The role of chronic muscle (in)activity on carnosine homeostasis: a study with spinal-cord injured athletes

Running title: Chronic muscle inactivity and muscle carnosine content

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Abstract

To examine the role of chronic (in)activity on muscle carnosine (MCarn) and how chronic (in)activity affects MCarn responses to β-alanine supplementation in spinal-cord injured athletes, sixteen male athletes with paraplegia were randomized (2:1 ratio) to receive β-alanine (n=11) or placebo (PL, n=5). They consumed 6.4 $g \cdot d^{-1}$ of β-alanine or PL for 28 days. Muscle biopsies of the active *deltoid* and the inactive *vastus lateralis* (VL) were taken before and after supplementation. MCarn in the VL was also compared with the VL of a group of individuals without paraplegia (n=15). MCarn was quantified in whole muscle and in pools of individual fibers by High-performance Liquid Chromatography. MCarn was higher in chronically inactive VL *vs.* well-trained *deltoid* (32.0±12.0 *vs*. 20.5±6.1 $mmol \cdot kg^{-1}$ DM; p=0.018). MCarn was higher in inactive *vs.* active VL (32.0±12.0 vs. 21.2±7.5 $mmol \cdot kg^{-1}$ DM; p=0.011). In type-I fibers, MCarn was significantly higher in the inactive VL than in the active deltoid (38.3±4.7 *vs.* 27.3±11.8 $mmol \cdot kg^{-1}$ DM, p=0.014). MCarn increased similarly between inactive VL and active *deltoid* in the β-alanine group (VL: 68.9±55.1%, p=0.0002; deltoid: 90.5±51.4%, p<0.0001), with no changes in the PL group. MCarn content was higher in the inactive VL than in the active *deltoid* and the active VL, but this is probably a consequence of fiber type shift (type I to type II) that occurs with chronic inactivity. Chronically inactive muscle showed an increase in MCarn after BA supplementation equally to the active muscle, suggesting that carnosine accretion following β-alanine supplementation is not influenced by muscle inactivity.

Keywords: muscle inactivity, muscle activity, carnosine, β-alanine, homeostasis.

Introduction

Carnosine is a multifunctional dipeptide abundantly expressed in human skeletal muscle, where it is thought to play important physiological roles, including pH regulation (1, 12) , Ca^{2+} handling (14) and reactive aldehyde detoxification (3, 9). The availability of β-alanine, the rate-limiting precursor of carnosine synthesis, is the most influential factor affecting muscle carnosine content (MCarn) (22). Studies have consistently shown that β-alanine supplementation increases MCarn (33), which has been associated with improved high-intensity exercise performance (34) and potentially improved health (2). Conversely, decreased MCarn during the washout period following β-alanine has been associated with the return of exercise tolerance to pre-supplementation levels (37).

While β-alanine intake, either ingested from food or supplements, increases MCarn in a dose-dependent, saturable fashion (33), it remains unclear whether other stimuli can also affect MCarn. There is evidence indicating that exercise can modulate carnosine homeostasis, with cross-sectional studies showing that sprint- and strength-trained athletes have higher MCarn than sedentary and endurance-trained individuals (31, 36). Although this is suggestive of an effect of high-intensity exercise on carnosine synthesis, possibly due to an effect of long-term exposure to acidosis as an adaptive trigger, several subsequent longitudinal studies did not confirm that chronic exercise training can increase MCarn (4, 15, 20, 27-29). The lack of control over the dietary intake of β-alanine, the lack of control for fiber type shifting alongside training, and purportedly insufficient training stimuli might, however, limit the interpretation of these studies. Type II fibers contains approximately 1.5 times more carnosine than type I fibers (21, 23), which highlights the importance of accounting for changes in fiber type distribution when fiber shift can occur.

De Salles Painelli et al. (11) showed that 12 weeks of high-intensity interval training increased MCarn in vegetarians, a population that consumes virtually no β-alanine in the diet. In contrast, Hoetker et al. (24) showed that low-intensity training increased muscle carnosine, while high-intensity training decreased muscle carnosine. The potential reasons for the disparity between these two studies are not immediately apparent and it is clear that there is a requirement to further investigate whether or not acute exercise can modulate the carnosine content in skeletal muscle.

It also remains uncertain whether exercise training or training status can affect carnosine accretion in response to β-alanine supplementation. Bex et al. (7) showed that

muscle groups under higher training loads respond to β-alanine supplementation with greater carnosine accretion in comparison with muscle groups under lower training loads. Bex et al. (6) showed increased carnosine accretion in response to β-alanine supplementation in individuals who undertook exercise training when compared with non-trained controls. In both studies (6, 7), muscle carnosine was measured with magnetic resonance spectroscopy, a method that has been shown to be less reliable than more direct measures of carnosine content, such as chromatography-based techniques from muscle biopsy samples (10). In contrast, using high-performance liquid chromatography (HPLC), Kendrick et al. (28) showed similar carnosine accretion between trained and untrained muscle groups in response to β-alanine supplementation. Out of the inconsistent findings in the literature, to date, it remains unknown whether physical activity modulates MCarn or whether it enhances MCarn responses to β-alanine supplementation.

To understand the role of muscle activity on muscle carnosine homeostasis, we examined the extremes of the muscle activity spectrum (*i.e.*, chronic long-term athletic training *vs.* chronic long-term muscle inactivity) by investigating well-trained athletes with spinal-cord injury. In these athletes we compared their chronically trained muscle (*deltoid*) with their chronically inactive muscle (*vastus lateralis*), both at baseline and in response to β-alanine supplementation. We hypothesized that paralyzed muscles would display lower carnosine levels at baseline and reduced carnosine loading in response to β-alanine supplementation, in comparison with active muscles.

Methods

Participants

Twenty-three men with spinal cord injury and paraplegia were screened for eligibility. Inclusion criteria were: *1)* participation in a structured exercise training program for ≥6 h per week for ≥6 months prior to participation; *2)* spinal-cord injury with loss of motor function of the lower limbs corresponding to the American Spinal Cord Injury Association Scale A or B for ≥1 year and *3)* age 18-45 years. Exclusion criteria were: *1)* diagnosed with a chronic disease that would preclude participation in the study and *2)* use of supplements containing creatine or β-alanine ≤6 months prior to participation. Before signing the written consent form, all participants were fully informed about benefits and risks involved with participation. All procedures were

approved by the Institutional Ethics Committee before the commencement of the study (#41495115.0.0000.5391) and complied with the Declaration of Helsinki.

One individual did not meet the inclusion criteria (sedentary) while six other individuals were unavailable for participation. Sixteen volunteers were then randomly allocated in a 2:1 ratio to receive β-alanine (n=11) or placebo (n=5). An unbalanced 2:1 allocation ratio was used due to the limited potential participant pool for this study, the invasive nature of the study and to increase the number of observations in the β-alanine group. Two participants (one from each group) dropped-out after the initial biopsy session and therefore did not participate in the post-supplementation analyses. We were unable to obtain usable muscle samples from the *vastus lateralis* of 3 participants (one from the β-alanine group and 2 from the placebo group).

The participants had been training for 10.5±3.7 years at the time of the study; all of them reported that they performed upper-body strength, arm crank, mobility and shoulder stability training 2-5 times per week in addition to their sport-specific training, totalling 573±102 min of training per week. All participants reported the use of passive leg exercises, but none of them used electrical stimulation of the lower limbs. Participant general characteristics are displayed in Table 1, while specific information about individual spinal cord injury is displayed in Table 2.

Table 1: Participant general characteristics.

	β-alanine (n=11)	**Placebo (n=5)**	**p**
Age (y)	36.1 ± 3.9	34.6 ± 8.1	0.62
Body mass (kg)	71.9 ± 11.8	67.8 ± 11.9	0.52
Length (cm)	176 ± 8.6	172 ± 6.9	0.38
BMI (kg/m^2)	23.2 ± 3.0	22.9 ± 3.8	0.89
Time since injury (y)	14.1 ± 3.7	13.4 ± 7.3	0.80
VO_{2peak} ($L \cdot min^{-1}$)	1.3 ± 0.6	1.7 ± 0.8	0.30
$Power_{max}$ (W)	71.2 ± 42.5	91.1 ± 53.7	0.44

BMI: body mass index; $Power_{max}$: maximum load attained in a progressive arm-crank exercise test to volitional fatigue. p-values refer to independent samples *t* tests.

Table 2: Individual spinal cord injury characteristics and training status of each participant.

Participant	ASIA classification	Lesion level	Training status	Sport
β-alanine				
1	B	C6-C7	Amateur athlete	Artistic Gymnastics
2	A	T12	Competitive athlete	Cross-training
3	B	T12-L1	Competitive athlete	Wheelchair basketball
4	B	L1-L2	Competitive athlete	Wheelchair basketball
5	B	C6-C7	Amateur athlete	Brazilian Jiu Jitsu
6	B	T3	Competitive athlete	Endurance hand-bike
7	B	C6-C7	Competitive athlete	Wheelchair rugby
8	B	T2-T3	Competitive athlete	Wheelchair rugby
9	A	C7	Competitive athlete	Wheelchair rugby
10	A	C5	Competitive athlete	Wheelchair rugby
11	A	C6-C7	Competitive athlete	Wheelchair rugby
Placebo				
12	B	T12-L1	Competitive athlete	Paralympic archery
13	B	T5-T6	Competitive athlete	Endurance hand-bike
14	B	L2	Competitive athlete	Wheelchair basketball
15	B	T9	Amateur athlete	Endurance hand-bike
16	B	C7	Competitive athlete	Wheelchair rugby

ASIA: American Spinal Cord Injury Association.

Experimental design

This study was designed to test the influence of chronic exercise training and muscle inactivity on *1)* MCarn and *2)* MCarn accumulation in response to β-alanine supplementation. Thus, we first performed a cross-sectional analysis to compare MCarn in trained (*deltoid*) *vs.* paralyzed (*vastus lateralis*) muscles in highly trained athletes with paraplegia. Since this within-individual analysis only allows for the comparison between different muscle groups (*i.e.*, *deltoid vs. vastus lateralis*), we conducted a second cross-sectional analysis to compare MCarn between paralyzed *vs.* active muscles in the same muscle group (i.e., *vastus lateralis*). Thus, we compared MCarn in athletes with paraplegia *vs.* MCarn in a group of physically active individuals without paraplegia (n=15 healthy, physically active men; age=28±5 y; body mass=76.2±17.3 kg;

178 BMI=24.8±3.9) who took part in another study that was being conducted at the same
179 time in our lab and was published elsewhere (37).
180
181 In addition to the cross-sectional analyses, we conducted a double-blind,
182 placebo-controlled trial with athletes with paraplegia to compare MCarn in response to
183 β-alanine supplementation in their trained *vs.* paralyzed muscle groups. To account for
184 the effects of chronic training and paralysis on fiber type distribution and for the
185 influence of the higher carnosine levels in type II fibers (11, 22), MCarn was
186 determined not only in whole muscle, but also in single muscle fibers. Groups were
187 equalized according to the $Power_{max}$ by ranking the participants within blocks of 4
188 individuals and using the block randomization method (www.random.org). All
189 participants with paraplegia were asked to attend to the laboratory on three different
190 occasions. In the first visit, body mass, body length and peak oxygen consumption were
191 measured. In a second visit, 2-7 days apart, muscle biopsies of the *deltoid* (trained
192 muscle) and the *vastus lateralis* (paralyzed muscle) were taken for pre-supplementation
193 (PRE) MCarn determination. A 28-day β-alanine supplementation period commenced
194 on the next day, after which (POST) another biopsy of the *deltoid* and the *vastus*
195 *lateralis* was taken. All athletes and coaches were instructed to maintain athletes'
196 training regimens and dietary habits across the supplementation period, which was
197 verbally confirmed upon their visit after supplementation. The experimental procedures
198 for the participants without paraplegia were described elsewhere (37), but in this study
199 we are only interested in their baseline MCarn as this will serve as a reference of typical
200 values in non-paralyzed *vastus lateralis*.
201
202 Preliminary Testing
203 Body mass was measured to the nearest 100 g on a digital scale (100 CH,
204 Welmy, São Paulo, Brazil). Wheelchair weight was first measured without the
205 volunteer. When stabilized, the scale display was reset and the volunteer was transferred
206 back to the wheelchair to determine body mass. Length was measured as the distance
207 between the top of the head of the bottom of the feet, with the individual laid down on a
208 gurney with their knees fully extended and their feet beside one another.
209 A maximal incremental test was conducted in a mechanically braked arm
210 ergometer (EB 4100, Cefise, Brazil) as previously described (32). The test was initiated
211 with a load corresponding to 25 W (0.3 kilopond at 85 $rev \cdot min^{-1}$), followed by 25 W

212 increments every minute until volitional exhaustion or until the participant could no
213 longer maintain 85 rev·min^{-1}. Breath by breath gas exchange was measured in a gas
214 analyzer (Quark CPET, Cosmed, Rome, Italy) calibrated according to manufacturer
215 instructions. VO_{2peak} was considered the arithmetic mean of the least 30 seconds of the
216 test and $Power_{max}$ was the maximum power attained.
217
218 Supplementation protocol
219 Participants consumed 6.4 g·d^{-1} of either β-alanine (SR CarnoSyn®, Natural
220 Alternatives International, Inc, Carlsbad, CA) or placebo (maltodextrin, Natural
221 Alternatives International, Inc, Carlsbad, CA) for 28 days, totalling an accumulated
222 dose of 179.2 g. The total daily dose was split into 4 individual doses of 1.6 g provided
223 in 0.8 g sustained release tablets, identical in number and appearance. All participants
224 completed a supplementation log; compliance with supplementation was 98±2% of the
225 total dose in β-alanine group and 97±1.5% in placebo group. At the end of the
226 supplementation period, the participants reported which substance they believed they
227 were taking. Three participants in the β-alanine group and 2 in the placebo groups
228 correctly guessed their treatments (Fisher's exact: p=0.6). Two out of the 16 participants
229 reported slight symptoms of paresthesia, one from β-alanine group and the other from
230 the placebo group.
231
232 Muscle biopsies
233 Approximately 70-100 mg of wet muscle were obtained from the mid portion of
234 the *vastus lateralis* and from the most lateral and voluminous part of the *deltoid* under
235 local anesthesia (3 ml lidocaine 1%), using the percutaneous Bergstrom needle biopsy
236 technique (5) modified with suction. Samples were obtained PRE and POST
237 supplementation from the same locations, as close as possible to one another, and
238 immediately frozen in liquid nitrogen. Before analyses, the samples were freeze dried
239 overnight for 16 h, and then all visible blood, fat and connective tissue were removed.
240
241 Quantification of MCarn in whole muscle
242 Carnosine was quantified in extracts of whole muscle by High-performance
243 Liquid Chromatography (LC-10vp, Shimadzu, Japan) coupled with ultraviolet detection
244 at 214 nm (SPD-10vp, Shimadzu, Japan), using the method described by Mora et al.
245 (30). Approximately 3-5 mg of freeze-dried muscle were manually powdered and

deproteinized in an acid extraction solution [0.5 M $HClO_4$, 1 mM EDTA; 1:50 sample weight (mg):volume (µl)] via intermittent vortex bursts for 10 min (30s on, 30 s resting on ice). The extracts were then centrifuged (3 min, 5000 g at 4° C) and the supernatant was then neutralized with 2.1 M $KHCO_3$ (1:4 v:v) and filtered through a 0.2 µm pore size centrifugal filter tube. Chromatographic separation was performed using an Atlantis HILIC silica column (4.6 × 150 mm, 3 µm; Waters, Massachusetts, USA) and an Atlantis Silica column guard (4.6 × 20 mm, 3 µm). Briefly, the method uses mobile phase A [0.65 mM ammonium acetate, in water/acetonitrile (25:75) (v/v)], and mobile phase B [4.55 mM ammonium acetate, in water/acetonitrile (70:30)], both adjusted to pH 5.5. The separation condition was: linear gradient from 0 to 100% of mobile phase B for 13 min at a flow rate of 1.4 mL·min^{-1}. All samples were analyzed in duplicate with the intra-assay coefficient variation being 2.03% and the coefficient of variation between different biopsies from the same site being 3.95% (10).

Quantification of MCarn in pools of individual fibers

Approximately 30-40 fibers were isolated and cut in two halves (0.5-1 mm). One half was used for MHC determination while the remaining piece was weighed on a quartz-fiber fish-pole balance to the nearest 0.01 µg, as previously described (11, 28). Fibers of the same MHC type were pooled and metabolite was extracted by adding ultrapure water and vortexing for 10 min (30-s bursts interspersed with 30-s cooling periods on ice). Carnosine was then quantified by HPLC (Hitachi, Schaumburg, IL) with pre-column derivatization coupled to a fluorescence detector as previously described (13). Metabolite separation was undertaken in a Hypersil ODS analytical column (3 µm, 150 x 4.6 mm I. D., Shandon, Runcom, UK) at 23 ºC. A binary gradient formed from solvent A [12.5 mM sodium acetate, pH 7.2 tetrahydrofuran (995:5, v/v)] and solvent B [12.5 mM sodium acetate, pH 7.2 - methanol-acetonitrile (500:350:150, v/v)] was used with the following gradient: 0 to 1.5 min, 0% solvent B; 1.5 to 10 min, 35% B; 10 to 26 min, 60% B; 26 to 30 min, 100% B; 30 to 35 min, 100% B; 35 to 45 min, 0% B. Flow-rate was 2.0 ml·min^{-1} in the initial 10 minutes, and gradually decreased to 1.0 ml·min-1 at 26 min until finish. Excitation and emission wavelengths were 340 nm and 450 nm. The derivatization reagent was kept in the dark at 2°C and prepared by mixing 80 µL of OPA (40mg) plus absolute ethanol (800 µL) to 4 µL of β-mercaptoethanol and 1 mL of a 0.4M borate buffer (pH 9.65). Extract and reagent (1:1 v:v) were reacted for 30 s prior to injection. Fresh derivatization reagent was used with

each new sample batch. All samples were analyzed in duplicate and the intra-assay coefficient of variation was 8.8% in pools of single fibers. Quantification of both chromatographic methods was performed by integrating peak areas.

Myosin heavy-chain isoform characterization

Myosin heavy-chain isoform was characterized in single fibers and in whole muscle by dissolving (mixing with a vortex) a half fiber or ~3 mg of freeze-dried muscle sample in extraction buffer (0.06 M tris-hydroxymethylaminomethane pH 6.8, 1% w/v sodium dodecyl sulphate, 0.6%w/v EDTA, 15% w/v glycerol, 5% v/v mercaptoethanol, and bromophenol blue) followed by SDS-PAGE electrophoresis, as described by Kendrick et al. (28). MHC isoform type was determined as described by Galpin et al (16). Ten micro-litters of the extract were loaded in a polyacrylamide gel and ran for ~28h at 4^{o}C. Protein was revealed using a silver staining kit (PlusOne, Cytiva). Band intensity was quantified in the ImageJ software and used for individual fiber typing or for whole muscle MHC distribution.

Dietary β-alanine intake assessment

β-alanine intake was assessed with three food diaries undertaken on 3 non-consecutive days (two weekdays and one weekend day) following the instructions of a registered nutritionist. β-alanine intake through consumption of fish, poultry and meat was estimated from the data of Jones et al. (25).

Statistical analysis

Participants general characteristics (age, body mass, length, body mass index, VO_{2peak}, $Power_{max}$, and time since injury) were compared between groups with unpaired t-tests. Linear mixed models (proc mixed, SAS University Edition) were used to compare whole muscle MCarn between *deltoid* and *vastus lateralis* before and after β-alanine or placebo supplementation. Group, time and muscle group were fixed factors, whilst participants were random factors. Linear mixed models were also used for dietary β-alanine intake data, with group and time being fixed factors. Four covariance matrices (unstructured, compound symmetric, autoregressive and toeplitz) were tested, with the best fit being chosen using the lowest BIC (Bayesian Information Criteria) value.

Hypothesis-driven, single degree-of-freedom contrast analysis was used to identify significant differences whenever a significant main interaction effect was observed. Delta post-pre supplementation was calculated and compared between muscle groups with Welch unpaired t-test for unbalanced samples (RStudio v.4.0.0).

For MCarn measured in pools of single fibers, linear mixed models did not converge (due to unbalanced sample sizes and missing sub-type fiber data, as most samples had no type I or type II MHC), meaning that data sets for each fiber type were analyzed separately. For the baseline data, β-alanine and placebo groups were pooled and a Welch unpaired t-test (RStudio v.4.0.0) for unbalanced sample sizes was used to compare MCarn between the *deltoid* and *vastus lateralis* muscles and to compare MCarn in the *vastus lateralis* between athletes with paraplegia and individuals without paraplegia; MCarn in type I *vs.* type II fibers were compared within the same muscle group of the same individuals with t-test for dependent samples (RStudio v.4.0.0). For the MCarn responses to β-alanine supplementation, fast MHC subtypes were pooled and delta post-pre supplementation was deemed as the response variable (data from the placebo group was excluded due to very low or zero number of observations in some instances) and compared with a two-way general linear model (type III, for unbalance samples) where muscle group (*deltoid vs. vastus lateralis*) and fiber type (type I *vs.* type II) were fixed factors (SPSS v.14). Data are reported as mean±SD and the significance level was set at $p<0.05$.

Results

Role of chronic muscle (in)activity on muscle carnosine

In the athletes with paraplegia, MCarn was significantly higher in the chronically inactive *vastus lateralis* muscle in comparison with their well-trained *deltoid* muscle (main effect of muscle group: $F=10.7$, $p=0.006$; figure 1, panel A). Higher MCarn was also shown in the inactive *vastus lateralis* when compared to the active *vastus lateralis* of individuals without paraplegia ($p=0.011$, 95%CI: 2.7–18.8; figure 1, panel B).

Figure 1: Individuals (white circles) and mean±SD group values for carnosine content in whole muscle. Panel A: cross-sectional analysis comparing the active *deltoid* muscle and the paralyzed *vastus lateralis* muscle in athletes with paraplegia (p-value refer to

the main effect of muscle group in the mixed models). Panel B: cross-sectional analysis comparing the paralyzed *vastus lateralis* muscle in athletes with paraplegia *vs* the active *vastus lateralis* in a group of physically active participants without paraplegia that took part in another study (data published in Yamaguchi et al. 2020 (37); p-value refer to Welch unpaired t-tests for unbalanced samples).
Note: data from athletes with paraplegia refer to baseline (pre-supplementation) β-alanine and placebo groups pooled.

Importantly, chronic muscle inactivity in spinal cord injury was associated with a substantive increase in fast MHC isoform distribution (Figure 2), which could influence whole muscle MCarn and become a confounding factor in our analysis, since MCarn is ~1.5 times higher in type II than in type I fibers (21, 23). Therefore, we also determined MCarn in single fibers. In type I fibers, MCarn was significantly higher in the inactive *vastus lateralis* muscle in comparison with the active *deltoid* muscle (p=0.014, 95%CI: -19.5–-2.6; figure 3, panel A). There were no significant differences in MCarn in type IIa between the active and inactive muscles (p=0.851, 95%CI: -12.7–15.2; figure 2, panel B), as was the case for hybrid type IIx/IIa fibers (p=0.53, 95%CI: -14.1–7.7; figure 3, panel C). We were unable to compare carnosine content in type IIx fibers because they were present in only 2 out of the 16 samples.

Figure 2. Representative images of silver-stained electrophoresis gel for MHC typing in whole muscle and in single fibers (panel A). MHC isoform distribution in the active deltoid muscle and in the paralyzed *vastus lateralis* muscle in athletes with paraplegia (β-alanine and placebo pooled) at baseline (panels B and C).

Figure 3: Individual (white circles) and mean±SD group values for muscle carnosine content in pools of single fibers in the paralyzed *deltoid* muscle and in the active *vastus lateralis* muscle in athletes with paraplegia. Panels A, B and C display comparisons between muscle groups. Panels D and E display comparisons within muscle groups.
Note: Data refer to baseline (pre-supplementation) β-alanine and placebo groups pooled. p-values for between-muscle group comparisons were calculated with Welch unpaired t-tests for unbalanced samples while p-values for within-muscle group comparisons were

calculated with t-test for dependent samples. Different sample sizes are due to the absence of fiber types in some of the samples.

Role of chronic muscle (in)activity on muscle carnosine responses to β-alanine supplementation

Significant increases in MCarn were shown in both *deltoid* and *vastus lateralis* muscles in the β-alanine, but not in the placebo group (group-by-time-by-muscle interaction: F=6.61, p=0.0045; figure 4, panel A). Between-group contrast analyses confirmed that post-supplementation MCarn was significantly higher in the β-alanine group than in placebo group (*deltoid*: p=0.0046; *vastus lateralis*: p=0.024). MCarn increased 68.9±55.1% in the *vastus lateralis* (within-group effect: p<0.0001) and 90.5±51.4% in the *deltoid* (within-group effect: p=0.0002) in the β-alanine group. No significant differences were shown for the delta changes between the *deltoid* and *vastus lateralis* in either the β-alanine (p=0.653; 95%CI:-10.6–6.9; figure 4, panel B) or placebo (p=0.623; 95%CI:-6.8–4.6; figure 4, panel B) group.

Figure 4: Individual (white circles) and mean±SD muscle carnosine content before and after β-alanine supplementation in *deltoid* and *vastus lateralis* in the β-alanine and placebo groups (panel A). Post-pre absolute delta changes in muscle carnosine in the β-alanine and placebo groups (panel B).
Note: In panel A, group-by-time-by-muscle interaction effect: F=6.61, p=0.0045; p-values were calculated with single-degree-of-freedom contrast analyses in the mixed models. In panel B, p-values were calculated with Welch unpaired t-tests for unbalanced samples.

To account for the effect of MHC isoform distribution on the MCarn responses to β-alanine supplementation, MCarn accretion was compared in pools of single fibers between the *deltoid* and *vastus lateralis*. No effects of fiber type (F=0.40, p=0.53), muscle group (F=0.19, p=0.68) or fiber type by muscle group interaction (F=2.37, p=0.13) were shown (figure 5).

Figure 5: Individual (white circles) and mean±SD single fiber muscle carnosine accretion in response to β-alanine supplementation in *deltoid* and *vastus lateralis.* Type II fibers represent IIa and hybrid IIa/IIx fibers pooled.
General linear model: fiber type (F=0.40, p=0.53); muscle group (F=0.19, p=0.68); fiber type by muscle group interaction (F=2.37, p=0.13).

Dietary β-alanine intake

The estimated β-alanine intake was similar between groups and across the study period (PRE: β-alanine=0.8±0.6g, placebo:0.4±0.3g; POST: β-alanine=1.1±1g; placebo:0.4±0.2g) (main effect of group: F=2.71, p=0.128; group by time interaction: F=0.2, p=0.664).

Discussion

By comparing a habitually highly active muscle (the *deltoid*) with a chronically inactive muscle (the *vastus lateralis*) in athletes with spinal cord injury, we have been able to show that not only chronic muscle inactivity does not result in a significant reduction in MCarn, but that the MCarn content in the chronically inactive *vastus lateralis* muscle was higher than in the highly active *deltoid* muscle. This result was further confirmed in a retrospective analysis that showed higher MCarn in the paralyzed v*astus lateralis* muscle of athletes with paraplegia than in the *vastus lateralis* muscle of physically active individuals without paraplegia. Whilst this result might not necessarily seem intuitive, previous studies have shown that prolonged muscle inactivity (>10 months) results in a dramatic muscle fiber type shift, from type I to type II (19, 35). Indeed, we showed that 95% of the fibers in the chronically inactive *vastus lateralis* muscle were fast MHC, whilst the distribution was closer to 50:50 (slow:fast MHC) in the highly active *deltoid* muscle and it is typically 40:60 (slow:fast) in non-paralyzed *vastus lateralis* (8). This suggests that higher MCarn content in the inactive *vastus lateralis* could be, at least in part, explained by a type I to II shift, suggesting that it is the predominance of type II muscle fibers in paralyzed muscles that could account for the elevated MCarn to a much greater extent than muscle paralysis *per se*. Increased type II fiber distribution can increase whole muscle MCarn because carnosine in type II fibers is ~1.5 times higher than in type I fibers (11, 21, 23). To account for this effect, we examined MCarn in pools of isolated fibers and showed no differences between

vastus lateralis and *deltoid* muscles in type II fibers.

Although the increase in whole muscle MCarn seemed to occur mainly due to fiber type shifts, in type I fibers a significant higher MCarn was shown, thereby suggesting that chronic muscle inactivity could increase MCarn, at least in type I fibers. Carnosine is typically higher in type II than in type I fibers (11, 21, 23), which we confirmed in the active deltoid muscle in the present study. Interestingly, in the paralyzed vastus lateralis, MCarn in type I fibers was not only comparable to type II fibers in the same paralyzed muscle, but also to the MCarn typically shown in type II fibers in other studies (11, 23). These data further reinforce the notion that chronic inactivity may increase MCarn in type I fibers only. Hence, increased MCarn in type I fibers can also account, although to a lesser extent, for the increased whole muscle MCarn. It is important to highlight, however, that type I fibers were not present in many of the samples obtained; in fact, only 4 samples had type-I fibers to be analysed and caution should be exercised in interpreting these data.

In spite of the limited strength of this evidence, we can speculate that the apparent higher carnosine levels in type I fibers in the inactive muscle might arise from an adaptive mechanism to help protect against excessive ROS formation that is typically found in atrophic conditions (18). This mechanism might involve improved efficiency of β-alanine transport into cells. Jung et al. (26) found that TauT expression, a major β-alanine transporter, is elevated in spinal cord motor neurons of Amyotrophic Lateral Sclerosis transgenic (G93A) mice, suggesting that TauT expression partially protects motor neurons by compensating for oxidative stress. Since oxidative stress is one of the main processes involved in muscle atrophy after spinal cord injury (18), these findings provide some support to the hypothesis that similar mechanisms can occur in chronically paralyzed skeletal muscle. We, unfortunately, were unable to assess protein and gene expression levels in our samples due to the very limited availability of usable material in the muscle specimens, which we acknowledge as a limitation of our study. Thus, this hypothesis remains to experimentally confirmed.

Importantly, β-alanine supplementation increased MCarn to a similar extent in both the active *deltoid* and the inactive *vastus lateralis*. This indicates that muscle (in)activity has no influence on the muscles ability to respond to β-alanine supplementation. This was confirmed in both type I and type II fibers, although the type I fiber dataset is limited due to the reduced number of type I fibers in the paralyzed

vastus lateralis. Our data are in agreement with the study of the Kendrick et al. (28) that showed similar carnosine accretion between trained and untrained muscle groups in response to β-alanine supplementation. On the other hand, our data disagrees with those by Bex et al. (7) who showed superior carnosine accretion in muscle groups under higher training loads in comparison with muscle groups under lower training loads. Our data also seem to contradict those by Bex et al. (6) who showed a tendency for increased carnosine accretion in response to β-alanine supplementation in individuals undergoing exercise training *vs*. non-trained controls. In their study, however, this difference was not confirmed when the two muscle groups (gastrocnemius and soleus) were analysed separately, suggesting a lack of consistency in the effects. In both studies (6, 7), MCarn was determined with magnetic resonance spectroscopy, a method that is less reliable for muscle carnosine quantification than the chromatographic methods used in our (10) and in Kendrick's studies (27, 28). Importantly, our study was the first to address this question using a single fiber approach, where differences in fiber type distribution are controlled. The similar increase between muscle groups and fiber types further indicates that chronic (in)activity has no effect on MCarn accretion in response to β-alanine supplementation, regardless of fiber type distribution. This corroborates early findings by Harris et al. (22) who showed that type I and type II fibers can equally increase MCarn during β-alanine supplementation in active muscle, and also expands this notion to a condition in which muscle activity is virtually absent.

The lack of difference in carnosine accretion between active and inactive muscles in response to β-alanine supplementation may seem to be in contrast with our hypothesis of increased β-alanine transport efficiency in type I fibers following chronic muscle inactivity, since the low availability of β-alanine to muscle cells is the most limiting factor for carnosine synthesis (22). However, this seems not to hold true when β-alanine is abundantly available due to supplementation (17). In cases where β-alanine availability is high, β-alanine transport efficiency seems no to influence carnosine synthesis rates and the activity of carnosine synthase appears to become a rate-limiting factor for intramuscular carnosine synthesis (17). This might explain why carnosine content is higher in type I fiber of paralyzed muscles under no β-alanine supplementation (hypothetically due to increased TauT expression), but no differences in carnosine loading was shown following supplementation, even in type I fibers.

To conclude, we demonstrated that MCarn is higher in an inactive than in an active, well-trained muscle. However, this is most likely a reflection of higher type II-fiber distribution resulting from muscle fiber shifts in the inactive muscle. Although we showed some evidence that chronic inactivity increases MCarn in type-I fibers, the strength of the evidence is limited due to the low number of observations. Finally, we provided robust evidence that carnosine accretion in chronically inactive muscle in response to β-alanine supplementation was similar to that of an active, well-trained muscle.

Perspectives and significance

By investigating athletes with spinal cord injury, resulting in paraplegia and lower limb immobilization, we demonstrated that sustained muscle (in)activity increases muscle carnosine content, which we suggest is most likely due to type I to type II fiber shift. We also provided some evidence that muscle inactivity might increase carnosine content in type I fibers, possibly as a compensatory effect for the increased oxidative stress that accompanies immobilization. Importantly, carnosine accretion was similar in active and inactive muscles in response to β-alanine supplementation, indicating that chronic (in)activity has no impact on the molecular machinery involved in carnosine synthesis and homeostasis in skeletal muscle. This also suggests that β-alanine supplementation, irrespective of exercise, is the main strategy to increase muscle carnosine.

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