Insulin does not stimulate β-alanine transport into human skeletal muscle

Running title: Effects of insulin on β-alanine uptake by muscle

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Abstract

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

Keywords: β-alanine; hyperinsulinemia; human skeletal muscle; carnosine; Taurine transporter.

Introduction

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

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

β-Alanine is thought to be transported into skeletal muscle cells via two different systems: *1)* a saturable process undertaken by TauT (Taurine transporter - SLC6A6), which is a Na^+ and Cl^- dependent transmembane transporter driven by transmembrane Na^+ flux (30) whose K_m (determined in primary cell culture of embryonic chick pectoral muscle) is ~40 μmol·L^{-1} (6); and *2)* by PAT1 (SLC36A1), which is a Na^+-independent, H^+-dependent transporter, whose activity is stimulated by the Na^+/H^+ exchanger (K_m for a β-dipeptidemimetic is ~40 mmol·L^{-1}) (22).

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

(42).

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

Methods

Ethical Approval

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

Experiments

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

physiological concentrations of β-alanine in plasma, allowing us to better assess the effects of insulin on the K_m of the transporters.

Experiment 1: β-alanine infusion to reach saturating concentrations

Participants

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

Experimental design

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

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

Main trials

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

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

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

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

Insulin and glucose infusion

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

β-alanine infusion

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

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

Muscle biopsies

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

Sample collection and insulin determination

Blood samples were collected in vacuum tubes (BD Vacutainer®) containing clot activator for plasma insulin determination, and K-EDTA for β-alanine determination. The samples for insulin were kept at room temperature until the end of the infusion procedure and then immediately taken to the Central Laboratory of the Clinical Hospital for processing and analysis using an immunofluorometric method. The samples for β-alanine determination were immediately centrifuged, and plasma was stored at -80°C for further analysis.

Determination of β-alanine in plasma, muscle and urine, and carnosine in muscle by HPLC-ESI^{+}-MS/MS

Twenty micro-liters of plasma were extracted in 980 µLof cold extraction buffer (methanol:acetonitrile:water 5:3:2 v/v). The extracts were then vortexed for 10 min (30-s bursts interspersed with 30s periods on ice) and centrifuged at 10,000*g* for 10 min at 4°C (36). The supernatants were subsequently submitted to analysis.

Approximately 3-4 mg of lyophilized muscle was powdered and deproteinized with 0.5M $HClO_4$, vortexed for 15 min and centrifuged at 5,000 g at 4°C for 3 min (19). Samples were neutralized with 2.1M $KHCO_3$, centrifuged at 5,000 g at 4°C for 3 min, and the supernatant stored at -80°C for further analysis.

Pure urine samples were diluted in water (1:100 v/v) for subsequent analysis. Plasma, muscle and urine samples and standards were quantified in duplicate and analyzed by on-line HPLC-ESI^+-MS/MS using CAR-d_4 as internal standard (11). Analysis was conducted in the positive mode and detection was realized on a triple quadrupole mass spectrometer API 6500 (Sciex, Washington D.C, WA), using selected reaction monitoring (SRM). For sample injection and cleanup, we used an Agilent HPLC system (Agilent Technologies, Santa Clara, CA) equipped with an autosampler (1200 High performance), a column oven set at 45°C (1200 G1216B), an automated high pressure flow switching valve, a 1200 Binary Pump SL and a Shimadzu10-AVp Isocratic Pump (Shimadzu, Tokyo, Japan) on two columns: Kinetex C18 column (100×4.6mm; particle diameter of 2.6 µm (Phenomenex, Torrance, CA) and Kinetex C18 column (100×2.1mm; particle diameter 2.6 µm (Phenomenex, Torrance, CA). The mobile phase consisted of 5mM ammonium acetate pH 5.5 (A) and acetonitrile (≥99.9% HPLC grade, Sigma-Aldrich) (B). Both solutions were filtered through a 0.22 µm PVDF membrane (Millipore, Bedford, MA). The separation condition was as follows: from 0 to 6 min, 10% acetonitrile and 150 µl/min; from 6 to 10 min, 10–90% acetonitrile and 150–300 µL/min; from 10 to 15 min, 90% acetonitrile and 300 µL/min; from 15 to 20 min, 90–10% acetonitrile and 300–150 µL/min allowing the first column to re-equilibrate until 30 min. A high-pressure flow switching valve composed of 2-positions and 6-ports was inserted between the two columns. The eluent from the first column was discarded by the valve up to the 3rd min of the run while keeping the second column supplied with a solution of water:acetonitrile (9:1, v/v) at a constant flow rate of 100 $\mu L \cdot min^{-1}$ (Shimadzu 10-AVp Isocratic Pump). After 3 min of the run, the valve switched position and the eluent of the first column entered the second column. Then, the samples were injected into the mass spectrometer. The valve returned to the starting position after 14 min of the run and both columns were rebalanced.

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

500°C, and the pressure of nitrogen in the collision cell was adjusted to high. The signal to noise ratio (S/N) of ≥7 was used as the quantification criteria. S/N transitions for β-alanine, carnosine and CAR*d4* were monitored using a dwell time of 150 ms.

β-alanine was not derivatized prior to analysis and the SRM transitions monitored were m/z 90→72 (quantification transition), m/z 90→45 and m/z 90→30 (confirmation transition). The quantification transition m/z 90→72 is unique to β-alanine and can be used to differentiate the α- from the β-isomer (38). SRM transitions monitored for carnosine were m/z 227→110 (quantification transition) and m/z 227→210 (confirmation transition). SRM transitions monitored for CAR*d4* were m/z 231→110 (quantification transition) and m/z 231→214 (confirmation transition). Representative chromatograms of plasma, urine and muscle samples are displayed in figure 2.

Paraesthesia evaluation scale

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

Dietary β-alanine intake assessment

Participants completed food diaries 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. (31).

Experiment 2: β-alanine ingestion to reach sub-saturating concentrations

Participants

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

Experimental design

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

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

Statistical Analysis

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

Results

Experiment 1: Effect of insulin on β-alanine transport at saturating concentrations

Plasma insulin

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

Plasma β-alanine

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

Muscle β-alanine

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

Muscle carnosine

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

Urinary β-alanine

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

Paraesthesia

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

Experiment 2: Effect of insulin on β-alanine transport at sub-saturating concentrations

Plasma insulin

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

Plasma β-alanine

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

Muscle β-alanine

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

Discussion

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

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

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

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

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

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

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

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

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

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

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

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

484 occur even after 24 weeks of supplementation (40). This is also in line with pharmacokinectic studies that
485 showed elevated whole-body retention rates (~97%) when typical doses of β-alanine were orally ingested,
486 along with only minimal losses in urine (27), and with the low rate of β-alanine-to-carnosine conversion in
487 the skeletal muscle (typically <6% of the total β-alanine ingested) (9, 40). This temporal mismatch would
488 result in free β-alanine accumulation in muscle, which would fit with the findings of Blancquaert et al. (9)
489 who showed that the excess of β-alanine becomes available to transaminases that degrade β-alanine in the
490 muscle and liver, ultimately directing β-alanine towards oxidation (9). If the activity of carnosine synthase
491 was the rate-limiting factor for carnosine synthesis when β-alanine is largely available in the skeletal muscle,
492 it would be questionable whether increasing β-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 β-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 $\mu mol \cdot l^{-1}$
498 (18) is far in excess of the muscle β-alanine concentrations (~5-8 $\mu mol \cdot l^{-1}$) attained following β-alanine
499 administration in our study. This means that much larger β-alanine concentrations would be required to
500 significantly elevate the activity of carnosine synthase, which somewhat contradicts the idea that further
501 increased β-alanine availability is not limiting for carnosine synthesis when β-alanine is already elevated. As
502 such, we cannot rule out the possibility that other strategies to increase β-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 β-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 β-alanine
509 transport into skeletal muscle, neither does it increase whole-body β-alanine retention. The lack of effect
510 was demonstrated under both physiological and supraphysiological β-alanine concentrations, suggesting that

511 insulin has no physiologically relevant effect on any of the β-alanine transporters. From an applied
512 perspective, our findings suggest that β-alanine ingestion does not need to be accompanied by a high-
513 carbohydrate meal; therefore, β-alanine supplementation strategies that manipulate insulin concentrations
514 seem not to be required.
515
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682 **Figure 1.** Experimental design and representation of the main trials in both experiments
683 where supraphysiological β-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 β-alanine (Experiment 2, right panel). HI=high insulin.
686
687 **Figure 2.** Representative chromatograms displaying monitored transitions of β-alanine,
688 carnosine*d4*, 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 β-alanine concentrations during the 180 min β-alanine
696 infusion period in both high (HI) and low insulin (LI) conditions (left chart), and area
697 under the β-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 β-alanine and carnosine in muscle extracts obtained
700 before and after β-alanine infusion in both HI and LI conditions. **Panel D**: Total β-
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.

706

707

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

712

713 **Figure 6. Panel A:** Plasma β-alanine concentrations during the 120 min after β-alanine
714 ingestion in both high (HI) and low insulin (LI) conditions (left chart), and area under
715 the β-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:** β-alanine concentrations
717 measured in muscle extracts obtained before and after β-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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735 **Disclosures:** All authors declare no competing interests in this study.

736

737 **Acknowledgements:** We thank Rafael P. da Silva and José Carlos de Lima Júnior for
738 their help with data collection.

739

740 **Grants:** This study was supported by: L.S.G: Coordenação de Aperfeiçoamento de
741 Pessoal de Nível Superior (CAPES); G.G.A: São Paulo Research Foundation - FAPESP
742 (#2014/11948-8); B.G: São Paulo Research Foundation - FAPESP (#2013/14746-4 and
743 #2017/13552-2); M.H.G.M: CEPID-Redoxoma-FAPESP (2013/07937-8) NAP-
744 Redoxoma (PRPUSP: Proc.2011.1.9352.1.8) and CNPq (301404/2016-0). This study
745 was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível
746 Superior - Brasil (CAPES- Finance Code 001).

747

748 **Author contributions:** L.S.Gonçalves, G.G.Artioli and B.Gualano designed reseacrh;
749 L.S.Gonçalves, C.Kratz, V.H.Carvalho, L.P.Sales, K.Nemezio, I. Longobardi,
750 L.A.Riani, M.M.O.Lima, T.Saito, A.L.Fernandes, J.Rodrigues performed research;
751 G.G.Artioli, B.Gualano, M.M.O.Lima, B.Geloneze and M.H.G.Medeiros contributed
752 new reagents or analytic tools.

753 L.S.Gonçalves, G.G.Artioli, B.Gualano, L. Santos, R.James, C. Sale, B.Geloneze and
754 M.H.G.Medeiros analyzed data.
755 L.S.Gonçalves, G.G.Artioli, B.Gualano, C. Sale and M.H.G.Medeiros wrote the paper.
756
757
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Fig.1

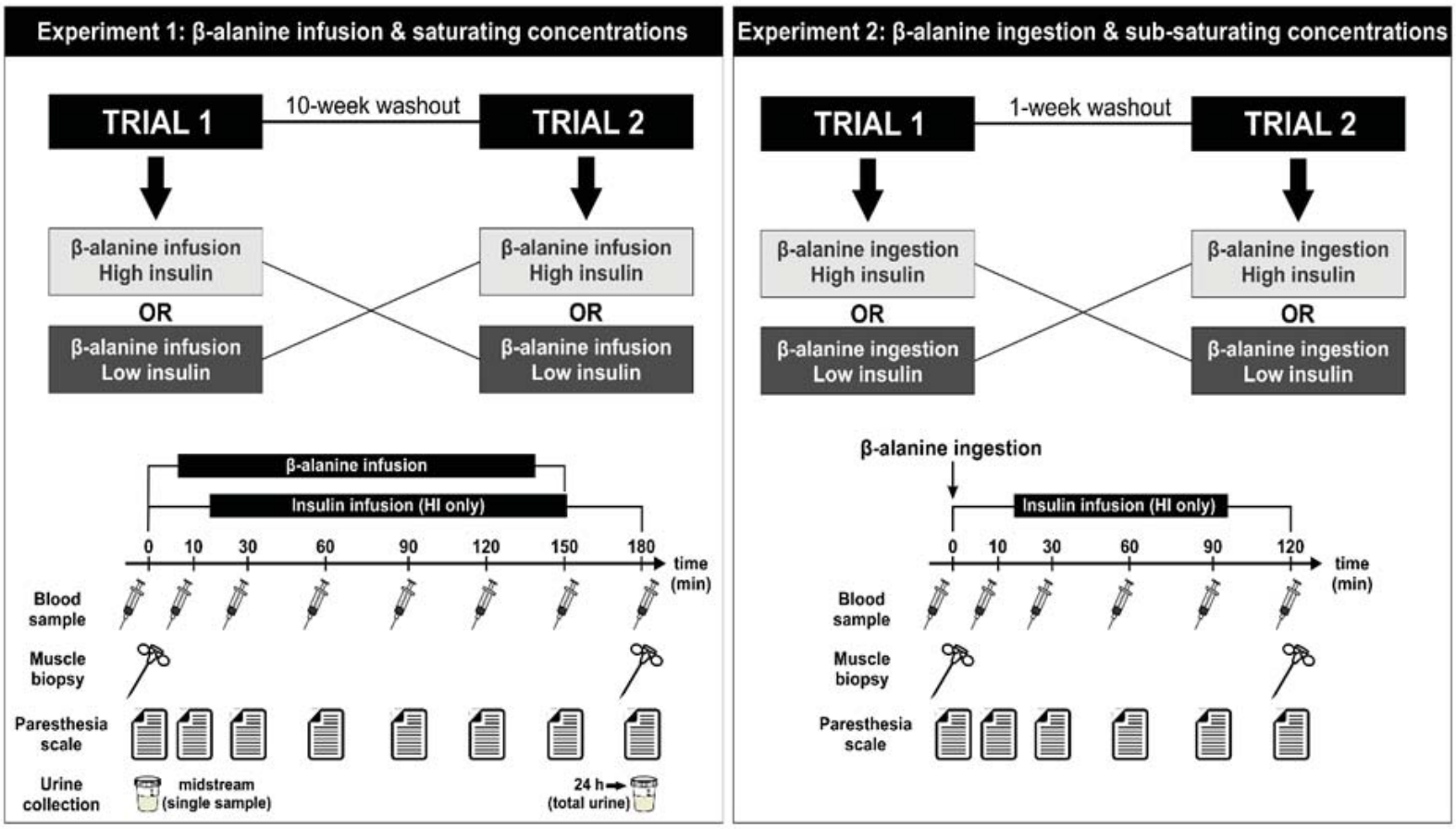
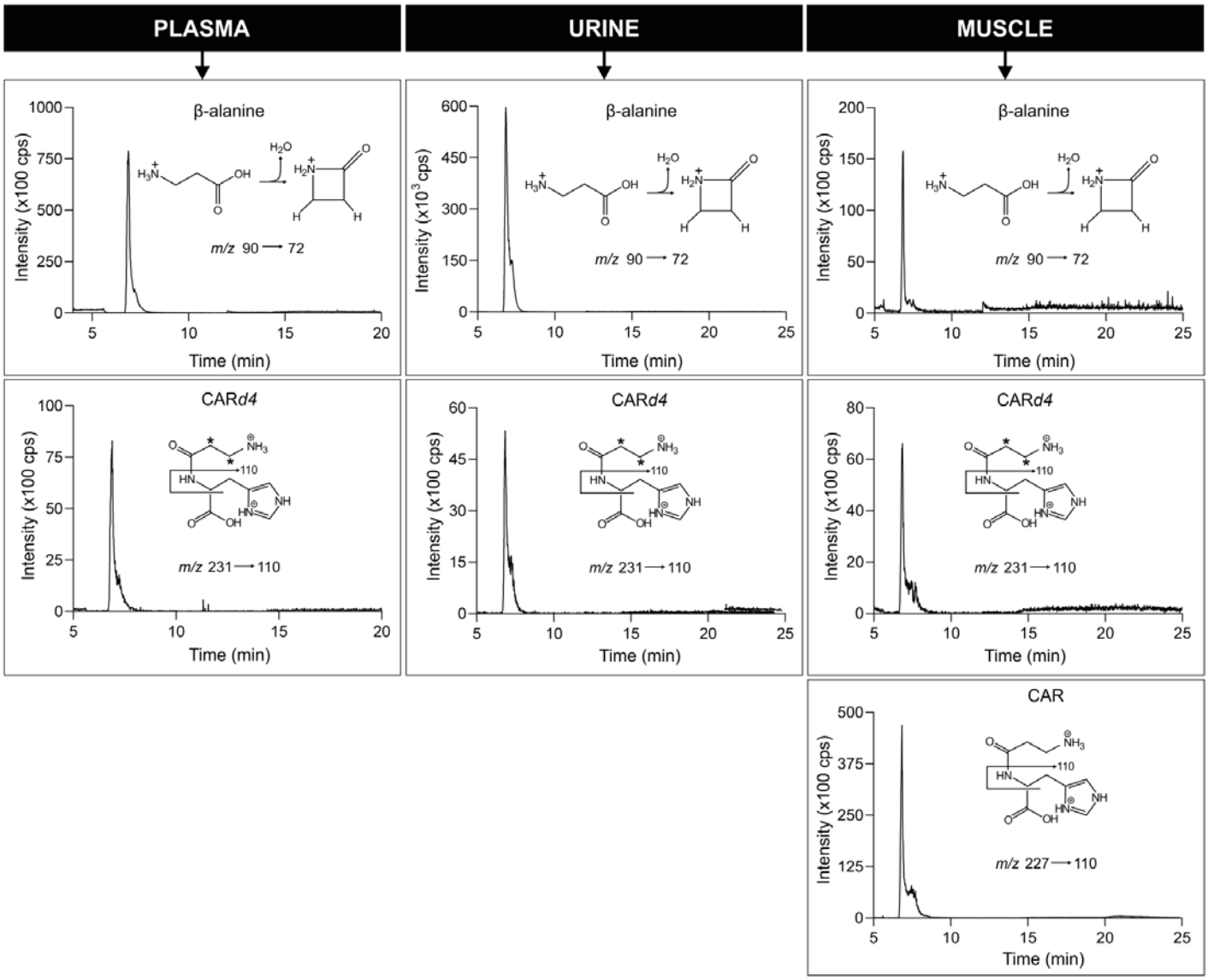


Fig.2



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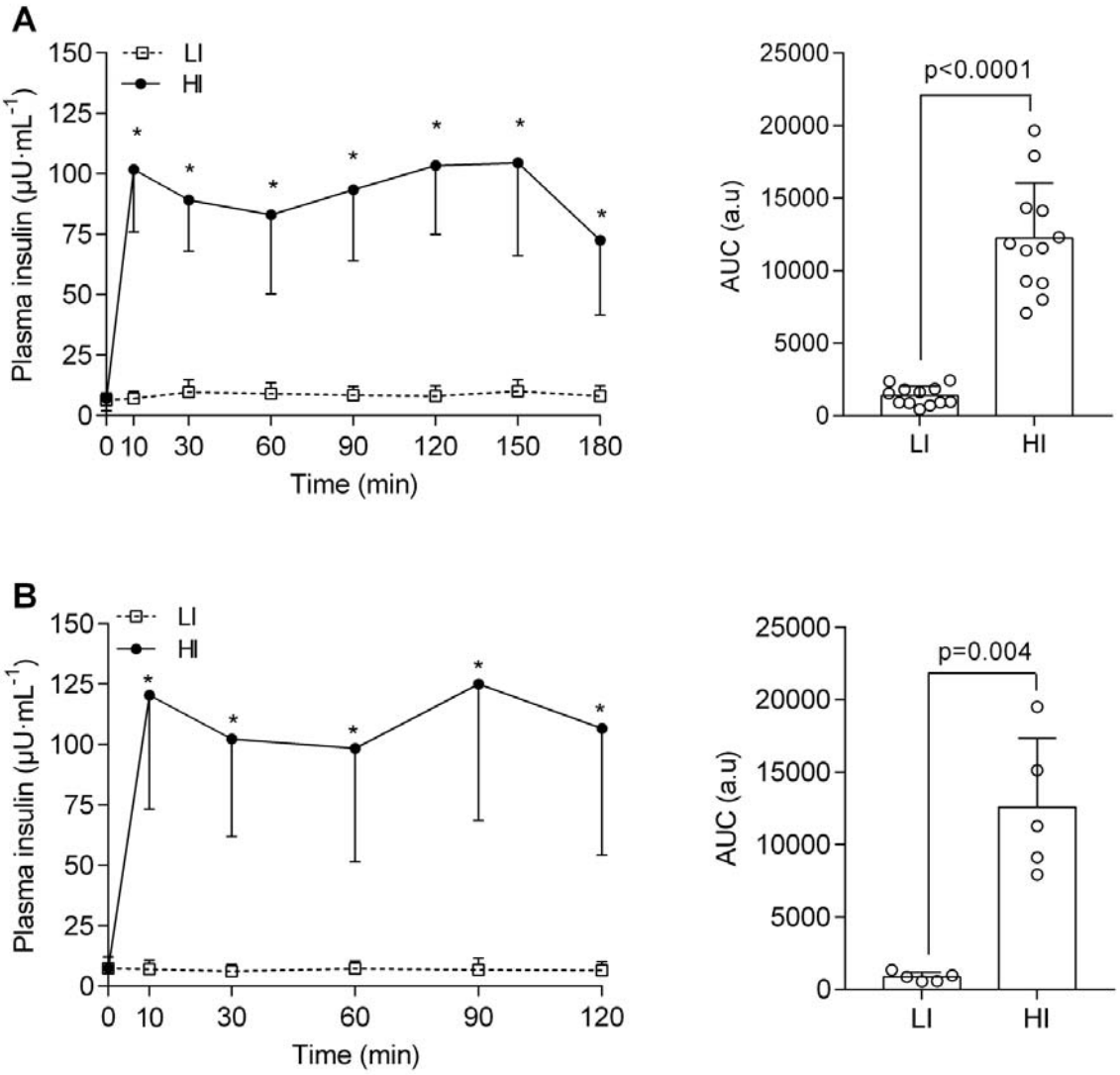

Fig.4

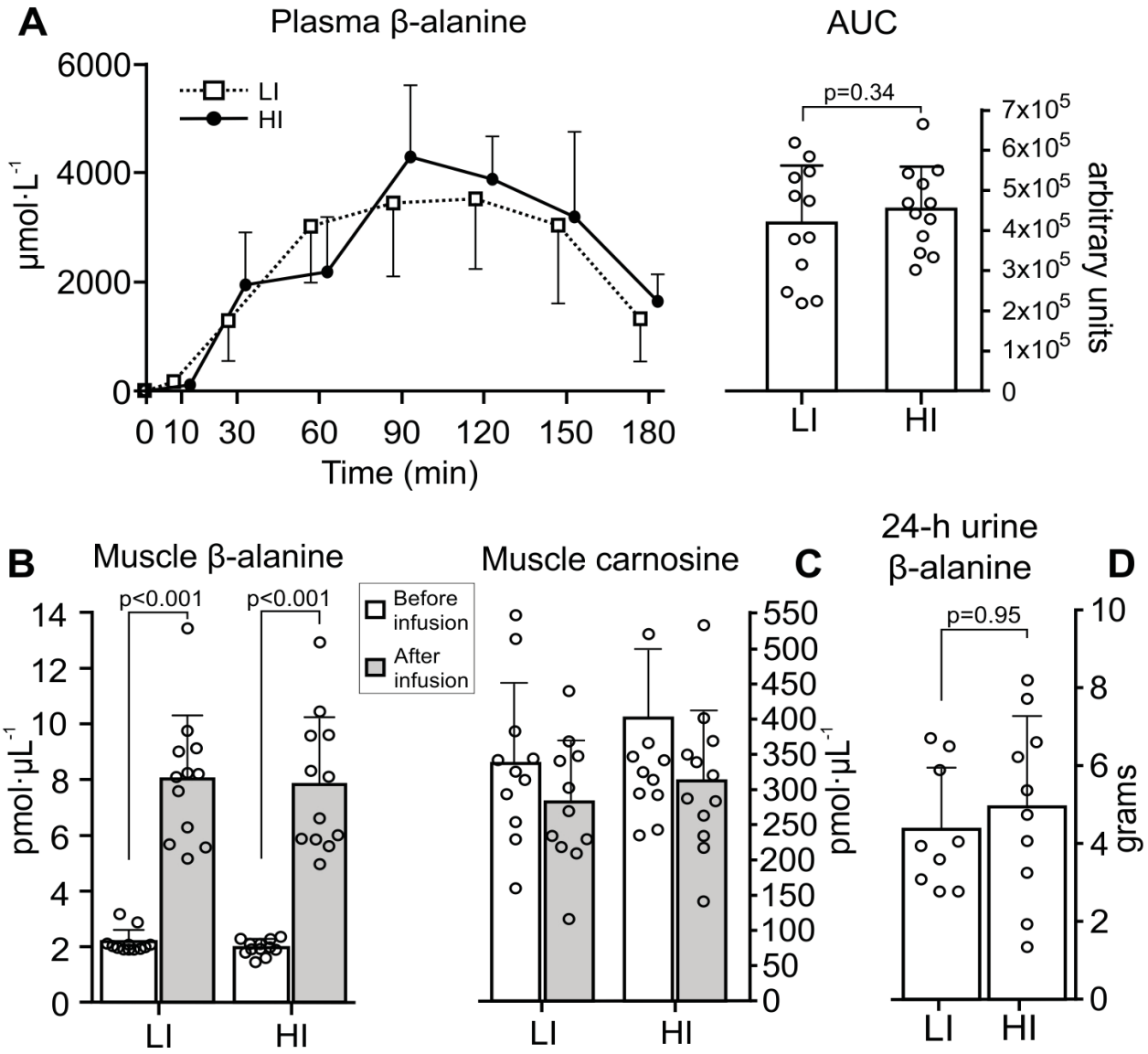

Fig.5

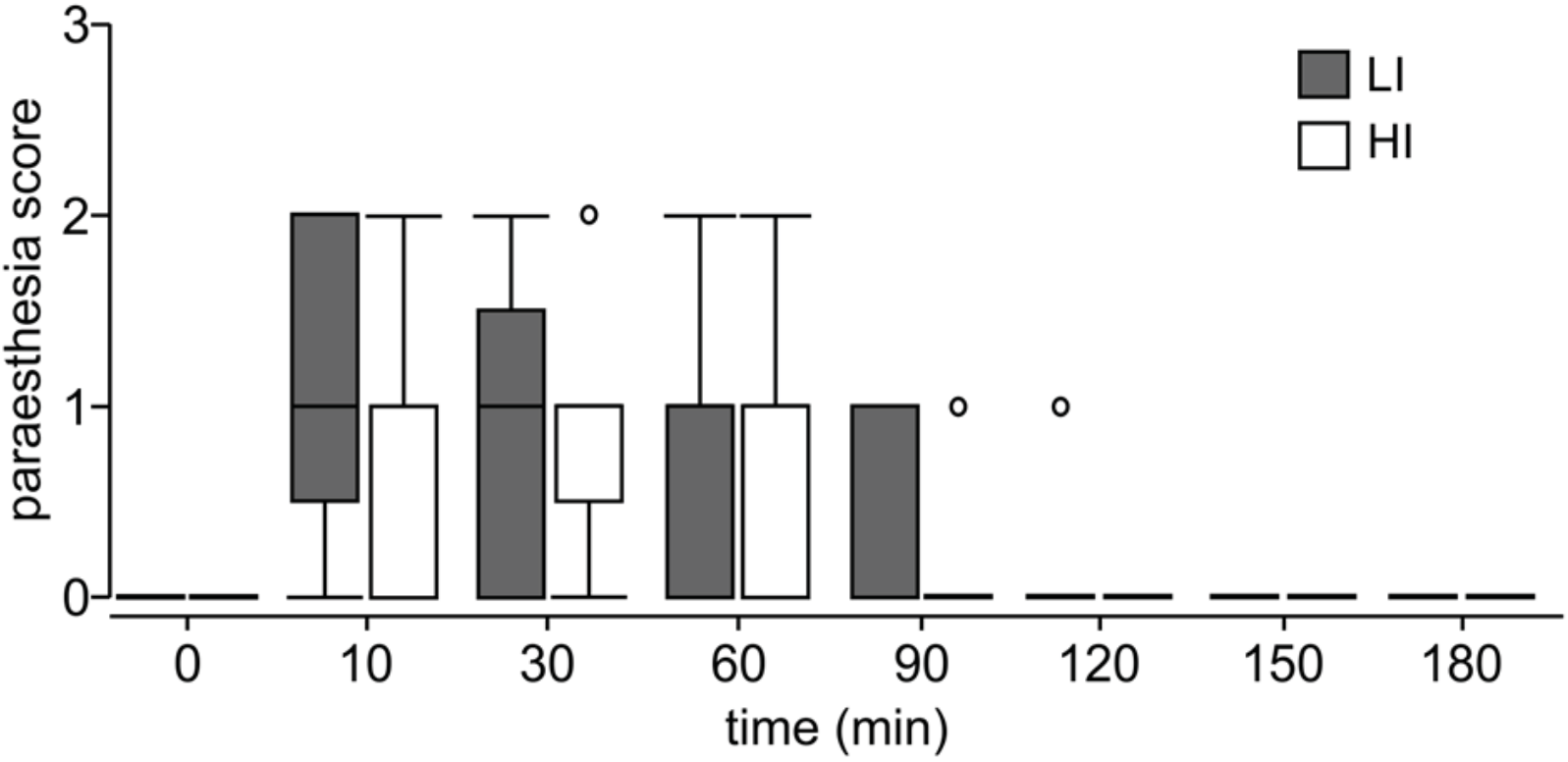

Fig.6