Role of histidyl dipeptides in contractile function of fast and slow motor units in rat skeletal muscle

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Kaczmarek D, Łochoński D, Everaert I, Pawlak M, Derave W, Celichowski J. Role of histidyl dipeptides in contractile function of fast and slow motor units in rat skeletal muscle. J Appl Physiol 121: 164–172, 2016. First published May 19, 2016; doi:10.1152/japplphysiol.00848.2015.—The physiological role of the muscle histidyl dipeptides carnosine and anserine in contractile function of various types of muscle fibers in vivo is poorly understood. Ten adult male Wistar rats were randomly assigned to two groups: control and supplemented for 10 wk with beta-alanine, the precursor of carnosine (~640 mg·kg body wt−1·day−1). Thereafter, contractile properties and fatigability of isolated fast fatigable (FF), fast resistant to fatigue (FR), and slow motor units (MUs) from the medial gastrocnemius were determined in deeply anaesthetized animals. The force resistance was tested with a 40-Hz fatigue protocol followed by a second protocol at 40 Hz in fast and 20 Hz in slow units. In the supplemented rats, histidyl dipeptide concentrations significantly increased (P < 0.05) by 25% in the red portion of the gastrocnemius, and carnosine increased by 94% in the white portion. The twitch force of FF units and maximum tetanic force of FR units were significantly increased (P < 0.05), and the half-relaxation time was prolonged in slow units (P < 0.05). FF units showed less fatigue during the first 10 s, and FR units showed higher forces between 10 and 60 s during the 40-Hz fatigue test. In slow units, forces declined less during the first 60 s of the 20-Hz test. In conclusion, this in vivo experiment demonstrates that an elevation in muscle histidyl dipeptide content elicits beneficial changes in MU contractile characteristics and consequently improve muscle performance. For instance, muscle carnosine concentration was increased by 60% after 4-wk and by 80% after 10-wk supplementation with beta-alanine, which improved exercise performance during a short cycle test in men (28). A metaanalysis by Hobson et al. (29) revealed that beta-alanine supplementation predominantly improves high-intensity activities lasting between 1 and 4 min and possibly also improves performance on activities that last slightly longer, such as 2-km rowing (2), thereby providing a rationale for its popularity as an ergogenic supplement in the sporting community.

Although several biochemical properties of carnosine and other histidyl dipeptides have been described (8), there is still a need for studies aimed at revealing a physiological role of these molecules in the whole skeletal muscle, as well as in slow and fast muscle fibers, under well-controlled physiological conditions. To date, several in vivo and in vitro approaches have been employed to address these issues. First, human in vivo studies on whole muscle contractility showed that muscle carnosine loading diminished the decrease in peak torque during repeated bouts of maximal isokinetic knee extensions (12). On the other hand, Hannah et al. (25) did not observe any change in the maximum or explosive voluntary force production capacity of knee extensors but noted a reduced half-relaxation time of evoked muscle contractions. However, even though whole muscle human studies allow the examination of the effects of interventions under physiological conditions (43), the contractile properties of different muscle fiber types cannot be distinguished, and it is difficult to identify underlying mechanisms (1). Another approach, using in vitro isolated rodent muscle preparations, revealed that beta-alanine supplementation in mice improved fatigue resistance in isolated slow soleus muscle (17). A leftward shift of the force-frequency curve in histidyl dipeptide-loaded muscle has been observed, but only in fast extensor digitorum longus (EDL) muscle. Unfortunately, the presence of hypoxia and extracellular gradients of O2, K+, or lactic acid occurring in this method may influence the results (5, 44), and only a mixture of fiber types can be studied. The latter issue can be resolved using mechan-
ically or chemically skinned single muscle fiber preparations. Such studies have demonstrated that carnosine can potentiate force in a concentration-dependent manner in fast and slow isolated human and rodent muscle fibers (15, 16). This effect was attributed to the ability of carnosine to increase Ca^{2+} sensitivity of the contractile apparatus, as the force-pCa curve was shifted to the left. However, these in vitro observations have not been confirmed in humans (25). Furthermore, it should be noted that in skinned fiber preparations, a loss of intracellular constituents may occur (1).

To overcome the limitations of previous approaches, we applied a neurophysiological technique, which enables an in vivo determination of the contractile function of isolated motor units (MUs) in the rat. To our knowledge, such a method has not been previously used to study the effects of nutritional interventions on muscular function. This approach provides physiological conditions even during long-lasting experiments and allows, in contrast to the human model, direct measurements of contractile properties in groups of muscle fibers of the same type (23). Importantly, rat medial gastrocnemius muscle was studied because it contains the entire spectrum of MU/muscle fiber types (19, 30), making it a good model to examine the effects of increased histidyl dipeptide levels both in fast and in slow fibers.

It is expected that beta-alanine supplementation would reduce fatigability in fast fatigable (FF) and fast resistant to fatigue (FR) MUs because it was shown that increased carnosine levels contribute to better pH buffering during high-intensity exercises (3, 28, 38). It has been previously suggested that in slow muscle fibers, carnosine can improve resistance to fatigue through enhancement of Ca^{2+} release. However, it is not known if this mechanism is manifested in vivo (16). To verify this hypothesis, a modified fatigue protocol involving the induction of low-frequency force decline in slow units was developed and applied in this study.

METHODS

Ethical approval. The care of animals and all treatment procedures were performed in accordance with the Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences, the European Union guidelines, and the Polish Law on the Protection of Animals. All experimental procedures were approved by the Local Ethics Committee for Animal Research.

Animals. Data were collected on 258 MUs from 10 adult male Wistar rats (outbred stock Amp: WIST) aged 6 mo and weighing 396 ± 32 (SD) g. Animals were housed in standard cages, two per cage, with a 12:12-h light-dark cycle and under controlled temperature (22 ± 2°C) and humidity (55 ± 10%) conditions. All animals were fed commercial rat chow (not containing carnosine or derivatives) ensuring a standard nutrient diet and had unrestricted access to water throughout the study period.

Study design. Animals were randomly assigned to either a control, nonsupplemented group (Con, n = 5) or beta-alanine-supplemented group (BA, n = 5). Rats in the Con group received only water whereas those in the BA group received a 1.0% wt/vol beta-alanine (Sigma Aldrich, Fluka, Japan) solution in water (10 g/l drinking water, corresponding to ~640 mg/kg body wt -1 day^-1^-1 for 10 wk. On the basis of the study by Everaert et al. (17), this was considered to be the most optimal procedure to achieve increases in muscle histidyl dipeptides and to prevent muscle taurine depletion. In both groups, drinking bottles were refreshed three times per week, and for each cage the drinking volume was measured in order to calculate mean water and solution intake per animal. In addition, each week the intake of chow per cage was controlled and body weight was recorded.

Surgical and electrophysiological procedures. Animals were anesthetized by an intraperitoneal administration of sodium pentobarbital (Morbital, initial dose 60 mg/kg) and maintained throughout the entire experiment with supplementary doses of 10 mg/kg approximately every hour. The depth of anesthesia was controlled by an observation of pinna and limb withdrawal reflexes. Experiments were terminated by administration of a lethal dose of pentobarbital (180 mg/kg).

The surgical preparation and electrophysiological procedures were performed using a previously described method (36). Briefly, the left medial gastrocnemius was dissected from surrounding tissues and L4 and L5 ventral roots were cut and exposed. The medial gastrocnemius muscle was connected to an inductive force transducer and stretched up to a passive force of 100 mN, which is optimal for a contraction of the majority of its MUs (10). Ventral roots were split into the thin filaments and stimulated by rectangular electrical pulses of 0.1-ms duration and variable voltage (up to 0.5 V), and the force of MU contractions was measured under isometric conditions. Isolation of MUs was confirmed when the action potential recorded with a bipolar silver wire electrode and the twitch force were of the “all-or-none” type and did not change in shape and size with an increase in the stimulus strength. To control the conditions of MU activation, animal body core temperature was automatically maintained by a closed-loop temperature controller at a constant level of 37 ± 1°C during the entire experiment. Moreover, electrical stimulation threshold and muscle passive preloading were continuously monitored during MU recordings.

Testing protocol and data analysis. The stimulation protocol consisted of four successive steps separated by 10-s intervals and two fatigue tests separated by 180 s and 30 s for fast and slow units, respectively. Five twitches, the unfused tetanus (500 ms, 40 Hz), the maximum tetanus (300 ms, 150 Hz), and contractions evoked by 10 successive trains of pulses (500-ms duration evoked at 1, 10, 20, 30, 40, 50, 60, 75, 100, and 150 Hz) were recorded. For each MU, the twitch force, the contraction time, and the half-relaxation time were calculated for the averaged twitch, whereas for the 150-Hz tetanus the maximum tetanus force was measured and the twitch-to-tetanus force ratio was then calculated. The peak forces for force recordings obtained at stimulation frequencies in the range of 1-150 Hz were used to plot the force-frequency curves, and the frequency required to evoke 60% of the maximum force and the slope at the steepest region of the curve were estimated by linear interpolation as previously described (36).

To test the fatigability of fast and slow MUs, the first (standardized) fatigue test protocol consisting of trains of stimuli evoked at 40 Hz, lasting 325 ms, and repeated once per second for 4 min was used. Then, after a 3-min break, the same fatigue protocol was applied again for 3 min in fast units. After a 30-s break, slow MUs were repeatedly stimulated once per second with 20-Hz frequency trains lasting 350 ms for 4 min. Verification of the type of MU was based on visual inspection of the force profile of the unfused tetanus evoked at 40 Hz. MUs with a sag in the force profile were classified as fast while those units without the sag were classified as slow (9, 23). Finally, seven postfatigue force trains of stimuli at 40 Hz for fast (lasting 325 ms) and 20 Hz for slow units (lasting 350 ms), repeated at 10-s intervals, were delivered after the second fatigue test. A 1-s break was implemented between the second fatigue test and the postfatigue recovery protocol.

The fatigue index was calculated as a reverse ratio of the peak tetanic force at the initial part of the first fatigue test to the peak force generated 2 min later (31). Fast units were further classified as FF when the fatigue index was below 0.5 or as FR when the index exceeded 0.5 (23, 31). For fast units the peak tetanic forces measured during the course of both fatigue tests were expressed as a percentage of the force of the first tetanus from the first fatigue test. For slow units, peak tetanic force responses were expressed in relation to the
peak force of the first tetanus in the first (40 Hz) and the second (20 Hz) fatigue tests, separately.

Muscle content of carnosine, anserine, and taurine. At the beginning of each experiment, the right medial gastrocnemius and soleus muscles were carefully dissected, any visible connective or fat tissues were removed, and the medial gastrocnemius muscle was divided into two parts (red and white)—one containing mainly type I muscle fibers and the other containing mainly type II muscle fibers. Muscle samples were weighed, placed in cryogenic vials (NUNC/Thermo Fisher Scientific), immediately frozen by immersion in liquid nitrogen, and kept at −80°C until analysis.

The muscle samples were subsequently dissolved in phosphate buffer saline solution (PBS, 20 μl/mg muscle) for homogenization. Muscle homogenates were deproteinized using 35% sulfosalicylic acid (SSA) and centrifuged (5 min, 14,000 g), and 100 μl of deproteinized supernatant were dried under vacuum (40°C). Dried residues were resolved with 40 μl of coupling reagent [methanol-triethylamine-H2O-phenylisothiocyanate (7:1:1:1)] and allowed to react for 20 min at room temperature. The samples were dried again and resolved in 100 μl of sodium acetate buffer (10 mM, pH 6.4). The same method was applied to the combined standard solutions of carnosine (Flamma), anserine (Sigma), and taurine (Sigma). The derivatized samples (20 μl) were applied to a Waters HPLC system with a Spherisorb ODS-2 column (4.6 × 150 mm, 5 μm) and UV detector (wavelength 254 nm). The column was equilibrated with buffer A (10 mM sodium acetate adjusted to pH 6.4 with 6% acetic acid), buffer B (60% acetonitrile-40% buffer A), and buffer C (100% acetonitrile) at a flow rate of 0.8 ml/min at 25°C. The limits of detection and quantification for carnosine were 3 and 10 μM, respectively. The coefficients of variation were 11.85, 11.60, and 7.86% for carnosine, anserine, and taurine, respectively.

Statistical analyses. Data are presented as means ± SD, and P < 0.05 was considered statistically significant. Data normality was assessed with the Shapiro-Wilk test. The entire population of MUs sampled for each studied group were pooled together, assigned to three MU categories, and compared with the use of the chi-squared test. Student’s t-test was used for statistical comparisons of body and muscle weights. Comparisons of MU contractile and force-frequency parameters were made with the use of Student’s t-test or Mann-Whitney U-test (BA vs. Con group).

The effects of time, group (BA vs. Con), and the interaction effect between the time and group on force profiles during the course of the fatigue tests and recovery period were tested with the two-way repeated measures analysis of variance (ANOVA). In fast MU, statistical analyses were performed within the three time periods: from 1 to 10 s (measured every second), from 10 to 60 s, and from 60 to 240 s (measured every 10 s) during the first and second 40-Hz fatigue tests. According to the theoretical model constructed by Baker et al. (4), these periods reflect the highest contribution of phosphagen, glycolysis, and mitochondrial respiration systems to the ATP resynthesis during skeletal muscle contractile activity, respectively. The fatigability of slow MUs was analyzed within the initial 20 s (measurements performed every second) and from 1 to 60 s and from 60 to 240 s (in both cases measurements performed every 10 s) for both 40- and 20-Hz fatigue tests. The course of the force-frequency curves was checked with the two-way ANOVA with group (BA vs. Con) and frequency (specified stimulation rates in TESTING PROTOCOL as factors). Mauchly’s sphericity method was used to assess the homogeneties of covariance. In all cases of measured effects the sphericity assumption was violated; therefore the data were corrected using the Greenhouse-Geisser procedure. Subsequently, the post hoc analysis for all comparisons was performed using the Bonferroni-corrected t-tests.

RESULTS

Body mass, muscle weight, and behavioral observations. The body mass of animals did not differ significantly between groups either at the beginning or at the end of the study. The body mass increase after 10 wk of intervention was 17.2 ± 4.4% in control and 16.9 ± 2.3% in the beta-alanine group (Table 1). Beta-alanine supplementation did not influence food intake. However, supplemented animals consumed ~14% less water than control rats (Table 1), which is in line with previous observations in mice orally treated with beta-alanine or carnosine (17).

Muscle content of carnosine, anserine, and taurine. The results concerning muscle carnosine, anserine, and taurine concentrations are presented in Table 2. In all studied muscles, anserine levels were generally significantly higher than carnosine levels both in the control as well as in the beta-alanine group. The concentration of histidyl dipeptides (defined as the sum of carnosine and anserine) was markedly lower in soleus than in gastrocnemius muscle. Oral supplementation with 1% beta-alanine resulted in significantly higher muscle carnosine levels compared with the control group in the red (+56%, P < 0.001) and white (+94%, P < 0.001) portions of the gastrocnemius, as well as in the soleus muscle (+112%, P < 0.001) (Table 2). Moreover, muscle anserine content was higher in the red gastrocnemius (+19%, P < 0.05) and the soleus muscle (+67%, P < 0.05) in animals supplemented with beta-alanine. Histidyl dipeptide levels were increased by 24% in the red gastrocnemius (P < 0.05) and by 76% in soleus (P < 0.05) in BA rats. The muscle taurine content was not significantly affected by BA supplementation and was 91, 94, and 97% of the control value in the red gastrocnemius, white gastrocnemius, and soleus, respectively.

Proportions and contractile properties of motor units. One hundred twenty six and one hundred thirty two MUs were investigated in control and supplemented rats, respectively. The percentage distribution of MUs did not differ between the two groups. Among all isolated MUs, FF, FR, and slow units constituted 38.1, 46.0, and 15.9% in the Con and 48.5, 39.4, and 12.1% in the BA animals, respectively (chi-squared test, P > 0.05).

The twitch contraction time and half-relaxation time in fast MUs did not differ between groups (Table 3). In slow MUs, the contraction time was not different but the half-relaxation time was significantly increased by 17% in supplemented animals compared with the control group (P < 0.05) (Table 3). Beta-alanine supplementation resulted in significantly higher twitching activity of phosphagen, glycolysis, and mitochondrial respiration systems to the ATP resynthesis during skeletal muscle contractile activity, respectively. The fatigability of slow MUs was analyzed within the initial 20 s (measurements performed every second) and from 1 to 60 s and from 60 to 240 s (in both cases measurements performed every 10 s) for both 40- and 20-Hz fatigue tests. The course of the force-frequency curves was checked with the two-way ANOVA with group (BA vs. Con) and frequency (specified stimulation rates in TESTING PROTOCOL as factors). Mauchly’s sphericity method was used to assess the homogeneties of covariance. In all cases of measured effects the sphericity assumption was violated; therefore the data were corrected using the Greenhouse-Geisser procedure. Subsequently, the post hoc analysis for all comparisons was performed using the Bonferroni-corrected t-tests.

| Table 1. Characteristics of control and beta-alanine-supplemented animals |
|------------------|------------------|------------------|
|                   | Control (n = 5)  | Beta-Alanine (n = 5) |
| BW start, g       | 392.50 ± 31.56   | 401.20 ± 36.48   | 0.69 |
| BW end, g         | 455.25 ± 44.40   | 468.88 ± 42.18   | 0.61 |
| BW Δ, g           | 67.30 ± 21.57    | 67.68 ± 10.09    | 0.63 |
| MG muscle weight, g | 1.19 ± 0.08     | 1.26 ± 0.11      | 0.30 |
| Muscle-body weight ratio, % | 0.26 ± 0.02 | 0.27 ± 0.01 | 0.59 |
| Food intake, g·rat−1·day−1 | 23.96 ± 2.14 | 24.76 ± 2.22 | 0.37 |
| Drinking volume, ml·rat−1·day−1 | 32.01 ± 4.24 | 27.52 ± 3.67 | 0.07 |
| Total ingested beta-alanine, g/rat | – | 19.26 ± 2.17 | – |

Values are means ± SD. BW, body weight; MG, medial gastrocnemius.
force (+24% vs. control) in FF units. On the other hand, in FR (+19%) and slow units (+16%) the BA-induced increase did not reach statistical significance (Table 3). In addition, there were no significant effects for frequency × group on the force-frequency relation either in FF (F(8,832) = 2.9; P < 0.05; Greenhouse-Geisser P > 0.05), FR (F(8,840) = 1.3; P > 0.05; Greenhouse-Geisser P > 0.05), or slow MUs (F(8,264) = 0.8; P > 0.05; Greenhouse-Geisser P > 0.05) (Fig. 1). Moreover, the frequency required to evoke 60% of the maximum force and the slope of the force-frequency curve did not differ between studied groups (data not shown).

The maximum tetanic force in FR MUs was significantly higher (+26%) in BA supplemented than in control animals (P < 0.05), while in FF (+10%) as well as in slow (+33%) MUs these differences were not significant. Finally, we did not observe any differences in the twitch-to-tetanus force ratio in either fast or slow MUs (Table 3).

Effects of beta-alanine supplementation on fatigability of motor units. To verify possible effects of beta-alanine supplementation on the resistance to fatigue of MUs, we performed detailed analysis of the peak force responses of repeated tetani in two consecutive fatigue tests and a subsequent recovery period, as described in METHODS.

**FF units.** There was a significant group × time effect (F(9,91) = 6.3; P < 0.000; Greenhouse-Geisser P < 0.05) on the relative force responses registered between the 1st and 10th second of the first fatigue test, which showed that force was transiently potentiated in FF units of BA rats (Fig. 2A, inset in panel at left). Thereafter, FF units of BA rats produced slightly but not significantly lower relative forces between the 150th and 240th seconds of the test (Fig. 2A). During the course of the whole second fatigue test, and during the recovery period, the relative forces of FF MUs did not differ between groups (Fig. 2A, panels in middle and at right). However, the absolute values of the peak forces evoked by all seven recovery trains performed just after the second fatigue test were significantly higher in FF units of beta-alanine-treated than in control animals (data not shown, significant group × time effect; F(6,606) = 5.8; P < 0.000; Greenhouse-Geisser P < 0.05).

**FR units.** The absolute forces of FR units between the 10th and 60th seconds were higher in supplemented rats compared with control (data not shown, significant group × time effect; F(5,495) = 4.2; P < 0.001; Greenhouse-Geisser P < 0.05). Moreover, FR units produced higher relative forces in the BA group between the 10th and 60th seconds of the first fatigue test (significant effect of group; F(1,69) = 6.3; P < 0.05 and significant group × time effect; F(5,495) = 3.8; P < 0.05; Greenhouse-Geisser P < 0.05) (Fig. 2B, panel at left). On the other hand, the relative forces during the entire course of the second fatigue test were comparable in both groups (Fig. 2B). Also, there were no differences in the forces induced by the recovery trains between the two groups (Fig. 2B, panels in middle and at right).

**Table 3. Contractile parameters of motor units**

<table>
<thead>
<tr>
<th>Group</th>
<th>Concentration</th>
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<tr>
<td></td>
<td>Red Gastrocnemius</td>
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<td>White Gastrocnemius</td>
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<td>Soleus</td>
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<tr>
<td>Control</td>
<td>2.93 ± 0.24</td>
<td>t(8) = −8.87</td>
<td>2.28 ± 0.79</td>
<td>U(8) = 0.00</td>
<td>1.93 ± 0.27</td>
<td>t(7) = −12.21</td>
</tr>
<tr>
<td>Beta-alanine</td>
<td>4.57 ± 0.34†</td>
<td></td>
<td>4.43 ± 0.28*</td>
<td>U(8) = 0.00</td>
<td>4.10 ± 0.26†</td>
<td>t(7) = −12.21</td>
</tr>
<tr>
<td>Control</td>
<td>15.46 ± 2.31</td>
<td>t(8) = −2.46</td>
<td>14.62 ± 4.64</td>
<td>t(8) = −1.37</td>
<td>7.18 ± 0.84</td>
<td>U(7) = 0.00</td>
</tr>
<tr>
<td>Beta-alanine</td>
<td>18.32 ± 1.19*</td>
<td></td>
<td>17.53 ± 1.01</td>
<td>t(8) = −2.10</td>
<td>11.96 ± 3.92*</td>
<td>U(7) = 0.00</td>
</tr>
<tr>
<td>Control</td>
<td>18.39 ± 2.43</td>
<td>t(8) = −3.56</td>
<td>16.90 ± 5.30</td>
<td>U(7) = 0.00</td>
<td>9.12 ± 1.07</td>
<td>U(7) = 0.00</td>
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<td>Beta-alanine</td>
<td>22.89 ± 1.44*</td>
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<td>21.95 ± 0.88</td>
<td>t(8) = −2.10</td>
<td>16.06 ± 3.77*</td>
<td>U(7) = 0.00</td>
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<tr>
<td>Control</td>
<td>14.08 ± 2.70</td>
<td>t(8) = 1.02</td>
<td>10.75 ± 2.98</td>
<td>t(8) = 0.47</td>
<td>21.63 ± 1.02</td>
<td>U(7) = 0.00</td>
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<tr>
<td>Beta-alanine</td>
<td>12.80 ± 0.69</td>
<td></td>
<td>10.09 ± 1.09</td>
<td>t(7) = 0.30</td>
<td>21.02 ± 3.79</td>
<td>U(7) = 0.00</td>
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Values are means ± SD. Calculated statistics: t value of the Student’s t-test (degrees of freedom); U value of the Mann-Whitney U-test (degrees of freedom).

Here, n, number of MUs studied within the particular groups; CT, twitch contraction time; HRT, twitch half-relaxation time; TwF, twitch force; Test, tetanus force; Tw/Tet, twitch-to-tetanic force ratio. *P < 0.05 indicates probability of Student’s t-test for equal variances and Mann-Whitney U-test for unequal variances for comparisons between beta-alanine-supplemented rats and the control animals.

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Slow units. In the first fatigue test, we did not observe any development of fatigue, and no differences in force profile were observed between the two groups. Interestingly, when the second fatigue test evoked at 20 Hz was analyzed, we observed that in both groups the absolute force initially declined up to 90 s and then remained stable up to the end of the test (significant effect of time; $F_{5,155} = 72.1; P < 0.001$; Greenhouse-Geisser $P < 0.001$). However, in MUs of BA rats, the relative force declined less (significant group × time effect; $F_{5,155} = 2.3; P < 0.05$; Greenhouse-Geisser $P < 0.05$) over the first 60 s of the test than in Con rats. This effect was even more pronounced for the initial 20 s of the test (significant group × time effect; $F_{19,589} = 2.6; P < 0.001$; Greenhouse-Geisser $P < 0.05$) (Fig. 3, inset in panel at left). Hence, in the BA group, the force dropped only to 80% while in the Con group, the force dropped to 68% of the initial value at the end of the test. Moreover, force during recovery was higher in BA rats (significant effect of group; $F_{1,31} = 4.6; P < 0.05$) (Fig. 3, panel at right).

DISCUSSION

A 10-wk supplementation period with beta-alanine in adult rats caused a substantial increase in muscle carnosine and anserine levels. The increases were more pronounced for carnosine than for anserine and also more pronounced in oxidative (soleus and red gastrocnemius) than in glycolytic (white gastrocnemius) muscles. These nutritionally induced metabolic changes were accompanied by several, generally beneficial, adaptations in contractile function in MUs of the rat gastrocnemius. BA supplementation improved the twitch force of FF units and the maximum tetanic force of FR units and prolonged the half-relaxation time in slow units. As expected, it reduced the fatigability of FF units but only during the first 10 s after initiation of repeated fatiguing activity. Moreover, it enhanced the force potentiation of FR units normally observed during a standard fatigue test. Finally, supplementation reduced force decline of slow units during the first minute of repeated contractions evoked by low-frequency stimulation and improved their ability to recover force.

This was the first study in rats to evaluate the effects of beta-alanine supplementation on muscle carnosine, anserine, and taurine content. We used a protocol proposed by Everaert et al. (17), who showed in mice that 8-wk supplementation with 1.2% beta-alanine caused a significant increase in carnosine and anserine level in hindlimb muscles. However, to avoid a decrease in taurine observed in that study, we used a 1% beta-alanine solution. Ten weeks of supplementation with ~640 mg of beta-alanine per kilogram of body weight per day increased carnosine levels by 112, 94, and 56% in soleus, white, and red medial gastrocnemius, respectively. So far, only two studies in rats have nutritionally evoked a substantial increase in carnosine concentrations in soleus (almost fivefold) and gastrocnemius (twofold), yet both studies examined the effect of 1.8% carnosine supplementation (37, 42).

Even though carnosine is considered a typical fast-twitch metabolite, on the basis of the fact that carnosine can increase Ca$^{2+}$ sensitivity of the contractile apparatus and possibly also Ca$^{2+}$ release in type I muscle fibers (16) it was expected that the ergogenicity of carnosine loading would originate from beneficial effects in more oxidative muscle fibers as well. An evaluation by Hobson et al. (29) demonstrated that supplemen-
Role of Muscle Histidyl Dipeptides in Motor Units • Kaczmarek D et al.

Fig. 2. Changes in the force of FF (A) and FR (B) MUs during the first (panels at left) and the second fatigue tests (panels in middle), as well as during seven postfatigue force recovery trains (panels at right). The values are presented as mean relative forces of unfused tetani evoked by 40-Hz stimulation. In both fatigue tests the peak forces are presented every 10 s and every second (during the first 10 s, insets in panels at left) and expressed as a percentage of the force generated at the beginning of the first fatigue test (the first tetanus). Vertical dashed lines in panels at left and in middle indicate three time windows in which the analysis with the two-way repeated measures ANOVA was performed. **P < 0.01 significant difference in relation to the 1-s CON value, †P < 0.01, ††P < 0.001 significant difference in relation to the 10-s BA value, post hoc Bonferroni-corrected t-test.

tation had the greatest effect on exercise events with durations of 1–4 min. Subsequent studies (6) have found similar results, suggesting an effect of BA supplementation on events lasting up to 10 min. Gross et al. (22) suggested that improved aerobic energy contribution to high-intensity exercise could help explain the ergogenic effects of BA. We now provide evidence that fatigue resistance and contractile modifications in muscle fibers of not only FF units but also FR units (mainly innervating type IIA muscle fibers) and slow units (mainly type I fibers) could indeed contribute to the improvement of muscle performance observed after beta-alanine supplementation. These findings are in line with the results of Everaert et al. (17), who demonstrated, in mice supplemented with ~1,300 mg of beta-alanine per kilogram of body weight per day, an increased fatigue resistance in the isolated soleus muscle, which is composed of type I (from 31 to 41%) and type IIA muscle fibers (13, 34), but not in EDL muscle, which consists predominantly of type IIB and IIA fibers (24, 32). Therefore our results seem to suggest that the ergogenic effect of beta-alanine supplementation is probably present in all three muscle fiber types. Still, the translation of the results obtained from animal studies to humans should be done with great caution.

The higher level of forces achieved during the first part of a fatigue test in FR units of beta-alanine-treated animals may be a result of increased fatigue resistance and/or increased force potentiation. In animal as well as in human MUs, during prolonged repeated contractions, two opposite mechanisms—i.e., fatigue and potentiation—occur simultaneously (18, 21). It is possible that after the early phase of force decrease (which is naturally observed) the force of FR units was more highly potentiated in animals with higher muscle histidyl dipeptide concentrations because of improved Ca\(^{2+}\) handling and therefore phosphorylation of regulatory light chains of myosin (39). In support of this hypothesis, improved Ca\(^{2+}\)
sensitivity by carnosine was first shown in chemically and mechanically skinned skeletal muscle fibers of animals (15, 33) and then confirmed in mechanically skinned type II and type I human muscle fibers (16). Alternatively, the enhanced force generation capacity of FR MUs might result from decreased contraction-induced acidosis caused by the enhanced concentration of histidyl dipeptides in myocytes (3, 28). Importantly, these two ergogenic mechanisms may be functionally linked (7). Swietach et al. (41) have elegantly demonstrated in cardiac myocytes that histidyl dipeptides act as local Ca$^{2+}$/H$^+$ exchangers, which may counteract the inhibitory action of H$^+$ ions and improve processes activated by calcium (40).

This study shows that 10 wk of beta-alanine supplementation induced an increase in the twitch force of FF units. Force potentiation in the presence of an elevated concentration of carnosine was also observed in mechanically skinned rat and human fibers and was explicable by the increase in the Ca$^{2+}$-sensitivity of the contractile apparatus (15, 16). In addition, we found that the maximal tetanic force in FR units was higher after beta-alanine supplementation, which was not observed in previous studies. For instance, tetanic and specific force were elevated by 10% beta-alanine supplementation (17), and only submaximal, not maximal, force of rat as well as human mechanically skinned muscle fibers was increased in the presence of elevated carnosine concentrations (15, 16). Also, in humans, no improvement in the maximum or explosive voluntary forces after BA supplementation was found (25). To date, only one study, by Goodman et al. (20), showed that similar to our results, a 40% increase in muscle taurome content after 2 wk of taurome supplementation resulted in significantly increased subtetanic and maximum tetanic specific force in rat EDL muscle. Therefore it appears that maximal tetanic force can be enhanced by the action of specific amino acids or peptides. As suggested by Gross et al. (22), a possible explanation for this phenomenon could be an increased muscle fiber cross-sectional area caused by the fluid influx in the presence of the higher concentration of histidyl dipeptides. Nevertheless, this assumption is speculative at present and requires further investigation.

No differences in the force-frequency relationship of motor units were noted after beta-alanine supplementation. This agrees with the observed lack of changes in the twitch time parameters, which strongly determine the course of the force-frequency relationship (11, 31, 35). Our results are in line with the findings of Hannah et al. (25), who did not observe any change in the force-frequency curve in humans supplemented with BA. On the other hand, these findings are contrary to previous studies where a leftward shift of the force-frequency relation was noted in isolated mouse EDL muscle after beta-alanine supplementation (17) and the force-pCa curve was shifted to the left in single chemically or mechanically skinned fibers in the presence of carnosine (15, 16, 33). As suggested by Everaert et al. (17), we may not have observed a similar effect because of the differences in contraction mechanisms that are present in various muscles or because of different conditions that may exist during in vitro and in vivo contractions.

One limitation of the current study is that even though a large total number of units (258) were sampled in this study (over 100 per group), the relatively small number of animals might influence the power of statistical analyses for some comparisons made, especially in the case of contractile parameters of slow motor units, which are naturally less abundant in the rat medial gastrocnemius.

In conclusion, we showed that 10-wk supplementation with 1% beta-alanine induces histidyl dipeptide loading in rat hindlimb muscles. This resulted in fiber type-specific contractile adaptations during in vivo single MU stimulation. Our data indicate that beta-alanine supplementation increases force-generating capacity and recovery during fatiguing repeated tetani in FR and slow MUs. In FF MUs, supplementation led to improved twitch force and force maintaining during the first 10 s of repeated tetani. Although only high-intensity exercise performance seems to benefit from BA supplementation, our data demonstrate an important function of histidyl dipeptides not only in fast but also in slow MUs. We demonstrate that this...
electrophysiological methodology is valuable in evaluating muscle contractile function and fatigue after nutritional interventions.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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