

1 **Insulin does not stimulate  $\beta$ -alanine transport into human skeletal muscle**

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3 **Running title:** Effects of insulin on  $\beta$ -alanine uptake by muscle

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**Abstract**

To test whether high circulating insulin concentrations influence the transport of  $\beta$ -alanine into skeletal muscle at either saturating or sub-saturating  $\beta$ -alanine concentrations, we conducted two experiments whereby  $\beta$ -alanine and insulin concentrations were controlled. Experiment 1: 12 men received supraphysiological amounts of  $\beta$ -alanine intravenously ( $0.11 \text{ g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$  for 150min), with or without insulin infusion.  $\beta$ -alanine and carnosine were measured in muscle before and 30 min after infusion. Blood samples were taken throughout the infusion protocol for plasma insulin and  $\beta$ -alanine analyses.  $\beta$ -alanine content in 24-h urine was assessed. Experiment 2: 6 men ingested typical doses of  $\beta$ -alanine ( $10 \text{ mg} \cdot \text{kg}^{-1}$ ) before insulin infusion or no infusion.  $\beta$ -alanine was assessed in muscle before and 120 min following ingestion. In experiment 1, no differences between conditions were shown for plasma  $\beta$ -alanine, muscle  $\beta$ -alanine, muscle carnosine and urinary  $\beta$ -alanine concentrations (all  $p > 0.05$ ). In experiment 2, no differences between conditions were shown for plasma  $\beta$ -alanine or muscle  $\beta$ -alanine concentrations (all  $p > 0.05$ ). Hyperinsulinemia did not increase  $\beta$ -alanine uptake by skeletal muscle cells, neither when substrate concentrations exceed the  $V_{max}$  of  $\beta$ -alanine transporter TauT, nor when it was below saturation. These results suggest that increasing insulin concentration is not necessary to maximise  $\beta$ -alanine transport into muscle following  $\beta$ -alanine intake.

**Keywords:**  $\beta$ -alanine; hyperinsulinemia; human skeletal muscle; carnosine; Taurine transporter.

## 55 Introduction

56 Carnosine ( $\beta$ -alanyl-L-histidine) is an intracellular dipeptide abundantly found in human skeletal  
57 muscle – content ranges from  $\sim 10$  to  $\sim 40$   $\text{mmol}\cdot\text{kg}^{-1}$  of dry muscle (40) – where it has important  
58 physiological roles, such as maintenance of acid-base homeostasis during high-intensity exercise (1, 4, 17),  
59 regulation of  $\text{Ca}^{2+}$  handling and sensitivity during muscle contraction (20, 26, 28), and scavenging of toxic  
60 products of lipid peroxidation (11). The rate-limiting factor for carnosine synthesis in skeletal muscle is the  
61 low availability of  $\beta$ -alanine (27), with  $\beta$ -alanine supplementation increasing muscle carnosine content by  
62  $\sim 40$  to 100% (5, 40).

63 Although  $\beta$ -alanine supplementation is the most effective known strategy to increase muscle  
64 carnosine content, previous studies showed that only  $\sim 6\%$  of the total dose is used to synthesize carnosine in  
65 the skeletal muscle (9, 40). Despite the low efficiency in the use of  $\beta$ -alanine for carnosine synthesis, there is  
66 evidence to suggest that this can be improved when circulating insulin levels are elevated (42). A potential  
67 explanation for the enhanced carnosine synthesis is a putative increase in the efficiency of  $\beta$ -alanine  
68 transport into skeletal muscle.

69  $\beta$ -Alanine is thought to be transported into skeletal muscle cells via two different systems: 1) a  
70 saturable process undertaken by TauT (Taurine transporter - SLC6A6), which is a  $\text{Na}^+$  and  $\text{Cl}^-$  dependent  
71 transmembrane transporter driven by transmembrane  $\text{Na}^+$  flux (30) whose  $K_m$  (determined in primary cell  
72 culture of embryonic chick pectoral muscle) is  $\sim 40$   $\mu\text{mol}\cdot\text{L}^{-1}$  (6); and 2) by PAT1 (SLC36A1), which is a  
73  $\text{Na}^+$ -independent,  $\text{H}^+$ -dependent transporter, whose activity is stimulated by the  $\text{Na}^+/\text{H}^+$  exchanger ( $K_m$  for a  
74  $\beta$ -dipeptidomimetic is  $\sim 40$   $\text{mmol}\cdot\text{L}^{-1}$ ) (22).

75 The  $\text{Na}^+/\text{K}^+/\text{ATPase}$  pump,  $\text{Na}^+$  influx and  $\text{Na}^+/\text{H}^+$  exchanger are stimulated by insulin (13, 32, 39,  
76 44), although the stimulating effect appears to be tissue-specific (34). The transport of other small nitrogen-  
77 containing molecules (i.e., creatine and carnitine) that, like  $\beta$ -alanine, are transported into skeletal muscle  
78 cells through  $\text{Na}^+$  flux driven mechanisms, is increased by elevated insulin concentrations (41, 43). Such an  
79 improvement in transport efficiency mediated by insulin may occur either via reduced  $K_m$  (14) or increased  
80  $V_{max}$  (34). Due to the similarity in transport mechanisms between  $\beta$ -alanine, creatine and carnitine, it has  
81 been postulated that  $\beta$ -alanine transport into skeletal muscle could be further stimulated by hyperinsulinemia

82 (42).

83 Chronic supplementation of carnitine in combination with high loads of high glycemic-index  
84 carbohydrates increased muscle carnitine retention (43), whilst hyperinsulinemia also stimulates creatine  
85 transport into skeletal muscle (41). As yet, however, no study has directly examined the influence of insulin  
86 on  $\beta$ -alanine transport into skeletal muscle, although Stegen et al. (42) showed that ingesting  $\beta$ -alanine along  
87 with meals over a period of 46 days ( $3.2 \text{ g}\cdot\text{d}^{-1}$  split into 4 daily doses of 800 mg) resulted in superior  
88 carnosine accretion in the *m. soleus* in comparison with the group that ingested  $\beta$ -alanine in between meals  
89 (i.e., low insulin condition). These data led to authors to speculate that an elevation in insulin concentrations  
90 induced by meal ingestion could mediate intramuscular  $\beta$ -alanine/carnosine accumulation (42), although  
91 they did not show the same effect in a different muscle (*m. gastrocnemius*) in the same study, causing  
92 uncertainty as to whether insulin truly enhances  $\beta$ -alanine uptake. To test the hypothesis that insulin levels  
93 influence  $\beta$ -alanine transport into skeletal muscle, we conducted two human studies using the  
94 hyperinsulinemic-euglycemic clamp technique, whereby both  $\beta$ -alanine and insulin concentrations were  
95 tightly controlled.

96

## 97 **Methods**

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### 99 *Ethical Approval*

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101 The study was approved by the Ethics Committee of the School of Medicine of the University of Sao  
102 Paulo (#1185971) and complies with the standards established by the Declaration of Helsinki.

103

### 104 *Experiments*

105 In experiment 1,  $\beta$ -alanine was intravenously infused to reach supraphysiological concentrations of  
106  $\beta$ -alanine in plasma to test whether insulin could stimulate  $\beta$ -alanine uptake when TauT was saturated. In  
107 order to confirm whether insulin could stimulate  $\beta$ -alanine uptake when both  $\beta$ -alanine transporters were not  
108 saturated, we then performed experiment 2, in which typical doses of  $\beta$ -alanine were ingested to reach

109 physiological concentrations of  $\beta$ -alanine in plasma, allowing us to better assess the effects of insulin on the  
110  $K_m$  of the transporters.

111

112 *Experiment 1:  $\beta$ -alanine infusion to reach saturating concentrations*

113

#### 114 Participants

115 Physically active male omnivores aged 18 to 35 years were eligible. Exclusion criteria were: use of  
116 dietary supplements containing  $\beta$ -alanine in the past 6 months, diagnosis of type I or type II diabetes  
117 mellitus, diagnosis of glucose intolerance, and the use of drugs known to interfere with insulin sensitivity or  
118 insulin secretion. We determined *a priori* that a sample size of 6 participants would be sufficient to detect a  
119 significant effect ( $\alpha=0.05$ ;  $1-\beta=0.8$ ) of insulin on muscle  $\beta$ -alanine content, assuming an effect size  $>$   
120 0.8 in a within-subject, repeated measures model (G\*Power 3.1.9.2). Forty-eight individuals were screened  
121 for eligibility, 24 of whom met all criteria. Fourteen of these agreed to participate in the study. Two  
122 individuals did not complete the study as they were unwilling to undertake muscle biopsies. Thus, 12  
123 participants completed the entire study (age:  $27\pm 5$  years, body mass:  $79.6\pm 7.4$  kg, height:  $1.80\pm 0.06$  m,  
124 habitual  $\beta$ -alanine intake in diet:  $482\pm 377$  mg $\cdot$ day $^{-1}$ ).

125

#### 126 Experimental design

127 This was a counterbalanced, crossover study. Participants attended the laboratory on two different  
128 days, at least 10 weeks apart (i.e., sufficient time to allow complete washout of the  $\beta$ -alanine infused), for  
129 the main trials. On each trial,  $\beta$ -alanine was intravenously infused for 150 min with circulating insulin  
130 concentrations being kept high in one trial (HI), and low in the other trial (LI). Neither the researchers nor  
131 the participants were blinded to the conditions, due to the required experimental set-up, although all samples  
132 were analysed blinded to the condition by the use of unique identifying codes. Skeletal muscle samples were  
133 collected before and 30 min after the infusion period for the determination of muscle  $\beta$ -alanine and  
134 carnosine concentrations. Blood samples were collected before, throughout and after the infusion period for  
135 the analyses of plasma insulin, glucose and  $\beta$ -alanine. A midstream urine sample was collected before the

136 infusion period, and a 24 h total urine collection was carried out starting from the first urination after the  
137 beginning of the infusion period and ending 24 hours after the first urination.

138

### 139 Main trials

140 Participants arrived at the laboratory following a 12 h overnight fast, having not consumed any meat,  
141 fish or poultry for the last 24 h. On the first day, body mass and height were measured to calculate body  
142 surface area, a required parameter for calculating insulin and glucose infusion rates. Prior to infusion, the  
143 participants were asked to void their bladders and to collect a midstream urine sample, which was stored at -  
144 80°C for further  $\beta$ -alanine analysis. Participants were then accommodated in a hospital bed for the insertion  
145 of cannulas. In the HI trial, two cannulas were inserted into different right arm veins for the infusion of  
146 insulin and  $\beta$ -alanine, whereas two cannulas were inserted into different veins of the left arm for glucose  
147 infusion and blood collection. In the LI trial, only  $\beta$ -alanine was infused; therefore, only one cannula was  
148 inserted into the right arm for the infusion of  $\beta$ -alanine and one cannula in the left arm for blood collection.  
149 The blood collection system was kept patent with the infusion of a saline solution, and a 55° C hand-  
150 warming blanket was placed on the left arm for venous blood arterialization (23).

151 After cannula insertion, a muscle sample was taken from the *m. vastus lateralis* by means of  
152 percutaneous needle muscle biopsy for  $\beta$ -alanine and carnosine determination. Following the biopsy,  $\beta$ -  
153 alanine was infused with/without insulin and glucose for 150 min, a sufficient amount of time for tissue  
154 uptake of  $\beta$ -alanine (27). Blood samples were taken for  $\beta$ -alanine and insulin analyses at the following time  
155 points: before, 10, 30, 60, 90, 120, and 150 min after the beginning of the infusion, and 30 min after the end  
156 of the infusion. Subjective assessment of paraesthesia was performed using a 0-to-3-point scale at the same  
157 time points. A post-infusion muscle biopsy was performed 30 min after the end of the infusion, in order to  
158 allow time for the infused  $\beta$ -alanine to be incorporated into bodily tissues. In the HI trial, insulin and glucose  
159 infusion were maintained for 30 min after the end of  $\beta$ -alanine infusion, in order to allow insulin to play its  
160 putative role to stimulate  $\beta$ -alanine uptake by the skeletal muscle.

161 Upon leaving the laboratory, all participants received a container and were requested to collect urine  
162 during the next 24 h and not to consume any type of meat during this period, with compliance being verbally

163 confirmed with all participants. On the next day, total urine volume was measured with the aid of glass  
164 measuring cylinders, the urine was homogenized and two 50 mL-aliquots were stored at  $-80^{\circ}\text{C}$  until  
165 analysis. Figure 1 (left panel) illustrates the experimental design.

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### 169 Insulin and glucose infusion

170 Insulin and glucose were only infused in the HI trials, following the same procedures described by  
171 DeFronzo et al. (16) for the hyperinsulinemic-euglycemic clamp. Insulin and glucose infusion rates were  
172 calculated according to individual body surface area. Insulin was then infused at a constant rate of  
173  $40\text{mU}\cdot\text{m}^2\cdot\text{min}^{-1}$  to reach the target concentration of  $\sim 100\mu\text{U}\cdot\text{mL}^{-1}$ . The infusion of 10% glucose started  
174 concomitantly with insulin infusion and was adjusted every 5 min to maintain blood glucose concentrations  
175 between 70-100  $\text{mg}\cdot\text{dL}^{-1}$ .

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### 179 $\beta$ -alanine infusion

180 Commercially available  $\beta$ -alanine in powder form ( $>99\%$  purity) was obtained from Sigma-Aldrich.  
181 Before commencing the study,  $\beta$ -alanine powder was sterilized with gamma radiation ( $^{60}\text{Co}$  multipurpose  
182 irradiator - IPEN-CNEN/SP, Brazil) in order to ensure safety and functionality of  $\beta$ -alanine, as previously  
183 described (24). Sterile  $\beta$ -alanine was diluted in 500 mL of saline solution. Infusion rates of  $\beta$ -alanine were  
184 adjusted according to individual's body mass, totalling  $0.11\text{g}\cdot\text{kg}^{-1}$  during the entire 150 min infusion period.  
185 This was determined to be the near the maximal tolerable amount for infusion within 150 min during pilot  
186 testing. These high amounts were chosen to elicit supraphysiological  $\beta$ -alanine concentrations and saturate  
187 TauT, so we could test whether insulin stimulates  $\beta$ -alanine transport when its concentration is above the  
188  $V_{max}$  of TauT.  $\beta$ -alanine was infused at increasingly high rates during the first 20 min; infusion rates were  
189 increased every 5 min, as follows: 0.25, 0.50, 0.60 and  $0.70\text{mg}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$  until they reached  $0.80\text{mg}\cdot\text{kg}^{-1}$

190  $\cdot \text{min}^{-1}$ , which was kept constant for the remaining 130 min of infusion. Mean $\pm$ SD  $\beta$ -alanine infusion rate  
191 was  $60.6 \pm 5.7 \text{ mg} \cdot \text{min}^{-1}$ , and mean $\pm$ SD total  $\beta$ -alanine infusion was  $9.16 \pm 0.78 \text{ g}$ .

192

### 193 Muscle biopsies

194 Muscle samples (~70-150 mg) were collected from the middle portion of the *vastus lateralis* using  
195 the percutaneous needle biopsy technique (8) with suction (37). A 1-cm wide incision was made on the skin  
196 and fascia under local anesthesia (3 mL, 2% xylocaine) to make muscle tissue accessible. Post-infusion  
197 biopsies were performed in an adjacent location, ~1 cm apart in the lateral axis, and at a similar depth.  
198 Immediately after sample collection, blood, fat and connective tissue were removed. The samples were snap  
199 frozen and stored in liquid nitrogen for further analyses.

200

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202

### 203 Sample collection and insulin determination

204 Blood samples were collected in vacuum tubes (BD Vacutainer®) containing clot activator for  
205 plasma insulin determination, and K-EDTA for  $\beta$ -alanine determination. The samples for insulin were kept  
206 at room temperature until the end of the infusion procedure and then immediately taken to the Central  
207 Laboratory of the Clinical Hospital for processing and analysis using an immunofluorometric method. The  
208 samples for  $\beta$ -alanine determination were immediately centrifuged, and plasma was stored at  $-80^\circ\text{C}$  for  
209 further analysis.

210

### 211 Determination of $\beta$ -alanine in plasma, muscle and urine, and carnosine in muscle by HPLC-ESI<sup>+</sup>-

#### 212 MS/MS

213 Twenty micro-liters of plasma were extracted in 980  $\mu\text{L}$  of cold extraction buffer  
214 (methanol:acetonitrile:water 5:3:2 v/v). The extracts were then vortexed for 10 min (30-s bursts interspersed  
215 with 30s periods on ice) and centrifuged at 10,000g for 10 min at  $4^\circ\text{C}$  (36). The supernatants were  
216 subsequently submitted to analysis.



217 Approximately 3-4 mg of lyophilized muscle was powdered and deproteinized with 0.5M HClO<sub>4</sub>,  
218 vortexed for 15 min and centrifuged at 5,000 g at 4°C for 3 min (19). Samples were neutralized with 2.1M  
219 KHCO<sub>3</sub>, centrifuged at 5,000 g at 4°C for 3 min, and the supernatant stored at -80°C for further analysis.

220 Pure urine samples were diluted in water (1:100 v/v) for subsequent analysis. Plasma, muscle and  
221 urine samples and standards were quantified in duplicate and analyzed by on-line HPLC-ESI<sup>+</sup>-MS/MS using  
222 CAR-*d*<sub>4</sub> as internal standard (11). Analysis was conducted in the positive mode and detection was realized  
223 on a triple quadrupole mass spectrometer API 6500 (Sciex, Washington D.C, WA), using selected reaction  
224 monitoring (SRM). For sample injection and cleanup, we used an Agilent HPLC system (Agilent  
225 Technologies, Santa Clara, CA) equipped with an autosampler (1200 High performance), a column oven set  
226 at 45°C (1200 G1216B), an automated high pressure flow switching valve, a 1200 Binary Pump SL and a  
227 Shimadzu 10-AVp Isocratic Pump (Shimadzu, Tokyo, Japan) on two columns: Kinetex C18 column  
228 (100×4.6mm; particle diameter of 2.6 μm (Phenomenex, Torrance, CA) and Kinetex C18 column  
229 (100×2.1mm; particle diameter 2.6 μm (Phenomenex, Torrance, CA). The mobile phase consisted of 5mM  
230 ammonium acetate pH 5.5 (A) and acetonitrile (≥99.9% HPLC grade, Sigma-Aldrich) (B). Both solutions  
231 were filtered through a 0.22 μm PVDF membrane (Millipore, Bedford, MA). The separation condition was  
232 as follows: from 0 to 6 min, 10% acetonitrile and 150 μL/min; from 6 to 10 min, 10–90% acetonitrile and  
233 150–300 μL/min; from 10 to 15 min, 90% acetonitrile and 300 μL/min; from 15 to 20 min, 90–10%  
234 acetonitrile and 300–150 μL/min allowing the first column to re-equilibrate until 30 min. A high-pressure  
235 flow switching valve composed of 2-positions and 6-ports was inserted between the two columns. The  
236 eluent from the first column was discarded by the valve up to the 3<sup>rd</sup> min of the run while keeping the  
237 second column supplied with a solution of water:acetonitrile (9:1, v/v) at a constant flow rate of 100 μL·min<sup>-1</sup>  
238 (Shimadzu 10-AVp Isocratic Pump). After 3 min of the run, the valve switched position and the eluent of  
239 the first column entered the second column. Then, the samples were injected into the mass spectrometer.  
240 The valve returned to the starting position after 14 min of the run and both columns were rebalanced.

241 Electrospray ionization (ESI) in the positive mode was used, and detection was made using selected  
242 reaction monitoring (SRM) on a triple quadrupole mass spectrometer. The Turbo Ionspray Voltage was kept  
243 at 5500 V, curtain gas at 15 psi and the nebulizer and auxiliary gas at 50 psi. The temperature was set to

244 500°C, and the pressure of nitrogen in the collision cell was adjusted to high. The signal to noise ratio (S/N)  
245 of  $\geq 7$  was used as the quantification criteria. S/N transitions for  $\beta$ -alanine, carnosine and *CARD4* were  
246 monitored using a dwell time of 150 ms.

247  $\beta$ -alanine was not derivatized prior to analysis and the SRM transitions monitored were m/z 90→72  
248 (quantification transition), m/z 90→45 and m/z 90→30 (confirmation transition). The quantification  
249 transition m/z 90→72 is unique to  $\beta$ -alanine and can be used to differentiate the  $\alpha$ - from the  $\beta$ -isomer (38).  
250 SRM transitions monitored for carnosine were m/z 227→110 (quantification transition) and m/z 227→210  
251 (confirmation transition). SRM transitions monitored for *CARD4* were m/z 231→110 (quantification  
252 transition) and m/z 231→214 (confirmation transition). Representative chromatograms of plasma, urine and  
253 muscle samples are displayed in figure 2.

254

#### 255 Paraesthesia evaluation scale

256 Because paraesthesia is a major side-effect of elevated plasma  $\beta$ -alanine concentrations (27), we  
257 monitored its occurrence and intensity as a safety measure at the following time points: before infusion, 10,  
258 30, 60, 90, 120, 150 min following the start of infusion, and 30 after the end of infusion, using a 0-3 scale  
259 adapted from Lingjaerde et al. (33), where zero means "I don't feel paraesthesia or I'm not sure what I feel",  
260 one means "I feel slight paraesthesia that hardly bothers me", two means "I feel moderate paraesthesia,  
261 which clearly bothers me", and three means "I feel intense paraesthesia, which bothers me a lot".

262

#### 263 Dietary $\beta$ -alanine intake assessment

264 Participants completed food diaries on 3 non-consecutive days (two weekdays and one weekend day)  
265 following the instructions of a registered nutritionist.  $\beta$ -alanine intake through consumption of fish, poultry  
266 and meat was estimated from the data of Jones et al. (31).

267

268

#### 269 *Experiment 2: $\beta$ -alanine ingestion to reach sub-saturating concentrations*

270

## 271 Participants

272 Six male omnivores (age:  $25\pm 3$  years, body mass:  $78.7\pm 10.1$  kg, height:  $1.76\pm 0.03$  m, habitual  $\beta$ -  
273 alanine intake in diet:  $636\pm 196$  mg $\cdot$ day $^{-1}$ ) were recruited to participate in this experiment, all of whom  
274 completed the entire study protocol. One of the participants also participated in experiment 1. Inclusion and  
275 exclusion criteria were the same of the experiment 1, as detailed above.

276

## 277 Experimental design

278 The experimental design is essentially the same as that used in the experiment 1, except for the oral  
279 ingestion of  $\beta$ -alanine (rather than infusion), the smaller amount of  $\beta$ -alanine provided, the shorter washout  
280 period, the shorter time-course, and the omission of urine collection. The shorter washout period is justified  
281 by the low total  $\beta$ -alanine administration, which is assumed to have little or no noticeable impact on muscle  
282 carnosine concentrations. The time-course was defined based on the pharmacokinetics of  $\beta$ -alanine  
283 appearance and disappearance from plasma after ingestion (Harris et al., 2006). In this counterbalanced,  
284 crossover study, participants attended the laboratory on two different days, 7 days apart (i.e., sufficient time  
285 to allow complete washout of the  $\beta$ -alanine ingested). On each trial,  $10$  mg $\cdot$ kg $^{-1}$  BM of pure powdered  $\beta$ -  
286 alanine was ingested, dissolved in 200 mL of tap water. Upon ingestion, circulating insulin concentrations  
287 was kept high in one trial (HI), and low in the other trial (LI) for 120 min, with trial order being  
288 counterbalanced. Neither the researchers nor the participants were blinded to the conditions, due to the  
289 required experimental set-up, although all samples were analysed blinded to the condition by the use of  
290 unique identifying codes. Skeletal muscle samples were collected before and after 120 min of insulin  
291 infusion (or no infusion) following  $\beta$ -alanine ingestion. Blood samples were collected before, throughout  
292 and after the infusion period for the analyses of plasma insulin, glucose and  $\beta$ -alanine. Figure 1 (right panel)  
293 illustrates the experimental design.

294 All procedures of the main trials, including blood collection, muscle biopsy, hyperinsulinemic-  
295 euglycemic clamp, sample processing and analyses were identical to those described in the experiment 1.

296

## 297 Statistical Analysis

298 Mixed model (proc mixed, SAS v. 9.3) procedure was used to compare muscle  $\beta$ -alanine and  
299 carnosine content between conditions, as well as plasma  $\beta$ -alanine and insulin concentrations and  
300 paraesthesia ratings between conditions. Condition (HI and LI) and time were included as fixed factors and  
301 participants were included as random factors. Tukey-Kramer adjustment for multiple comparisons was  
302 performed whenever a significant F-value was obtained. The area under the curve (AUC) was calculated  
303 using the linear trapezoidal method. Paired *t*-tests were used to compare the AUC of plasma insulin and  
304 plasma  $\beta$ -alanine, and  $\beta$ -alanine in the 24-h urine samples. Statistical significance was accepted at  $p \leq 0.05$ .  
305 Data are presented as mean  $\pm$  1 standard deviation.

306

## 307 **Results**

308

### 309 *Experiment 1: Effect of insulin on $\beta$ -alanine transport at saturating concentrations*

310

#### 311 Plasma insulin

312 Insulin concentrations were significantly higher in HI than in LI (condition-by-time interaction:  
313  $p < 0.0001$ ) at all time-points (pairwise analyses: all  $p < 0.0001$ ), with the exception of baseline ( $p = 0.8$ )  
314 (Figure 3, panel A). A significantly higher insulin AUC was shown in HI than in LI ( $p < 0.0001$ ;  
315 95%CI=8596-13112). These data confirm that the clamp technique was able to sustain physiological  
316 hypersulinemia throughout the entire procedure.

317

#### 318 Plasma $\beta$ -alanine

319 Plasma  $\beta$ -alanine concentrations increased following  $\beta$ -alanine infusion and remained elevated  
320 throughout the infusion period (main effect of time:  $p = 0.0001$ ). Although a significant condition-by-time  
321 interaction was shown ( $p = 0.03$ ), no significant differences between LI and HI were shown at any time point  
322 (pairwise comparisons: all  $p > 0.05$ ). Similarly, no significant differences between conditions were shown for  
323 the area under the plasma  $\beta$ -alanine curve ( $p = 0.34$ ; 95%CI=-177130 – 67097) (figure 4, panel A).

324

325 Muscle  $\beta$ -alanine

326  $\beta$ -alanine infusion resulted in a ~2.5 to 3.0-fold increase in muscle  $\beta$ -alanine content (main effect of  
327 time:  $p < 0.0001$ ), with no significant differences between HI and LI conditions (condition-by-time  
328 interaction:  $p = 0.85$ ) (figure 4, panel B).

329

330 Muscle carnosine

331 Muscle carnosine concentrations did not significantly change following the infusion of  $\beta$ -alanine,  
332 irrespective of condition (main effect of time:  $p = 0.08$ ; main effect of condition:  $p = 0.75$ ; condition-by-time  
333 interaction:  $p = 0.18$ ) (figure 4, panel C).

334

335

336 Urinary  $\beta$ -alanine

337  $\beta$ -alanine concentrations were below the limit of detection in all midstream urine samples collected  
338 before infusion in both trials (therefore, data are not shown). Large amounts of  $\beta$ -alanine were, however,  
339 detected in the 24 h urine samples following infusion, but no significant differences between conditions  
340 were shown ( $p = 0.95$ ; 95%CI = -3.1 – -2.9) (figure 4, panel D).

341

342 Paraesthesia

343 A significant main effect of time ( $p < 0.0001$ ) was shown for self-reported ratings of paraesthesia  
344 throughout the main trials, indicating that  $\beta$ -alanine infusion elicited some degree of paraesthesia. No  
345 significant differences between conditions were, however, shown (main effect of condition:  $p = 0.68$ ;  
346 condition-by-time interaction:  $p = 0.06$ ) (figure 5).

347

348 *Experiment 2: Effect of insulin on  $\beta$ -alanine transport at sub-saturating concentrations*

349

350 Plasma insulin

351 Insulin concentrations were significantly higher in HI than in LI (condition-by-time interaction:  
352  $p < 0.0001$ ) at all time-points (pairwise analyses: all  $p < 0.001$ ), except at baseline ( $p > 0.999$ ) (Figure 3, panel  
353 B). A significantly higher AUC was shown in HI than in LI ( $p = 0.004$ ; 95%CI = -17260 – -6214). These data  
354 confirm that the clamp technique was able to sustain physiological hypersulinemia throughout the entire  
355 procedure.

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357

### 358 Plasma $\beta$ -alanine

359 Plasma  $\beta$ -alanine concentrations rapidly increased following  $\beta$ -alanine ingestion and tended to return  
360 to pre-ingestion levels towards to the end of the 120 min period (main effect of time:  $p < 0.0001$ ). No  
361 differences were shown between LI and HI (condition-by-time interaction:  $p = 0.58$ ; pairwise analyses: all  
362  $p > 0.05$ ) and there were no significant differences between conditions for the  $\beta$ -alanine AUC ( $p = 0.23$ ; 95%  
363 CI = -935 – 3070) (figure 6, panel A).

364

365

### 366 *Muscle $\beta$ -alanine*

367  $\beta$ -alanine ingestion resulted in a ~1.5-fold increase in muscle  $\beta$ -alanine content (main effect of time:  
368  $p = 0.0003$ ), with no significant differences between HI and LI (main effect of condition:  $p = 0.37$ ; condition-  
369 by-time interaction:  $p = 0.32$ ) (figure 6, panel B).

370

## 371 **Discussion**

372 This study aimed to test the hypothesis that hyperinsulinemia could stimulate  $\beta$ -alanine transport to  
373 skeletal muscle. Our experimental setup allowed tight control over blood insulin concentrations (figure 3)  
374 while testing different concentrations of plasma  $\beta$ -alanine. Our findings provide compelling evidence that  
375 hyperinsulinemia does not increase  $\beta$ -alanine uptake by muscle cells, neither when substrate concentrations  
376 exceeded the  $V_{max}$  of TauT, nor when it was at a level below the saturation of  $\beta$ -alanine transporters.  $\beta$ -  
377 alanine transport across membranes can occur through three different protein transporters, namely TauT

378 (SLC6A6), PAT1 (SLC36A1) and  $ATB^{0,+}$  (SLC6A14) (3). However, only the genes encoding TauT and  
379 PAT1 have been shown to be expressed in human skeletal muscle (21), with only the former being  
380 characterised in terms of physical-chemical properties in skeletal muscle cells (7). Thus, very little is  
381 currently known about their specific mechanisms in human skeletal muscle.

382 TauT is a high-affinity, low-capacity transporter that carries  $\beta$ -alanine and taurine across membranes  
383 in a  $Na^+$  and  $Cl^-$  dependent manner in a ratio of 1:1:2 (3, 30). Due to this ionic dependency, TauT operates as  
384 a secondary active transport system, whereby the transporting energy is obtained through an electrochemical  
385 gradient of  $Na^+$ , which is maintained by the activity of the  $Na^+/K^+$ /ATPase pump (25). PAT1 is a  $Na^+$ -  
386 independent,  $H^+$ -dependent, low-affinity, high-capacity transport system of zwitterionic amino acids,  
387 including  $\beta$ -alanine, taurine and GABA (10, 12). Although the PAT1 transporter is not dependent on  $Na^+$ ,  
388 evidence indicates that  $Na^+$  can stimulate PAT1 activity (2). This suggests that optimal PAT1 activity is  
389 coupled to the activity of the  $Na^+/H^+$  exchanger, since the  $Na^+/H^+$  exchanger increases the  $H^+$  gradient across  
390 the membrane, thereby increasing the driving force for PAT1 (29).

391 Studies using the *m. sartorius* of frog (35) and the *m. soleus* of rat (14) suggest that insulin stimulates  
392  $Na^+/K^+$ /ATPase pump activity by increasing intracellular  $Na^+$  affinity. In human fibroblasts, Longo (34)  
393 showed that insulin increased pump activity and  $Na^+/K^+/Cl^-$  co-transport by increasing the  $V_{max}$  of the  
394 transporters without affecting their  $K_m$ . Because the activity of the  $Na^+/K^+$ /ATPase pump (13, 39, 44) and  
395 the  $Na^+/H^+$  exchanger are stimulated by insulin (32), it has been hypothesised that hyperinsulinemia could  
396 stimulate the transport of  $\beta$ -alanine into human skeletal muscle, ultimately leading to increased muscle  
397 carnosine accrual in response to  $\beta$ -alanine supplementation (42). From an applied standpoint, higher  
398 amounts of  $\beta$ -alanine could be transported into skeletal muscle if the  $V_{max}$  of TauT were increased, which  
399 could potentially increase the  $\beta$ -alanine-to-carnosine conversion inside the muscle cells over a  $\beta$ -alanine  
400 supplementation period. This, at least in theory, would mean that either a) the individual could attain a  
401 greater increase in muscle carnosine stores during any given supplementation period (optimising its effects)  
402 or b) the individual could reduce the required supplementation time (making  $\beta$ -alanine supplementation  
403 more user friendly).

404 Mechanisms of transport can be enhanced by increasing either the maximum velocity of the  
405 transporter ( $V_{max}$ ) or the affinity between the transporter and its substrate (decreased  $K_m$ ). Increased transport  
406 capacity is observable when substrate concentrations are above the saturation limit of the transporter,  
407 whereas increased affinity is observable when substrate concentrations are below transporter saturation. In  
408 our first experiment, the rate of  $\beta$ -alanine infusion was virtually the maximum tolerable, considering the  
409 degree of paraesthesia elicited. The elevation in plasma  $\beta$ -alanine concentrations ( $\sim 3000\text{-}4000 \mu\text{mol}\cdot\text{L}^{-1}$ )  
410 have certainly exceeded the maximum capacity of TauT (whose  $K_m$  is reported to be  $\sim 100$ -fold lower), but  
411 were probably below the saturation of PAT1 (whose  $K_m$  for a  $\beta$ -dipeptidemimetic is  $\sim 10$ -fold higher) (22).  
412 Thus, it seems impossible to create an experimental condition that would allow the determination of whether  
413 insulin could further increase the already high capacity of the PAT1 transporter. Attaining larger  
414 concentrations of  $\beta$ -alanine in plasma is too far from any physiological condition and, therefore, of little  
415 practical relevance. Since we did not show any evidence of the stimulatory effect of insulin on  $\beta$ -alanine  
416 transport in experiment 1, it seems safe to conclude that insulin does not increase the  $V_{max}$  of TauT, or the  $K_m$   
417 of PAT1.

418 Although experiment 1 quite conclusively showed no effect of insulin on the  $K_m$  of PAT1 or the  $V_{max}$   
419 of TauT, it does not rule out the possibility of insulin enhancing transport when  $\beta$ -alanine concentrations are  
420 below the saturation of TauT. The hypothesis in this case was that insulin could increase the affinity  
421 between  $\beta$ -alanine and TauT, thereby decreasing its  $K_m$ . To test this remaining hypothesis, we then  
422 conducted experiment 2, where the experimental approach was essentially the same, but the  $\beta$ -alanine  
423 concentrations were much lower. This was attained using a typical dose of orally ingested  $\beta$ -alanine; one  
424 advantage of this design is to test the stimulatory effects of insulin under conditions that are similar to  
425 typical physiological conditions. The results of experiment 2 further confirmed the lack of an effect of  
426 insulin on  $\beta$ -alanine transport.

427 Collectively, the results of this *in vivo* human study suggest that insulin does not decrease the  $K_m$  of  
428 PAT1, increase the  $V_{max}$  of TauT, or decrease the  $K_m$  of TauT. Although the effects on the  $V_{max}$  of PAT1  
429 remain untested, the physiological plausibility of such a high  $\beta$ -alanine concentration in plasma is virtually  
430 non-existent, so that it has very little practical relevance. The lack of effects on PAT1 transport activity can



431 be explained based upon the fact that  $\text{Na}^+$  increases PAT1 activity via an increased proton gradient only  
432 when intracellular pH is acidic, ranging from 5.5 to 6.0 (10, 12, 15, 29). Since these conditions are one order  
433 of magnitude away from the physiological pH of resting muscle (pH  $\sim$ 7.0), we speculate that any putative  
434 effect of insulin on the  $\text{Na}^+/\text{H}^+$  exchanger, and ultimately on PAT1 activity, would not be observable under  
435 physiological conditions. Although it is difficult to provide a clear explanation for the lack of effects of  
436 insulin on TauT activity, we can only speculate that TauT could be less sensitive to increases in the  
437 intramembrane  $\text{Na}^+$  gradient, brought about by increased  $\text{Na}^+/\text{K}^+/\text{ATPase}$  activity, than other transporters,  
438 which might be a consequence of the high-affinity and the rapid saturation of TauT. Alternatively, any  
439 effects of insulin on TauT activity at the molecular level could be so minor that they do not translate into  
440 physiological effects that are observable in whole-body fully integrated systems. Therefore, despite the use  
441 of highly sensitive methods in this study, no stimulatory effects of insulin were detected, meaning that they  
442 are either non-existent or too small to be of any physiological significance.

443 Our findings contradict those by Stegen et al. (42), who showed that supplementation of  $3.2 \text{ g}\cdot\text{d}^{-1}$  of  
444  $\beta$ -alanine (4 daily doses of 800 mg) for 46 days significantly increased muscle carnosine concentration in the  
445 group that supplemented  $\beta$ -alanine along with meals when compared to the group that consumed  $\beta$ -alanine  
446 in between meals. Although this study was not designed to assess  $\beta$ -alanine transport or the effects of insulin  
447 directly, the increased muscle carnosine accretion led to the suggestion that insulin could stimulate  $\beta$ -alanine  
448 transport into skeletal muscle. This effect was, however, only shown in the *m. soleus* and not in the *m.*  
449 *gastrocnemius*, casting some doubt upon this purported effect. The current study provides clear experimental  
450 support to show that there is no stimulatory effect of insulin on  $\beta$ -alanine transport into skeletal muscle. One  
451 alternative explanation for the findings by Stegen et al. (41) is that, instead of an effect of hyperinsulinemia,  
452 food intake might have increased the availability of substrates for the enzymes that degrade  $\beta$ -alanine (4-  
453 aminobutyrate-2-oxoglutarate transaminase and alanine-glyoxylate transaminase), which would have  
454 increased the competition for the binding site of the enzymes, thereby resulting in greater  $\beta$ -alanine  
455 availability for synthesising carnosine. This hypothesis, however, remains highly speculative and requires  
456 experimental confirmation.

457 It is interesting to note that a large increase in muscle  $\beta$ -alanine was shown 2-3 hours following  $\beta$ -  
458 alanine administration in both experiments. Although this rapid increase points to the efficiency of the  
459 transport systems, the administration of  $\sim$ 10-fold larger doses in experiment 1 vs. experiment 2 resulted in  
460 muscle  $\beta$ -alanine accretion of comparable magnitudes, thereby suggesting a saturation in  $\beta$ -alanine transport.  
461 The excess of  $\beta$ -alanine that does not enter the muscle seems to be, at least in part, eliminated in the urine, as  
462 indicated by the high amount ( $\sim$ 50% of the total administered) of  $\beta$ -alanine appearing in the urine in  
463 experiment 1. In the experiment 2, we did not collect 24-h urine samples to measure  $\beta$ -alanine losses, but  
464 Harris et al. (2006) (27) showed a much smaller ( $\sim$ 3% of total administered)  $\beta$ -alanine loss in urine with the  
465 same dose (800 mg) of  $\beta$ -alanine (taken orally) as we used in experiment 2. These results appear to be  
466 consistent with a high-affinity, low-capacity transporting system, and might suggest that TauT (high-  
467 affinity, low-capacity) is the major  $\beta$ -alanine transporter in the skeletal muscle whilst PAT1 (low-affinity,  
468 high-capacity) might play a secondary role. Further studies are needed to precisely characterise the specific  
469 roles of TauT and PAT1 on muscle  $\beta$ -alanine uptake.

470 The saturation of  $\beta$ -alanine transport appears to occur at plasma concentrations close to those elicited  
471 by typical supplemental doses of  $\beta$ -alanine, which indicates that the ingestion of larger doses will probably  
472 not result in substantial increases in  $\beta$ -alanine uptake by the skeletal muscle but, instead, will likely result in  
473 larger losses in urine. The rapid and marked increase in muscle  $\beta$ -alanine in only 2-3 hours  
474 following/alongside  $\beta$ -alanine administration contrasts with the lack of increase in muscle carnosine in the  
475 same period.

476 One possible explanation for the mismatch between  $\beta$ -alanine transport (rapid) and carnosine  
477 synthesis (slow) could be related to the  $\beta$ -alanine transport systems, which, despite being saturable, appear to  
478 be relatively more efficient than the carnosine synthase enzyme. This could suggest that  $\beta$ -alanine transport  
479 is not the limiting factor for carnosine synthesis, at least when ample  $\beta$ -alanine is available. Under these  
480 circumstances, the activity of carnosine synthase, rather than  $\beta$ -alanine availability, could be limiting for  
481 carnosine synthesis. This notion is supported by a cell culture study where an excess of  $\beta$ -alanine availability  
482 led to the saturation of carnosine synthase, making it the rate-limiting factor for  $\beta$ -alanine-to-carnosine  
483 conversion (6), and by chronic  $\beta$ -alanine supplementation studies showing that carnosine saturation may not

484 occur even after 24 weeks of supplementation (40). This is also in line with pharmacokinetic studies that  
485 showed elevated whole-body retention rates (~97%) when typical doses of  $\beta$ -alanine were orally ingested,  
486 along with only minimal losses in urine (27), and with the low rate of  $\beta$ -alanine-to-carnosine conversion in  
487 the skeletal muscle (typically <6% of the total  $\beta$ -alanine ingested) (9, 40). This temporal mismatch would  
488 result in free  $\beta$ -alanine accumulation in muscle, which would fit with the findings of Blancquaert et al. (9)  
489 who showed that the excess of  $\beta$ -alanine becomes available to transaminases that degrade  $\beta$ -alanine in the  
490 muscle and liver, ultimately directing  $\beta$ -alanine towards oxidation (9). If the activity of carnosine synthase  
491 was the rate-limiting factor for carnosine synthesis when  $\beta$ -alanine is largely available in the skeletal muscle,  
492 it would be questionable whether increasing  $\beta$ -alanine uptake into skeletal muscle would result in more  
493 carnosine synthesis unless this were accompanied by an increase in the activity of carnosine synthase. If we  
494 assume this hypothesis to be correct, then it would make sense to find ways to increase carnosine synthase  
495 activity, rather than  $\beta$ -alanine availability, as a means to maximise carnosine accretion in the skeletal  
496 muscle.

497 On the other hand, the experimentally determined  $K_m$  of carnosine synthase, which is  $90 \mu\text{mol}\cdot\text{l}^{-1}$   
498 (18) is far in excess of the muscle  $\beta$ -alanine concentrations ( $\sim 5\text{-}8 \mu\text{mol}\cdot\text{l}^{-1}$ ) attained following  $\beta$ -alanine  
499 administration in our study. This means that much larger  $\beta$ -alanine concentrations would be required to  
500 significantly elevate the activity of carnosine synthase, which somewhat contradicts the idea that further  
501 increased  $\beta$ -alanine availability is not limiting for carnosine synthesis when  $\beta$ -alanine is already elevated. As  
502 such, we cannot rule out the possibility that other strategies to increase  $\beta$ -alanine transport to skeletal muscle  
503 could result in further increased muscle carnosine synthesis. Our study clearly demonstrates that  
504 hyperinsulinemia is not one of these strategies. It also seems that increasing plasma  $\beta$ -alanine concentrations  
505 above those already achieved with currently used supplemental doses have little potential to further enhance  
506 carnosine synthesis. Future studies could, however, focus upon other ways to maximise carnosine accretion  
507 and the molecular mechanisms underlying such effects should they exist.

508 In conclusion, the present study showed that high insulin concentrations do not stimulate  $\beta$ -alanine  
509 transport into skeletal muscle, neither does it increase whole-body  $\beta$ -alanine retention. The lack of effect  
510 was demonstrated under both physiological and supraphysiological  $\beta$ -alanine concentrations, suggesting that

511 insulin has no physiologically relevant effect on any of the  $\beta$ -alanine transporters. From an applied  
512 perspective, our findings suggest that  $\beta$ -alanine ingestion does not need to be accompanied by a high-  
513 carbohydrate meal; therefore,  $\beta$ -alanine supplementation strategies that manipulate insulin concentrations  
514 seem not to be required.

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518

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682 **Figure 1.** Experimental design and representation of the main trials in both experiments  
683 where supraphysiological  $\beta$ -alanine concentrations were attained with intravenous  
684 infusion (Experiment 1, left panel) and physiological concentrations were attained with  
685 the ingestion of typical doses of  $\beta$ -alanine (Experiment 2, right panel). HI=high insulin.  
686

687 **Figure 2.** Representative chromatograms displaying monitored transitions of  $\beta$ -alanine,  
688 carnosine<sup>d4</sup>, and carnosine in plasma, urine and skeletal muscle extracts.  
689

690 **Figure 3.** Plasma insulin concentrations during 180 min of intravenous insulin infusion  
691 (HI) or basal fasting insulin (LI) in experiment 1 (**panel A**) and during the 120 min of  
692 insulin infusion or basal fasting in experiment 2 (**panel B**).

693 AUC: area under the curve ( $p < 0.0001$ ).

694

695 **Figure 4. Panel A:** Plasma  $\beta$ -alanine concentrations during the 180 min  $\beta$ -alanine  
696 infusion period in both high (HI) and low insulin (LI) conditions (left chart), and area  
697 under the  $\beta$ -alanine curve (AUC) in both conditions (right chart). No differences  
698 between LI and HI were shown at any time point (all  $p > 0.05$ ). **Panels B and C:**

699 Intramuscular concentrations of  $\beta$ -alanine and carnosine in muscle extracts obtained  
700 before and after  $\beta$ -alanine infusion in both HI and LI conditions. **Panel D:** Total  $\beta$ -  
701 alanine recovered in the 24-h urine samples collected after infusion in both HI and LI  
702 conditions. All results refer to data obtained in the experiment 1.

703 p-values on panels B and C refer to condition-by-time interaction.

704 Two participants were assessed per trial, meaning that a total of 12 independent  
705 experiments were conducted, totalising 24 observations.

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708 **Figure 5.** Subjective ratings of paraesthesia reported before and throughout  $\beta$ -alanine  
709 infusion in experiment 1. All participants reported no paraesthesia in experiment 2 at  
710 any time point following  $\beta$ -alanine ingestion (therefore, data are not shown). No  
711 significant differences between conditions were shown.

712

713 **Figure 6. Panel A:** Plasma  $\beta$ -alanine concentrations during the 120 min after  $\beta$ -alanine  
714 ingestion in both high (HI) and low insulin (LI) conditions (left chart), and area under  
715 the  $\beta$ -alanine curve (AUC) in both conditions (right chart). No differences between LI  
716 and HI were shown at any time point (all  $p > 0.05$ ). **Panel B:**  $\beta$ -alanine concentrations  
717 measured in muscle extracts obtained before and after  $\beta$ -alanine ingestion in both HI  
718 and LI conditions. All results refer to data obtained in the experiment 2.  
719 p-values on panel B refer to condition-by-time interaction (above) and Tukey-Kraemer  
720 adjusted pairwise comparison *vs.* before ingestion.

721 Two participants were assessed per trial, meaning that a total of 6 independent  
722 experiments were conducted, totalising 12 observations.

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747

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755 L.S.Gonçalves, G.G.Artioli, B.Gualano, C. Sale and M.H.G.Medeiros wrote the paper.

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Fig.1

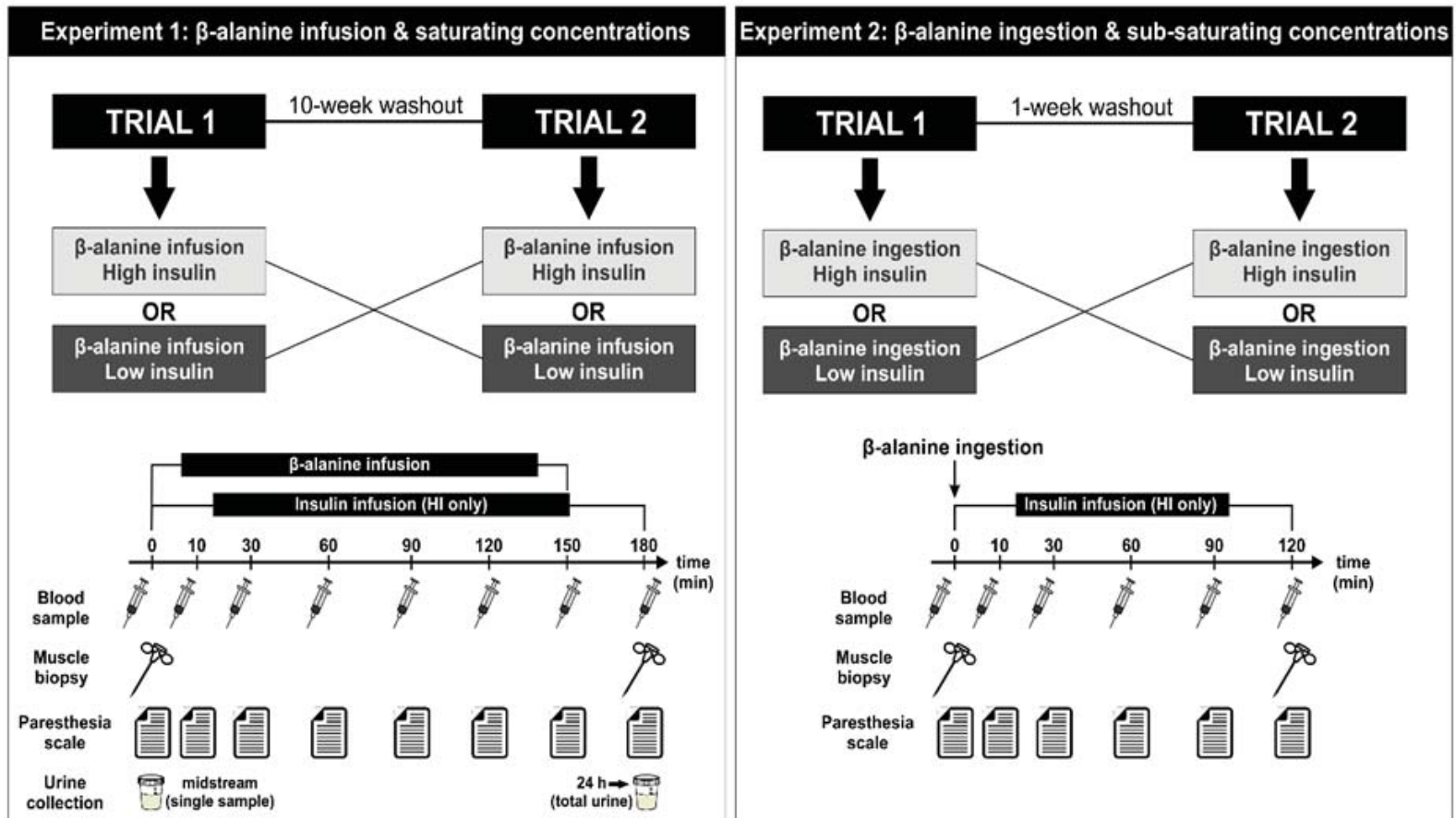


Fig.2

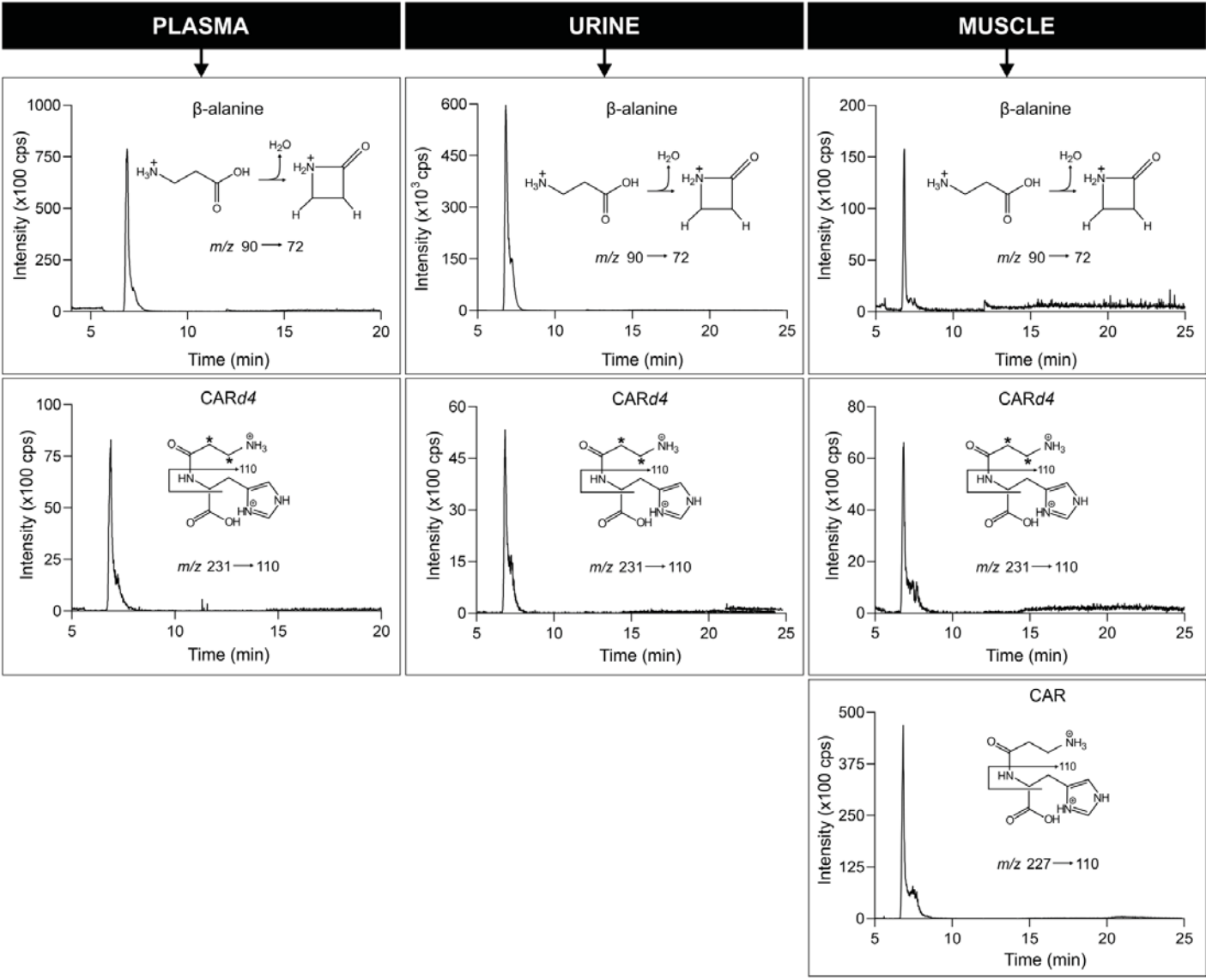


Fig.3

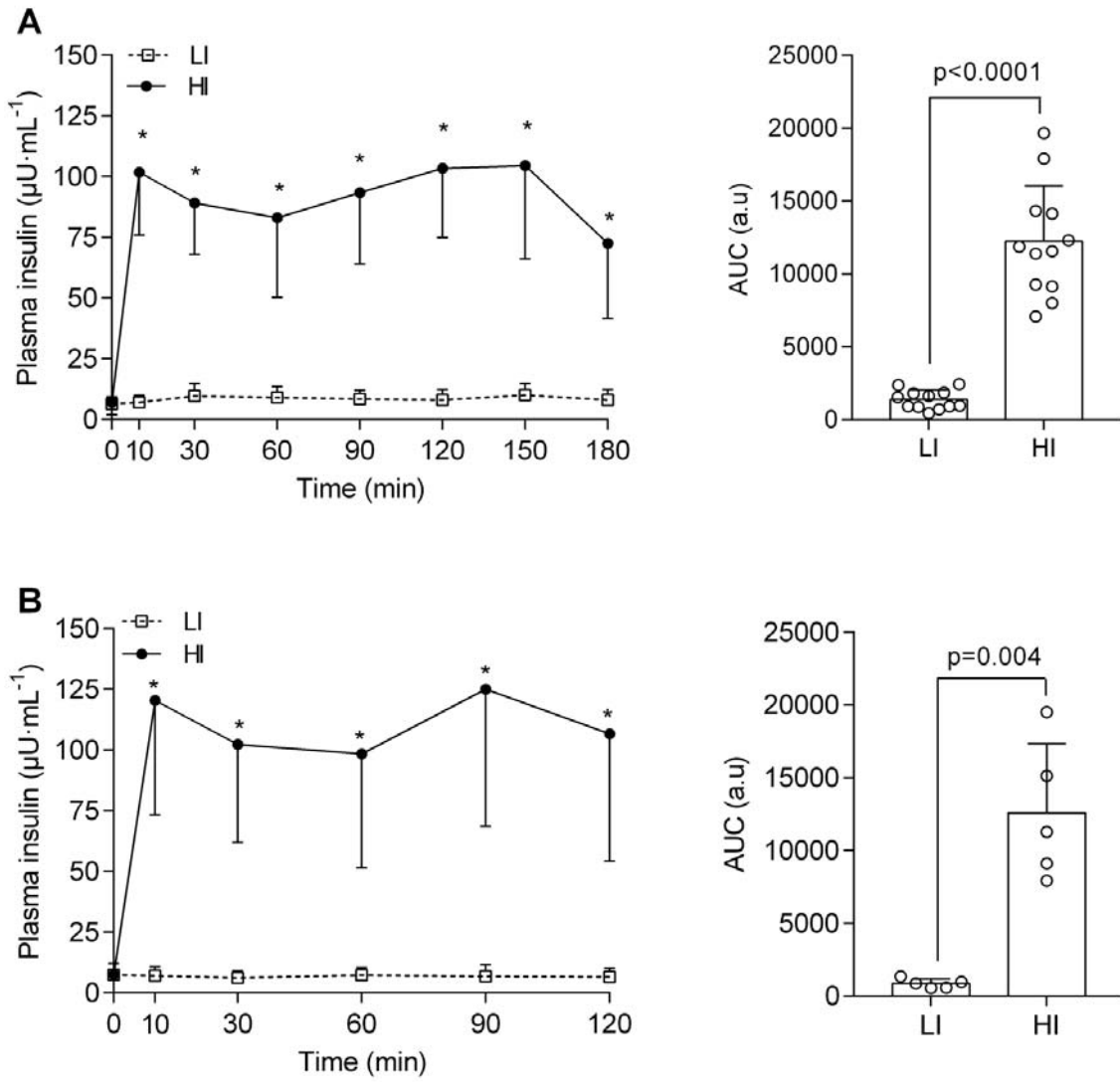


Fig.4

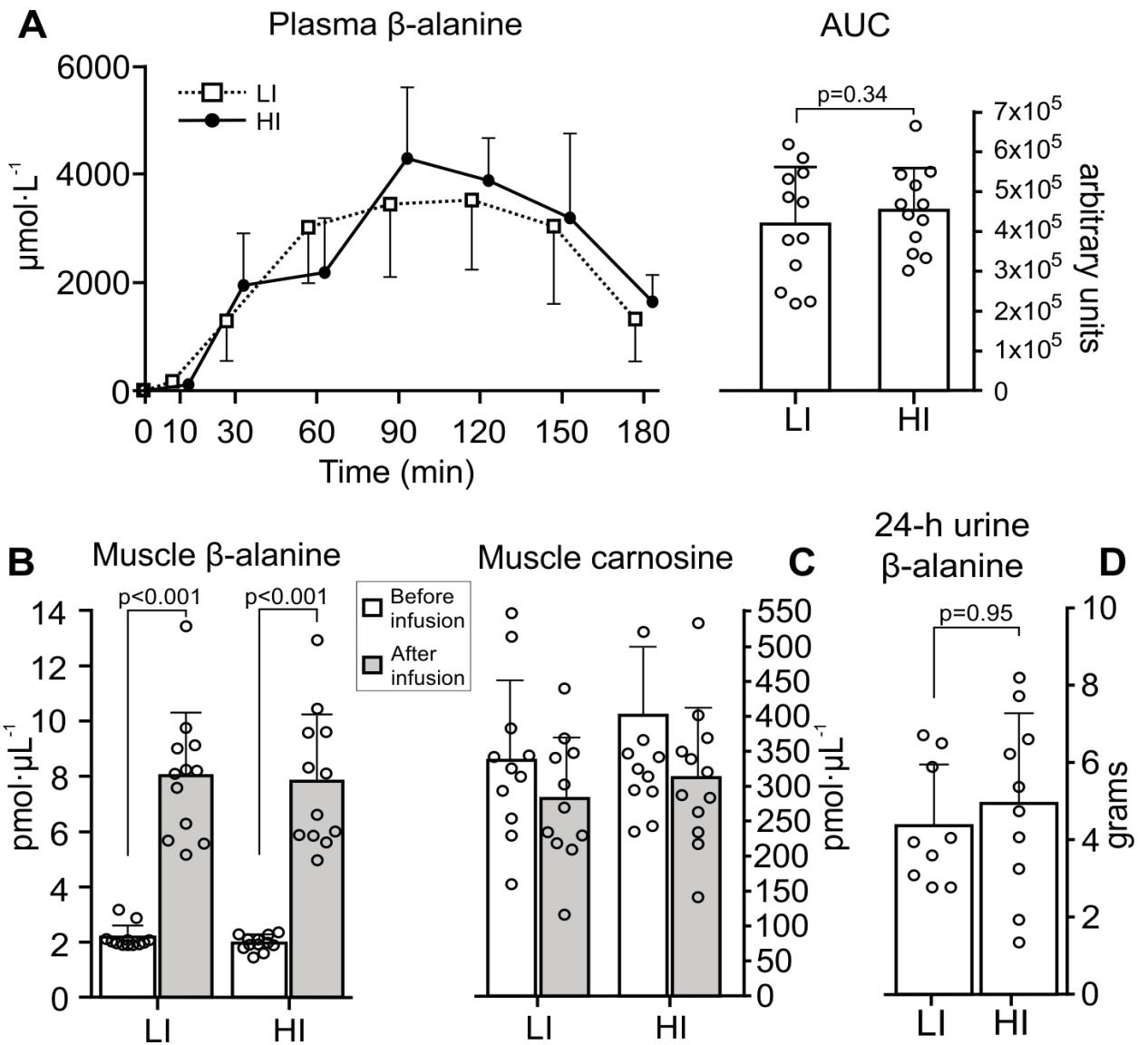




Fig.5

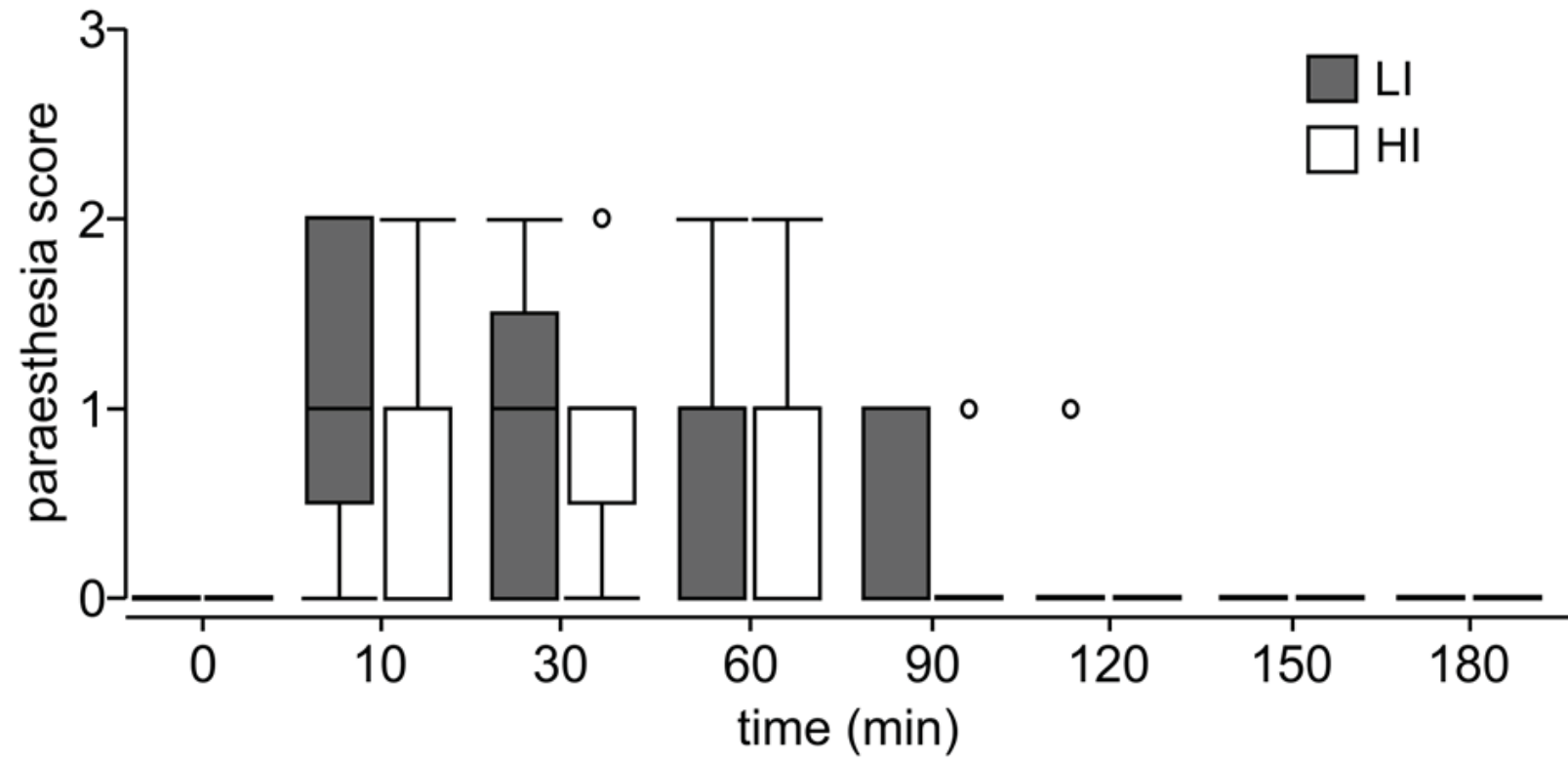


Fig.6

