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ORIGINAL ARTICLE

Effect of two β-alanine dosing protocols on muscle carnosine synthesis and washout

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Abstract Carnosine (β-alanyl-L-histidine) is found in high concentrations in skeletal muscle and chronic β-alanine (BA) supplementation can increase carnosine content. This placebo-controlled, double-blind study compared two different 8-week BA dosing regimens on the time course of muscle carnosine loading and 8-week washout, leading to a BA dose–response study with serial muscle carnosine assessments throughout. Thirty-one young males were randomized into three BA dosing groups: (1) high–low: 3.2 g BA/day for 4 weeks, followed by 1.6 g BA/day for 4 weeks; (2) low–low: 1.6 g BA/day for 8 weeks; and (3) placebo. Muscle carnosine in *tibialis-anterior* (TA) and *gastrocnemius* (GA) muscles was measured by ^{1}H-MRS at weeks 0, 2, 4, 8, 12 and 16. Flushing symptoms and blood clinical chemistry were trivial in all three groups and there were no muscle carnosine changes in the placebo group. During the first 4 weeks, the increase for high–low (TA 2.04 mmol/kg$_{ww}$, GA 1.75 mmol/kg$_{ww}$) was ~twofold greater than low–low (TA 1.12 mmol/kg$_{ww}$, GA 0.80 mmol/kg$_{ww}$). 1.6 g BA/day significantly increased muscle carnosine within 2 weeks and induced continual rises in already augmented muscle carnosine stores (week 4–8, high–low regime). The dose–response showed a carnosine increase of 2.01 mmol/kg$_{ww}$ per 100 g of consumed BA, which was only dependent upon the total accumulated BA consumed (within a daily intake range of 1.6–3.2 g BA/day). Washout rates were gradual (0.18 mmol/kg$_{ww}$ and 0.43 mmol/kg$_{ww}$/week; ~2%/week). In summary, the absolute increase in muscle carnosine is only dependent upon the total BA consumed and is not dependent upon baseline muscle carnosine, the muscle type, or the daily amount of supplemented BA.

Keywords β-alanine · Carnosine · Muscle · Synthesis · Washout · Dose–response

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Introduction

Carnosine has been described since the 1930s as a potent intra-muscular buffer due to its nitrogen containing side imidazole ring, which can directly accept and buffer H^+ ions at a pKa constant of 6.83 (Bate-Smith 1938), thus slowing the decline in pH during intense exercise (Baguet et al. 2010b). Beyond the imperative role that carnosine plays in intra-muscular buffering during high-intensity exercise, a multitude of other physiological roles for carnosine has been demonstrated with in vitro models, including acting as an antioxidant (Boldyrev et al. 1993), a regulator of muscle excitation–contraction coupling via increasing calcium sensitivity (Batrukova and Rubtsov 1997), a source of histidine for histamine synthesis for wound healing (Flancbaum et al. 1990) and protecting proteins against glycation during aging (Hipkiss 2005). The main source of dietary carnosine in humans is via meat consumption (Abe 2000), of which most omnivores consume ~50 to 300 mg of carnosine per day (Baguet et al. 2009) and as much as ~500 mg if they consume 100 g of turkey. Conversely, it has recently been demonstrated that vegetarians have ~22% lower muscle carnosine content (Everaert et al. 2011). Dietary carnosine is rapidly broken down in plasma due to the presence of the hydrolyzing

enzyme carnosinase (Asatoor et al. 1970), which yields β-alanine (BA) and L-histidine with minimal carnosine detectable in the plasma (Park et al. 2005). In contrast, carnosine is synthesized in human skeletal muscle and cells of the CNS from the essential amino acid L-histidine and the non-proteinogenic amino acid β-alanine (Bakardjiev and Bauer 1994).

Since the concentration of L-histidine in muscle and plasma is high relative to the low intramuscular concentration of BA, which exhibits a much higher K_m for the enzyme carnosine synthetase (Bakardjiev and Bauer 1994), it was hypothesized that BA may be limiting to carnosine synthesis. Supporting this premise, Harris et al. (2006) were the first to show that prolonged BA supplementation ($\sim$3 to 6 g/day) in humans results in a significant 40–65% increase in muscle carnosine content and have subsequently shown that this can increase high-intensity exercise performance via total work done at 110% of cycling wattage maximum (Hill et al. 2007), which was recently confirmed by Sale et al. (2011). It has previously been shown that when subjects are given a single BA dose of more than 800 mg (corresponding to $\sim$10 mg/kg body weight) there are ever increasing paresthesia symptoms (minor "pins and needles", skin vasodilation, flushing, over $\sim$60 to 120 min; Harris et al. 2006). But, the required acute daily BA needed to increase muscle carnosine is $\sim$tenfold higher than normal dietary intake. Given this, most studies have implemented small repeated daily doses of $\leq$800 mg (as much as 8 individual doses; up to 6.4 g/day) to minimize paraesthesia symptoms (Derave et al. 2007; Hill et al. 2007; Kendrick et al. 2008). Recently, a commercialized slow-release tablet of BA has been produced and it was demonstrated that a single 1.6 g BA dose resulted in a blunting of the peak plasma curve with no reports of any paresthesia symptoms in any subjects (Decombaz et al. 2011; Harris et al. 2008). However, the efficacy of muscle carnosine synthesis with the slow-release BA tablet remains to be investigated.

Recently, there has been considerable interest from the scientific and clinical research communities relating to the impact that augmented muscle carnosine may have on health and sports performance outcomes (for reviews see: Artioli et al. 2010; Derave et al. 2010; Sale et al. 2010), and all previous studies have shown significant increases in muscle carnosine pre to post-BA supplementation (Baguet et al. 2009, 2010a; Derave et al. 2007; Harris et al. 2006; Hill et al. 2007; Kendrick et al. 2008, 2009). However, only one of these previous studies utilized two different BA doses and showed that a total of 89.6 g BA for 4 weeks resulted in a 7.8 mmol/kg dry mass (dm) increase in muscle carnosine, whereas 145.6 g BA for 4 weeks resulted in a 11.1 mmol/kg dm increase (Harris et al. 2006), suggesting some degree of linearity to the dose-response. Thus, there remains to be a comprehensive dose–response study examining the effect of several BA doses with serial muscle carnosine assessments throughout to clearly delineate the synthesis and wash-out rates in several muscle groups. Furthermore, no studies have yet examined whether a slow-release BA tablet, which circumvents paresthesia symptoms, is also effective in raising muscle carnosine content and only one previous study has examined standardized clinical chemistry measures pre and post prolonged BA supplementation (Harris et al. 2006). Consequently the optimal BA dosing regimen to augment intramuscular carnosine content remains to be clarified. Therefore, this placebo-controlled, double-blind study was designed to compare two different 8 week BA dosing regimens on the time course of muscle carnosine loading and subsequent 8 week washout. Non-invasive ^{1}H-magnetic resonance spectroscopy (MRS) was utilized to undertake six serial muscle carnosine measures throughout. Our hypotheses were that carnosine synthesis would show a progressive dose–response dependent upon daily BA intake (either 1.6 or 3.2 g/day) with no signs of intramuscular saturation.

Methods

Subjects

Thirty-one healthy male subjects with a body mass index (BMI) $>$ 18 and $<$25 kg/m^2 participated in this study. Subjects were excluded if they had a soy, fish or crustacean allergy, had extremely adverse paresthesia sensitivity to a pure 400 mg β-alanine (BA) acute supplementation (as assessed during the initial visit) and if they were regular consumers of sports foods/supplements in the last 3 months. Furthermore, subjects were also excluded if their baseline muscle carnosine content was $>$1 standard deviation above the average baseline muscle carnosine as assessed by preceding measurements combined from a pilot study and the current study (excluded if the average of *gastronemius* (GA) and *tibialis anterior* (TA) eventually was $>$8.95 mmol/kg$_{ww}$ (kg wet weight)). Altogether, 43 subjects were initially tested, 9 were excluded due to carnosine contents that were $>$1 standard deviation above the average baseline, and 3 withdrew during the course of the experiment for scheduling or personal reasons. There were no differences in average age (24.8 $\pm$ 4.5 year), weight (76.1 $\pm$ 8.0 kg), height (181.2 $\pm$ 6.2 cm) or BMI (23.2 $\pm$ 1.8 kg/m^2) between any of the three experimental groups. An initial lifestyle questionnaire that examined subjects habitual dietary (meat and fish consumption) and exercise routines and baseline muscle carnosine content showed no relationship (correlation between baseline

muscle carnosine and meat consumption ($r = 0.16$) or training/exercise load ($r = 0.11$)). All subjects gave their informed consent, and the study was approved by the local Ethics Committee of the Kanton Bern (KEK #117/08), and registered on ClinicalTrials.gov (#NCT 00813553).

Experimental design

This study was designed as a placebo-controlled, double-blind, randomized, parallel-design, single center study with three intervention groups (Fig. 1). The primary purpose was to examine the dose–response of muscle carnosine synthesis to two different β-alanine supplementation regimes compared to placebo over 8 weeks, and subsequent washout (weeks 8–16). Subjects were computer randomized (Trialsys program) into one of 3 supplementation groups as follows:

1. Group high–low ($n = 10$): High dose BA for 4 weeks (3.2 g BA/day), followed by low dose BA for 4 weeks (1.6 g BA/day), followed by 0 g BA/day for 8 weeks.
2. Group low–low ($n = 11$): low dose BA for 8 weeks (1.6 g BA/day), followed by 0 g BA/day for 8 weeks.
3. Group placebo ($n = 10$): 0 g BA/day for 8 weeks, no washout measurements.

As further described below, muscle carnosine and creatine quantification by ^{1}H-MRS was performed at baseline (week 0) and at weeks 2, 4, 8, 12 and 16 in both GA and TA lower leg muscle groups for the HL and LL groups. After week 8, all ^{1}H-MRS data were entered into a controlled database prior to knowing what subjects were in the PL group, who did not have any further ^{1}H-MRS measures made on week 12 or 16. A questionnaire based self-assessment of possible paraesthesia symptoms was conducted during the MRS measurement visits at weeks 0, 2, 4 and 8 (Fig. 1). A wide range of standard blood parameters (for full list and lab normal reference values see Table 1) were also assessed by the clinical chemistry laboratory at Inselspital-Bern University Hospital from a 15 ml blood draw at baseline and post-supplementation (week 8). The Inselspital clinical chemistry lab is accredited STS 259 according to the international norm ISO/IEC 17025.

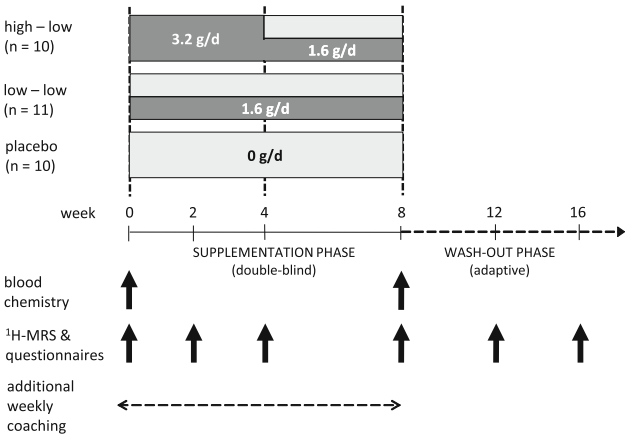

Fig. 1 Schematic representation of study protocol, which was a placebo-controlled, double-blind, randomized, parallel-design, single center study with three groups supplementing either β-alanine or placebo over 8 weeks: (1) high–low; (2) low–low; and (3) placebo

β-alanine and placebo supplements

The BA and the placebo were prepared in two batches in the form of identical ~1,200 mg tablets by Natural Alternative International, Inc. (NAI). Active or placebo blister packs of tablets were then filled by an external non-study related certified co-packer using blinded study coding lists. Each active tablet contained 800 mg of β-alanine, along with hydroxypropyl, methylcellulose, stearic acid, magnesium stearate and silicon dioxide, in a novel controlled slow-release form. Batch BA purity and individual tablet dose was determined by ion exchange chromatography and quantified using dual wavelength photometric detection following ninhydrin post column derivatization (Biochrom 30, Biochrom Ltd, Cambridge, UK; PEEK Li columns, Laborservice Onken GmbH, D-Gründau), and each tablet was measured to be 92–98% of target theoretical 800 mg dose. All subjects consumed two daily servings of 2-tablets/serving (4 tablets/day) throughout the entire 8 weeks supplementation period with meals. Subjects met with investigators regularly to receive their blister pack of supplements (Fig. 1), and compliance was verbally checked each week and ensured by subjects returning the empty blister packs. Subject's compliance to consuming all required supplements was nearly 100%.

MRS acquisition and spectral fitting

Each subjects planned versus actual visit for their MRS scan was extremely precise, with 91% of MRS scans being achieved on scheduled days ($n = 151$), 97% within 2 days of schedule ($n = 10$), and the rest of the scans achieved within 5 days of scheduled date ($n = 5$). For MRS scheduling/availability, nearly all measurements were done at the same time of the day. As outlined above, a time course of carnosine and creatine concentrations in both TA and GA muscles was measured by ^{1}H-MRS using a 3 Tesla MR system (TRIO, SIEMENS Erlangen, Germany). Following the acquisition of a localizer series by the body coil for the adjustment of the spectroscopy voxel relative to the tibia plateau, a high-resolution imaging series of the calf (Fast spin echo, echo train 19, TR = 2660 ms, TE 13 ms, slice 4 mm, pixel 0.625 mm × 0.625 mm) was acquired for the definitive placement of the MRS voxel (Fig. 2a, b). A standard flexible surface coil was used to obtain the

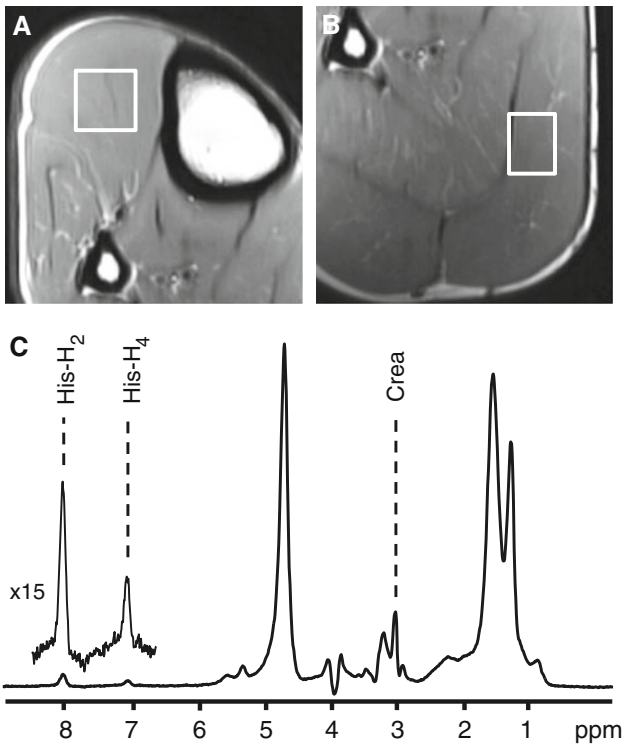

Fig. 2 MR images with localized volume of interest (*white box*) in *m. tibialis anterior* (**a**) and *m. gastrocnemius* (**b**). Image **c** shows a water-suppressed ^{1}H-MR spectrum obtained from an 18 × 18 × 30 mm^3 voxel located in the *m. tibialis anterior* (male, 22 years). The labeled resonances are Histidine-H_2 (His-H_2, used for quantification of carnosine), Histidine-H_4 (His-H_4), and central line of the creatine-CH_3 resonance (Crea, triplet due to dipolar coupling)

high-resolution images and subsequent PRESS spectra ("point-resolved-spectroscopy", TR = 3000 ms, TE = 30 ms). The default voxel size was set to 18 × 18 × 30 mm (Left–Right, Anterior–Posterior, Head–Feet), but was adjusted in LR and AP direction in the case of small TA or GA muscles or fatty infiltrations (Fig. 2a, b). Ninety-six scans with the central frequency at the carnosine-H_2 position (8.0 ppm, PRESS sequence of the vendor adapted) were followed by an unsuppressed water scan ($n = 1$) with the central frequency shifted to the water position to correct for the chemical shift displacement and to acquire exactly the same voxel for the metabolites and the water standard. A second spectrum with a smaller voxel and the central frequency shifted to creatine was acquired at the same position to measure the region between 0.5 and 4.5 ppm. During the measurement, the leg was fixed in all three directions by a home-built fixation device. Quantitation of the carnosine-H_2 and the water resonance was done in jMRUI-3.0 (Naressi et al. 2001) by a batch job with optimized prior knowledge (known relations between resonances and limitations for the fitting process) without human interaction: (1) Removal of the resonances between 0 and 6.0 ppm by a HLSVD process (Hankel-Lanczos Singular Value Decomposition) (2) Copy of the unsuppressed water resonance multiplied by 0.001 to −1,000 Hz from the original position in the spectrum. This resonance was then used to fix the line-width of the carnosine resonances (3) Fitting of the spectra using the AMARES algorithm with the following prior knowledge: line-width carnosine-8 = line-width water * 0.83. This ratio was determined from an unrestricted automatic fit from all spectra. Phase and frequency of the resonances were limited with soft constraints. The carnosine-H_2 (Fig. 2c) resonance at 8.0 ppm was used for quantitation since it is less affected by dipolar coupling, resulting in broadening and apparent T2 shortening, than the carnosine-H_4 resonance (Baguet et al. 2009; Boesch and Kreis 2001; Derave et al. 2007; Ozdemir et al. 2007; Schroder and Bachert 2003). Calculation of absolute carnosine concentrations [mmol/kg_{ww}] was based on the unsuppressed water signal and on corrections for the effect of relaxation times (Ozdemir et al. 2007). The central peak of the creatine-CH_3 resonance (Fig. 2c) was quantified without preceding application of HLSVD to estimate relative changes of muscular creatine. The coefficients of variances (CV) were determined previously and in pilot testing, and for carnosine was 8.0% and for creatine to be 12.8% (data not shown).

Paresthesia symptoms questionnaires

Any paresthesia side-effects (e.g., flushing, vasodilation, tingling) and any associated mood-related influences were monitored by a series of questionnaires (Fig. 1). These questionnaires included a body surface symptoms score (SSS), profile of mood states (POMS) and a state anxiety inventory (SAI), and are fully described by Decombaz et al. (2011). The flushing symptoms questionnaire (FSQ) was developed specifically for this study, and was a 10-question retrospective questionnaire focusing on any unusual skin or paresthesia symptoms, and the intensity and duration of symptoms, in the previous 24 h. Questions on "warmth", "redness", "pins and needles" and "itching" on a 5-point scale (from none to weak to moderate to strong or extremely strong) were included. The SSS questionnaire was about identifying potential unusual sensations in relation to spatial characteristics on the body surface. It was developed to identify the body site locations most affected by unusual sensations. It consisted of a schematic image of the body, front and back sides, with 23 areas marked by rectangular areas. Subjects were instructed to identify as many areas where symptoms were perceived, or to select the closest rectangle if symptoms fell outside. For POMS, a German adaptation (Albani et al. 2005) of the abridged POMS (Shacham 1983) was used. It consists of 37 descriptors, each belonging to one of 6 different mood states: depression, anxiety-tension, anger-hostility, fatigue-

inertia, confusion, and vigor activity. The Spielberger version of the SAI was used (Spielberger et al. 1983) containing 20 statements of which half comprise positive feelings and half comprise negative feelings. The SAI is the sum of all scores and ranges from 20 to 80. The higher the SAI, the more anxious the subject is.

Statistics

A univariate general model was used to detect an influence of the muscle type and any differences that could have occurred with the arbitrary assignment to the treatment groups. An analysis of covariance (ANCOVA, covariate being the level at week 0) was performed on log-transformed data between treatments at weeks 2, 4, and 8, followed by a post-hoc comparison of the groups with Bonferroni correction. A paired *t* test was used to compare blood clinical chemistry outcomes. For the dose–response analysis, a univariate general linear model (full factorial with "starting levels" as covariate and the factors "muscle", "treatment regime", "accumulated BA", including all interaction terms) was used to detect significant differences for absolute and relative dose response. All statistical analysis was performed with PASW 18.0.0 (SPSS Inc., Chicago, IL, USA). All data are reported as mean ± standard deviation (SD) with significance assumed at $p < 0.05$, unless otherwise indicated.

Results

Muscle carnosine synthesis and washout

The time course of GA and TA carnosine synthesis and washout are highlighted in Fig. 3a, b, respectively. Baseline carnosine at week 0 was not different between the three groups for either TA (5.75 ± 1.09 mmol/kg_{ww}; range: 3.97–7.48) or GA muscles (8.84 ± 1.54 mmol/kg_{ww}; range: 5.32–11.89), but TA had significantly lower ($p < 0.001$) baseline carnosine than GA. There was no significant increase in muscle carnosine in the placebo trial for either muscle group.

Week 2 Although highly variable, already after 2 weeks, the high–low treatment regime [total of 44.8 g β-alanine (BA)] lead to a significant increase in carnosine in both muscles (17.4 ± 9.6% in TA, $p < 0.001$; 9.7 ± 10.8% in GA, $p = 0.030$). In addition, just 22.4 g of total BA consumed (low–low group) significantly increased carnosine in TA muscle (11.8 ± 7.4%, $p = 0.005$), and there was a trend for an increase in GA (8.1 ± 11.5%, $p = 0.082$).

Week 4 During the first 4 week, the low–low group supplemented a total of 44.8 g BA, while high–low group supplemented double that amount (89.6 g BA). Accordingly, there was a ~twofold greater increase (TA $p = 0.011$ vs. GA $p = 0.030$) in muscle carnosine in both muscle groups for the high–low group (TA 2.04 mmol/kg_{ww} vs. GA 1.75 mmol/kg_{ww} increase) compared to the low–low group (TA 1.12 mmol/kg_{ww} vs. GA 0.80 mmol/kg_{ww} increase).

Week 8 At the end of 8 weeks, low–low resulted in a 21.9 ± 14.4 and 35.5 ± 13.3% increase in carnosine in TA and GA muscles, respectively. Equally, high–low BA supplementation resulted in a 30.3 ± 14.8 and 44.5 ± 12.5% increase in carnosine in TA and GA muscles, respectively, which was significantly greater ($p < 0.001$ for each muscle) than placebo. However, differences between the two treatment regimes high–low versus low–low at 8 weeks were no longer statistically significant (GA $p = 0.261$, TA $p = 0.269$).

Washout From weeks 8 to 16, the decay rates were modest and between 0.09 and 0.22 mmol/kg_{ww} per week (~2 to 3% of the baseline value per week) with the muscular carnosine levels remaining ~40% of the BA-induced increase after the 8 weeks wash-out period (Fig. 3).

Muscle carnosine dose–response regression analysis and correlations

Given the current study's design of multiple BA doses and muscle carnosine measures over time, an in-depth dose–response analysis using a univariate general linear model was undertaken examining the absolute (mmol/kg_{ww}; Fig. 4a) and relative (%, Fig. 4b) increase in muscle carnosine content compared to the total grams of BA consumed. Thus, eight separate dose–response analyses could be made (two doses, two muscle groups at weeks 2, 4 and 8). A univariate general model (full factorial model with the covariate "starting levels" and factors "muscle", "consumed BA dose", "treatment regime", and all interaction terms) of the absolute carnosine levels (Fig. 4a) revealed a highly linear dependence with a R^2 of 0.921 and showed that consumed BA dose ($p < 0.001$) and starting carnosine levels ($p < 0.001$) were responsible for ~80% of data variation in absolute carnosine levels. A linear regression of the absolute carnosine increases showed a dose–response of a carnosine increase of 2.01 mmol/kg_{ww}

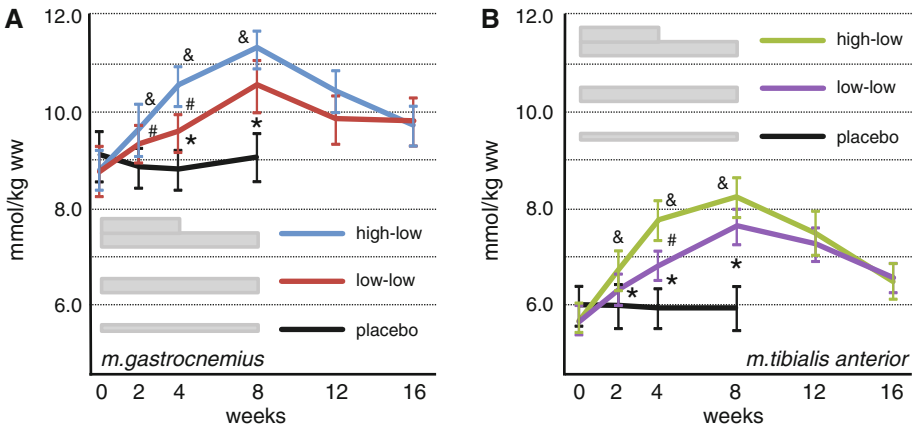

Fig. 3 Synthesis and washout of muscle carnosine concentrations [mmol/kg_{ww}] over time in *m. gastronemius* (**a**) and *m. tibialis anterior* (**b**) following either *β*-alanine or placebo over 8 weeks. #low–low significantly different than placebo, &high–low significantly different than placebo, *low–low significantly different than high–low

per 100 g of consumed BA dose (95% confidence interval 1.83–2.19 mmol/kg_{ww}). From all the other factors, only muscle type showed a tendency to contribute to the dose–response observations, but did not reach significance ($p = 0.055$). In particular, the daily BA treatment regime ($p = 0.941$) was unrelated to the dose-response. In contrast, when the same univariate general model was applied to relative carnosine levels (Fig. 4b), muscle ($p < 0.001$), consumed BA dose ($p < 0.001$), and dose*muscle ($p = 0.004$) were significant while the daily BA treatment regime was still unrelated to the observed dose–response ($p = 0.799$). The differences between the univariate analyses of absolute and relative carnosine increases were due to the fact that there was ~50% more ($p < 0.001$) baseline muscle carnosine in GA versus TA muscle groups (Figs. 3, 5), thus reducing the relative increase in GA.

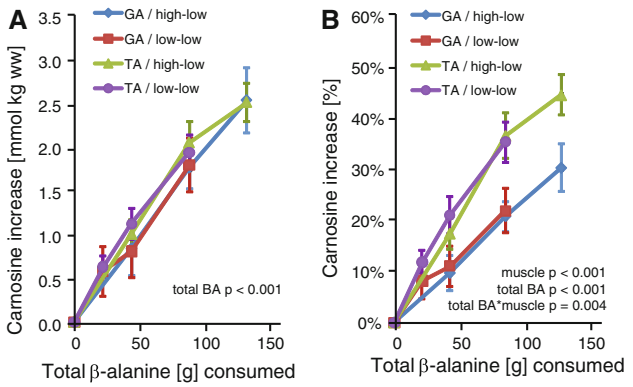

Fig. 4 Dose–response characteristics of *β*-alanine on the muscle carnosine levels in *gastronemius* (GA) and *tibialis anterior* (TA) muscles following two different *β*-alanine supplementation protocols of either high–low or low–low regime over 8 weeks shown as (**a**) absolute carnosine increases [mmol/kg_{ww}] and (**b**) relative carnosine increases (% increase)

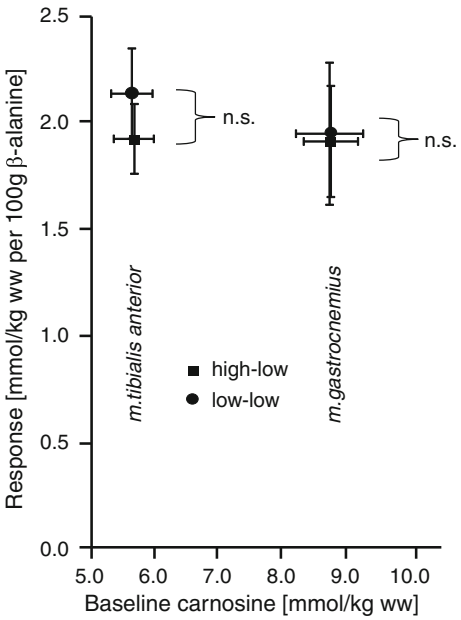

Fig. 5 Average absolute increases in muscle carnosine following two different *β*-alanine supplementation protocols of either high–low or low–low over 8 weeks in relation to baseline starting muscle carnosine values in *m. gastronemius* and *m. tibialis anterior*, respectively. The overlapping *error bars* indicate that there is neither a difference in carnosine increases between the two treatment regimes nor between the two muscle types

Figure 5 shows the average absolute increase in muscle carnosine due to BA supplementation in relation to the baseline pre-BA supplementation muscle carnosine values in both TA and GA muscles. At baseline, GA muscle carnosine was ~3 mmol/kg_{ww} greater ($p < 0.001$) than TA. There was no association between baseline carnosine levels and subsequent muscle carnosine increases with BA supplementation for either muscle group or for the two supplementation regimes ($p = 0.342$ for all data points).

Muscle creatine concentrations

Creatine was also accessed by means of ^{1}H-MRS (data not shown). There was no treatment or time effect on muscle creatine (central resonance fitted in individual spectra) throughout the entire intervention, as the placebo group had similar values compared to either high–low or low–low.

Blood clinical chemistry

Pre- and post-supplementation analysis of whole blood and plasma parameters for each treatment group revealed no significant effects of time or any treatment (Table 1). At baseline, there were no differences between the groups and when collapsed there were 3.8% of measurements that were outside clinical norms (2.9% above norms, 0.9% below norms). There was no significant difference between treatment groups post-BA supplementation (low–low vs. high–low), and when collapsed together 5.3% of measurements was outside norms (4.4% above norms, 0.9% below norms), with no significant difference from pre-supplementation ($p = 0.215$). No clear trend was shown and the variations between subjects were larger than any effect of treatment.

Chronic paresthesia symptom and mood assessments

The FSQ and the SSS questionnaires assessed any unusual body surface paresthesia symptoms and the location of the symptoms, respectively, while the POMS and SAI investigated any associated mood/temperament associated with such symptoms. The percentage of unusual sensation via FSQ (ten questions per assessment, with six separate tests, Fig. 1) was generally very low, and even without correction for multiplicity, there was no statistical difference between the groups (16.4, 11.6 and 20.0% reported unusual symptoms for placebo, low–low and high–low, respectively). Of these unusual symptoms, the SSS questionnaire

Table 1 Blood hematology and clinical chemistry for each treatment group pre and post 8 week β-alanine or placebo supplementation

Parameter	Normal ref.	High–low ($n = 10$)		Low–low ($n = 11$)		Placebo ($n = 10$)	
	range	PRE	POST	PRE	POST	PRE	POST
Na (mmol/l)	132–142	140.8 ± 1.8	141.2 ± 1.3	141 ± 1.6	141.7 ± 1.7	140.7 ± 1.3	140.6 ± 1.4
K (mmol/l)	3.5–4.7	3.8 ± 0.4	3.8 ± 0.3	3.9 ± 0.1	3.9 ± 0.4	3.8 ± 0.3	3.9 ± 0.3
Creatinine (mol/l)	59–104	81.5 ± 7.6	83.9 ± 13.6	79.6 ± 10.5	80.2 ± 7.3	75.8 ± 8	78.3 ± 8.7
Urea (mmol/l)	2.9–7.7	5.6 ± 0.9	5.9 ± 1.1	5.5 ± 1.4	5.8 ± 1.2	5.5 ± 0.9	5.1 ± 1.0
Total protein (g/L)	60–82	74.4 ± 3.7	76.4 ± 2.3	76.3 ± 2.8	76.5 ± 4.1	75.7 ± 4.2	77.3 ± 3.3
Albumin (g/L)	30–52	43.6 ± 1.9	43.0 ± 3.7	45.5 ± 2.9	43.9 ± 2.7	45.4 ± 3.4	45.7 ± 2.4
Total bilirubin (μmol/L)	3–26	12.0 ± 6.6	12.1 ± 7.2	13.4 ± 9.0	12.8 ± 4.8	8.3 ± 4.4	11.7 ± 6.9
AST (U/L)	10–41	36.9 ± 28.6	39.3 ± 23.4	23.6 ± 3.4	24.7 ± 2.9	31.0 ± 8.8	30.7 ± 9.3
Alkaline phosphatase (U/L)	36–108	67.4 ± 18.8	67.0 ± 19.3	62.3 ± 16.6	62.2 ± 14.3	64.6 ± 16.7	62.6 ± 17.2
Bicarbonate (mmol/l)	22–29	25.0 ± 2.2	25.0 ± 1.4	25.6 ± 1.3	25.8 ± 2.4	24.6 ± 1.8	25.3 ± 2.1
CS (cardiac) troponin (μg/l)	0.00–0.01	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
Hgb (g/l)	135–168	155.7 ± 7.1	152.5 ± 6.9	152.1 ± 11.2	151.0 ± 11.3	151.3 ± 7.5	152.0 ± 9.7
Hct (%, l/l)	40–50	45 ± 2	45 ± 2	44 ± 3	45 ± 3	44 ± 2	44 ± 2
RBC (T/l)	4.2–5.7	5.1 ± 0.3	5.0 ± 0.2	5.0 ± 0.3	5.0 ± 0.4	5.0 ± 0.3	5.0 ± 0.3
MCV (fl)	80–98	88.2 ± 2.2	88.8 ± 2.3	88.9 ± 2.3	88.8 ± 2.7	89.3 ± 5.1	89.4 ± 4.9
MCH (pg)	27–33	30.4 ± 1.1	30.4 ± 0.8	30.4 ± 1.2	30.3 ± 1.1	30.6 ± 1.6	30.8 ± 2.3
MCHC (g/l)	320–360	343.6 ± 7.5	343.7 ± 6.1	341.5 ± 5.2	340.5 ± 6.4	342.3 ± 3.7	342.7 ± 7.2
WBC (G/l)	3.5–10.5	7.5 ± 1.7	6.5 ± 1.2	6.8 ± 1.3	6.3 ± 1.0	6.8 ± 2.2	6.6 ± 1.5
Neutrophils (G/l)	1.6–7.4	4.3 ± 1.4	3.5 ± 0.8	4.0 ± 1.1	3.8 ± 1.2	3.7 ± 1.6	3.7 ± 1.3
Eosinophils (G/l)	0.02–0.40	0.21 ± 0.18	0.17 ± 0.11	0.16 ± 0.06	0.15 ± 0.06	0.12 ± 0.11	0.16 ± 0.15
Basinophils (G/l)	0.00–0.15	0.04 ± 0.03	0.03 ± 0.01	0.04 ± 0.03	0.04 ± 0.02	0.03 ± 0.01	0.03 ± 0.01
Monocytes (G/l)	0.20–0.93	0.58 ± 0.15	0.55 ± 0.19	0.56 ± 0.12	0.51 ± 0.12	0.54 ± 0.17	0.55 ± 0.13
Lymphocytes (G/l)	1.1–3.5	2.4 ± 0.7	2.2 ± 0.7	2.0 ± 0.4	1.8 ± 0.5	2.3 ± 0.5	2.2 ± 0.4
Platelets(G/l)	140–380	228.1 ± 49.0	215.6 ± 34.5	233.9 ± 87.0	255.8 ± 105.4	249.1 ± 58.3	243.7 ± 32.5
Gamma-Globulin (kU/l)	0–100	72.3 ± 69.6	96.4 ± 124.2	99.7 ± 241.4	84.5 ± 195.0	56.8 ± 45.6	59.1 ± 54.4

All data reported as mean ± SD

Ref reference, *AST* aspartate-aminotransferase, *Hct* hematocrit, *Hgb* hemoglobin, *K* potassium, *MCH* mean corpuscular hemoglobin, *MCHC* mean corpuscular hemoglobin concentration, *MCV* mean cell volume, *Na* sodium, *RBC* blood cell count, *WBC* white blood cell count

identified that the most frequent symptom location, independent of treatment, was the arms and shoulders. When analyzing the mood questionnaires (POMS and SAI), although minor, the placebo group reported significantly more negative POMS and SAI ratings compared to either treatment (POMS 0.39–0.27; SAI 2.90–3.05), but there was no difference between low–low or high–low treatments. There were no systematic or significant changes in the POMS or SAI ratings across any treatment group throughout the duration of the study.

Discussion

Since this study utilized different β-alanine (BA) doses and multiple muscle carnosine measures over time, it adds significantly to our dose–response understanding of BA supplementation. The key findings are that, within a wide range of total consumed BA used in this study, the absolute increase in muscle carnosine is only dependent upon the total BA consumed, albeit within a daily intake range of 1.6–3.2 g BA/day. Interestingly, it is not dependent upon baseline muscle carnosine, the muscle type, or the daily amount of supplemented BA. In addition, as little as 1.6 g BA/day can significantly increase muscle carnosine stores already within 2 weeks of supplementation and can induce small, but continual, rises in already augmented muscle carnosine stores.

Muscle carnosine dose–response to BA supplementation

In agreement with the current data, every single previous study has demonstrated significant increases in muscle carnosine during a BA supplementation protocol via either quantification of muscle biopsies (Harris et al. 2006; Hill et al. 2007; Kendrick et al. 2008, 2009) or ^{1}H-MRS analysis (Baguet et al. 2009, 2010a; Derave et al. 2007). However, this is the first study to examine the synthesis and washout of muscle carnosine with two different BA dosing protocols with multiple muscle carnosine assessments over time. Our study design allowed for a univariate test to examine various factors, such as daily BA treatment regimes, muscle types, and their interaction that may influence the extent of intramuscular carnosine accumulation. The univariate general model clearly demonstrates a highly linear dependency ($R^2 = 0.921$) based on the total grams of BA consumed, when either 1.6 or 3.2 g BA/day are consumed. It is shown that 100 g BA increased the absolute carnosine levels by 2.01 mmol/kg$_{ww}$ (95% confidence interval 1.83–2.19 mmol/kg$_{ww}$). Thus, the absolute carnosine dose–response is solely dependent upon the total grams of BA consumed, and there is no impact on the muscle type or the daily BA supplementation regime. These findings result in a greatly simplified BA prescriptive application to augment muscle carnosine. Moreover, the continued apparent linearity (or the non-saturation) of this correlation suggests that maximal muscle carnosine levels that are potentially attainable remain to be identified via a long-term (>6 months) BA dosing study. A limitation of this analysis is the fact that a simultaneous wash-out effect during BA intake has not been considered. However, as discussed below, the washout is very slow and thus should not interfere dramatically with the BA loading. In addition, a linear washout as suggested by Baguet et al. (2009) would just reduce the effect of the intake, which would not principally change the statistical model that has been applied.

^{1}H-MRS allows for different muscle groups to be analyzed for carnosine to ascertain whether muscles with more fast-twitch (e.g., GA) or slow-twitch (e.g., TA) muscle fiber profiles display a differential response to BA supplementation. Baseline carnosine content has previously been shown to be greater in muscle with a greater proportion of type II fibers (fast-twitch; e.g., GA) as compared to type I [slow-twitch; e.g., TA (Baguet et al. 2009)] or in single fiber quantification from muscle biopsies of the *vastus lateralis* (Dunnett and Harris 1995; Hill et al. 2007; Kendrick et al. 2009), which is supported by the current data showing baseline GA carnosine content of $\sim$9 mmol/kg$_{ww}$ compared to $\sim$6 mmol/kg$_{ww}$ for TA (Fig. 3a, b). However, since muscle fiber orientation influences ^{1}H-MR spectra (see references in Ozdemir et al. 2007), variations of absolute metabolite levels between different muscles have to be interpreted with caution. C2 resonance of carnosine reduces this influence as compared to the C4 signal. The causative explanation for higher muscle carnosine at baseline and post-BA supplementation in muscle dominated by fast-twitch fibers remain to be clarified. However, given that carnosine contributes to intra-muscular buffering (Bate-Smith 1938), it is interesting to note that sprinters and rowers, as compared to marathon runners, not only have a greater percentage of type II fibers but also greater muscle carnosine concentrations, muscle buffering capacity and high-intensity exercise performance (Parkhouse and McKenzie 1984). Nevertheless, our data are in agreement with others (Baguet et al. 2009) in that we demonstrated the same absolute carnosine increase in both TA and GA muscles ($\sim$2.5 mmol/kg$_{ww}$; Fig. 4a). This could suggest that there are no fiber type differences in either the BA transporter, changes in transporter expression throughout the supplementation period, and/or differences in muscle carnosine synthase (Bakardjiev and Bauer 1994), as one might then expect a divergence in fiber type specific muscle carnosine synthesis during BA supplementation. However, these mechanism(s) remain to be elucidated.

We also examined whether there was any relationship between baseline carnosine content and the subsequent increase in carnosine due to BA supplementation. Figure 5 shows that there was no statistically detectable effect that lower baseline carnosine levels lead to greater subsequent muscle carnosine increases ($n = 21$). Conversely, Baguet et al. (2010a) found a positive correlation between high baseline muscle carnosine and the increase in muscle carnosine after supplementation [$n = 8$; (Baguet et al. 2010a)]. This difference may be due to some methodological differences of the studies (inclusion of soleus muscle in the Baguet study, exclusion of subjects with high carnosine levels in the current study). Interestingly, muscle creatine increases have been shown to be negatively correlated to high baseline creatine values (Harris et al. 1992), which suggest a saturation, or "muscle full", response and helps to potentially explain responders and non-responders to creatine supplementation. Even if most of the published data showing increases in muscle carnosine from BA supplementation do not yet appear to show significant carnosine saturation, perhaps by excluding subjects with high-baseline carnosine in the current study we predisposed all subjects to show significantly large increases in muscle carnosine, thus negating any "ceiling effect" of subjects with high-baseline carnosine. Accordingly, until it is determined what the maximal attainable muscle carnosine levels are with prolonged BA supplementation (muscle saturation point), correlations between baseline carnosine and increases in carnosine appear less relevant. Furthermore, whether continually supplementing with BA until a maximal muscle carnosine saturation point is achieved comes with continued increases in high-intensity exercise performance and positive health/clinical outcomes, or whether there are potential negative side-effects, remains to be elucidated.

For the first time, the current study also measured muscle carnosine changes after just 2 weeks of BA supplementation with the lowest daily BA dose (1.6 g BA/day) reported in the literature. Interestingly, both groups of high–low and low–low received the same 1.6 g BA/day from weeks 4 to 8, and both groups showed a very consistent and almost equal continual carnosine increase of ~7 and 11% for high–low and low–low, respectively (average for both muscle groups). Therefore, within the range of muscle carnosine contents found within this study (3.91–12.67 mmol/kg_{ww}) at the point when the lower "maintenance" dose was initiated (week 4), the lowest required BA dose to at least maintain augmented muscle carnosine stores appears to be less than the 1.6 g BA/day, as this dose still caused slow, but significant, increases in muscle carnosine from weeks 4 to 8 (Fig. 3a, b). Whether this low daily dose of BA would also maintain even higher levels of muscle carnosine remains to be investigated. Although highly variable between subjects, already after just 2 weeks, there were small (~8 to 12%), but significant increases in muscle carnosine (Fig. 3a, b). Again, these absolute increases were highly linear and dependent upon total grams of BA consumed, but it remains unlikely that these small increases in carnosine could result in a significant performance and/or health benefit.

To our knowledge, this is also the first study to measure increases in muscle carnosine synthesis after subjects consumed a slow-release BA supplement, to eliminate paresthesia symptoms. It has previously been demonstrated that this slow-release tablet of BA results in a blunting of peak plasma BA with similar area under the curve compared to pure BA (Decombaz et al. 2011; Harris et al. 2008). This slow-release BA still reaches plasma concentrations above the BA transporter K_m of ~40 μM (Bakardjiev and Bauer 1994) and thus would be hypothesized to be effective for muscle carnosine synthesis. Accordingly, this study clearly demonstrates that, despite altered plasma pharmacokinetics, slow-release BA is highly efficacious for muscle carnosine synthesis. Correspondingly, our 89.6 and 134.4 g of total supplemented slow-release BA caused carnosine increases of 37 and 45% in TA muscle with high–low dosing protocol, respectively. This is in near agreement with the 40 and 46% increase that Harris et al. (2006) demonstrated with 89.6 and 145.6 g of non slow-release BA, despite the added variability due to different methods for measuring muscle carnosine.

Muscle carnosine washout

This is the second study to examine post-BA muscle carnosine washout kinetics and confirms that once augmented, muscle carnosine appears to be one of the most stable muscle metabolites ever examined (Baguet et al. 2009). Unlike creatine which has a washout of ~4 weeks (Hultman et al. 1996), the washout of augmented skeletal muscle carnosine after the termination of BA supplementation is very slow, as the current data show a washout time of ~15 to 20 weeks after a ~30 to 45% increase in muscle carnosine. These modest decay rates (~2%/week; between 0.18 and 0.43 mmol/kg_{ww}/week) resulted in muscle carnosine levels that were still ~50% above baseline values after a 4 week washout period (Fig. 3a, b). This washout rate is ~40% slower than the one observed by Baguet et al. (2009), who showed a washout of ~2 to 4% per week. Similar to Baguet's observation of higher carnosine increase with higher baseline levels, this difference may be due to methodological differences of the studies (exclusion of subjects with high carnosine levels in the current study). In addition, the numbers of included subjects (Baguet, $n = 8$, current study, $n = 21$) may be too small to draw far-reaching conclusions. Taken together, both Baguet

et al. (2009) and this study show that once muscle carnosine is increased, any potential performance and/or health benefits resulting from augmented muscle carnosine would appear to be continually realized up to even 4–6 weeks post-supplementation due to the stability of muscle carnosine levels and the slow washout profile.

Muscle creatine

To our knowledge, this is the first study to concurrently assess muscle creatine stores during a chronic BA supplementation protocol, displaying no effect of BA on creatine concentrations, as there were no treatment or time changes throughout the entire intervention. This result is not surprising given that creatine and BA do not share the same skeletal muscle transporter and elicit unique physiological effects. Accordingly, several studies have demonstrated emerging positive performance outcomes when subjects undertook concurrent BA and creatine supplementation (Hoffman et al. 2006; Stout et al. 2006; Zoeller et al. 2007). However, more well-controlled and well-powered performance studies are required to fully ascertain any synergies with these two supplements.

Effect of β-alanine supplementation on blood chemistry and chronic paresthesia symptoms

Despite the recent upsurge of studies involving BA supplementation, only a single previous study has reported routine blood clinical chemistry and hematology measures to assess safety (Harris et al. 2006). The current studies' high–low group consumed a total of 134.4 g of slow-release BA over 8 weeks, while subjects over 4 weeks in the Harris et al. (2006) study consumed a total of 145.6 g BA. Regardless of the dosing protocol, time period or type of BA, both studies demonstrate no significant changes pre- to post, or between placebo or BA groups, in any of the routine blood parameters (Table 1). It has previously been demonstrated that when subjects are acutely supplemented with BA at levels greater than 800 mg/dose it results in moderate to significant paraesthesia (Harris et al. 2006; Hill et al. 2007). Given this, BA supplementation studies have generally utilized small repeated daily doses of $\leq$800 mg doses to minimize paraesthesia symptoms (Derave et al. 2007; Hill et al. 2007; Kendrick et al. 2008). Recently, a commercialized slow-release tablet of BA has been produced and a single acute 1.6 g dose resulted in a blunting of the peak plasma BA curve with no reports of any paresthesia symptoms (Decombaz et al. 2011; Harris et al. 2008). In accordance, the current study also found no significant paraesthesia effect between treatments when 1.6 g of slow-release BA was given acutely at study commencement (week 0). However, to our knowledge, not a single study has examined paresthesia symptoms when BA is chronically supplemented over weeks, while muscle carnosine is concurrently increasing. Cell culture and rodent studies have demonstrated some impact of altering carnosine (β-alanyl-L-histidine) content on total L-histidine, which may impact precursor availability for histamine production and release (Flancbaum et al. 1990; Shen et al. 2008). Thus, it could be hypothesized that changing carnosine content over time might potentially impact upon associated whole-body histamine kinetics and paraesthesia symptoms. However, this study clearly demonstrates no effect of acute or chronic slow-release BA supplementation on paraesthesia symptoms or blood clinical chemistry and hematology measures over 8 weeks. However, long-term studies (>10 weeks) are still needed to definitively know the maximal muscle carnosine contents, and any potential side-effects from long-term BA supplementation.

Conclusion

Similar to creatine (Kley et al. 2011), BA supplementation to augment muscle carnosine is poised for potential application beyond the niche exercise and performance enhancement field and into other prospective roles in myocellular homeostasis and muscular myopathies as well (Derave et al. 2010; Sale et al. 2010). The impressively linear dose-response, which is not dependent upon daily dose (within 1.6–3.2 g/day) or muscle type, is completely dependent on the total amount of consumed BA. The data of this study result in a greatly simplified BA prescriptive application to augment muscle carnosine, which appear remarkably stable post-supplementation. This study also demonstrates that as little as 1.6 g BA/day can be efficacious to increase muscle carnosine, or at the very least maintain augmented carnosine concentrations, in healthy young subjects. Moreover, slow-release BA does not cause acute or chronic paresthesia or impact upon blood clinical chemistry over 8 weeks. Hence, the current study brings a much improved understanding of the BA dose-response, minimal efficacious dose for carnosine increases, carnosine washout and data to show both acute and chronic safety in the utilization of slow-release BA.

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