a sample size of n=8 athletes to reject the null hypothesis in a 2 tailed paired T-test.

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