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Abstract

Carnosine (β-alanyl-L-histidine) plays an important role in exercise performance and skeletal muscle homeostasis. Dietary supplementation with the rate-limiting precursor β-alanine leads to an increase in skeletal muscle carnosine content, which further potentiates its effects. There is significant interest in carnosine and β-alanine across athletic and clinical populations. Traditionally, attention has been given to performance outcomes with less focus on the underlying mechanism(s). Putative physiological roles in human skeletal muscle include acting as an intracellular pH buffer, modulating energy metabolism, regulating Ca^{2+} handling and myofilament sensitivity, and scavenging of reactive species. Emerging evidence shows that carnosine could also act as a cytoplasmic Ca^{2+}–H^{+} exchanger and form stable conjugates with exercise-induced reactive aldehydes. The enigmatic nature of carnosine means there is still much to learn regarding its actions and applications in exercise, health and disease. In this review, we examine the research relating to each physiological role attributed to carnosine, and its precursor β-alanine, in exercising human skeletal muscle.

Key words: metabolism; buffer; fatigue; calcium; antioxidant; detoxification

Introduction

Carnosine (β-alanyl-L-histidine) is a multifunctional histidine-containing dipeptide (HCD) occurring naturally in high concentrations in mammalian skeletal muscle (1). It is also present in relatively large quantities in the central nervous system, particularly the olfactory epithelium and bulb (2), and in smaller amounts in the kidney (3) and myocardium (4). A large body of research shows that elevating skeletal muscle carnosine (m-carn) contents can improve anaerobic exercise capacity and performance (5). While its ergogenic effects are clear, the actions of carnosine are complex. Putative physiological roles in human skeletal muscle include acting as an intracellular pH buffer (pH_i) (6), direct modulation of energy metabolism (7), regulation of Ca^{2+} handling and myofilament sensitivity (8), quenching of reactive species, and detoxification of reactive aldehydes (9). Several recent publications provide new insights into these roles, which warrant a detailed discussion. In this review, we examine the research relating to each physiological role attributed to carnosine, and its rate-limiting precursor β-alanine, in exercising human skeletal muscle.

Carnosine and β-Alanine Metabolism

β-alanine availability is influenced by diet and, to a lesser extent, endogenous production from uracil degradation in the liver (10). A typical omnivorous diet provides ~300-550 $mg \cdot day^{-1}$, whereas a vegetarian diet provides virtually no β-alanine (11,12). Figure 1 depicts carnosine and β-alanine absorption and transport. Following ingestion, carnosine reaches the small intestine where it enters enterocytes via peptide transporter 1 (PEPT1). Tissue carnosinase (CN2), mainly present in the jejunal mucosa, hydrolyses carnosine into its constituent amino acids β-alanine and L-histidine (13). Small amounts of carnosine remain intact and enter the bloodstream via a basolateral peptide transporter, whereas, β-alanine enters via a basolateral amino acid transporter. Highly active serum carnosinase (CN1) rapidly hydrolyses much of the remaining carnosine (13). Small amounts (≤14%) can be detected in urine during the 5-hours following ingestion

(14); meaning that a portion of the ingested carnosine circulates intact for a short period. Park et al. (15) confirmed this by showing a small increase in plasma carnosine, which peaked 3.5 hours following consumption of cooked beef. Individuals with homozygosity for the $(CTG)_5$ allele display lower CN1 activity which is associated with higher circulating plasma carnosine levels (16) and a reduced risk of developing diabetic nephropathy (17).

Everaert et al. (18) and Saunders et al. (19) have respectively identified mRNA transcripts of peptide-histidine transporter 1 (PHT1) and peptide transporter 2 (PEPT2) in human skeletal muscle; however, direct uptake or release of carnosine has not yet been shown. Uptake of β-alanine into skeletal muscle occurs via proton-assisted amino acid transporter 1 (PAT1) and taurine transporter (TauT) (18). Subsequently m-carn is synthesised *in situ*, catalysed by carnosine synthase (20). Drozak et al. (21) identified the encoding gene as ATP-grasp-domain-containing protein 1 (ATPGD1), which shows a greater affinity for L-histidine (K_m ~0.37 mM) than for β-alanine (K_m ~0.09 mM). Due to low substrate affinity and availability, endogenous synthesis of carnosine is rate-limited by β-alanine availability (10); and high-dose L-histidine supplementation alone does not augment m-carn contents in humans (22).

Humans exhibit m-carn concentrations in the millimolar range (Figure 2), similar to levels of adenosine triphosphate, phosphorylcreatine, and taurine (23). Several factors determine m-carn contents, with diet being the easiest to modify. The absence of dietary carnosine or β-alanine does not appear to influence m-carn contents in the short-term (≤6 month vegetarian diet) (24). However, long-term (≥1y and ≥8y) vegetarians display ~17–26% and 35% lower m-carn compared with omnivores, respectively (25,26). Harris et al. (10) first showed, in humans, that β-alanine supplementation (4-weeks, 6.4 $g.day^{-1}$) results in a substantial (~60%) increase in m-carn content. A response since shown to be reproducible, with an initial linear increase in response to the total β-alanine dose supplemented (Figure 3). Saunders et al. (19) supplemented 1075g over 24 weeks, representing the largest total β-alanine dose in the literature, which led to a 119 ± 42%

increase in m-carn from baseline and a plateau from 20 to 24 weeks. The average time to reach maximal m-carn content was 18 ± 6 weeks, which may indicate a putative saturation point. Some participants continued to exhibit higher m-carn contents between weeks 20 and 24. Therefore, a longer duration or higher dose of β-alanine is likely required to maximise m-carn contents in certain individuals.

Once augmented, total m-carn has a recorded washout time of ~2-4% per week (11,27). It remains unclear whether this stability is due to a low or high turnover rate. Although CN2 is present in skeletal muscle, carnosine degradation is believed to be minimal due to low enzyme activity at intramuscular pH (~7.1) (28). Studies utilising the microdialysis technique show a large elevation of carnosine in skeletal muscle interstitium following trauma to the sarcolemma from the insertion probe (29). This suggests an efflux of m-carn secondary to muscle damage; however, there is no measurable loss of m-carn immediately following high-intensity interval training (HIIT) (9). At present, the rate of m-carn decay appears to depend on the muscle location, fibre-type, and dietary β-alanine intake, but further investigations into m-carn turnover are warranted.

In humans, the type I:II ratio for m-carn in fibres from *m. vastus lateralis* is 1.3:2 (30–32). Absolute increases in m-carn from β-alanine supplementation are comparable for both fibre-types, which can lead to a doubling of m-carn in type I fibres and a ~50% increase in type II fibres (30). A possible explanation for lower type I contents is greater rate of degradation, efflux from the muscle cell, or a lower synthetic rate. Conversely, higher type II m-carn contents may be an adaptive response to anaerobic demands and perturbations in local pH. The latter is supported by cross-sectional data showing higher m-carn in sprinters compared with aerobic athletes and healthy controls (6). Recently a longitudinal study in vegetarians observed a ~25% increase in m-carn following 12 weeks of HIIT (26). Similar studies, reporting null findings, are all short-term (≤6 weeks) with a lower weekly training volume and fail to control for training-

induced changes in muscle fibre distribution (33) or dietary intake of β-alanine (32). The precise mechanism of the training-induced increase in m-carn remains unknown, but recent evidence shows an increase in ATPGD1 mRNA by ~28% in response to a 6-week HIIT regime (34). Furthermore, Hoetker et al. (35) showed m-carn contents and ATPDG1 gene expression concomitantly fluctuate throughout different phases of exercising training; suggesting that the amount of carnosine synthesis is important regulator of m-carn homeostasis. Changes in hepatic β-alanine synthesis and β-alanine transport into muscle fibres are also likely important to provide sufficient substrate for carnosine synthesis to occur.

Less is known regarding the physiological roles of β-alanine, independent of its role in synthesising m-carn. Ingested β-alanine is rapidly absorbed and plasma concentrations peak 30-60 minutes post-supplementation (10,36), with small losses occurring via urinary excretion (~1-3%) and through incorporation into m-carn (~3-6%) (10,37). Supplementing with a sustained-release β-alanine formulation results in greater retention and higher m-carn contents (38); this presents a more efficient supplementation strategy for use in future studies. The large proportion of remaining β-alanine is thought to be rapidly deaminated and oxidised. In an early rodent model, Pihl and Fritzson (39) described the metabolic fate of C^{14}-labelled β-alanine following intraperitoneal injection. The cumulative excretion of C^{14} in expired CO_2 over 5-hours was 93%, 60%, and 77% of the dose administered, respective to the carbon atom labelled. In humans, β-alanine transamination results in the formation of the keto-acid malonate semialdehyde (40). A reaction, according to rodent data, catalysed by two mitochondrial enzymes, β-alanine-2-oxoglutarate transaminase (GABA-T) and alanine-glyoxylate transaminase (AGXT2) (4). Human hepatocytes express both enzymes and GABA-T is additionally present in kidney and brain tissue, which are putative sites of β-alanine metabolism. Malonate semialdehyde undergoes oxidative decarboxylation to acetyl-CoA, which provides a substrate for the tricarboxylic acid cycle (Figure 1). Skeletal muscle β-alanine contents may increase up to ~98% in response to

supplementation (41). However, β-alanine does not appear to undergo transamination in skeletal muscle and instead primarily contributes to carnosine synthesis (20). The role of the excess β-alanine is muscle unclear, but it may contribute to physiological function within the cell (*e.g,* molecular signalling) or be transported to another organ where it is metabolised.

Skeletal Muscle Metabolism

Direct Effects on Glycolysis and Aerobic Metabolism

Early research showed carnosine regulates enzyme activity and chelates heavy metal glycolytic inhibitors in skeletal muscle (42), leading to an increase in glycolytic flux (43). Despite showing an ability to exert a direct influence on energy metabolism *in vitro*, data in human skeletal muscle are equivocal.

An increase in glycolytic flux or capacity, independent of oxidative capacity, is quantifiable by higher lactate accumulation during and following exercise. Some β-alanine supplementation studies have demonstrated this, with higher post-exercise plasma lactate values following the special judo fitness test (12) and 4 x 30s upper-body Wingate tests (44). However, in these studies total mechanical work was not matched between pre- and post-supplementation trials. Direct effects on glycolysis cannot be separated from indirect effects, which are an increase in total work performed (*e.g.*, due to increased buffering capacity). We also note many studies have failed to show a difference in delta lactate, despite not matching mechanical work (45–47).

To our knowledge, only two studies have quantified the effects of β-alanine supplementation on energy system contribution with matched total mechanical work (7,48). da Silva et al. (48) showed no change in oxidative, glycolytic, or ATP-PCr contribution during 4 x 60s cycling bouts performed with a constant cadence at 110% maximal aerobic power output. In stark opposition to the glycolytic activation hypothesis, Gross et al. (7) observed a decrease in post-exercise muscle lactate and oxygen deficit following a 90s fixed-power cycling test.

Activity of the rate-limiting glycolytic enzyme, phosphofructokinase, was also reduced. This occurred alongside a small (~1.3%), but significant, increase in the estimated aerobic energy contribution; although aerobic enzyme activities were unchanged. β-alanine supplementation may also delay the onset of blood lactate accumulation during treadmill running (49), indicative of an improvement in oxidative capacity. Despite this, increasing m-carn contents does not appear to alter ventilatory threshold or VO_2max/peak (50).

In a cell model, treatment of C2C12 murine skeletal myotubes with 800μM of β-alanine led to induction of several markers of mitochondrial biogenesis (51). While interesting, several limitations preclude acceptance of these findings. Intracellular carnosine was not quantified so it is unclear whether the effects are due to β-alanine or carnosine. Furthermore, evidence that β-alanine supplementation evokes favourable oxidative and mitochondrial adaptations is not supported by longitudinal studies (7,33). The proposed small benefit to aerobic metabolism, shown by Gross et al. (7), is difficult to reconcile with evidence that exercise capacity and performance exceeding 10 minutes does not typically improve following β-alanine supplementation (5). Despite showing an ability to interact with metabolic pathways *in vitro*, the influence of carnosine as a direct modulator of energy metabolism in whole skeletal muscle appears less pronounced.

Intramyocellular pH Buffering

The early works of Bate Smith (52) and Deutsch & Eggleton (53) first proposed the role of carnosine as a pH_i buffer. The addition of β-alanine to L-histidine raises the pKa of the histidine imidazole ring from 6.1 (free histidine) to 6.83 (carnosine), causing it to act as a buffer over the exercise-induced pH_i transit range (pH ~7.1 to 6.5) (10). This feature is consistent across species, whereby the highest HCD concentrations are found in animals with the greatest anaerobic energy demands, *e.g.* due to prolonged sprinting (locomotion) or hypoxia (diving) (54). Furthermore, species with a highly oxidative phenotype and contractile properties (*i.e.*, hummingbirds) possess

low HCD contents (55). This suggests HCDs are non-essential to aerobic metabolism and muscle contractility, and instead, supports the primary physiological role as a pH_i buffer. Due to these functions, there is widespread interest in β-alanine supplementation and m-carn in situations of exercise-induced acidosis.

The role of acidosis in peripheral fatigue during short-duration, high-intensity exercise has been debated. Recent data show elevated levels of H^+ (pH ~6.2) and P_i (~30 mM) act synergistically to depress cross-bridge function by inhibiting isometric force, shortening velocity, peak power, and the low to high-force transition of the cross-bridge cycle (56). Regardless of the specific mechanism, increasing m-carn, via β-alanine supplementation, improves exercise capacity and performance in exercise durations of 30s to 10 minutes (5). This outcome is consistent with acting as a pH_i buffer, as H^+ accumulation is at its highest and more likely contributor to fatigue than with shorter or longer exercise durations. Assessments of exercise capacity (*e.g.*, time to exhaustion) show the greatest benefit from increasing m-carn contents (5). Whereas, performance-based tests (*e.g.*, time trials) show a smaller benefit, likely due to being highly influenced by pacing strategy. As such, the physiological milieu at the end of a performance-based task may not represent volitional fatigue or severe acidosis.

Several studies have used the Henderson-Hasselbalch equation (Figure 4) to estimate the effect of m-carn on non-bicarbonate total muscle buffering capacity (βm) in muscle homogenates. Mannion et al. (57) first estimated that m-carn contributes ~7% to βm. This likely underestimates the *in vivo* buffering contribution of m-carn (βm-*carn*) due to methodological limitations, discussed herein. Measurements are recorded in a metabolic composition close to that of rigor mortis and, upon homogenisation, there is a complete loss of adenosine triphosphate and phosphorylcreatine (58). As a result, estimates of βm do not include dynamic buffering via rephosphorylation of adenosine diphosphate by phosphorylcreatine. Instead, it encompasses histidine residues of proteins and dipeptides, inorganic phosphate, and hexose monophosphates

(58); and *βm* is quantified without measuring the concentrations of these non-HCD buffers. Lastly, carnosine is a mobile buffer, freely dissolved in the cytoplasm, whereas proteins are fixed buffers. Such mobility allows carnosine to contribute to the prevention of local pH_i gradients (59), which likely encompass greater effects than estimated from its proportion of *βm* alone.

Exercise models that induce fatigue under solely anaerobic conditions may provide a better estimation of *βm-carn in vivo*. Several studies show β-alanine supplementation increases isometric knee extension time to fatigue (+11.1–17.2%) when performed at 45% maximal voluntary isometric contraction (MVIC) (60–62). To our knowledge, when performing identical methods, only one study has yielded null results (63). The test is estimated to cause fatigue in ~78s, a duration and intensity accompanied by the largest increase in pyruvate and lactate (64). Moreover, an intensity of 45% MVIC raises intramuscular pressure sufficient to prevent muscle reoxygenation (65). This creates a local hypoxic environment and greater reliance on intracellular buffers (*e.g.*, carnosine). These differences partly explain why the studies did not reproduce the earlier findings of Derave et al. (45). Participants recorded an isometric knee extension time to fatigue of ~173s and ~201s, substantially longer than the subsequent times of ~75s (62) and ~55s (60). To sustain a longer contraction the intensity was likely <45% MVIC(64). The results are further confounded by differences in the knee extension measurement angle of 45^o (45) and 90^o (60,62). Despite both positions producing comparable MVIC values, the time to fatigue is ~62% longer at a shorter quadriceps muscle length (50^o vs. 90^o) (66). The lower relative force results in less intramuscular pressure and only a partial restriction of tissue oxygenation (65). Therefore, some circulation would have been maintained to enable H^+ efflux from the working muscles, reducing the demand on intracellular buffers.

Hill et al. (30) investigated 4 and 10-weeks β-alanine supplementation on cycling to exhaustion at 110% VO_{2peak} ($CCT_{110\%}$), a test designed to cause fatigue in ~150s. M-carn contents increased by ~59% and ~80%, leading to improvements in exercise capacity of 13% and

16.2%, respectively. Sale et al. (46) replicated these methods and reported a 12.1% improvement in $CCT_{110\%}$ following 4-weeks supplementation. The magnitude of improvement is remarkably consistent across the isometric knee extension hold and $CCT_{110\%}$ protocols and shows a dose-response to β-alanine supplementation (30). Both protocols induce severe acidosis, however, it is challenging to isolate these effects from concomitant changes in Ca^{2+} handling that may also be causative in fatigue (see following section). A limitation to the pH_i buffer perspective is the absence of evidence showing a carnosine-mediated attenuation of the exercise-induced decline in muscle pH. Gross et al. (7) showed no effect of β-alanine supplementation on post-exercise muscle pH following a 90s fixed-power cycling test. To contrast using a similar model, the findings did not replicate the mild (~0.1 pH units) alkalising effect of elevated plasma bicarbonate on muscle acid-base balance during exercise (67). One explanation is that, because H^+ buffered by m-carn remains within the muscle, on extraction, the protonated carnosine dissociates from its H^+ with the cation returning to the muscle homogenate. Furthermore, it is possible the increase in m-carn was insufficient to improve *βm* by a detectable amount. The low supplementation dose (38 days, 3.2 $g \cdot d^{-1}$) induced a 24% elevation in *m. vastus lateralis* carnosine content (7); whereas a more typical β-alanine dose (*e.g.,* 4-6 weeks, 6.4 $g \cdot d^{-1}$) can lead to a ~2-3 fold higher increase (Figure 2).

The shortcomings in our understanding are likely to persist until reliable methods are available to quantify real-time changes in muscle pH during exercise. However, by triangulating findings from controlled human trials, *in vitro* research, and comparative physiology, there is robust evidence to support a key physiological role of carnosine as a pH_i buffer in skeletal muscle.

Ca^{2+} Handling and Muscle Contractility

Ca^{2+} Release, Reuptake and Myofilament Ca^{2+} Sensitivity

Several studies show that carnosine influences Ca^{2+} handling, which could partly explain the ergogenic effects of m-carn. Decreases in Ca^{2+} release from the sarcoplasmic reticulum (SR), myofilament Ca^{2+} sensitivity, and Ca^{2+} reuptake into the SR occur during fatiguing contractions

(68). These factors act synergistically with the accumulation of metabolic by-products (namely H^+ and P_i) to cause loss in muscle function during intense contractile activity.

Early studies in chemically skinned skeletal muscle fibres proposed a role for carnosine, and other HCDs, in facilitating Ca^{2+} release from the SR and increasing myofilament Ca^{2+} sensitivity (69,70). The chemically skinned muscle fibre model has since been criticised as it results in disruption to normal coupling between Ca^{2+} release channels and voltage-sensor proteins (dihydropyridine receptors) (71). Furthermore, the positive results were from experiments performed in the presence of sub-physiological concentrations of cytoplasmic Mg^{2+}, a potent inhibitor of Ca^{2+} release.

Dutka and Lamb (71) showed no effect of carnosine on Ca^{2+} release from the SR when performed in mechanically skinned muscle fibres under conditions that corrected for the previous shortcomings. However, they did confirm adding carnosine to muscle preparations lowers the amount of Ca^{2+} ions required to produce half-maximum tension, with minimal change in the maximum Ca^{2+}-activated force. Dutka et al. (8) replicated these findings in human skeletal muscle samples. In a concentration-dependent manner, 8 mM and 16 mM carnosine increased the pCa_{50} (-log[10] of Ca^{2+} concentration at half-maximal force) by ~0.07 and 0.12 pCa units in type I fibres, and by ~0.06 and 0.1 pCa units in type II fibres, respectively. This equates to a leftwards shift of the force-pCa relationship (the *in vitro* analogue of the force-frequency relationship), whereby a fibre producing ~40% of maximal force in the absence of carnosine would produce ≥60% in the presence of 16 mM carnosine (Figure 5) (8). In the studies discussed, experiments involve heavily buffered preparations that maintain pH at ~7.1 and therefore do not assess carnosine on Ca^{2+} handling throughout the range of exercise-induced acidosis (*e.g.,* pH ~7.1 to ~6.5). This is important as muscle pH and Ca^{2+} handling are inextricably linked. For example, the sarco/endoplasmic reticulum-ATPase (SERCA) pump rate,

responsible for Ca^{2+} uptake, declines ~2-fold over a pH drop from 7.1 to 6.6 (72). The ability of m-carn to buffer pH_i may indirectly improve Ca^{2+} handling in the muscle cell.

Human β-alanine supplementation studies show varied responses to *in vivo* measures of Ca^{2+} handling. Gross et al. (73) reported a ~7% improvement in maximum and average power during a countermovement jump, despite no change in maximal jump height. The authors attributed this to an increase in contraction velocity secondary to enhanced myofilament Ca^{2+} sensitivity. While interesting, subsequent human studies disagree. Hannah et al. (74) and Jones et al. (63) showed no effect of β-alanine supplementation on peak force, time to peak tension, and maximum or explosive force production in voluntary and electrically evoked contractions in fresh and fatigued muscle; refuting earlier *in vitro* data (8) and the observation of a leftwards shift in the force-frequency curve in mice (75). In whole, contracting human skeletal muscle, carnosine may be less important in sensitising the myofilaments to Ca^{2+} than detected in *in vitro* and rodent models. This is possibly due to differences in relative muscle excitability between species (for a review, see 76). Furthermore, β-alanine supplementation does not typically enhance maximal force production *in vitro* (8,71) or in human studies (63,73,74,77).

Interestingly, both Hannah et al. (74) and Jones et al. (63) showed a significant decrease in knee extensor half-relaxation time, highlighting a potential interaction between m-carn and Ca^{2+} handling. Resting and potentiated twitches were recorded prior to and following three sets of MVIC, while supramaximal octets (eight impulses at 300 Hz) assessed explosive performance of the musculotendinous unit. β-alanine supplementation decreased half-relaxation time in resting (–12%) and potentiated (–7%) twitches in fresh muscle (74); in resting (–19%) and potentiated (–2%) twitches in fatigued muscle, and supramaximal octets in fresh (–20%) and fatigued (–11%) muscle (63). Everaert et al. (75) reported an attenuation of the slowing in relaxation rate, in predominantly slow-twitch muscle (soleus), during the first 3 minutes of a fatiguing protocol in mice. This presents a contrast from human data, where the reduction in

relaxation time occurred in resting twitch and explosive contractions (63), implying the response is similar in both muscle fibre types. The reason for these differences between studies is unclear, but it may be due to the abundance of anserine in rodents. In human skeletal muscle, anserine is either absent or accounts for only a minor (~2%) portion of the total HCD content (10,35); whereas, anserine content is higher than carnosine in mice (ratio ~1:2.1) and rats (ratio ~1:2.4), which increase further with β-alanine supplementation (75). Anserine and carnosine show differences in their Ca^{2+} handling properties (69); therefore, the changes in muscle function in rodents are due to the total increase in HCD content and not carnosine alone.

The slowing of relaxation can limit performance in exercise where rapidly alternating movements are performed (68). However, repeated resisted muscle contractions to fatigue (*e.g.*, strength-endurance exercise) show mixed responses to β-alanine supplementation. Derave et al. (45) showed an attenuation of fatigue in repeated isokinetic knee extensions performed at $180^{o}{\cdot}s^{-1}$ (5 x 30 repetitions). Raising m-carn by ~37-47% led to greater average peak torque in sets 3-5 compared with the control group. More recently, Bassinello et al. (60) could not reproduce these results, despite using the same experimental methods. There was also no change in total repetitions performed during high volume (8 sets, 70%1RM) smith-machine bench press and 45^{o} leg press exercise. This is consistent with data that show no improvement in fatigue resistance during exhaustive arm curl exercise (~20-40 repetitions), even with a ~59% increase in m-carn (77). At present, there is inconsistency between results from *in vitro*, animal, and human studies. The ability to influence Ca^{2+} handling and myofilament Ca^{2+} sensitivity may be important chemical properties of carnosine. However, if these were primary physiological roles in skeletal muscle we might expect improvements in exercise over a wider range of modes and durations. The findings of a decrease in half-relaxation time in humans are interesting and warrant further investigation. Lastly, our discussion is specific to skeletal muscle and the role of carnosine-Ca^{2+} interactions in other tissues (*e.g.*, cardiomyocytes) may differ.

Cytoplasmic Ca^{2+}–H^+ Exchanger

Emerging evidence suggests that m-carn may function as a diffusible cytoplasmic Ca^{2+}–H^+ exchanger in cardiomyocytes (59). This combines elements of the two previously discussed roles: Ca^{2+} handling and pH_i buffering. The interrelationship between H^+ and Ca^{2+} is important in exercising skeletal muscle. H^+ can compete with Ca^{2+} at the troponin-binding site, thereby limiting the ability of the muscle contractile machinery to operate effectively (78). Both Ca^{2+} and H^+ competitively bind to carnosine, which can cause unloading of Ca^{2+} in areas of high H^+ production (*e.g.*, local glycolytic metabolism) and unloading H^+ in areas of high Ca^{2+} production (*e.g.*, efflux from the RyR1 channels) (59). Through these actions, carnosine is able to regulate highly compartmentalised ionic microdomains and potentially improve contractile function through increasing myofilament Ca^{2+} sensitivity.

The evidence that an increase in m-carn can reduce half-relaxation time could explain a role of the Ca^{2+}-H^+ exchanger in human skeletal muscle (63,74). Relaxation time is influenced by the rate of dissociation of Ca^{2+} from troponin; translocation of Ca^{2+} to a site close to the SR; and reuptake of Ca^{2+} into the SR by SERCA pumps (72). Only one in one hundred Ca^{2+} ions is free to diffuse, and the diffusivity of the remaining Ca^{2+} depends on the mobility of the Ca^{2+}-buffer complex (59). During Ca^{2+} uptake by SERCA, H^+ are counter-transported from the SR lumen to the cytosol, simultaneously, H^+ are transported from the cytosol to the lumen during Ca^{2+} release (79), leading to H^+ and Ca^{2+} non-uniformity. As a mobile buffer, carnosine may translocate Ca^{2+} closer to the SERCA pump for Ca^{2+} reuptake (Figure 6). While the cytoplasmic Ca^{2+}–H^+ exchanger is an alluring concept, the model proposed by Swietach et al. (59) was demonstrated in rat ventricular cardiomyocytes, where spatiotemporal responses in Ca^{2+} sparks differ to skeletal muscle (80). It is conceivable that the role also occurs in human skeletal muscle, but validation is required before drawing strong conclusions.

Redox Activity

Through its diverse chemical properties, carnosine has the ability to scavenge reactive species, form adducts with reactive aldehydes, and chelate metal ions (81). These actions may confer a benefit against oxidative stress and deleterious modifications to biomolecules, including proteins, lipids, and DNA. Here, consistent with the theme of our review, we focus on these roles within the context of exercising skeletal muscle. For a clinical perspective, we direct the reader to a recent review by Artioli et al. (82).

Scavenger of Reactive Species

The production of reactive oxygen species (ROS) increases in skeletal muscle during exercise (83). Despite endogenous defences, high rates of ROS production can exceed the antioxidant capacity of muscle fibres, leading to oxidative stress. Carnosine, and other HCDs, can quench superoxide anions, hydroxyl radicals, and peroxyl radicals (81) thereby reducing intracellular oxidative stress. It is unknown, however, whether carnosine contributes to redox homeostasis in whole human skeletal muscle. Further complicating the issue is evidence that ROS exert a beneficial or detrimental effect on contractile function depending upon the magnitude and duration of increase, localisation of accumulation and the type of ROS produced (83).

In two similar human studies, Smith et al. (84) and Smith-Ryan et al. (85) supplemented recreationally trained participants with β-alanine (4-weeks, 4.8 $g \cdot d^{-1}$) and recorded plasma markers of oxidative stress in response to a 40-minute treadmill run. SOD activity, total antioxidant capacity, reduced glutathione, and 8-isoprostane were all unaffected by supplementation at baseline and post-exercise. The additional interpretation of confidence intervals suggested a likely beneficial effect of supplementation in reducing post-exercise 8-isoprostane (84). However, assessing the activity of redox enzymes in plasma is strongly discouraged and the use of non-specific assays, several of which are inherently flawed (for a review, see 86), means that few conclusions can be drawn from these data.

In a novel comparative physiology model, Dolan et al. (55) examined *m. pectoralis* samples from two avian species with distinct metabolic phenotypes: hummingbirds (highly oxidative) and chickens (highly glycolytic). Due to their oxidative and contractile demands, hummingbirds have a well-developed primary antioxidant system to neutralise the by-products of aerobic metabolism. Interestingly, total skeletal muscle HCD content was negatively correlated (R^2 = 0.7899) to SOD activity and positively correlated (R^2 = 0.8659) to βm capacity. A finding that suggests HCDs are non-essential to mitigate oxidative stress in skeletal muscle. Therefore, while HCDs exhibit chemical properties of an antioxidant, the importance of this *in vivo* appears limited due to well-developed primary antioxidant defences. It is therefore possible that carnosine is in fact more effective at binding and removing secondary redox products, namely saturated and unsaturated aldehydes.

Formation of Adducts with Reactive Aldehydes

Carnosine, and other HCDs, contain highly reactive nucleophilic amines that can form stable conjugates with highly toxic lipid peroxidation products (*e.g.,* malondialdehyde, 4-hydroxynonenal (HNE), and acrolein) in skeletal muscle (87). Lipid peroxidation products accumulate following intense exercise, which may amplify and prolong tissue damage under conditions of oxidative stress. To minimize protein modification reactions, most tissues metabolise aldehydes via enzymatic pathways catalysed by aldehyde dehydrogenases and aldo-keto reductases. Emerging evidence shows that m-carn plays an important role in non-enzymatic detoxification of reactive aldehydes, an effect that β-alanine supplementation potentiates.

Following a ~50% increase in m-carn contents, Carvalho et al. (9) detected a >2-fold greater formation of carnosine-acrolein adducts in muscle biopsy samples taken immediately after a HIIT session (4 x 30s Wingate tests with 3 minutes recovery between efforts). There was no effect for exercise- or supplementation-alone, indicating that m-carn conjugated the acrolein generated during exercise. Other markers of lipid peroxidation, carnosine-HHE (4-hydroxy-

hexanal) and carnosine-HNE, were either undetectable or did not change with supplementation or exercise, respectively. Using similar outcomes, Hoetker et al. (35) put participants through a periodized 9-week exercise block that included multiple modes of testing, endurance cycling training, and a 6-week HIIT regime. At the end of the training block, individuals receiving β-alanine supplementation had greater formation of carnosine-aldehyde adducts in post-exercise skeletal muscle samples. Compared with the placebo group formation of carnosine-HNE, and reduced carnosine-acrolein conjugates: carnosine-propanal and carnosine-propanol (88), increased by ~58%, ~119%, and ~86%. Interestingly, and in contrast to Carvalho et al. (9), there were no changes in carnosine-adducts after the first session of HIIT, at which point m-carn contents were elevated by ~51% compared with an elevation of ~127% at the end of the 6-week HIIT program (35).

The reason for the differences between studies is unclear, but both studies showed acrolein-based adducts in higher quantities than with other reactive aldehydes. One explanation is that the bimolecular rate constant for carnosine with acrolein is ~8-fold higher than for carnosine with HNE (89), hence its propensity to form favourably with acrolein or its derivatives. The carnosine-aldehyde adducts are subsequently eliminated from the body via urinary excretion (88). Given that lipid peroxidation products in skeletal muscle are lowest in endurance-trained and highest in sedentary, obese individuals (90), the role of m-carn as a detoxifying agent may be particularly important in clinical populations. Consistent with this hypothesis, carnosine scavenging of damaging reactive aldehyde species has recently been shown to enhance glucose uptake into myotubes, thereby protecting these cells against cellular dysfunction driven by oxidative stress (91).

Perspectives

The enigmatic nature of carnosine makes it challenging to draw a unified conclusion on its physiological roles in human skeletal muscle. However, we have discussed key research in the

field, using a combination of *in vitro*, animal models, comparative physiology, and human intervention studies to show the best available evidence for each role. In the context of exercise physiology, the most robust evidence shows m-carn functions as a pH_i buffer; however, this does not exclude the potential of other functions that may be additive to, or in place of, its buffering actions. The possibility that m-carn acts as a cytoplasmic Ca^{2+}–H^+ exchanger is an exciting new paradigm; coupled with emerging evidence that m-carn detoxifies reactive aldehydes, shows that there is still much to learn regarding the physiological roles of carnosine in skeletal muscle.

It is important to note that, outside of skeletal muscle, the actions of carnosine may markedly differ to those we have discussed. Indeed, roles that are less important in skeletal muscle may be of primary importance in other organs and vice-versa. Major pathways of β-alanine metabolism and its turnover require further investigation in humans. It is possible the elevation in skeletal muscle β-alanine, as occurs with high-dose supplementation, plays a functionally relevant role within the muscle cell or in its transport to another organ. Further knowledge from animal and cell models is likely to come from approaches that involve knocking-out or overexpressing genes involved in carnosine metabolism. Whether carnosine can influence gene expression or epigenetics is of interest and will undoubtedly be an area of future research.

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Conflict of Interest

JJM, GGA, and MDT collectively declare that they have no competing interests. CS has received β-alanine supplements free of charge from Natural Alternatives International (NAI) for use in experimental investigations; NAI have also supported open access page charges for some manuscripts. The review is presented honestly, and without fabrication, falsification, or inappropriate data manipulation. The viewpoints expressed in the review do not constitute endorsement by the American College of Sports Medicine.

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Figure Captions

Figure 1. A schematic depicting carnosine and β-alanine absorption and metabolism. The presence of carnosine in the blood is dependent on the ingested dose and CN 1 activity. Specific transporters on the basolateral membrane have not been fully characterised. β-alanine metabolism in the liver represents a pathway observed in rodents, putative in humans, but requires validation. β-alanine transport is sodium and chloride-dependent. Carnosine transport into and out of skeletal muscle remains to be demonstrated in humans. See the corresponding text for the accompanying references. CN1; serum carnosinase, CN2; tissue carnosinase, MSA; malonate semialdehyde, PAT1; proton-assisted amino acid transporter 1, PEP1/2; peptide transporter 1/2, PHT1; peptide-histidine transporter 1, TauT; taurine transporter, TCA; tricarboxylic acid.

Figure 2. Representative skeletal muscle carnosine concentrations, specific to fibre-type, across different demographics. All samples obtained using the muscle biopsy technique from *m. vastus lateralis*. ● young, healthy males and mixed gender samples; ■ young, healthy females; □ young, healthy vegetarian males (pre- and post-HIIT training intervention); ▲ elderly (~70y) males and females. TI; type I muscle fibres, TII; type II muscle fibres (based on data from: 10,26,30,31,92).

Figure 3. Relative increase in skeletal muscle carnosine contents following b-alanine supplementation. Values obtained using the muscle biopsy technique or proton magnetic resonance spectroscopy (^{1}H-MRS). Data presented are changes in mixed-muscle fibre-types from 13 independent studies (total 54 samples: 7,10,11,19,22,27,30,32,37,41,45,47,77).

Figure 4. During a pH change from 7.1 to 6.5, m-carn concentrations of 10.5 $mmol \cdot kg^{-1} \cdot dm^{-1}$ and 49.7 $mmol \cdot kg^{-1} \cdot dm^{-1}$ provide the capacity to sequester ~3.5 $mmol \cdot H^{+} \cdot kg^{-1} \cdot dm^{-1}$ and 16.5 $mmol.H^{+} \cdot kg^{-1} \cdot dm^{-1}$ (■), respectively. Corresponding to a *βm-carn* contribution to total *βm* of 4.5% and 18.2% (▲). Data show values in omnivore TI muscle fibres at baseline (23) and TII muscle fibres following 28d β-alanine supplementation (10). Non-bicarbonate *βm* was determined by

titrating muscle homogenates against HCl at 37^{o}C and expressed as the μmol H^+ required to change the pH of 1g free-dried muscle from pH 7.1 to 6.5. This encompasses the contribution made by the static buffers (proteins, dipeptides and phosphates) to *in vivo* buffering. A derivation of the Henderson-Hasselbalch equation was used to estimate the contribution of m-carn to total buffering ($\beta m\text{-}carn$ = {[m-carn]/(1 + $10^{(6.5\ -pKa)}$)} – {[m-carn]/(1 + $10^{(7.1\ -pKa)}$)}). The linear relationship shown may not reflect pH changes *in vivo*, where reductions can be non-linear.

Figure 5. Schematic representation of the force-Ca^{2+} relationship in skeletal muscle. A leftwards shirt indicates an increase in myofilament Ca^{2+} sensitivity (red line). The result is a large increase in submaximal force (dashed line) with little effect on maximal force.

Figure 6. Schematic depicts the proposed role of Ca^{2+}-H^+ exchanger in skeletal muscle. Ca^{2+} release and reuptake from the SR, combined with H^+ production from glycolysis, results in ionic microdomains and local pH_i gradients. Carnosine is able to bind, transport, and deposit H^+/Ca^{2+} in the cytoplasm to regulate local pH_i. Subsequently, H^+ appearing in the blood are buffered by bicarbonate (HCO_3^-) and expired as CO_2. Primary pathways involve the (1) sodium-hydrogen exchanger, (2) sodium bicarbonate cotransporter, and (3) monocarboxylate transporter (MCT) 1 and 4. Key membrane transporters and organelles involved in H^+ and Ca^{2+} homeostasis are depicted: Cl^- channel isoform 1 (CIC-1), dihydropyridine receptors (DHPR), monocarboxylate transporters 1/4 (MCT1/4), sodium-bicarbonate cotransporter (NBC), Na^+–Ca^{2+} exchanger (NCX), Na^+–H^+ exchanger (NHE), plasmalemmal Ca^{2+}–H^+–ATPase pumps (PMCA), ryanodine receptor 1 (RyR1), sarcoplasmic reticulum (SR), sarco-endoplasmic reticulum Ca^{2+}–H^+–ATPase pumps (SERCA).

Figure 1

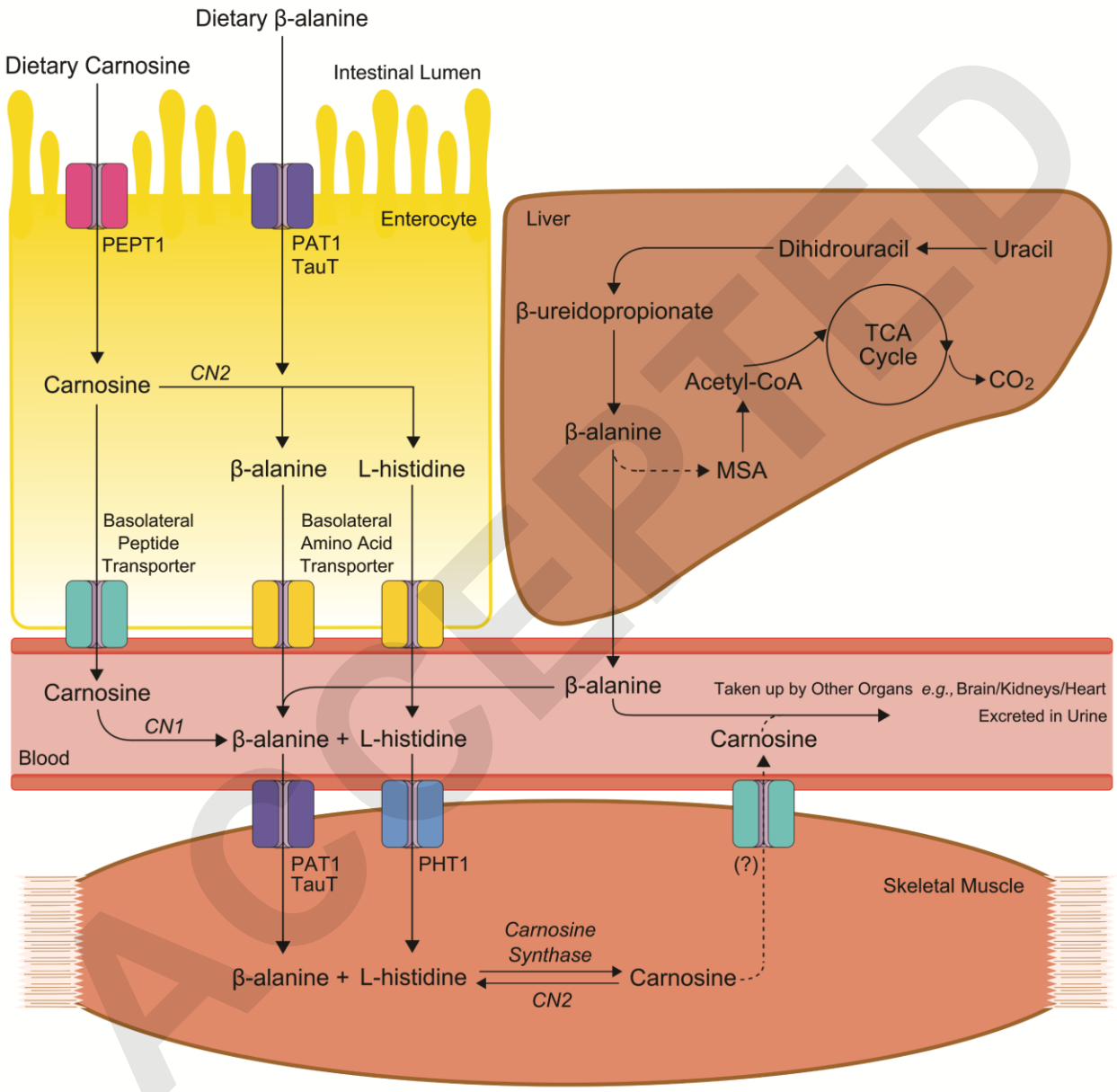

Figure 2

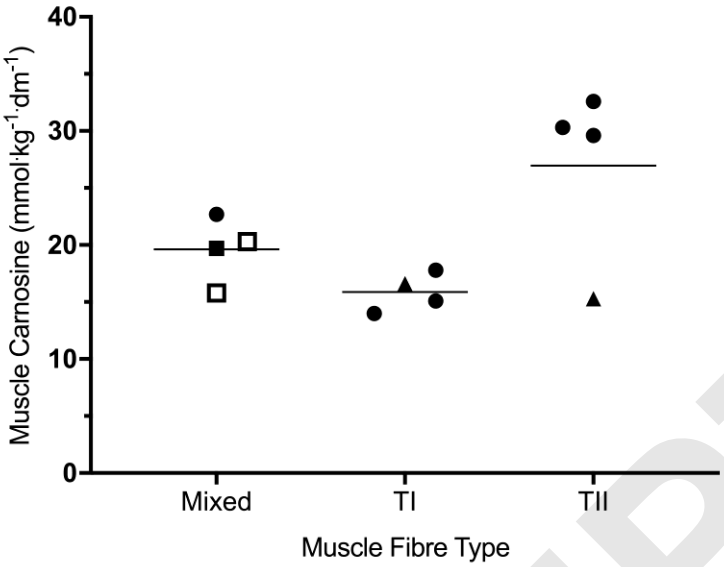

Figure 3

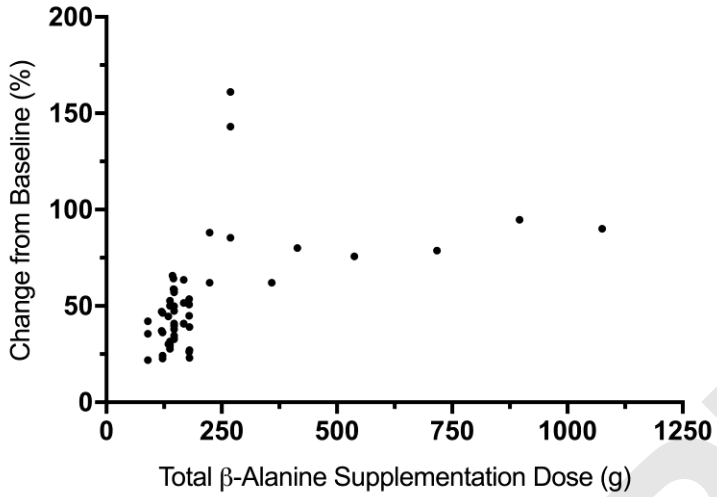

Figure 4

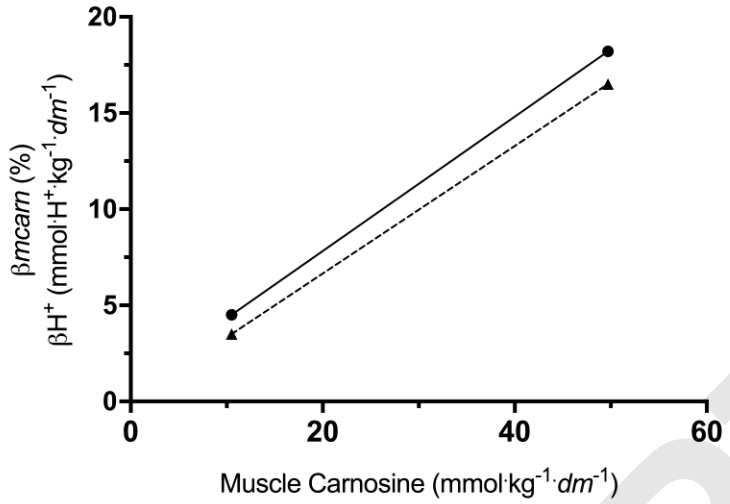

Figure 5

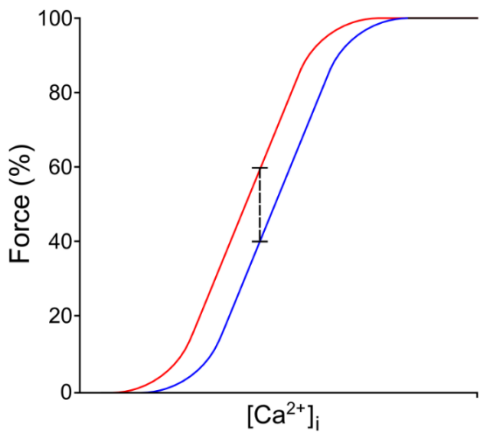

Figure 6