

## Gene expression of carnosine-related enzymes and transporters in skeletal muscle

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**Abstract** Chronic oral beta-alanine supplementation can elevate muscle carnosine (beta-alanyl-L-histidine) content and improve high-intensity exercise performance. However, the regulation of muscle carnosine levels is poorly understood. The uptake of the rate-limiting precursor beta-alanine and the enzyme catalyzing the dipeptide synthesis are thought to be key steps. The aims of this study were to investigate the expression of possible carnosine-related enzymes and transporters in both human and mouse skeletal muscle in response to carnosine-altering stimuli. Human gastrocnemius lateralis and mouse tibialis anterior muscle samples were subjected to HPLC and qPCR analysis. Mice were subjected to chronic oral supplementation of beta-alanine and carnosine or to orchidectomy (7 and 30 days, with or without testosterone replacement), stimuli known to, respectively, increase and decrease muscle carnosine and anserine. The following carnosine-related enzymes and transporters were expressed in human and/or mouse muscles: carnosine synthase (CARNS), carnosinase-2 (CNDP2), the carnosine/histidine transporters PHT1 and PHT2, the beta-alanine transporters TauT and PAT1, beta-alanine transaminase (ABAT) and histidine decarboxylase (HDC). Six of these genes showed altered expression in the investigated interventions. Orchidectomy led to decreased muscle carnosine content, which was paralleled with

decreased TauT expression, whereas CARNS expression was surprisingly increased. Beta-alanine supplementation increased both muscle carnosine content and TauT, CARNS and ABAT expression, suggesting that muscles increase beta-alanine utilization through both dipeptide synthesis (CARNS) and deamination (ABAT) and further oxidation, in conditions of excess availability. Collectively, these data show that muscle carnosine homeostasis is regulated by nutritional and hormonal stimuli in a complex interplay between related transporters and enzymes.

**Keywords** Orchidectomy · Beta-alanine supplementation · Beta-alanine aminotransferase · Testosterone

### Introduction

Although carnosine (beta-alanyl-L-histidine) has been discovered as early as in 1900 by Vladimir Gulevitch, the biological role and metabolism of this dipeptide and its methylated analog anserine is still not fully understood (Derave et al. 2010). Carnosine is abundant in skeletal muscle (20 mmol/kg dw, Mannion et al. 1992) and gender (Mannion et al. 1992; Everaert et al. 2011), age (Baguet et al. 2012), muscle fiber type composition (Hill et al. 2007) and diet (Harris et al. 2006; Everaert et al. 2011) have been identified as determinants of muscle carnosine content. Furthermore, muscle carnosine levels have been shown to be positively modified by long-term beta-alanine and carnosine supplementation (Harris et al. 2006) and negatively by orchidectomy in male mice (Penafiel et al. 2004).

Enhanced muscle carnosine levels contribute to better high-intensity exercise performance (Hobson et al. 2012),

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possibly due to the potential of carnosine to act as an intracellular pH buffer (Baguet et al. 2010; Parkhouse et al. 1985) and calcium regulator (Dutka and Lamb 2004; Dutka et al. 2011; Batrukova and Rubtsov 1997). However, the regulation of muscle carnosine levels, e.g. transcription, translation and kinetics of enzymes and transporters, is poorly understood. This is mainly due to the fact that several of the enzymes and transporters which regulate its homeostasis have only very recently been molecularly identified, such as carnosine synthase (Drozak et al. 2010), or even still await to be discovered, such as carnosine methyltransferase.

Harris et al. (2006) were the first to show that the synthesis of carnosine is limited by the availability of beta-alanine in skeletal muscle. Therefore, an important point of regulation is the transsarcolemmal beta-alanine transport from blood into the muscle. Bakardjiev and Bauer (1994) showed that this active uptake is sodium and chloride dependent and has a  $K_m$  value of 40  $\mu\text{M}$ . Several transporters are able to transfer beta-alanine (TauT, PAT1, ATB<sup>0,+</sup>) of which only ATB<sup>0,+</sup> expression has not been investigated in skeletal muscle (Pierno et al. 2012; Drummond et al. 2010). Following uptake in skeletal muscle cells, beta-alanine will be combined with histidine by the carnosine synthase enzyme (CARNS; Drozak et al. 2010) to form carnosine. The availability of the precursors beta-alanine and histidine in skeletal muscle cells for carnosine synthesis also depends on the presence and/or activity of the alternative metabolic pathways in which these amino acids take part. Therefore, we also measure mRNA content of beta-alanine transaminase (ABAT), the enzyme which catalyzes the transamination of beta-alanine into malonate semi-aldehyde and further metabolism into the citric acid cycle, and histidine decarboxylase (HDC), which catalyzes the formation of histamine from histidine.

As muscle carnosine levels are quite stable (Baguet et al. 2009) and as pH in the myocytes is not optimal for degradation (Teufel et al. 2003), it is believed, however never established, that no carnosine-degrading enzyme is present in mammalian skeletal muscles. Another metabolic pathway likely involved in the homeostasis of carnosine is its transport across the muscle membrane. The four members of the proton-coupled oligopeptide transporters family (POT: PEPT1, PEPT2, PHT1 and PHT2) are able to transfer di- and tripeptides across biological membranes, including carnosine and anserine. Using a mouse knock-out model of PEPT2, Kamal et al. (2009) showed that endogenous muscle carnosine levels were increased 50 % in the absence of PEPT2. As it has been shown that PEPT2 is expressed, although weakly, in skeletal muscle of rodents (in contrast to PEPT1; Lu and Klaassen 2006), this transporter may be involved in muscle carnosine transport. However, it remains to be elucidated which of the above-mentioned

transporters are expressed in mammalian skeletal muscle in general, and human skeletal muscle in particular.

The first aim of this study was to elucidate which of the suggested carnosine-related transporters and enzymes are actually expressed in skeletal muscle of adult mice and humans. Investigated genes are related to following possible metabolic pathways of carnosine and constituent amino acids: (1) beta-alanine transport (TauT, PAT1, ATB<sup>0,+</sup>), (2) carnosine synthesis (CARNS), (3) other metabolic pathways of the precursors of carnosine [beta-alanine transaminase (ABAT); histidine decarboxylation (HDC)], (4) carnosine hydrolysis (CNDP1, CNDP2), (5) carnosine and/or histidine transport (PEPT1, PEPT2, PHT1, PHT2). The second aim was to explore the transcriptional events in mouse skeletal muscles in response to stimuli known to modulate the muscle histidine-containing dipeptide (HCD, i.e. carnosine and anserine) levels. These conditions include: (a) enhanced HCD content, evoked by long-term beta-alanine and carnosine supplementation and (b) decreased HCD levels, evoked by orchidectomy (Penafiel et al. 2004) and its reversal by testosterone replacement.

## Methods

### Humans

Muscle biopsies were taken from 22 healthy non-vegetarian subjects (12 males and 10 females,  $21.2 \pm 1.5$  years). All subjects were physically active (weekly 2–3 h of sport), but were not involved in sport competition or organized training. Subjects gave their informed consent and the study was approved by the local Ethical Committee (Ghent University Hospital, Belgium).

The muscle biopsies were taken from the right gastrocnemius lateralis at rest, with a 14 Gauge true-cut biopsy needle (Bard Magnum Biopsy gun; Bard, Inc., NJ, USA). Under ultrasonographic guidance (Ultrasonography Pro Sound SSD-5000, ALOKA Co., Ltd., Tokyo, Japan with probe UST-5545, frequency 5–13 MHz), two muscle samples ( $\sim 15$  mg each; one for HPLC analysis and one for qPCR) were taken under local anesthesia (lidocaine 1 %, Linisol<sup>®</sup>). The samples were immediately frozen in liquid nitrogen and stored at  $-80$  °C until subsequent analysis.

### Animals

In order to reduce the number of animals, muscle samples of earlier performed experiments were used to investigate the effect of supplementation (Everaert et al. 2012) and

orchidectomy (De Naeyer et al., unpublished data) on gene expression of carnosine-related enzymes and transporters.

#### Nutritional supplementation study

A total of 32 male NMRI mice (mean  $\pm$  SD BW at the end of supplementation  $44.6 \pm 6.4$  g) were used to study the effect of supplementation on muscle HCD and gene expression of carnosine-related enzymes and transporters. Two times nine mice received either 1.8 % beta-alanine (Sigma) or 1.2 % carnosine (Flamma) during 8 weeks in their drinking water, while 14 control mice received only water.

#### Orchidectomy study

To study the effect of orchidectomy, a total of 60 male C57BL/6 mice were operated under isoflurane inhalation: (a) sham-operation (sham), (b) orchidectomized (ORX\_V) or (c) orchidectomized and subsequent treatment with testosterone (ORX\_T). Testosterone was administered using 0.5 cm subcutaneous silastic implants (Silclear tubing  $1.57 \times 2.41$  mm, Degania, Israel) in the cervical region. A physiological rate of hormone release was obtained by diluting testosterone with cholesterol (1/2) as calculated by Vanderschueren et al. (2000), releasing 11.5  $\mu$ g of testosterone daily. Empty silastic tubes were implanted in the ORX\_V group. The efficacy of orchidectomy and androgen treatment was verified by measurement of the seminal vesicles mass. Animals were killed at 1 week (9 mice/group) or 4 weeks (sham  $n = 10$ , ORX\_V  $n = 12$ , ORX\_T  $n = 11$ ) after surgery.

All animals were allowed free access to food (standard chow) and water at room temperature and were exposed to a light cycle of 12 h per day. At the end of each intervention period, mice were anaesthetized by an intraperitoneal infusion of 80 % Rompun–20 % Ketalar. After careful dissection of the tibialis anterior muscle, mice were killed by cervical dislocation. Any visible connective or fat tissue was removed from tibialis anterior and the muscle was quickly frozen in liquid nitrogen and stored at  $-80$  °C. The experimental protocol was approved by the Ethics Committee for Animal Research at University Hospital Ghent and followed the “Principles of laboratory animal care”.

#### mRNA isolation, cDNA synthesis, qPCR

Total RNA from both human and mouse muscles was isolated using the TriPure Isolation Reagent (Roche) followed by a purification with the RNeasy Mini Kit (Qiagen). An on-column DNase treatment was performed using the RNase-Free DNase Set (Qiagen). RNA was quantified

using a Nanodrop 2000C spectrophotometer (Thermo Scientific) and RNA purity was assessed using the A260/A280 ratio. Using a blend of oligo(dT) and random primers, 500 ng RNA was reversed transcribed with the iScript cDNA Synthesis kit (Bio-Rad), according to the manufacturer’s instructions. qPCR was carried out on a Lightcycler 480 system (Roche) using an 8  $\mu$ L reaction mix containing 3  $\mu$ L template cDNA (1/10 dilution), 300 nM forward and reverse primers and 4  $\mu$ L SYBR Green PCR Master Mix (Applied Biosystems, Belgium). The cycling conditions comprised a polymerase activation at 95 °C for 10 min, followed by 45 cycles at 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s. Primer sequences of the genes of interest (Table 1) were mainly newly designed using Primer Express software 3.0 (Applied Biosystems), except when primer sequences were already available in the literature. Sequence specificity was confirmed using NCBI Blast analysis (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). To control the specificity of amplification, data melting curves were inspected and PCR efficiency was calculated for expressed genes (between 90 and 110 for all genes, except for mouse CNBP2: 88 and mouse CARNS: 85). Normalized gene expression values were calculated by dividing the raw gene expression values for each sample by the expression values of the (geometric mean of the) most stable reference gene(s) (mouse: Ppia, Rplp0 and B2M; human: EEF1A1). Relative gene expression levels were calculated via the  $\Delta\Delta C_t$  method (Derveaux et al. 2010).

#### Quantification of muscle histidine-containing dipeptides by means of HPLC

Human and NMRI mice skeletal muscles were freeze-dried (dw), while wet weight (ww) muscles of C57BL/6 mice were used. Muscles were subsequently dissolved in phosphate buffer (PBS, 100  $\mu$ L/1 mg dw muscle for NMRI mice and human, 10  $\mu$ L/1 mg ww muscle for C57BL/6 mice) for homogenization. Muscle homogenates were deproteinized using 35 % sulfosalicylic acid (SSA) and centrifuged (5 min, 14,000g). 100  $\mu$ L of deproteinized supernatant was dried under vacuum (40 °C). Dried residues were resolved with 40  $\mu$ L of coupling reagent: methanol/triethylamine/H<sub>2</sub>O/phenylisothiocyanate (PITC) (7/1/1/1) and allowed to react for 20 min at room temperature. The samples were dried again and resolved in 100  $\mu$ L of sodium acetate buffer (10 mM, pH 6.4). The same method was applied to the combined standard solutions of carnosine (Flamma), anserine (Sigma) and taurine (Sigma). The derivatized samples (20  $\mu$ L) were applied to a Waters HPLC system with following parameters: hypersilica column ( $4.6 \times 150$  mm, 5  $\mu$ m), UV detector (wavelength 210 nm), two buffers [buffer A (10 mM sodium acetate adjusted to pH 6.4 with 6 % acetic acid)

**Table 1** Primers used in qPCR analysis

Function	Official name	Trivial name	Species	Forward primer (5'–3')	Reverse primer (5'–3')	Gene ID	Source
Carnosine synthesis	CARNS1	CARNS	Mouse	TGA-TAG-GCC-CCT-ACT-GAG-TAA-GGT	TCA-GTG-TCC-TTG-TGC-GCA-GGG-TAT	107239	Primer express
			Human	GGC-GTC-AGC-AAG-AAG-TTC-GT	CCG-GTG-CTC-TGT-CAT-GTC-AA	57571	Primer express
Carnosine hydrolysis	CNDP1	CNDP1	Mouse	AGG-AGT-GGG-TGG-CCA-TTGA	CAA-GGC-CAT-CAT-CTG-GAA-GAG	338403	Primer express
	CNDP2	CNDP2	Human	CGG-AGG-TAG-ACG-GGA-AAC-TTT	CGC-TCA-CAG-CAT-TGA-TCC-AA	84735	Primer express
Carnosine transport			Mouse	GGA-GAT-ACC-ACT-TCC-TCC-CAT-TC	CGT-CCA-GGT-GCC-CGT-AAA-T	66054	Primer express
			Human	TTG-CTG-ATG-GGC-TCT-TTG-GT	TCG-ATG-TCG-TCG-TAC-AGC-TTG-T	55748	Primer express
	SLC15A1	PEPT1	Mouse	CTT-GGA-GCC-ACC-ACA-ATG-G	ACA-GAA-TTC-ATT-GAC-CAC-GAT-GA	56643	Ma et al. (2011)
	SLC15A2	PEPT2	Human	CCA-ACG-CCA-CAC-GCT-TCT-A	CCC-TTT-TTC-TGG-CTT-CTG-GTT	6564	Primer express
			Mouse	TGG-CTG-GGA-AAA-TTC-AAG-ACA	ATG-GCA-CCC-AAA-GAC-TTG-AAAT-AC	57738	Primer express
Carnosine/histidine transport			Human	GGT-ACA-GTG-GGC-AGA-ATT-CA	CCC-CGC-ATA-TCC-TCT-GTC-TTT	6565	Primer express
			Human	TCA-CTT-CCA-CCT-GAA-ATA-TCA-C	CTG-TAT-CCT-TTA-CCA-TCA-TGC-T		Agu et al. (2011)
	SLC15A4	PHT1	Mouse	CAT-GTG-TCC-GTG-ATT-GAG	GCG-TGG-TCT-AAC-TGC-CAA-TCT	100561	Primer express
			Human	GGT-TAT-GCG-ATC-CCC-ACT-GT	ATC-AGG-AGG-CIT-GGT-GAT-GAA	121260	Primer express
	SLC15A3	PHT2	Mouse	GCT-GAC-CAG-GTG-ATG-GAT-CTC	AAT-ATG-GCA-CCC-AGG-TTG-ATG	65221	Primer express
Beta-alanine transport			Human	TTT-GCC-ACC-CCC-GTC-TT	CAG-TTT-TGG-AGA-GCG-AGC-TTA-AG	51296	Primer express
			Human	GAT-CAG-TGA-GAT-CIT-TGC-CA	ATT-GTT-GAT-GTT-CCC-AAA-GTC-C		Agu et al. (2011)
	SLC6A6	TauT	Mouse	TGG-CCG-ACA-GCA-TTC-CA	GCC-TTC-TCT-AAG-GTG-CCF-TCC-T	21366	Primer express
			Human	CGT-ACC-CCT-GAC-CTA-CAA-CAA-A	CAG-AGG-CGG-ATG-ACG-ATG-AC	6533	Iruloh et al. (2007)
	SLC36A1	PAT1	Mouse	TCT-GCT-GTG-TCT-ACT-TCG-TGT-TTC-T	GGA-TCAC-CGGT-CAC-ATT-GTT-GTT	215335	Primer express
	SLC6A14	ATB <sup>0,+</sup>	Human	CAT-AAC-CCT-CAA-CCT-GCC-CAA-C	GGG-ACG-TAG-AAC-TGG-AGT-GC	206358	Anderson et al. (2009)
Beta-alanine transaminase	Abat	ABAT	Mouse	TCA-ATT-CTC-AGC-ACC-AAG-GTT-ACT	TGT-GTA-TGT-GCA-CCA-TGA-GCA-T	56774	Primer express
			Human	CTT-CTG-GCT-TGG-CTC-ATA-G	TGA-AGC-ACC-CTC-CAG-AGT-TGC	11254	Anderson et al. (2009)
			Mouse	CCT-TCA-TGG-GTG-CTT-TCC-A	CAA-AGG-AAG-GGA-TGT-CAA-TCT-TG	268860	Primer express
			Mouse	CTG-GAT-TCT-GGG-TCA-AGG-ACA-A	AGA-GTT-GGC-ATG-TCG-GAG-GTA	15186	Primer express
Histamine synthesis	HDC	HDC	Mouse	CTG-GAT-TCT-GGG-TCA-AGG-ACA-A	AGA-GTT-GGC-ATG-TCG-GAG-GTA	15186	Primer express
	Rplp0	Rplp0	Mouse	GGA-CCC-GAG-AAG-ACC-TCC-TT	GCA-CAT-CAC-TCA-GAA-TTT-CAA-TGG	11837	RTprimerDB
	B2m	B2m	Mouse	CAT-GGC-TCG-CTC-GGT-GAC-C	AAT-GTG-AGG-CGG-GTG-GAA-CTG	12010	RTprimerDB
	Ppia	Ppia	Mouse	CAA-ATG-CTG-GAC-CAA-ACA-CAA-ACG	GTT-CAT-GCC-TTC-TTT-CAC-CTT-CCC	268373	RTprimerDB
	EEF1A1	EEF1A1	Human	CTG-GCA-AGG-TCA-CCA-AGT-CT	CCG-TTC-TTC-CAC-CAC-TGA-T	1915	RTprimerDB

(Ma et al. 2011; Agu et al. 2011; Iruloh et al. 2007; Anderson et al. 2009)

and buffer B (60 % acetonitrile–40 % buffer A)], flow rate of 0.8 mL/min, room temperature. Limit of detection and quantification were, respectively, 3 and 10  $\mu\text{M}$ . A slightly modified method was employed in C57BL/6, in order to further improve sensitivity.

## Statistics

Data are presented as mean  $\pm$  SD and significance level was set at  $p \leq 0.05$ . The effects of supplementation and orchidectomy (with or without testosterone administration) on muscle HCD and taurine levels and mRNA expression of carnosine-related enzymes and transporters were evaluated with a one-way analysis of variance (ANOVA) and subsequently post hoc LSD test in case of significant group effect. Correlations between gene expression levels and HCD muscle levels were obtained by means of Pearson correlations.

## Results

### Expression of carnosine-related enzymes and transporters in skeletal muscle

Table 2 summarizes the expression of all investigated carnosine-related enzymes and transporters. As expected,

**Table 2** Overview of mRNA expression of carnosine-related enzymes and transporters in human (gastrocnemius) and mouse (tibialis anterior) skeletal muscle and Pearson correlations between muscle HCD levels and gene expression levels

Gene	Expression		Pearson correlation $r$		
	Human	Mouse	Human	Mouse	
			Carnosine	Carnosine	Anserine
CARNS	++	+	0.07	−0.24	−0.27
CNDP1	0	0(+)	N/A	N/A	N/A
CNDP2	++	+	0.01	−0.35 <sup>S</sup>	−0.22
PEPT1	0	0	N/A	N/A	N/A
PEPT2	0(+)	0(+)	N/A	N/A	N/A
PHT1	++	++	−0.05	−0.10	−0.06
PHT2	0(+)	+	N/A	0.14	0.00
TauT	++	++	0.04	−0.41*	−0.37*
PAT1	++	++	−0.09	−0.12	−0.06
ATB <sup>0,+</sup>	0	0(+)	N/A	N/A	N/A
ABAT		++	N/A	−0.28	−0.25
HDC		++	N/A	−0.10	−0.20

Division for expression is based on efficiency and quality of duplo experiments: ++ very good, + good, 0(+) doubtful, 0 no expression

N/A not applicable

Correlation: \*  $p < 0.05$ , <sup>S</sup> $p > 0.05$  and  $< 0.1$

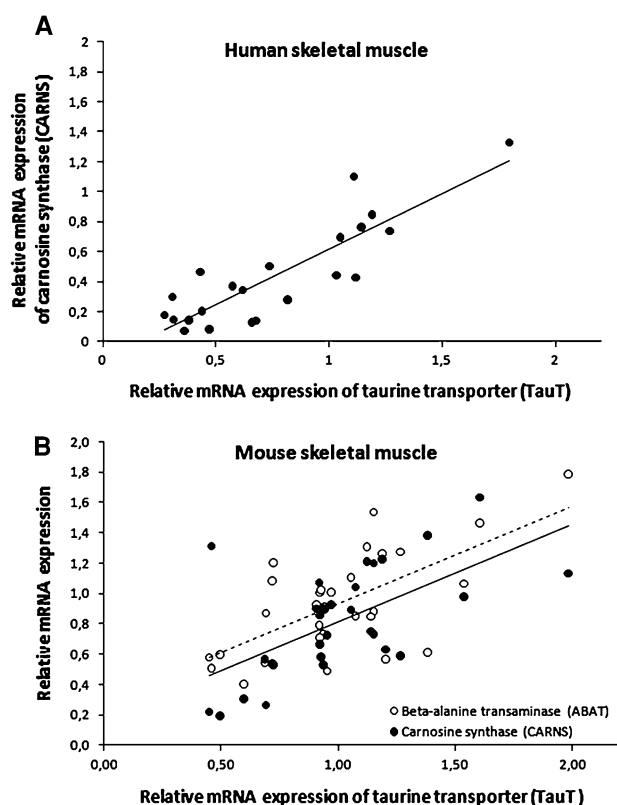
CARNS was expressed both in human and mouse skeletal muscle. CNDP1 is close to absent in skeletal muscle, but the cytosol non-specific dipeptidase (CNDP2) seems to be expressed in skeletal muscle of both species. Concerning carnosine transporters, PEPT1 is absent from skeletal muscle and we were not able to reliably quantify PEPT2 mRNA in skeletal muscles of both species. The carnosine/histidine transporter PHT1 is abundant in both species, but PHT2 is only expressed in mouse skeletal muscle. Two of the three beta-alanine transporters, namely TauT and PAT1, are expressed in skeletal muscle of both species. The presence of two possible alternative metabolic pathways for the precursors of carnosine is confirmed, as both ABAT and HDC are clearly expressed in skeletal muscle.

Baseline mRNA levels of the expressed transporters and enzymes were not different ( $p > 0.1$ ) between men (CARNS:  $0.41 \pm 0.36$ , TauT:  $0.70 \pm 0.46$ , PAT1:  $0.59 \pm 0.34$ ; PHT1:  $0.56 \pm 0.38$ , CNDP2:  $0.48 \pm 0.27$ ) and women (CARNS:  $0.48 \pm 0.33$ , TauT:  $0.83 \pm 0.34$ , PAT1:  $0.74 \pm 0.35$ ; PHT1:  $0.59 \pm 0.19$ , CNDP2:  $0.62 \pm 0.29$ ). Further, we tested for possible correlations between the mRNA expression data of different genes in humans and animals who did not undergo any dietary, surgical or pharmacological intervention (the controls of the dietary study and the sham-operated mice from the androgen study) and thus display a normal muscle HCD content. We observed a positive correlation between TauT and both CARNS and ABAT mRNA expression levels, as displayed in Fig. 1a (human) and b (mouse). CARNS mRNA expression is also positively correlated with CNDP2 (human:  $r = 0.68$ ; mouse:  $r = 0.72$ ;  $p < 0.01$ ), PAT1 (human:  $r = 0.76$ ; mouse:  $r = 0.66$ ;  $p < 0.01$ ) and ABAT (mouse:  $r = 0.57$ ) mRNA expression, but not with muscle HCD levels (Table 2). Furthermore, the gene expression levels of both beta-alanine transporters TauT and PAT1 are positively correlated with each other (human:  $r = 0.66$ ; mouse:  $r = 0.53$ ;  $p < 0.01$ ).

### Beta-alanine and carnosine supplementation

#### HCD content

As displayed in Fig. 2a, muscle carnosine levels of beta-alanine (1.2 %, 8 weeks) and carnosine (1.8 %, 8 weeks) supplemented mice were significantly higher (+168 %,  $p < 0.001$  and +48 %,  $p = 0.05$ , respectively) compared to control mice ( $2.41 \pm 0.96$  mmol/kg dw). Muscle anserine content was only enhanced as a result of beta-alanine supplementation (+42 %,  $p = 0.012$  vs. control:  $8.03 \pm 2.68$  mmol/kg dw) and not after carnosine supplementation. Taurine content was lower in beta-alanine (−18 %,  $p = 0.006$ ) and tended to be lower in carnosine (−11 %,  $p = 0.08$ ) supplemented mice compared to control ( $131.82 \pm 17.36$  mmol/kg dw, data not shown).



**Fig. 1** Correlation between the relative mRNA expression of TauT and CARNS in human muscle (**a**  $r = 0.864$ ,  $p < 0.001$ ) and correlation of TauT with CARNS and ABAT in skeletal muscles of mice who did not undergo any intervention (**b** CARNS:  $r = 0.74$ ,  $p < 0.001$ , ABAT:  $r = 0.55$ ,  $p < 0.001$ )

#### Enzymes and transporters (Fig. 2b)

In beta-alanine supplemented mice, CNBP2 (+27 %,  $p = 0.046$ ), TauT (+28 %,  $p = 0.024$ ), ABAT (+40 %,  $p = 0.035$ ) mRNA levels were higher and CARNS mRNA content tended to be higher (+42 %,  $p = 0.063$ ) compared to control. Carnosine supplementation decreased CNBP2 (−39 %,  $p = 0.01$ ) and PHT2 (−34 %,  $p = 0.005$ ) mRNA levels and tended to increase the expression of TauT (+21 %,  $p = 0.083$ ). PAT1, PHT2 and HDC expression were not affected by carnosine nor beta-alanine supplementation (data not shown).

#### Orchidectomy with or without testosterone replacement

##### HCD content (Fig. 3)

Seven days of orchidectomy did not affect HCD levels (sham\_7d: carnosine  $1.66 \pm 0.24$  mmol/kg ww and anserine  $6.09 \pm 0.42$  mmol/kg ww), although castrated mice with testosterone replacement had significantly higher carnosine levels compared to castrated mice without testosterone (7 days, +18 %,  $p = 0.009$ ). Carnosine, but not

anserine levels, were decreased 30 days after orchidectomy (−47 %,  $p < 0.001$  vs. sham\_30d:  $1.93 \pm 0.65$  mmol/kg ww), being reversed by testosterone administration ( $p = 0.001$  vs. ORX\_V\_30d). Taurine levels were not affected by orchidectomy with or without testosterone replacement (data not shown).

#### Enzymes and transporters (Fig. 3)

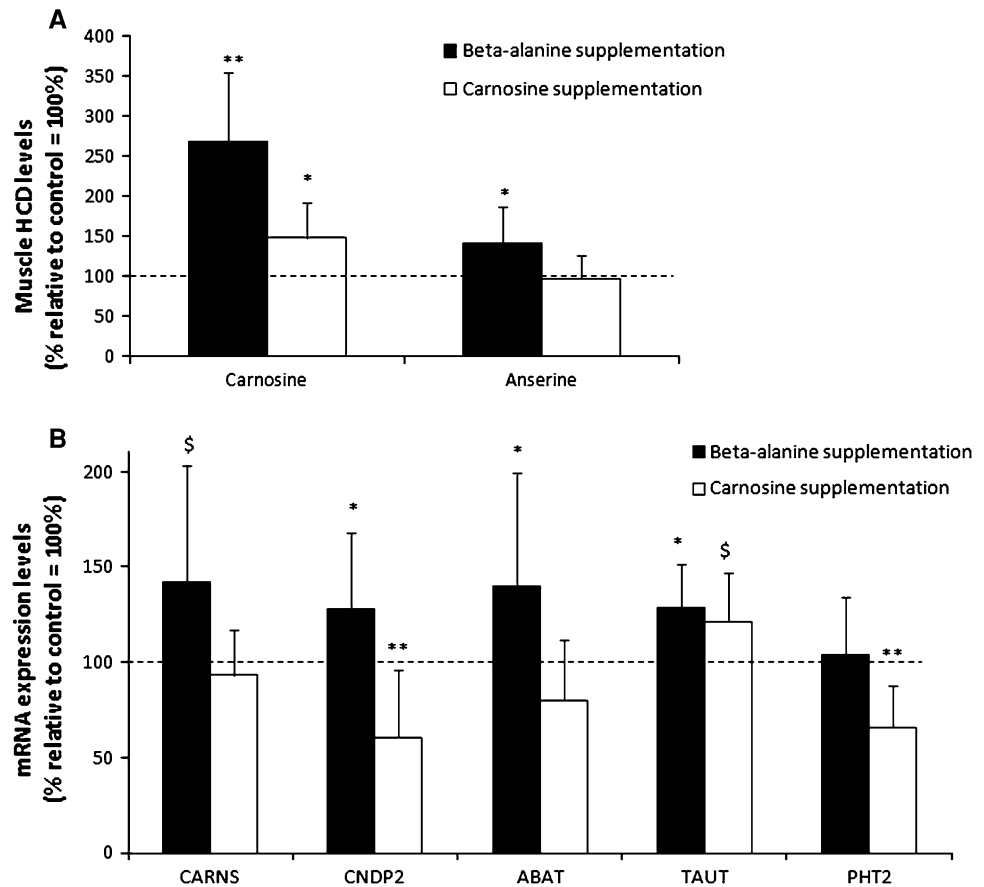
Seven days: CARNS (+43 %,  $p = 0.001$ ) mRNA levels were upregulated, whereas CNBP2 (−18 %,  $p = 0.044$ ) and TauT (−21 %,  $p = 0.014$ ) were downregulated and ABAT (−29 %,  $p = 0.068$ ) mRNA expression tended to decrease after 7 days of orchidectomy. The changes in CNBP2 (−31 %,  $p = 0.001$  vs. sham) and ABAT gene expression (−29 %,  $p = 0.068$  vs. sham) in castrated mice were not reversed by testosterone replacement ( $p > 0.05$ ), while the decrease in TauT expression disappeared by administration of testosterone ( $p > 0.05$  vs. sham). There was a ~3-fold difference in CARNS between castrated mice with and without testosterone replacement, being highest in the latter ( $p < 0.001$ ).

Thirty days: after 30 days of orchidectomy, mRNA expression levels of CARNS (+57 %,  $p = 0.011$ ) were still increased and PHT1 (−30 %,  $p = 0.044$ , data not shown) mRNA content was decreased. Castrated mice with testosterone replacement showed no differences in expression profiles with control mice, whereas the expression levels of CARNS (−74 %,  $p < 0.001$ ) mRNA were lower and TauT (+20 %,  $p = 0.086$ ), PHT1 (+45 %,  $p = 0.001$ , data not shown) and PHT2 (+24 %,  $p = 0.034$ , data not shown) expression levels were higher or tended to be higher compared to castrated mice without testosterone administration.

## Discussion

We aimed to explore the gene expression of the enzymes and transporters related to carnosine in skeletal muscle. From the literature, we defined five possible regulatory steps, involving 12 relevant genes: (1) beta-alanine uptake (TauT, PAT1, ATB<sup>0,+</sup>), (2) carnosine synthesis (CARNS), (3) other metabolic pathways of the precursors of carnosine [beta-alanine transaminase (ABAT); histidine decarboxylation (HDC)], (4) carnosine hydrolysis (CNBP1, CNBP2), (5) carnosine and/or histidine transport (PEPT1, PEPT2, PHT1, PHT2). In this study, we have now identified mRNA expression of seven of them (CARNS, CNBP2, PHT1, TauT, PAT1, ABAT and HDC) by qPCR in both mouse and human muscle biopsies, and one of them in mouse muscle (PHT2). For several of these (CNBP2, PHT1, PHT2, ABAT), our study demonstrates for the first

**Fig. 2** The effects of 8 weeks of beta-alanine (1.2 %) and carnosine (1.8 %) supplementation on mouse muscle **a** carnosine and anserine levels and **b** carnosine-related enzymes and transporter expression (mean  $\pm$  SD, one-way ANOVA: \*\* $p < 0.01$  vs. control, \* $p < 0.05$  vs. control,  $^{\$}p > 0.05$  and  $< 0.1$  vs. control)



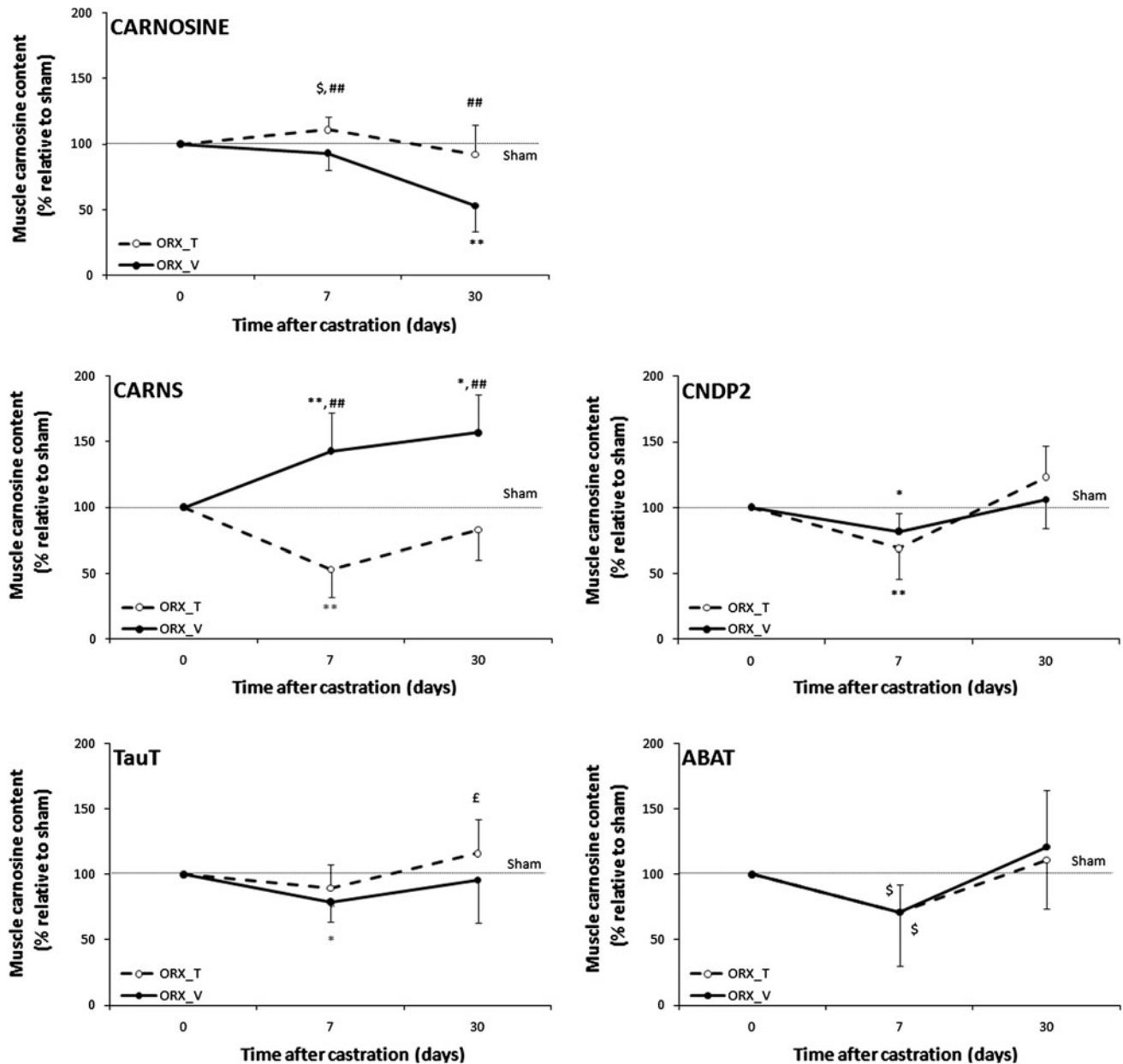
time gene transcripts in mammalian skeletal muscle (qPCR).

The landmark studies of Harris and co-workers in horses (Dunnett and Harris 1999) and humans (Harris et al. 2006) defined beta-alanine availability as the major rate-limiting factor in muscle carnosine synthesis and content. The proteins involved in this rate-limiting role of beta-alanine are less well characterized and could either involve the transsarcolemmal transport of beta-alanine (TauT and PAT1), the carnosine synthesis reaction itself (CARN5) or alternative metabolic pathways of beta-alanine (ABAT). In order to dissect the possible involvement of these genes, we explored their expression in various interventions that can alter muscle carnosine content.

As shown in Fig. 4a, increased circulating beta-alanine concentration (through oral supplementation) stimulates gene expression of both TauT, CARN5 and ABAT. This signifies that during increased beta-alanine concentrations, the large muscle organ helps to eliminate beta-alanine from the blood (TauT) and metabolize it toward both increased oxidation (through ABAT) and increased dipeptide synthesis (CARN5). Our study provides the first indication for such an alternative metabolic pathway for beta-alanine in skeletal muscle, as 4-aminobutyrate transaminase (ABAT), which is able to convert beta-alanine to malonate semi-

aldehyde (deamination reaction), is expressed in skeletal muscle and is increased upon excess beta-alanine availability. Similarly, beta-alanine supplementation in rats also resulted in increased mRNA expression of beta-alanine transaminase in kidney (Ito et al. 2001). We hypothesize that the muscular enzyme expression of ABAT evolved in omnivores and carnivores where beta-alanine constitutes a significant portion of the dietary amino acids and can contribute to the macronutrient energy delivery. The contribution of amino acid oxidation to total ATP production in resting or contracting skeletal muscle is generally very low, but can increase—even up to 10 % of the total energy delivery—in conditions of increased amino acid availability (Brooks 1987).

In Fig. 4b, we summarize the transcriptional events that take place during orchidectomy in male mice, an intervention which has been shown to decrease rodent muscle carnosine content (Penafiel et al. 2004) and which suggests an important regulatory role of testosterone. Orchidectomy induces a decreased expression of the TauT. It is possible that this resulted in a decreased sarcoplasmic availability of beta-alanine and that this is the cause of lower muscle carnosine levels following orchidectomy. Indeed, when orchidectomy is antagonized by testosterone replacement (Fig. 3), no such decrease in TauT is observed, and



**Fig. 3** The effects of 7 and 30 days of orchidectomy with or without testosterone replacement on mouse muscle carnosine content and expression of related enzymes and transporters [mean  $\pm$  SD, one-way ANOVA: \*\* $p$  < 0.01 vs. sham, \* $p$  < 0.05 vs. sham, \$ $p$  > 0.05 and

<0.1 vs. sham, ## $p$  < 0.01 between ORX\_T and ORX\_V, € $p$  > 0.05 and <0.1 vs. ORX\_V, lines are drawn to indicate time effects (no interaction measurements were made)]

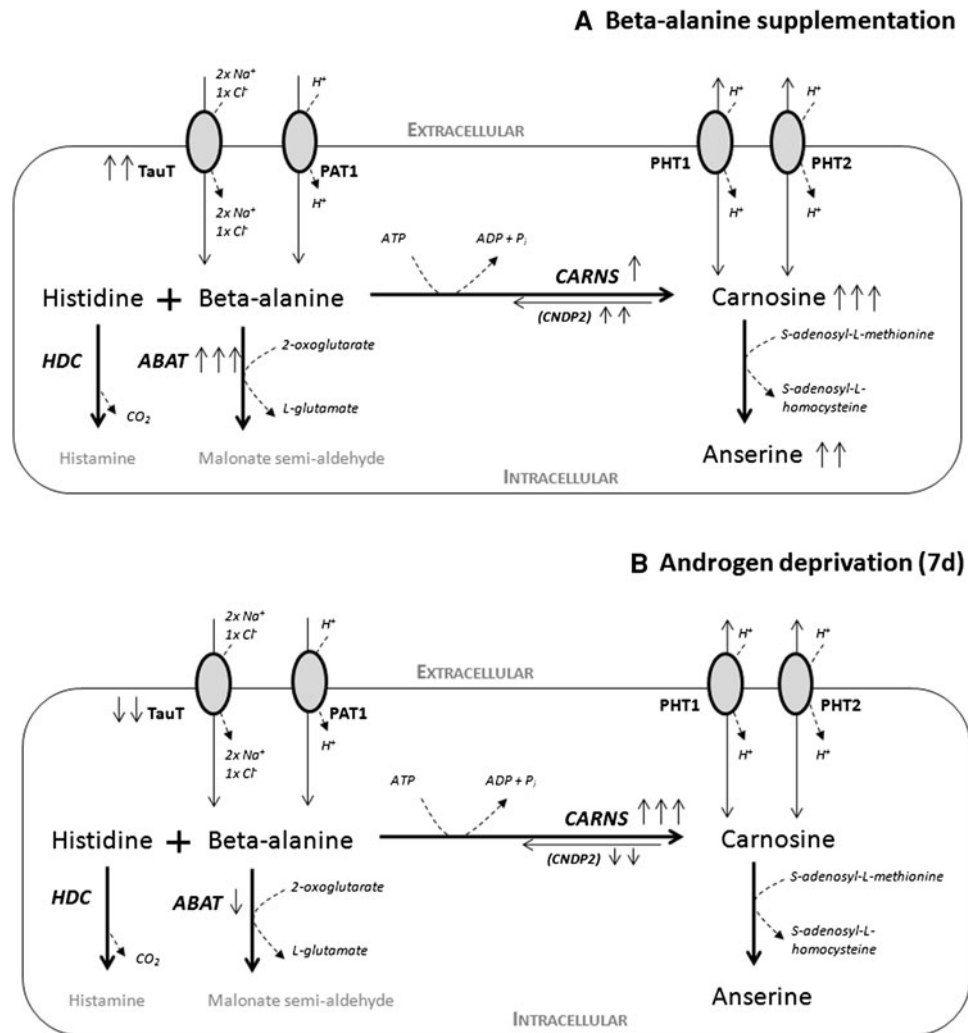
accordingly, no carnosine deficiency occurs. These data suggest that the TauT is under direct or indirect androgenic control and could be partly responsible for the observed effects of testosterone on muscle carnosine content. Future investigations will have to examine whether TauT is involved in the conditions that have been attributed to androgens, such as the sudden increase and resulting gender dimorphism in human muscle carnosine content emerging during male puberty (Baguet et al. 2012), the very high muscle carnosine concentrations in body-

builders, who may have used anabolic steroids (Tallon et al. 2005), or even the aging-related decline in muscle carnosine content (Baguet et al. 2012; Del Favero et al. 2012).

When further analyzing the events during orchidectomy (Fig. 4b), we observed a differential regulation of ABAT and CARNs. Interestingly, in this condition of presumed reduced sarcoplasmic beta-alanine availability, priority seems to be given to the carnosine synthesis, as CARNs is strongly upregulated (threefold higher in orchidectomy



**Fig. 4** Schematic overview of mRNA expression of carnosine-related enzymes and transporters after long-term and beta-alanine supplementation (**a** 1.2 % of drinking water, 8 weeks) and after 7 days of orchidectomy (**b**). *One arrow*:  $p > 0.05$  and  $< 0.1$  versus control or sham, *two arrows*:  $p < 0.05$  versus control or sham, *three arrows*:  $p < 0.01$  versus control or sham



without compared to with testosterone replacement), whereas the enzyme responsible for directing beta-alanine toward oxidation is downregulated (ABAT). This observation could further support our current main working hypothesis that the priority role of beta-alanine is to serve as the precursor of carnosine synthesis, and that the role of beta-alanine as a fuel is only used in conditions of excess availability. The strong positive correlations between TauT expression on the one hand and CARNS and ABAT expression on the other are in further agreement with this scheme (Fig. 1).

Based on the evidence that plasma taurine levels are elevated after acute beta-alanine supplementation (10–40 mg/kg BW, Harris et al. 2006) and that the uptake of beta-alanine is Na<sup>+</sup> and Cl<sup>-</sup> dependent (Bakardjiev and Bauer 1994), the taurine transporter (TauT) has been suggested to be responsible for beta-alanine transport into the muscle. Our study provides evidence for an additional beta-alanine transporter in skeletal muscle, namely PAT1. Interestingly, the expression levels of both beta-alanine

transporters (TauT and PAT1) are positively correlated to each other. As only TauT is modified by supplementation (Fig. 4a) and orchidectomy (Fig. 4b) and as beta-alanine transport by PAT1 is not Na<sup>+</sup> and Cl<sup>-</sup> dependent (Anderson et al. 2009), we now suggest that TauT is, albeit not the only, yet probably the most dominant transporter for the uptake of beta-alanine in skeletal muscle.

Although a few studies randomly investigated the presence of the four members of the POT-family in skeletal muscles of different species (Botka et al. 2000; Lu and Klaassen 2006; Doring et al. 1998), this is the first study which systematically investigated their expression in human and mice skeletal muscles (with or without altered muscle carnosine levels). Our results indicate that PHT1 and PHT2 are the main transporters expressed in skeletal muscle, as PEPT1 is not expressed and PEPT2 can in our hands not be reliably quantified with qPCR in skeletal muscle of both investigated species. PHT1 and PHT2 have been shown to differ from PEPT1 and PEPT2 in that they recognize, next to carnosine and other oligopeptides, also

the amino acid L-histidine as substrate (for review see Daniel 2004). Although this study demonstrated the expression of PHT1 and PHT2 in skeletal muscle, the changes in their mRNA expression did not systematically parallel changes in muscle carnosine content and therefore the contribution of these transporters of the POT-family to muscle carnosine metabolism still remains to be elucidated.

As Drozak et al. (2010) defined CARNS as a sluggish enzyme, with a turnover rate ( $=k_{\text{cat}}$ ) lower than  $1 \text{ s}^{-1}$ , it was expected that the synthesis of carnosine would be limited by the expression of this enzyme. However, CARNS mRNA expression was increased in conditions of decreased muscle carnosine content (after orchidectomy) and decreased in conditions of increased muscle carnosine content (when testosterone was administered to castrated mice). Thus, we can refute the hypothesis that the reducing effect of androgen deprivation on carnosine levels results from a downregulation of CARNS, as previously suggested by Penafiel et al. (2004). On the contrary, our results would suggest that CARNS is trying to prevent changes in muscle carnosine levels, except when beta-alanine is abundant in blood during beta-alanine supplementation (Fig. 4a).

We found that HDC, the enzyme that converts L-histidine to histamine, is expressed in mouse skeletal muscle, as previously shown by Nijjima-Yaoita et al. (2012). Carnosine is hypothesized by some to be a histamine precursor and a non-mast cell reservoir for histidine and histamine, although this function is uncertain in skeletal muscle. Nagai et al. (2012) suggested that muscle carnosine can influence blood pressure and other autonomic nervous system-regulated processes through a pro-histaminic effect. As both CNDP2 and HDC are expressed in skeletal muscle, the possibility of carnosine degradation to histidine and subsequent decarboxylation inside the skeletal muscle cell cannot be excluded. However, it seems unlikely that HDC in itself contributes to the regulation of carnosine content in skeletal muscle, as HDC expression did not change in any of the carnosine-altering interventions.

Our results demonstrate the presence of CNDP2 mRNA in skeletal muscle. Whether CNDP2 is able to actively degrade carnosine in skeletal muscle is doubtful. Lenney et al. (1985) showed by in vitro experiments that carnosine can be degraded to a very small extent in skeletal muscle, with  $\sim 30$  times lower carnosinase activity compared to kidney tissue. Furthermore, the optimal pH for carnosine hydrolysis by CNDP2 has been shown to be 9.5 (Lenney et al. 1985) and it is therefore believed that carnosine degradation in skeletal muscle (pH 7.1) by CNDP2 is minimal.

In conclusion, this study suggests that carnosine homeostasis in skeletal muscle is met through a complex interplay between different transporter isoforms and enzymes, interacting with carnosine and/or its constituent amino acids. We now identified eight such enzymes and

transporters with mRNA expression in human and/or mouse skeletal muscle. We suggest that not carnosine synthase (CARNS), but rather the taurine transporter (TauT), responsible for transsarcolemmal beta-alanine uptake, constitutes a major point of regulation of muscle carnosine synthesis. In conditions of excess availability (beta-alanine supplementation), beta-alanine may also be deaminated for further oxidation through ABAT. Yet, when beta-alanine availability is scarce, priority may be given to convert it into carnosine and avoid its oxidation.

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**Conflict of interest** The authors declare that there is no conflict of interest.

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