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Article *in* AJP Cell Physiology · November 2023

DOI: 10.1152/ajpcell.00485.2023

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Defining ketone supplementation: the evolving evidence for post-exercise ketone supplementation to improve recovery and adaptation to exercise

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Keywords: training adaptations, exercise recovery, β-hydroxybutyrate, acetoacetate, post-exercise ketosis

Running title: Post-exercise ketosis to improve athletic performance

List of abbreviations

βHB: β-hydroxybutyrate

AcAc: acetoacetate

KE: ketone ester

PEKS: post-exercise ketone supplementation

BW: body weight

BDH1: β-hydroxybutyrate dehydrogenase

MCT1 : monocarboxylate transporter 1

SIRT1: sirtuin 1

SIRT3: sirtuin 3

HAT: histone acetyltransferase

HDAC: histone deacetylase

PGC-1α: peroxisome proliferator-activated receptor gamma coactivator 1α

H3K9: histone 3 lysine 9

H3K14: histone 3 lysine 14

p53: tumor protein p53

MyoD: myoblast determination protein 1

HIF-1α: hypoxia-inducible factor 1-alpha

Kbhb: histone lysine β-hydroxybutylation

FOXO1: forkhead box protein O1

BDNF: brain-derived neurotrophic factor

CS: citrate synthase

miRNAs: MicroRNA

mTOR: mammalian target of rapamycin

AMPK: 5' AMP-activated protein kinase

GLUT-4: glucose transporter type 4

Akt: phosphorylation of protein kinase B

GDF15: growth differentiation factor 15

GPCRs: G-protein coupled receptors

FFAR2: free fatty acid receptor 2

MEF2A: myocyte-specific enhancer factor 2A

CaMKKß: Ca^{2+}/calmodulin-dependent protein kinase beta

FFAR3: free fatty acid receptor 3

PUMA-G: protein upregulated in macrophages by interferon-gamma receptor

ANP: atrial natriuretic peptide

SOD2: superoxide dismutase 2

CAT: catalase

Nrf2: nuclear factor erythroid 2-related factor 2

NF-kB: nuclear factor kappa-light-chain-enhancer of activated B cells

TNF- α: tumor necrosis factor alpha

iNOS: inducible nitric oxide synthase

IL-β: interleukine-1 beta

NLRP3: NLR family pyrin domain containing 3

GABA: gamma-aminobutyric acid

NREM: non-rapid eye movement

VGLUT: vesicular glutamate transporters

EPO: erythropoietin

SGLT2: sodium-glucose co-transporter 2

VEGF: vascular endothelial growth factor

eNOS: endothelial nitric oxide synthase

Abstract

Over the last decade, there has been a growing interest in the use of ketone supplements to improve athletic performance. These ketone supplements transiently elevate the concentrations of the ketone bodies acetoacetate (AcAc) and D-β-hydroxybutyrate (βHB) in the circulation. Early studies showed that ketone bodies can improve energetic efficiency in striated muscle compared to glucose oxidation and induce a glycogen-sparing effect during exercise. As such, most research has focused on the potential of ketone supplementation to improve athletic performance via ingestion of ketones immediately before or during exercise. However, subsequent studies generally observed no performance improvement, and particularly not under conditions that are relevant for most athletes. However, more and more studies are reporting beneficial effects when ketones are ingested after exercise. As such, the real potential of ketone supplementation may rather be in their ability to enhance post-exercise recovery and training adaptations. For instance, recent studies observed that post-exercise ketone supplementation (PEKS) blunts the development of overtraining symptoms, and improves sleep, muscle anabolic signaling, circulating erythropoietin levels, and skeletal muscle angiogenesis. In this review, we provide an overview of the current state-of-the-art about the impact of PEKS on aspects of exercise recovery and training adaptation, which is not only relevant for athletes but also in multiple clinical conditions. In addition, we highlight the underlying mechanisms by which PEKS may improve exercise recovery and training adaptation. This includes epigenetic effects, signaling via receptors, modulation of neurotransmitters, energy metabolism, and oxidative and anti-inflammatory pathways.

Word count: 243

Introduction

Ketone bodies – namely D-ß-hydroxybutyrate (ßHB), acetoacetate (AcAc), and acetone – are molecules that are continuously produced from the breakdown of free fatty acids. Ketogenesis primarily occurs in the liver mitochondria, and to a lesser extent in astrocytes and kidney cells (1). The production of ketone bodies is upregulated during periods of reduced carbohydrate availability and increased lipolysis, such as starvation, fasting, or by following a low-carbohydrate, high-fat 'ketogenic diet' (1). The belief that increasing blood ketone bodies may be advantageous for athletic performance is already long-standing. This was primarily sparked by studies in the mid and the end of the 20th century reporting that ketone bodies (i) inhibit glycolytic activity (2, 3), (ii) act as an alternative energy substrate (2, 4), and (iii) possess thermodynamic advantages over glucose oxidation by enhancing the free energy release upon ATP hydrolysis (5). Nevertheless, these benefits of ketone bodies do not seem to outweigh the negative effect of the low carbohydrate availability on high-intensity performance that is inherent to strategies that increase endogenous ketone production (6). However, the advent of ketone supplements, in particular the ketone monoester (R)-3-hydroxybutyl (R)-3-hydroxybutyrate, which enabled the induction of a transient state of ketosis regardless of the availability of other macronutrients, has sparked a renewed interest in the potential of ketone bodies to improve athletic performance.

Despite this renewed interest and a seminal paper showing that ingestion of this ketone monoester improved 30 min cycling time-trial performance following a 2h submaximal exercise bout (7), multiple studies with higher ecological validity have recently reported either no (8–15) or even a slightly negative effect (16–19) of ketone ester (KE), ketone salt (20, 21), or ketone precursor (22–24) ingestion on acute exercise performance. In contrast to the diminished evidence for an acute ergogenic effect, a growing body of research suggests that the true potential of exogenous ketosis may be in their ability to improve post-exercise recovery and the adaptive response to exercise (25). Therefore, the aim of this review is to discuss the current state-of-the-art about the various physiological mechanisms by which post-exercise ketone supplementation (PEKS) can potentially augment exercise recovery and long-term training adaptations. Specific focus will be directed towards the various signaling effects resulting either directly from ketone bodies or from ketone body metabolism.

Exogenous ketosis: the best of both worlds?

The physiological effects of ketosis have been traditionally explored through endogenous ketosis induced by either fasting or adherence to a low-carbohydrate, high-fat ketogenic diet. These nutritional interventions increase the reliance on fat *vs.* glucose oxidation to generate ATP (6, 26, 27). However, fasting is obviously undesirable for athletes due to the generated energy deficit and the unfavorable metabolic perturbations (28). Adherence to a ketogenic diet has been associated with training

adaptations that can be considered as beneficial such as increased fat oxidation during exercise (29). However, these elevated fat oxidation rates are accompanied by negative adaptations including a drop in exercise economy (e.g. requiring a higher oxygen demand for a given exercise load) (30), impaired training capacity (6), and diminished performance improvements (30).

Interestingly, the concentration of circulating ketone bodies can also be increased via the intake of ketone precursors such as ketone salts or ketone esters (see figure 1 for an overview of the different ketone supplements). These ketone supplements were developed because of the acidic nature of AcAc and βHB making direct administration unviable (31). Nevertheless, it is noteworthy that a recent study has shown oral intake of pure βHB (10g) to be well tolerated in healthy humans. However, blood βHB levels were not assessed in that study, and blood acid-base balance was unaffected, making it unclear whether pure βHB effectively raised blood ketone body levels (32).

To overcome the acidity issue of AcAc and βHB, researchers initially used the oral administration or intravenous infusion of AcAc and βHB bound to a cation, typically sodium or potassium. This method allowed for the investigation of the physiological effects of ketone bodies without the confounding physiological milieu of endogenous ketosis. However, ketone salts are not ideal for athletes due to the high salt content and high incidence of gastrointestinal distress (33). Similarly, ketone precursors like (R,S)-1,3-butanediol elevate blood ketone levels via hepatic conversion to βHB and AcAc (34). Yet, due to their rapid oxidation in the liver through alcohol metabolism, high doses of (R,S)-1,3-butanediol can result in hypoglycemia (31). Additionally, their intake is often associated with side effects such as gastrointestinal distress and nausea (23).

These issues can be avoided by the creation of an ester bond between a ketone body precursor and a ketone body. The first synthesized KE was monoacetoacetin, a water-soluble ketone monoester composed of glycerol and acetoacetate which was developed by Birkhahn et al. (35) in 1978. Their research demonstrated that upon intravenous administration in rats, this KE is hydrolyzed into glycerol and AcAc, resulting in increased levels of circulating ketone bodies. Nevertheless, to our knowledge, this KE has never been used in clinical studies. It was until 2012 that the first KE – namely the previously mentioned ketone monoester (R)-3-hydroxybutyl (R)-3-hydroxybutyrate – underwent human safety and tolerability trials (36). Upon oral administration, carboxylesterases and esterases in the gastrointestinal tract, blood, liver, and other organs completely hydrolyze the KME to ßHB and the ketone body precursor (R,S)-1,3-butanediol. Subsequently, alcohol- and aldehyde dehydrogenases in the liver metabolize (R,S)-1,3-butanediol into ßHB and AcAc. Pharmacokinetic investigations have showed that a single bolus of 282 mg.kg body weight^{-1} (BW) of this KE leads to a rapid and transient increase in ketone bodies, with ßHB peaking at ~2.8 mM and AcAc peaking at 0.7 mM after 1 to 2h under fasted conditions (36). This KE has also been shown to effectively induce ketosis during and after exercise, as well as in combination with the intake of other macronutrients. However, under these

conditions, the degree of ketosis is significantly lower compared to resting conditions or isolated intake (7, 36). In contrast to ketone salts, the ingestion of this KE neither provokes significant gastrointestinal distress, nor a significant elevation of the S-isoform of βHB (36–38). As such, this KE is highly suitable to determine the effects of ketosis on athletic performance and health in an ecologically valid manner.

Since 2012, other KE have been used in clinical trials including the (R,S)-1,3-butanediol acetoacetate diester (16) and the bis hexanoyl (R)-1,3-butanediol diester (39–41). All these KE are rapidly metabolized, resulting in a rapid and robust increase in blood ketone body concentrations. However, each of these esters are metabolized into different amounts of ketone molecules and ßHB isoforms. For instance, the AcAc diester provides a racemic equivalent of βHB in contrast to the R-ßHB of the ketone monoester and the bis hexanoyl (R)-1,3-butanediol diester (40). This is important because the R-βHB enantiomer is the typical endogenous product of ketosis resulting from the last step of ketogenesis in which β-hydroxybutyrate dehydrogenase (BDH1) reduces AcAc to R-βHB. In contrast, S-βHB is metabolized more slowly and exerts fewer signaling effects than R-βHB (38, 42). Furthermore, endogenous ketosis yields higher concentrations of βHB relative to AcAc in the blood (typically 2:1 to 4:1 βHB:AcAc ratio) (43). This heightened βHB *vs.* AcAc concentration is likely evolutionary advantageous, as current research suggests that βHB exerts more signaling functions compared to AcAc (44).

To fully exploit the signaling functions of ketone bodies, it seems therefore crucial that the KE produces a significant increase in R-βHB levels. Nonetheless, the selection of a specific KE may primarily depend on the desired effect. For example, a study involving the injection of either AcAc or βHB into the brain of mice found that only AcAc increased the amount of slow-wave sleep (45).

Post-exercise ketosis

As previously mentioned, blood ketone body levels can be increased via starvation, fasting, adherence to a ketogenic diet or by prolonged endurance exercise. After exercise, and especially following endurance exercise, blood ketone body concentrations can increase up to ~0.3 – 1.8 mM (46, 47). This increase was first observed in 1909 by Forssner (48), who reported an increased presence of acetone in his urine on days when he engaged in walking activities, which has been used later on as an index of performance (49). However, only from the second half of the 20th century onwards the underlying physiological mechanisms of post-exercise ketosis were investigated (50).

These studies reported that the rise in ketone body concentration following exercise is dictated by different factors (figure 2). For instance, glucose ingestion 2h post-exercise blunts post-exercise ketosis, whereas such inhibitory effect does not occur when glucose is ingested immediately after exercise (51). Moreover, the degree of post-exercise ketosis appears to be inversely related to liver glycogen content, suggesting that post-exercise ketosis is a result of energetic stress (52). This is

supported by the observation that high carbohydrate intake before exercise blunts post-exercise ketosis, whereas high fat intake rather elevates post-exercise ketosis (53). The extent of post-exercise ketosis is also influenced by both exercise intensity and duration, with post-exercise ketosis being predominantly increased following moderate exercise of long duration (54). Based on these observations, it has been suggested that post-exercise ketosis early after exercise is determined by liver glycogen depletion and glycolytic flux, while later in recovery (e.g. after several hours) it is regulated by insulin levels and free fatty acid concentrations (33). Furthermore, cross-sectional data also indicates that the extent of post-exercise ketosis tends to increase with age, although with high interindividual variation (53).

The degree of post-exercise ketosis is higher in untrained individuals compared to trained athletes, although the underlying physiological mechanism is not yet elucidated (46). Most likely, the production of ketone bodies in athletes is inhibited due to a lower mobilisation of liver glycogen during exercise (55). Furthermore, training enhances the capacity to oxidize ketones through upregulation of the monocarboxylate transport 1 (MCT1) and ketolytic enzymes (33). Earlier research also indicated that the ketotic response to a high-fat meal (56) and to fasting (57) appears to be higher in females compared to males. This suggests that the extent of post-exercise ketosis may also be higher in females but this has not yet been investigated. However, the opposite may also be true given that women for instance show a relatively lower reliance on liver glycogen during moderate intensity exercise (58).

The elevation of blood ketone body concentration following exercise implies a potential physiological role for these molecules in post-exercise recovery and exercise adaptation. However, post-exercise ketosis resulting from endogenous production is rapidly diminished by standard post-exercise nutritional practices such as the intake of a protein-carbohydrate recovery drink (59). Moreover, emerging evidence indicates that AcAc and βHB elicit effects similar to those typically observed in response to exercise and which are implicated in the adaptive response to exercise training. These effects include among others the inhibition of histone deacetylation and the upregulation of sirtuins 1 (SIRT1) and 3 (SIRT3) (discussed below in more detail). This suggests that PEKS may induce a unique physiological milieu to enhance post-exercise recovery and exercise adaptation as it allows to benefit from the potential beneficial effect of post-exercise ketosis in combination with other nutritional exercise recovery strategies (e.g., carbohydrate-protein recovery drink). Therefore, in the following section, we will outline the metabolic and signaling effects of AcAc and βHB that are potentially relevant in the context of exercise recovery and training adaptation. For an overview of the general metabolic or signaling roles of ketone bodies, the reader is referred to earlier reviews (1, 42, 44, 60).

Mechanisms by which KBs can impact exercise recovery and training adaptation

Ketone bodies are pleiotropic signaling molecules that affect cellular pathways in a wide variety of tissues. Therefore, in the next section, we provide an overview of the cellular mechanisms by which ketone bodies can potentially impact exercise recovery and adaptation. A graphical overview of these mechanisms is provided in figure 3.

Epigenetics

Histone lysine acetylation

Emerging evidence indicates that chromatin remodeling plays a significant role in controlling the expression of genes that underly exercise adaptations. A crucial post-translational modification governing chromatin structure is the acetylation or deacetylation of histone proteins. The degree of histone acetylation is regulated by the balance between histone acetyltransferase (HAT) and histone deacetylase (HDAC) activities, with an increased acetylation level generally associated with enhanced transcriptional activity (61). Research indicates that the inhibition of especially class IIa HDACs plays a crucial role in exercise adaptation, as (i) these enzymes are highly expressed in skeletal muscle, (ii) are markedly inhibited by endurance exercise, and (iii) given that HDAC inhibition increases exercise responsive genes such as peroxisome proliferator-activated receptor gamma coactivator 1α (PGC-1α) (62). The importance of HDAC inhibition for skeletal muscle plasticity has been demonstrated in experiments with HDAC inhibitors, such as Scriptaid. Acute administration of this potent class IIa HDAC inhibitor results in an upregulation of oxidative genes and improves exercise capacity in rodents (63).

Interestingly, ketone bodies have the potential to mimic aspects of exercise adaptation in a similar manner by inhibiting HDACs and by increasing the intracellular acetyl-CoA pool, thereby increasing the substrate availability for HATs (1, 42). Both βHB and AcAc inhibit class I and class IIa HDACs, although βHB has a stronger effect on HDAC activity. Specifically, βHB has been shown to inhibit HDAC1, HDAC3, and HDAC4 in HEK293T cells with IC_{50} values of 5.3 mM, 2.4 mM, and 4.5 mM, respectively. These concentrations are achievable during both endogenous and exogenous ketosis (64). However, IC_{50} values for HDAC6 (48.5 mM) and those for AcAc were much higher (*e.g.,* 11.0 mM, 8.3 mM, and 29.0 mM for HDAC1, 4 and 6, respectively) which questions their physiological relevance. Furthermore, increasing blood βHB concentrations either via a 24-hour fast (βHB: ~1.5m M), caloric restriction (βHB: ~0.5 mM), or intraperitoneal administration of βHB, has been shown to increase the acetylation of histone 3 lysine 9 (H3K9) and H3K14 in various tissues in mice including kidney, brain and liver tissue (64). In agreement, lysine acetylation in skeletal muscle has been shown to increase in mice upon a 2 month ketogenic diet (65). While histones represent the primary target of HDACs, the inhibition of HDAC activity can also increase acetylation and activation of non-histone

proteins involved in exercise adaptation, such as tumor protein p53 (p53), myoblast determination protein 1 (MyoD) and hypoxia-inducible factor 1-alpha (HIF-1α) [1,31,39].

Histone lysine β-hydroxybutylation

Recent studies have demonstrated that βHB can also induce post-translational histone modifications through histone lysine β-hydroxybutylation (Kbhb). This has been shown for the first time by Xie et al., (66) who revealed that Kbhb levels increased in a dose-dependent manner in HEK293 cells upon exposure to increasing βHB concentrations (e.g. 2, 5 and 10 mM). Furthermore, increasing blood βHB endogenously either via a 48h fast or streptozotocin-induced diabetic ketoacidosis increased Kbhb levels in the liver of mice. The specific effects of Kbhb in exercise adaptation are not yet elucidated, but recent studies suggest that Kbhb is associated with increased transcription of genes involved in the adaptive response to exercise. Notably, elevated concentrations of βHB mitigate the development of aortic endothelial cell injury, potentially by promoting the transcription of vascular endothelial growth factor (VEGF) via H3K9bhb (67). Furthermore, βHB acting through Kbhb, enhances mRNA expression of important genes such as forkhead box protein O1 (FOXO1), brain-derived neurotrophic factor (BDNF), PGC-1α, and citrate synthase (CS). These findings suggest that Kbhb may represent a crucial mechanism by which ketone bodies influence the adaptive response to exercise (68, 69).

Regulation of sirtuins

A key factor in regulating the adaptive response to exercise training involves the decrease in myocellular energy status during exercise. This decrease results from the increased turnover of energy molecules such as ATP and NADH, leading to elevated intramyocellular concentrations of AMP and NAD^+ (70). Elevated concentrations of NAD^+ activate NAD^+-dependent sirtuins, which, in turn, deacetylate both histone and non-histone proteins. Research has shown that exercise-induced elevations of especially SIRT1 and SIRT3 are potentially important for exercise adaptation, as both SIRT1 and SIRT3 enhance the expression of genes such as PGC-1α and p53 that are associated with an improved skeletal muscle oxidative phenotype (70–72).

Mechanistically, ketone bodies may increase the activity of SIRT1 and SIRT3 indirectly by elevating the cytoplasmic NAD^+/NADH ratio compared to glucose oxidation. The oxidation of βHB requires only two NAD^+ molecules during the conversion of βHB to acetyl-CoA, while glycolysis requires four NAD^+ molecules. Furthermore, βHB exclusively consumes NAD^+ molecules in the mitochondria, whereas glycolysis consumes two NAD^+ molecules in the cytosol (42). Nonetheless, it remains unclear whether ketone bodies can effectively increase the NAD^+/NADH ratio in skeletal muscle. One study reported that a ketogenic diet in 14-month-old mice increases the activity of SIRT1 and SIRT3 in skeletal muscle (65), while another study found that the administration of R-β-hydroxybutyric acid sodium salt had no effect on SIRT1 and SIRT3 levels in cultured myotubes (73). In contrast, stronger evidence indicates that ketone bodies increase the NAD^+/NADH ratio in neurons, as well as the

expression of SIRT1 and SIRT3 (74–76). This suggests that ketone bodies may indeed be capable of altering the NAD^+/NADH ratio and increase sirtuin activity.

DNA methylation

Limited evidence indicates that ketone bodies modify gene expression through inhibition of DNA methylation, a process typically associated with increased gene transcription (77). Multiple studies in murine models (78, 79) and humans (80) demonstrated that one of the primary mechanisms by which a ketogenic diet improves epilepsy is through inhibition of DNA methylation in the brain. This inhibition is thought to be a result of the observed decrease in adenosine kinase levels in response to a ketogenic diet. The decrease in adenosine kinase subsequently leads to elevated adenosine levels, a crucial end product required for transmethylation, which ultimately results in the inhibition of DNA methylation (79).

Interestingly, research indicates that both aerobic and resistance exercise primarily induce DNA hypomethylation, leading to a more functional transcriptive state. For example, aerobic exercise has been demonstrated to reduce DNA methylation in the promotor region of the PGC-1α gene, and this decrease is inversely correlated with PGC-1α expression (77, 81). However, is remains to be identified if DNA hypomethylation induced by a ketogenic diet is a direct result of ketogenesis, or results from other metabolic changes induced by the diet. Additionally, it is unclear whether these effects also occur in skeletal muscle tissue. Nonetheless, a 12-week ketogenic diet has been shown to induce a genome-wide decrease in whole-blood methylation in epileptic patients. Interestingly, these changes were not only associated with genes involved in epilepsy, but also with fat metabolism and transcriptional regulation (80). Further research is needed to investigate the specific role of ketone bodies in DNA methylation and their interaction with exercise adaptation.

MicroRNAs

MicroRNA (miRNAs) are non-coding RNA strings that bind to mRNA via a complementary sequence, thereby inhibiting mRNA translation. A growing body of evidence indicates that miRNA expression is regulated by both aerobic and resistance exercise and is also involved in the adaptive response to exercise (82). Recent research shows that a ketogenic diet alters circulating miRNA levels (*e.g.,* hsa-let-7b-5p, hsa-miR-143-3p, hsa-miR-143-3p, hsa-miR-504-5p) in humans following a 6-week ketogenic diet (βHB: ~0.5 mM). Interestingly, the specific miRNAs altered by the ketogenic diet are known to target specific genes implicated in mammalian target of rapamycin (mTOR), PPAR, insulin, and inflammatory signaling pathways (83). Consequently, further research is warranted to determine the impact of ketone bodies on miRNA expression and whether this constitutes a potential mechanism through which ketone bodies improve exercise adaptations.

Cellular energy status

Studies in rats (84) and humans (85, 86) indicate that ketone bodies enhance the restoration of cellular energy status after exercise. These studies found that PEKS blunts the increase in 5' AMP-activated protein kinase (AMPK) phosphorylation in skeletal muscle following exercise. (85). Mechanistically, the attenuation of the post-exercise induced increase in AMPK activation by ketone bodies, likely results from the potential of ketone bodies to serve as an additional energy substrate for ATP production [30]. In this perspective, we observed that KE ingestion attenuates the increase in serum growth differentiation factor 15 (GDF15) – a biomarker of cellular stress – both immediately after exercise as well as for up to at least 1h post-exercise (14, 15). Furthermore, GDF15 has recently been identified to activate AMPK in skeletal muscle, allowing us to speculate that at least part of the inhibitory effect of ketone bodies on post-exercise AMPK activation occurs via an attenuation of GDF15 (87).

The specific consequences of ketone body-induced inhibition of post-exercise AMPK phosphorylation are not known. Yet, this may impact several processes involved in exercise recovery and adaptation. First, AMPK activation is required to promote post-exercise fatty acid oxidation and skeletal muscle glucose uptake, likely by delaying glucose transporter type 4 (GLUT-4) endocytosis which in turn increases sarcolemmal GLUT-4 abundance (88). In addition, the activation of AMPK that occurs in response to exercise is also indispensable for the improved muscle insulin sensitivity that occurs for up to 48h after exercise (89). Interestingly, this effect is most likely mediated via phosphorylation of AMPK's downstream factor AS160 (90). Given that Takahisha et al. (84) observed an increase *vs.* a decrease in AS160 upon incubation with ßHB for 15 min vs. 2h suggests that ketone bodies first increase AS160 activation via phosphorylation of protein kinase B (Akt) early in the post-exercise recovery period, whereafter AS160 decreases via an inhibition of AMPK phosphorylation. Beyond its role in post-exercise energy metabolism, AMPK also regulates the expression of genes implicated in exercise adaptation. For instance, heightened AMPK activity enhances mitochondrial biogenesis and the expression of mitochondrial enzymes (91). Consequently, it is equally possible that downregulation of AMPK activity by KE ingestion negatively impacts certain adaptations to exercise.

G-receptor signaling

G-protein coupled receptors (GPCRs) mediate cellular responses to a wide variety of external agents. AcAc and βHB are known to interact with at least three GPCRs that are involved in metabolic regulation. First, AcAc functions as an agonist for the GPR43 receptor, also known as free fatty acid receptor 2 (FFAR2). In murine models, it has been demonstrated that AcAc-induced GPR43 signaling increases lipolysis by upregulating lipoprotein lipase activity under ketotic conditions such as fasting or a ketogenic diet. GPR43 deficient mice show also a disturbed lipid profile and reduced weight loss during fasting compared to wild-type mice (92). Furthermore, in rat L6 myotubes, activation *vs.*

278 silencing of GPR43 respectively increased *vs.* decreased the expression of genes and proteins that are
279 increased upon exercise including myocyte-specific enhancer factor 2A (MEF2A), PGC-1α, and
280 Ca^{2+}/calmodulin-dependent protein kinase beta (CaMKKß) (93). It has also been demonstrated that
281 GPR43 activation by a low dose of sodium butyrate (0.2 mM) stimulates neovascularization, increases
282 VEGF levels, and extracellular matrix remodeling in a model of granulation tissue formation in mice
283 (94).

284 Second, βHB interacts with two GPCRs, namely GPR41 and GPR109A. Just like GPR43, GPR41 (or
285 FFAR3) is also involved in the regulation of energy homeostasis (95). Of particular interest, a study by
286 Kimura et al. (96) demonstrated that βHB acts as a potent antagonist of GPR41. Administration of
287 βHB at a dose of 500 mg.kg BW^{-1} in mice reduces heart rate and mitigates the propionate-induced
288 elevation in both oxygen consumption and extracellular signal-regulated protein kinase 1/2 (ERK1/2)
289 activity. This suggests that βHB directly influences energy balance by inhibiting GPR41-mediated
290 sympathetic activation. However, it is important to note that the precise role of βHB in this context
291 remains unclear, as another study showed that βHB functions rather as an agonist for GPR41 (97).
292 Nevertheless, it is possible that mediation of GPR41 activity is involved in the ability of PEKS to
293 alleviate sympathetic overactivity during overload training (98).

294 In addition, βHB acts as a potent agonist of the nicotinic acid receptor GPR109a (EC_{50} = 0.7 mM),
295 also known as the protein upregulated in macrophages by interferon-gamma receptor (PUMA-G)
296 which is highly expressed in adipocytes and results in the inhibition of lipolysis. This function stands
297 in contrast to the lipolytic effect induced by AcAc through GPR43 signaling. It is conceivable that the
298 lipolytic effects of AcAc are counteracted by the inhibitory impact of βHB on lipolysis through
299 GPR109a signaling. This hypothesis is supported by consistent observations of reduced plasma free
300 fatty acids upon KE administration, both at rest (38) and during exercise (14, 15, 18, 99). Furthermore,
301 we recently demonstrated that exogenous ketosis during exercise decreased plasma atrial natriuretic
302 peptide (ANP) levels, which may also contribute to the anti-lipolytic effect of ketone bodies (13).

303 **Oxidative stress and inflammation**

304 Both AcAc and βHB have been shown to exhibit anti-inflammatory and anti-oxidative properties (1,
305 100). The suppression of exercise-induced inflammation and oxidative stress can be beneficial for
306 acute exercise recovery following extreme exercise. However, prolonged suppression of inflammation
307 and oxidative stress is potentially detrimental for training adaptations, as it reduces the activation of
308 pathways involved in exercise adaptation (101, 102).

309 βHB acts directly as an antioxidant for hydroxyl radicals and indirectly activates the cellular
310 antioxidant system through various mechanisms (103). For instance, by increasing the NAD^+/NADH
311 ratio, it activates SIRT1 and SIRT3. SIRT1, in turn, activates the transcription factor FOXO3, which
312 promotes the expression of antioxidant enzymes such as superoxide dismutase 2 (SOD2) and catalase

(CAT). SIRT3 also increases the activation of SOD2 and other anti-oxidative genes (103). Additionally, increased histone acetylation resulting from inhibition of class I HDACs, enhances the transcription of FOXO1 and FOXO3 (64, 103, 104). Furthermore, elevated ketone body oxidation rates increase the activity of nuclear factor erythroid 2-related factor 2 (Nrf2) through increased fumarate production. These transcription factors collectively promote the expression of several proteins involved in the cellular antioxidant system (103). FOXO1, FOXO3, and Nrf2 also stimulate signaling pathways involved in fatty acid oxidation, mitochondrial function, and skeletal muscle angiogenesis (105, 106).

Moreover, by activating the GPR109a receptor, βHB suppresses nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) mediated pro-inflammatory signaling and downregulates the expression of tumor necrosis factor alpha (TNF- α), inducible nitric oxide synthase (iNOS), and interleukine-1 beta (IL-β) (1, 107). βHB has also been shown to inhibit the NLR family pyrin domain containing 3 (NLRP3) inflammasome, resulting in reduced cytokine release (108). This indicates that AcAc and βHB primarily activate anti-inflammatory and anti-oxidative pathways. Yet, in several in vitro experiments in which high concentrations of βHB and AcAC were used, ßHB and AcAc rather increased oxidative stress and inflammatory signaling (1).

Neurotransmitters

Exercise modulates the release of several neurotransmitters involved in our perception of fatigue, mood, motivation and cognition (109, 110). Dysregulations in neurotransmitter levels are implicated in the development of central fatigue, overtraining, and sleep disturbances in athletes (109). Interestingly, it has been shown that raising brain ketone body levels influences the concentration of multiple neurotransmitters in the brain which may be a possible explanation for the observed mitigation of exercise-induced decrements in cognitive performance (10, 86) and sleep (45, 111) by KE.

A magnetic resonance spectroscopy study conducted in healthy humans demonstrated that the administration of 395 $mg.kg^{-1}$ body weight KE reduced the levels of glutamate and gamma-aminobutyric acid (GABA) in the brain cortex compared to glucose administration (112). A similar reduction in glutamate levels has also been observed in mice upon injection of lithium AcAc, but not sodium ßHB (45). Such reduction may improve sleep by suppressing neuronal excitability through inhibition of glutamatergic pathways (113), and this link is supported by the fact that the decrease in glutamate upon lithium AcAc injection was accompanied by increased slow-wave activity during non-rapid eye movement (NREM) sleep. The specific mechanism by which ketone bodies reduce glutamate levels is not yet fully understood. One possible explanation is the allosteric competition between chloride ions and AcAc for binding to vesicular glutamate transporters (VGLUT), which are responsible for storage and release of glutamate in synaptic vesicles. Chloride ions allosterically

regulate the uptake of glutamate into synaptic vesicles via VGLUT. However, AcAc competes with chloride for allosteric binding to VGLUT, which in turn reduces the uptake and subsequent release of glutamate (114). Other possible mechanisms include βHB-induced activation of kynurenic acid (115), increased cerebral ketone body oxidation (116), and anaplerotic use of glutamate under ketogenic conditions (112, 117).

Additionally, it has been shown that a ketogenic diet increases extracellular adenosine levels by downregulating the expression of the enzyme adenosine kinase (114, 118). Adenosine is considered as a potent homeostatic regulator of sleep, as extracellular concentrations increase during prolonged wakefulness and induce sleep and especially slow-wave sleep by acting on adenosine A1 receptors (113). Furthermore, animal experiments have shown that a ketogenic diet increases dopamine activity in the brain (119). This finding is further supported by research from our group demonstrating that KE during ultra-endurance exercise (86) and before sleep (111) increases dopamine levels respectively in plasma and urine. Such increases in dopamine levels could be important for athletes, as low dopamine levels are associated with central fatigue (109), resilience (120) and impaired cognitive performance (121), and given that dopamine signaling is involved in the regulation of NREM and REM sleep (119).

State of the art: impact of ketone bodies on exercise recovery and training adaptations

In the next section, we discuss the current state-of-the-art regarding the impact of PEKS on aspects that are implicated in exercise recovery and training adaptation. A graphical overview of these physiological mechanisms can be found in figure 4.

Muscle glycogen resynthesis

Immediately following exercise, there is a high priority to restore intra- and extra-muscular energy stores. This typically includes the replenishment of muscle glycogen as one of the key priorities, given that muscle glycogen content is positively related with high intensity exercise performance (122). After exercise, and with adequate carbohydrate provision, this is usually accomplished within 24h via a dramatic increase in glycogen synthase activity, glucose transport and enhanced sensitivity of skeletal muscle to insulin (123).

First evidence that PEKS improves net muscle glycogen resynthesis was provided in humans using a KE (124). Following an intense cycling session that depleted muscle glycogen, well trained athletes ingested either KE or a non-caloric control drink followed by a 2h hyperglycemic clamp (10 mM glucose). Interestingly, muscle glycogen repletion during the initial 2h post-exercise recovery period was ~60% higher in the KE *vs.* placebo condition. This was accompanied by a 32% higher whole-body glucose uptake which the authors attributed to the twofold higher insulin concentrations throughout the recovery period. These data are in line with a recent *ex-vivo* study in which sodium (R,S)-ßHB dose-dependently (1 to 4 mM) increased post-exercise glycogen repletion in the anconeus epitrochlearis muscles of mice that were incubated with 8 mM glucose and 60 $\mu U.mL^{-1}$ insulin (84). These authors also observed an increased phosphorylation of Akt^{Thr308} and its downstream protein Akt substrate AS160 in the obtained muscles. Given that this pathway plays a central role in insulin-stimulated glucose uptake further supports the idea that ketones enhance muscle glycogen synthesis in an insulin-dependent manner. Interestingly, these increased phosphorylation statuses occurred only following 15 min of incubation, whereas the phosphorylation status of both Akt and AS160 was decreased after 2h of incubation. This is in line with earlier reports indicating that R-ßHB but not S-ßHB inhibits insulin-mediated glucose uptake in skeletal muscle upon exposures longer than 9 h. This effect was also more pronounced in oxidative compared to glycolytic muscles suggesting that mitochondrial metabolism may be involved in the inhibitory effect on glucose transport (125).

Despite these promising findings, some important caveats should be noted. First, the control drink used in the first study was non-caloric, while the KE provided ~4.7 $kcal.g^{-1}$. Second, the authors in the first study attributed the higher glycogen resynthesis to the KE-induced doubling of insulin concentrations. However, when glycogen concentration is below 150 $mmol.kg^{-1}$ dry weight, glycogen synthesis occurs independently of insulin concentration during the first 30-60 minutes post-exercise (126). Third, glucose levels were standardized in both studies and were maintained at a

supraphysiological level (10 mM) in the human study, while a recent meta-analysis concluded that KE ingestion decreases blood glucose by ~0.5 mM (127). Fourth, both studies were performed after an overnight fast which is known to enhance glucose uptake and glycogen synthesis in skeletal muscle due to lower glycogen levels (126).

A more recent study performed by our research group assessed the effect of PEKS on post-exercise muscle glycogen resynthesis in a more ecologically valid design involving a pre-exercise carbohydrate-rich breakfast (85). Following a single leg glycogen depletion protocol, participants received a recovery drink providing 1 g.kg $BW^{-1}.h^{-1}$ carbohydrates and 0.3 g.kg $BW^{-1}.h^{-1}$ hydrolyzed whey-protein concentrate. They received in addition either a KE drink of 0.5 g.kg BW^{-1} immediately after exercise, followed by 0.25 g.kg $BW^{-1}.h^{-1}$, or an isocaloric placebo drink at similar timepoints. The KE resulted in slightly lower blood glucose levels throughout the recovery period (~6 mM in KE *vs.* ~7 mM in placebo). However, muscle glycogen concentrations, as well as plasma insulin levels were unaffected by KE during the recovery period. The most plausible explanation for these conflicting results is the diverse effect on glucose and insulin levels between both studies. In this perspective, the insulinotropic effect of ketone bodies are not observed when blood glucose concentrations are below 8 mM in healthy humans (85, 128–130). A potential mechanism behind the insulinotropic effect of ketones involves the utilization of βHB and AcAc as metabolic intermediates for anaplerosis within β-cell mitochondria (131). This explains why elevated insulin levels following KE ingestion are exclusively observed in situations of high glucose concentrations, coinciding with an increased demand for insulin production and secretion.

These data indicate that PEKS can increase muscle glycogen resynthesis during the early post-exercise recovery period in an insulin-dependent manner. Nevertheless, such effect is overruled by appropriate carbohydrate and protein provision post-exercise. As such, there is currently no clear evidence to support that PEKS can enhance the restoration of muscle glycogen after exercise under conditions that are relevant for athletes.

Muscle protein turnover

Achieving a net positive muscle protein balance is important for maximizing training adaptations in both strength and endurance athletes (132). Interestingly, ketone bodies have been demonstrated to possess both anti-catabolic and anabolic properties (133). Anti-catabolic effects of ketone bodies have been evidenced in healthy individuals (134), as well as under various catabolic conditions in both humans (e.g. starvation, acute inflammation) (135, 136) and rodents (e.g. cancer cachexia) (137). The anti-catabolic effect of ketone bodies has traditionally been attributed to their role as an alternative energy source during fasting and starvation, thereby reducing the reliance on gluconeogenic substrates such as muscle proteins, for ATP production (138). Furthermore, research indicates that ketone bodies can prevent muscle atrophy by downregulating the two major proteolytic pathways, *i.e.* the ubiquitin

proteasome system and the autophagy-lysosomal system. Downregulation of these pathways is achieved through ketone body mediated inhibition of Akt-Foxo3a signaling (139) and the inhibition of NF-kB via ßHB-induced activation of GPR109a (140). Additionally, AcAc and βHB may also prevent muscle protein degradation by inhibiting class I and class IIa HDACs (137, 141). However, most evidence for the anti-catabolic effects of ketone bodies arises from studies under severe catabolic conditions. Therefore, it remains unclear whether ketone bodies also reduce skeletal muscle protein degradation immediately following exercise.

To our knowledge, only a single study has investigated the impact of PEKS on post-exercise anabolic signaling. This study demonstrated that PEKS increases markers that are indicative for heightened mammalian target of rapamycin complex 1 (mTORC1) activity, the main controller of post-exercise muscle protein synthesis (142). This increased activity was evidenced by an elevated phosphorylation of mTORC1's downstream targets, ribosomal protein S6 kinase 1 (S6K1), and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) in skeletal muscle tissue following 5h of recovery. Mechanistically, the heightened mTORC1 signaling likely resulted from the KE-induced inhibition of AMPK activation that was observed after 90 mins of recovery. As such, more rapid restoration of cellular energy homeostasis post-exercise upon KE, and the resulting deactivation of AMPK likely enabled the initiation of the anabolic signaling effect observed following KE ingestion. Such elevated mTORC1 activity was also observed in response to βHB administration in mice following two-weeks of hindlimb unloading (139) an in adult mice following a ketogenic diet (143). Furthermore, it has been demonstrated that the intravenous infusion of βHB in healthy subjects following an overnight fast leads to a decrease in leucine oxidation and an elevation in leucine incorporation, which is indicative for enhanced muscle protein synthesis (134).

The available evidence indicates that ketosis plays an important role in attenuating protein degradation while simultaneously promoting muscle protein synthesis. This suggests that ketone bodies are a potent strategy to (i) increase muscle mass in response to exercise, (ii) and to attenuate muscle wasting during periods of inactivity (*e.g.,* injury). Further studies are required to identify the precise impact of ketosis on muscle protein synthesis and degradation during exercise training.

Erythropoietin and red blood cell mass

Increasing circulating erythropoietin (EPO) is pivotal to improve endurance exercise performance as well as to optimize training adaptations (144). In this perspective, Evans et al. recently demonstrated that PEKS increased circulating EPO levels by ~25% during at least the first 4h of post-exercise recovery (145). Also, in a recent study by our group, consistent post-exercise and pre-sleep KE ingestion during a 3-week overload period resulted in a similar increase in circulating EPO. Interestingly, EPO levels were assessed at least 9h after the last KE dosage was provided. This suggests that elevations in circulating EPO can persist for many hours following ketone ingestion.

However, in this study the overload training period also resulted in the development of multiple overreaching symptoms (*e.g.,* decrease in maximal heart rate, energy deficiency) in the placebo group but not in the KE group. Also tolerated training load was ~15% higher in the KE *vs.* placebo group during the last training week (98). Consequently, it is plausible that KE enabled the normal adaptive response to exercise training, whereas an increase in EPO levels was abolished in the control condition due to the development of fatigue/overreaching. However, the data of Evans et al., (145) and the fact that an earlier study did not observe an increase in EPO levels after a 3-week training camp at sea level (146) suggests that the increase in EPO in our study resulted directly from KE. This is further supported by a study wherein intravenous infusion of ~4-5 mM ßHB increased EPO release by ~40% in healthy subjects in the fasted state (147).

The performance enhancing effect of EPO has traditionally been considered to result from its ability to stimulate erythropoiesis, which together with an EPO-induced reduction in plasma volume increases hematocrit and oxygen transport capacity (144). Currently, it has not been identified if the observed changes in EPO post-exercise are indeed sufficient to induce an improvement in hemoglobin mass and oxygen transport capacity in humans, and whether these effects are additive to stimuli that are frequently used by athletes to increases EPO such as hypoxia. Preliminary data suggest that such increase in EPO may be sufficient to elevate hemoglobin mass given that administration of sodium-glucose co-transporter 2 (SGLT2) inhibitors – which causes an increase in endogenous ketosis – for either 4 or 12 weeks increased blood EPO concentration and elevated hematocrit, hemoglobin levels, and red cell mass in patients with type 2 diabetes (148, 149). Besides the hematopoietic impact, the observed increase in EPO upon ketosis may also impact exercise performance and mental fatigue via non-hematopoietic effects such as improved neural processing and cognitive function, suppression of inflammation, angiogenesis and skeletal muscle regeneration (144).

The precise physiological mechanism underlying ketone body-induced upregulation of EPO is currently unknown, but most likely involves epigenetic regulation via histone H3 lysine 9 (H3K9) acetylation in kidney cells. Physiological concentrations of ßHB (~1-2 mM) have been shown to increase histone H3K9 acetylation in the kidneys of mice via inhibition of class I and IIa HDACs (64). A comparable increase in histone H3K9 acetylation has also been observed in murine tissue upon exposure to hypoxia, and resulted in the upregulation of genes implicated in EPO production and release (150).

There is growing evidence that ketone bodies increase the endogenous production of EPO in humans after exercise. Future studies should elucidate whether the ketone body-induced increase in EPO also leads to hematological improvements and whether these effects occur in elite athletes and in combination with other hematopoietic strategies such as altitude exposure.

Skeletal muscle capillarization

As indicated earlier, EPO is not only involved in hematopoiesis but also in other beneficial processes induced by exercise such as skeletal muscle angiogenesis. In this perspective, the study by Poffé et al. (151) that reported KE to increase circulating EPO during a 3-week overload training period, also observed that KE increased skeletal muscle capillarization by ~40%. This increase in capillarization was accompanied by elevated levels of the pro-angiogenic factors vascular endothelial growth factor (VEGF) and endothelial nitric oxide synthase (eNOS), both at the protein and mRNA level. It is conceivable that angiogenesis was directly stimulated by elevated ketone body levels as previous research demonstrated that βHB promotes VEGF synthesis in mouse brain endothelial cells (152), in the aorta of diabetic rats (67), as well as enhanced lymphangiogenesis in mice (153). Furthermore, incubation of murine cardiac endothelial cells with either βHB or AcAc enhanced cell proliferation rates, which is a prerequisite for blood vessel formation (154). Both AcAc and βHB also promoted capillary sprouting in a 3D angiogenesis assay (154). These effects appear to be directly dependent on ketone body oxidation, as the angiogenic response to increased concentrations of AcAc and βHB was absent in murine cardiac endothelial cells lacking the enzyme succinyl-CoA:3-oxoacid transferase, a key enzyme in ketone body oxidation (154).

An increased angiogenic response is potentially one of the key mechanisms by which PEKS stimulates long-term training effects. Such increase in angiogenesis not only improves the supply of oxygen and nutrients to the muscle, but is also considered a prerequisite for other training adaptations to occur, such as a shift to a more oxidative fiber type (155). The observed increase in angiogenesis is most likely mediated by increased endothelial ketone body oxidation (154). However, ketones may also increase angiogenesis through other mechanisms, such as activation of GPR43 by AcAc (97), stimulation of EPO release (144), and increased activation of Nrf2, FoxO3 and SIRT1 (105, 106, 155).

Mitochondrial biogenesis and function

Mitochondrial biogenesis and mitochondrial function are critical metabolic adaptations to (endurance) exercise. Emerging evidence suggests that ketone bodies, and in particular a ketogenic diet, enhances mitochondrial biogenesis and function. In mice, a ketogenic diet has been shown to increase PGC-1α, the master regulator of mitochondrial biogenesis (65, 156), citrate synthase activity (157), mitochondrial mass (158), and electron transport chain proteins (65, 156, 157). These mitochondrial adaptations can explain at least in part the positive effects of a ketogenic diet on muscle mass, muscle strength, endurance performance, and longevity in mice (143, 156). However, this primarily stems from experiments with aged, sedentary mice or mice afflicted with myopathies, which thus already exhibit reduced mitochondrial quality and function. Furthermore, these effects seem to be tissue-

specific, as a ketogenic diet and the administration of βHB inhibit mitochondrial biogenesis in cardiomyocytes (159).

A ketogenic diet is also associated with multiple metabolic changes independent of ketosis, making it challenging to attribute the effects on mitochondrial biogenesis and function exclusively to endogenous ketosis. For example, increased dependence on mitochondrial respiration increases ROS formation which can lead to chronic activation of PGC-1α (104). Nevertheless, these mitochondrial effects can also directly result from ketosis. In this perspective, improvements in mitochondrial function upon a ketogenic diet coincide with an increase in acetylated lysine protein levels (65, 143). As previously described, βHB directly elevates protein acetylation through inhibition of HDACs and increased production of acetyl-CoA (42, 64).

Enhanced acetylation is a critical component for mitochondrial biogenesis (160). In this perspective, pharmacological inhibition of HDAC activity has been shown to increase PGC-1α levels as well as proteins of the oxidative phosphorylation complexes (63). Furthermore, the elevated cellular acetylation state also increases acetylation of p53, which is crucial to maintain basal mitochondrial content and to stimulate mitochondrial biogenesis (161, 162). Furthermore, AcAc, through the activation of the MEK-ERK 1/2 signaling pathway, induces a dose-dependent upregulation of MEF2 (163), a transcription factor that enhances the activity of PGC-1α (164). Additionally, ketogenic diets have been observed to upregulate the expression of SIRT1 and SIRT3 (65, 156). Both of these sirtuins increase skeletal muscle mitochondrial biogenesis via stimulation of PGC-1α (72).

These studies demonstrate that endogenous ketosis induces favorable morphological and functional changes in skeletal muscle mitochondria, especially in mice with reduced mitochondrial health. However, it remains to be determined whether a ketogenic diet or the intake of ketone supplements elicits similar mitochondrial changes in humans, particularly among (endurance) athletes who already possess high mitochondrial respiratory capacity and efficiency (165).

Anti-inflammatory and oxidative stress

The relationship between oxidative stress, inflammation, and exercise adaptation is complex and multifaced. On the one hand, exercise triggers the production of free radicals and inflammatory cytokines, which is essential to maximally stimulate cellular pathways involved in exercise adaptation and to increase the capacity of the anti-oxidative and anti-inflammatory system. However, on the other hand, unaccustomed exercise can disturb the delicate balance of these systems, which may potentially contribute to muscle fatigue and overtraining (166). Consequently, depending on the specific context, agents that counteract oxidative stress and inflammation can have both beneficial and detrimental effects on exercise adaptation (102). To date, only a few studies investigated the effects of PEKS on inflammation and oxidative stress following exercise in humans.

In a first study PEKS suppressed circulating monocyte chemoattractant protein-1 (MCP-1) levels but did not alter the increase in other circulating cytokines (e.g. IL-6, TNF- α), nor induced functional effects during recovery following a single session of eccentric exercise-induced muscle damage (167). In agreement, ACTH, cortisol and IL-6 levels were also not altered by post-exercise and pre-sleep KE ingestion during a 3-week endurance training overload period (98). In contrast, a recent study performed by our research group demonstrated that KE inhibited the infiltration of macrophages in skeletal muscle 36h after an ultramarathon (168). This inhibition is potentially mediated by reduced MCP-1 levels induced by increased ketone body levels (167, 169). Macrophages are involved in tissue regeneration by activating growth factors and cytokines. Nevertheless, excessive macrophage infiltration can potentially result in fibrosis (170). Therefore, it is currently unknown whether ketone-induced inhibition of macrophages is a beneficial or detrimental effect.

To our knowledge, only a single study has evaluated the impact of ketosis on the oxidative response to exercise. This study reported that supplementation with ketone salts during a 1-week period neither affected the plasma levels of SOD2, CAT, total antioxidant capacity nor red blood cell levels of glutathione and SOD immediately after, 30 minutes after, and 24 hours after a 35-min cycling bout (171). Taken together, the limited evidence that is currently available indicates that ketone bodies only inhibit skeletal muscle macrophage infiltration in response to extreme exercise but does not impact inflammation or oxidative responses to less ‘extreme’ exercise.

Sleep

The adaptive response to exercise training is primarily dictated by the sequential metabolic and signaling responses occurring after each individual training stimulus. Often neglected in this process is the contribution of sleep which represents a significant recovery window. The importance of sleep is evidenced by the detrimental consequences of sleep deprivation including an impairment in exercise recovery (172), reduced myofibrillar protein synthesis (173), compromised glucose tolerance (174), and increased cardiovascular stress (174). Furthermore, sleep is known to deteriorate following acute increases in training load (175), intensified training periods (176), and multi-day competitions (177). Hence, optimizing sleep in athletes is not only important to facilitate acute recovery but also to promote beneficial long-term training effects.

Previous studies reported that ketosis induced via a ketogenic diet increases sleep quality and rapid eye movement sleep (REM) in healthy humans (178), as well as in children diagnosed with epilepsy (179). However, it remained unclear whether these effects were a result of elevated ketone body levels or other metabolic changes associated with a ketogenic diet. Therefore, we recently investigated if similar beneficial effects are also present upon KE. More specific, we assed the impact of KE following a day of strenuous training that was designed to disturb sleep. Interestingly, post-exercise and pre-sleep KE ingestion improved sleep efficiency by ~2%, and entirely counteracted the exercise-

induced decrease in REM sleep and wakefulness after sleep onset (111). This indicates that the sleep improvements that were earlier observed in response to a ketogenic diet are likely a direct result of ketosis rather than from the stark decrease in carbohydrate intake or increased fat intake. Such statement is supported by earlier animal data indicating that cerebral injection of AcAc, but not βHB, increases slow-wave sleep in a dose-dependent manner and slightly decreases REM sleep. This suggests that the beneficial effect of ketosis on sleep is likely caused by AcAc and not by ßHB (180). Of importance, the contrasting effect of ketosis on REM sleep between both studies likely resulted from the fact that lithium acetoacetate was used in the animal study. Indeed, lithium carbonate has previously been shown to decrease REM sleep in rats [115].

There are several potential physiological mechanisms via which ketone bodies can improve sleep quality. One possible mechanism is an increase in dopaminergic activity during the night following KE ingestion (119). Such increase in cerebral dopamine is known to facilitate the transition from NREM to REM sleep (181), which could explain the observed improvement in REM sleep upon ketosis. Besides dopamine, ketone bodies may also improve sleep via other mechanisms such as via an inhibition of glutamatergic activity (45, 112). This proposed mechanism could potentially explain the findings by Chikahisa et al. (45) as improved sleep and decreased brain glutamate levels were only observed following AcAc administration, and not following βHB injection.

The available evidence indicates that ketosis counteracts exercise-induced dysregulation in sleep quality and quantity. However, it is important to stress that recent data from our group indicates that ketone bodies fail to improve ‘normal sleep’ in humans with already good sleep quality (Robberechts et al., unpublished observation).

Where to go next?

In conclusion, existing research has demonstrated that PEKS may have the potential to enhance exercise recovery and promote training adaptations through a wide range of physiological mechanisms in multiple tissues. This suggests that the ergogenic potential of ketone supplements rather lies in enhancing post-exercise recovery instead of acutely improving exercise performance. Nonetheless, it is worth noting that research into the ergogenic effects of PEKS is still in its infancy.

One notable area where further research is warranted involves the need for more ecologically relevant studies. Currently, the longest study investigating the effects of PEKS in athletes is three weeks during a period of overtraining (98). Hence it remains unclear if PEKS can yield similar advantages during standard training periods, characterized by a good balance between exercise and recovery. Nevertheless, we expect that PEKS is still beneficial under such conditions, as emerging evidence indicates that ketone bodies upregulate cellular pathways implicated in exercise recovery by their signaling activities rather than by suppressing overtraining symptoms. Furthermore, it remains to be investigated if long-term chronic consumption of ketone supplements could potentially diminish

certain beneficial effects of PEKS on exercise recovery and adaptation. In addition, almost all studies investigated the effects of PEKS in recreationally active subjects/athletes. Given that elite athletes for instance already possess higher skeletal muscle capillarization and mitochondrial mass and function, it is most likely that the effects of PEKS are at least less extensive in elite athletes (165, 182). Future studies should also investigate the synergistic effects of PEKS with commonly used training methods such as altitude training camps and heat training, that often increase the demand for exercise recovery.

There is also a growing clinical interest in the therapeutic use of ketone supplements. Most specifically, there is a sparked interest in the use of ketone bodies in the treatment of patients with neurodegenerative diseases (183), cancer (184), cardiovascular diseases (185), and traumatic brain injury (186). However, the therapeutic potential of ketone bodies may be even broader. Indeed, exercise is nowadays encouraged as standard clinical care for a high number of diseases, and recovery from exercise typically takes longer in these individuals compared to healthy subjects. As such, analogous to athletes, PEKS can potentially enhance the effectiveness of exercise therapy by improving recovery and amplifying training adaptations. This could be particularly beneficial in patients with exercise intolerance or chronic fatigue syndrome, where the balance between exercise and recovery is of utmost importance to prevent the development of additional fatigue (187, 188)

Acknowledgements

Figures were created in Biorender.

Grants

This work was supported by Research Fund Flanders (Fonds voor Wetenschappelijk Onderzoek – Vlaanderen; research grant no. G089221N). C.P. is supported by an FWO Postdoctoral Research Grant (12B0E24N).

Disclosures

The authors declare that they have no conflicts of interest.

Author contributions

RR and CP conceived and designed the article; RR and CP drafted the manuscript; RR and CP prepared figures. All authors critically evaluated the manuscript and approved for submission. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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Figures

Figure 1. Overview of the different ketone supplements and their pharmacokinetics.

Pharmacokinetics were calculated based on the doses used in the following studies in healthy participants. (i) 28g of medium chain triglycerides + 150 mL fruit drink after breakfast (189); (ii) 3 x 11.5 g of (R)-1,3-butanediol at 0 min, 30 min and 60 min after an overnight fast (190); (iii) 282 mg.kg BW^{-1} of a combination of (R,S)-sodium β-hydroxybutyrate and (R,S)-potassium β-hydroxybutyrate + 6 g sweetener diluted in 300 ml water after an overnight fast (38); (iv) 282 mg.kg BW^{-1} (R)-3-hydroxybutyl (R)-3-hydroxybutyrate after an overnight fast (38); (v) 25g bis hexanoyl (R)-1,3-butanediolin a fasted state (40); (vi) two doses of 250 mg.kg BW^{-1} (R,S)-1,3-butanediol acetoacetate diester following respectively 45 min and 90 min after breakfast (16). Peak R-βHB: highest blood R-βHB concentration; time to peak R-βHB, time from intake of the ketone supplement to peak R-βHB blood concentration, R-βHB > 0.5, time that blood R-βHB concentration is above 0.5 mM; %R-βHB over total βHB, percentage of total R-βHB blood concentration over total βHB blood concentration ([R-βHB] + [S-βHB]), %AcAc over R-βHB, percentage of AcAc blood concentration over R-βHB at peak R-βHB blood concentration, βHB, β-hydroxybutyrate; AcAc, acetoacetate; BW, body weight; KME, ketone monoester.

Figure 2. Factors affecting post-exercise ketosis.

The following factors have been shown to increase the degree of post-exercise ketosis: low training status, ageing, prolonged moderate exercise, adherence to a low-CHO diet, low CHO intake before exercise, or decreased liver glycogen content. The following factors decrease the degree of post-exercise ketosis: post-exercise alanine intake, glucose intake 2 hours post-exercise, and high CHO intake before exercise. CHO, carbohydrates.

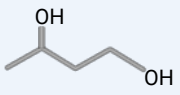
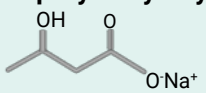
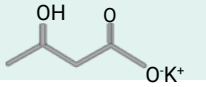
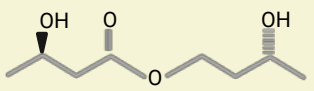
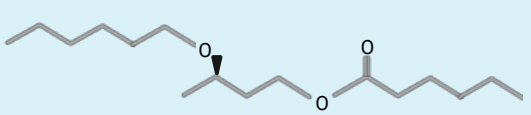
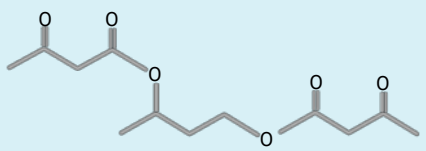
Figure 3. Potential mechanisms by which ketone bodies impact exercise and recovery.

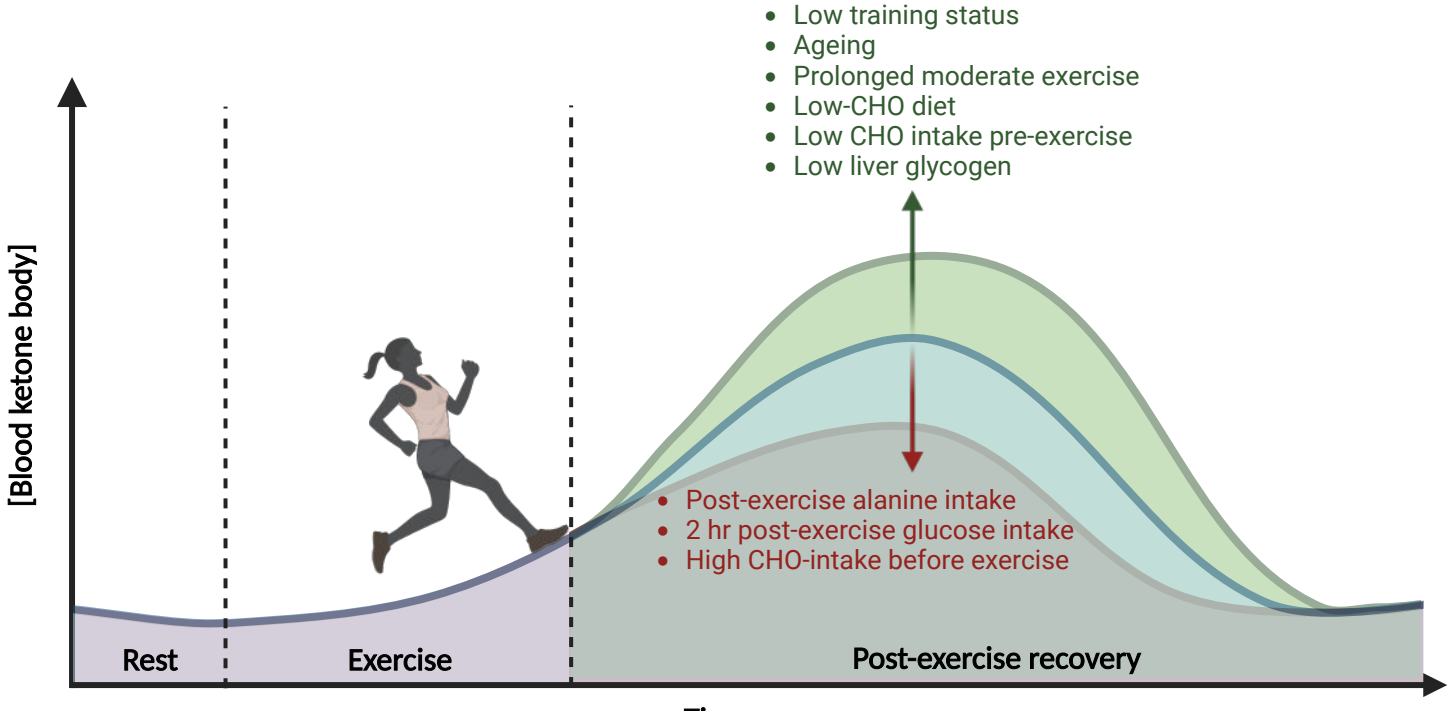
Overview of the potential physiological mechanisms by which the ketone bodies β-hydroxybutyrate and acetoacetate can improve exercise recovery and training adaptations. These mechanisms include hormonal and epigenetic regulation, alteration of energy metabolism, rapid recovery of cellular energy status, G receptor signaling, and modulation of anti-oxidative and anti-inflammatory pathways, and brain neurotransmitter levels.

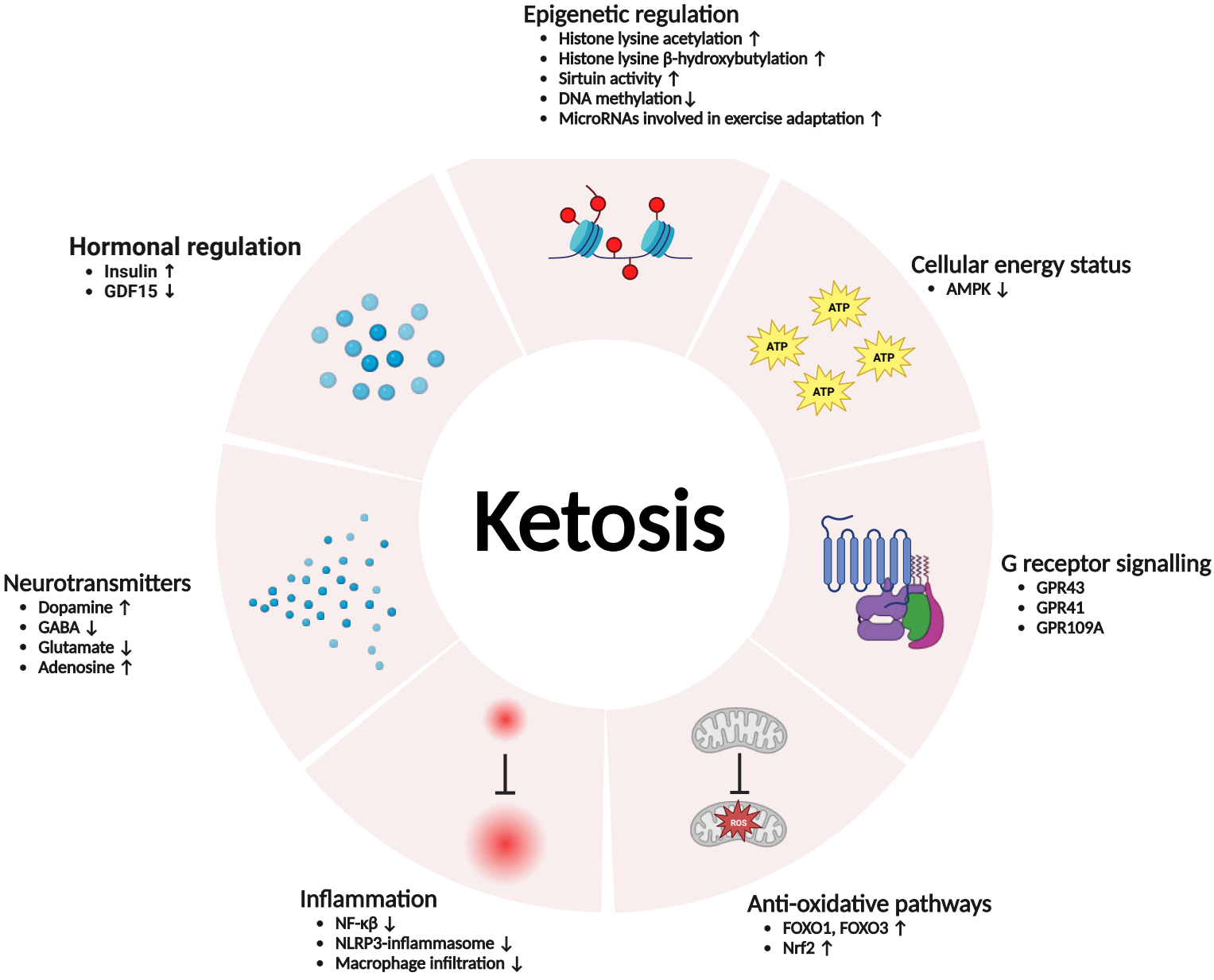
Figure 4. Different aspects of exercise recovery and adaptation that are impacted by ketosis.

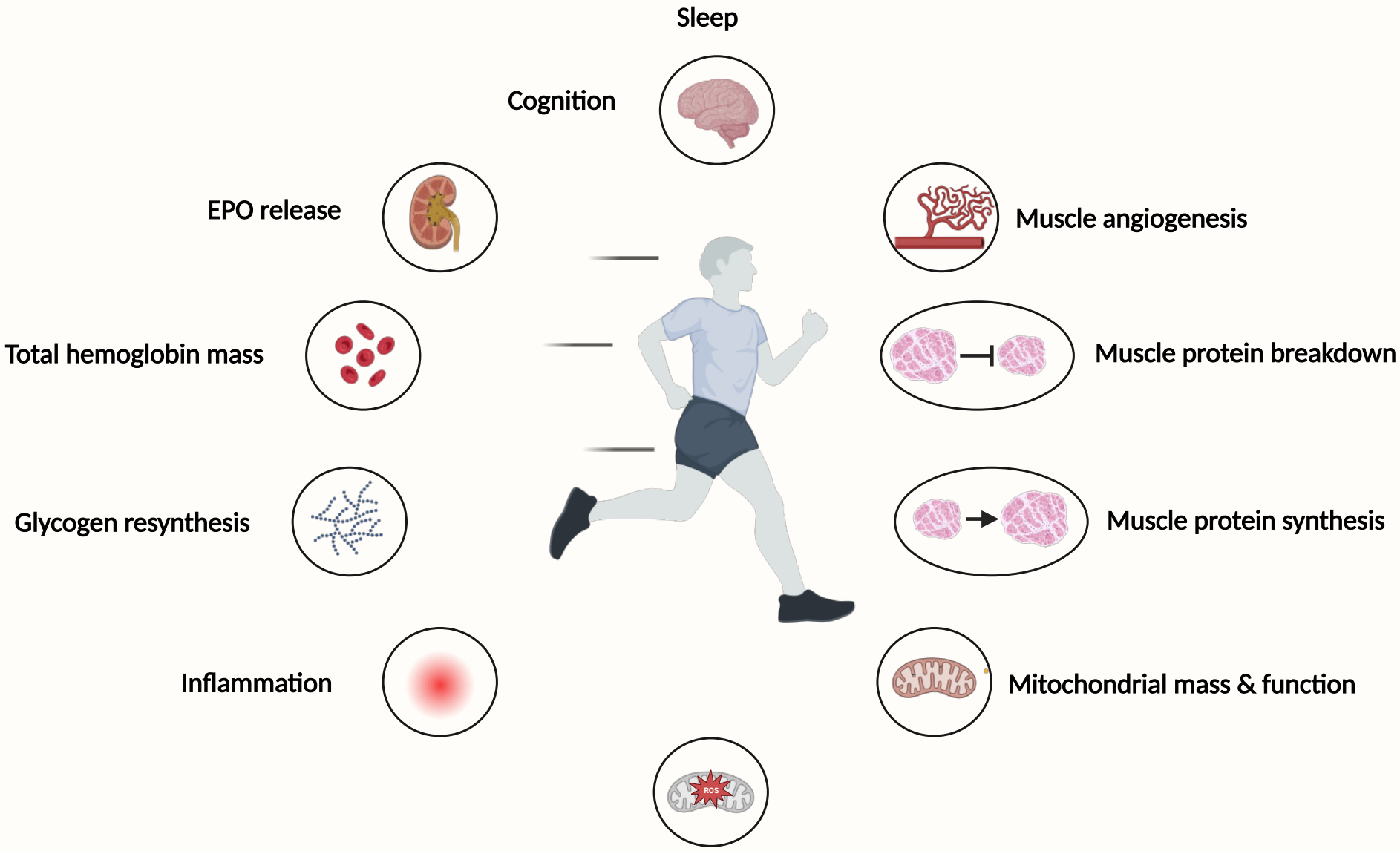
Overview of the different physiological effects by which post-exercise ketosis can potentially improve exercise recovery and training adaption. Scientific evidence indicates that ketone bodies may (i)

improve sleep and cognition, (ii) increase skeletal muscle angiogenesis, muscle protein synthesis, glycogen resynthesis and mitochondrial mass and function (iii) reduce skeletal muscle protein breakdown, oxidative stress and inflammation, (iv) and increase circulating EPO levels and total hemoglobin mass.

	Ketone supplement	Pharmacokinetics					Opinion
		Peak R-βHB	Time to peak R-βHB	R-βHB > 0.5	%R-βHB over total βHB	%AcAc over R-βHB	
Ketone precursors	**Medium chain triglycerides** 	0.5 ± 0.1 mM	2.5 - 3.5 hrs	1 - 2 hrs	100 %	Unknown	Not recommended due to low degree of ketosis and high incidence of side effects (e.g. stomach cramping, nausea, gastrointestinal discomfort).
		β-oxidation → Acetyl-CoA; Ketogenesis → R-βHB / AcAc					
	(R)-1,3-butanediol 	2.1 ± 1.0 mM	1.5 - 2.5 hrs	> 5 hrs	R: 100 %	Unknown	Recommended because it induces a prolonged ketosis. However, it achieves peak [βHB] more slowly compared to the KME and has undergone minimal research.
		Alcohol metabolism → R,S-βHB					
Ketone salts	**(R,S)-sodium β-hydroxybutyrate**  **(R,S)-potassium β-hydroxybutyrate** 	~1 mM	1 - 2 hrs	2 - 3 hrs	R: 100 % (R,S): 50%	Unknown	Not recommended because nearly all KS are racemic. Pure (R)-enantiomeric KS would theoretically yield an equivalent amount of R-βHB as KME, but consumption is still associated with a high amount of electrolytes, and incidence of side effects are higher compared to KME.
		Acid - base reaction → (R,S)-βHB					
Ketone mono-esters	**(R)-3-hydroxybutyl (R)-3-hydroxybutyrate** 	2.8 ± 0.1 mM	30 - 60 min	3 - 5 hrs	100 %	~20 %	Currently, the most promising available ketone supplement due to a rapid induction of ketosis, characterized by a sustained elevation of R-βHB above 1 mM for multiple hours with no side effects. Furthermore, it is currently the most investigated ketone ester supplement.
		Hydrolysis → R-βHB; (R)-1,3-butanediol → Alcohol metabolism → R-βHB					
Ketone di-esters	**Bis hexanoyl (R)-1,3-butanediol** 	~1.6 ± 0.4 mM	1 - 2 hrs	3 - 5 hrs	> 96 %	Unknown	A possible alternative to the KME, albeit peak R-βHB concentrations are lower and drop quicker below 1 mM compared to KME. Furthermore, caution is required, as one study indicates that side effects are relatively high (e.g. gastrointestinal distress).
		Hydrolysis → Hexanoic acid → β-oxidation → Acetyl-CoA → Ketogenesis → R-βHB / AcAc; (R)-1,3 butanediol → Alcohol metabolism → R-βHB					
	(R,S)-1,3-butanediol acetoacetate diester 	Only studied during exercise, [BHB] < 0.5 mM after two times 250 mg.kg BW^{-1}			50%	~60 %	Not recommended due to low degree of ketosis and high incidence of side effects.
		Hydrolysis → AcAc + AcAc; (R,S)-butanediol → Alcohol metabolism → (R,S)-βHB					







Defining ketone supplementation: the evolving evidence for post-exercise ketone supplementation to improve recovery and adaptation to exercise