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ORIGINAL ARTICLE

Dietary GABA induces endogenous synthesis of a novel imidazole peptide homocarnosine in mouse skeletal muscles

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Abstract

Carnosine (β-alanyl-L-histidine) is an imidazole dipeptide present at high concentrations in skeletal muscles, where it plays a beneficial role. However, oral intake of carnosine or β-alanine to increase skeletal muscle carnosine levels has disadvantages such as low efficiency and side effects. Therefore, we proposed homocarnosine (γ-aminobutyryl-L-histidine) as a novel alternative imidazole peptide for skeletal muscle based on its structural similarity to carnosine. To induce endogenous homocarnosine synthesis in skeletal muscles, mice were fed a basal diet mixed with 0, 0.5, 2, or 5% γ-aminobutyric acid (GABA) for 6 weeks. As expected, in the control group (0% GABA), GABA and homocarnosine were present in trace concentrations. Skeletal muscle homocarnosine levels were significantly increased in the 2% and 5% GABA intake groups (tenfold, $P < 0.01$ and 53-fold, $P < 0.01$; respectively) relative to those of the control group, whereas 0.5% GABA intake induced no such effect. GABA intake had no effect on the levels of carnosine, anserine, and β-alanine. Vigabatrin (inhibitor of GABA transaminase (GABA-T)) administration to mice receiving 2% GABA intake for 2 weeks led to GABA-T inhibition in the liver. Subsequently, a 43-fold increase in circulating GABA levels and a tendency increase in skeletal muscle homocarnosine levels were observed. Therefore, skeletal muscle homocarnosine synthesis can be induced by supplying its substrate GABA in tissues. As GABA availability is tightly regulated by GABA-T via GABA degradation, inhibitors of GABA or β-alanine degradation could be novel potential interventions for increasing skeletal muscle imidazole dipeptides.

Keywords Imidazole dipeptide · Carnosine · Homocarnosine · Skeletal muscle · GABA · β-Alanine

Introduction

Imidazole peptides are naturally occurring peptides specifically present in skeletal muscles of animals in high (millimolar) concentration (Boldyrev et al. 2013). The most common imidazole peptides found in animal skeletal muscles are β-alanine-containing imidazole peptides such as carnosine (β-alanyl-L-histidine), anserine (β-alanyl-1-methylhistidine), and ophidine or balenine (β-alanyl-L-3-methylhistidine) (Boldyrev et al. 2013) as shown in Fig. 1. In stark contrast to animals, carnosine is the predominant imidazole peptide present in human skeletal muscles with a small extent of anserine (two orders of magnitude lower) (Boldyrev et al. 2013; Hoetker et al. 2018; Johnson et al. 2016). Carnosine has been reported to exert antioxidant and anti-fatigue effects crucial for muscle recovery and function, which has been claimed to enhance exercise performance in humans (Boldyrev et al. 2013). With respect to pathology, carnosine supplementation has been reported to play a protective role

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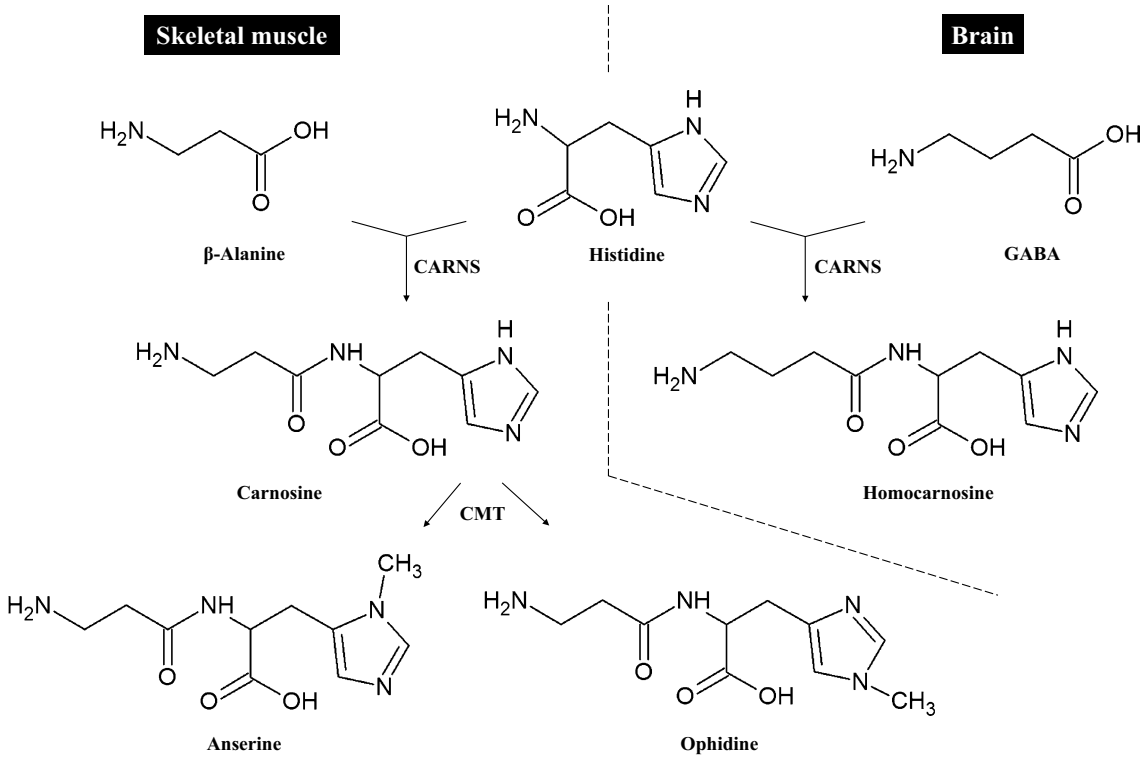

Fig. 1 Chemical structures of naturally occurring imidazole dipeptides exclusively present in skeletal muscles and brains of animals. Carnosine synthase (CARNS) is the enzyme responsible for carnosine and homocarnosine synthesis. Carnosine *N*-methyltransferase (CMT) is the enzyme believed to be responsible for the transfer of a methyl group to carnosine to form anserine (or ophidine)

in a wide range of diseases such as diabetes, cancer, brain diseases, heart diseases, and inflammatory diseases (Hipkiss 2009; Boldyrev et al. 2013; Derave et al. 2019). Because of versatile beneficial functions of carnosine and being the main imidazole peptide in human skeletal muscles, in recent years, various interventions have been proposed for in vivo increase of this peptide, including prescribed intake of carnosine, its precursor β-alanine, and other amino acids. However, these interventions are associated with disadvantages such as low efficiency and side effects (Décombaz et al. 2012; Liu et al. 2012a; Blancquaert et al. 2016). Thus, the present study aimed to establish a novel imidazole peptide as a viable alternative to the β-alanine-containing imidazole peptide carnosine in skeletal muscles.

Homocarnosine is an imidazole peptide found predominantly in brains, but not in skeletal muscles (Bauer 2005). It is composed of γ-aminobutyric acid (GABA) and histidine and has a similar structure to carnosine, with the only difference being an extra carbon atom in GABA compared to that in β-alanine of carnosine (Fig. 1). In our previous study, metabolomic analysis revealed that vitamin B6 deficiency significantly decreased the levels of carnosine, anserine, and homocarnosine in rat heart muscle by regulation of the in vivo availability of the precursor β-alanine and GABA (Kumrungsee et al. 2019). Taken together with the fact that homocarnosine biosynthesis is regulated by the enzyme (carnosine synthase and CARNS) involved in carnosine biosynthesis (Boldyrev et al. 2013), this finding led us to hypothesize that lack of the substrate GABA in skeletal muscles leads to absence of skeletal muscle homocarnosine biosynthesis. Thus, the specific objective of the present study was to determine if dietary GABA can induce endogenous homocarnosine synthesis in skeletal muscles. Despite sharing structural similarities to β-alanine and being a substrate of the same enzyme CARNS, GABA has never been considered as a dietary factor for enhancing imidazole peptide concentration in skeletal muscles. Recent studies have reported various beneficial effects of homocarnosine, such as antioxidant and anti-inflammatory actions (Kohen et al. 2006; Huang et al. 2018), prevention of DNA damage (Kang 2005), and inhibition of advanced glycation end-product formation (Alhamdani et al. 2007). In addition, homocarnosine has been shown to possess greater tolerance to degradation by serum carnosinase relative to carnosine (Teufel et al. 2003; Peters et al. 2010), this study may inspire a new idea for inducing new imidazole peptides to skeletal muscle instead of depending on only β-alanine-containing imidazole peptide carnosine for enhancing the muscle functions.

Materials and methods

Animal care, diets, and experimental protocol

Male ICR mice (5 weeks old, Charles River Japan, Hino, Japan) were maintained in accordance with the Guide for the Care and Use of Laboratory Animals established by Hiroshima University and approved by the Ethics Committee of the University (Ethical Approval No. C17-19). Mice were housed in metal cages in groups of two in a temperature-controlled room (24 ± 1 °C) in a 12-h light/dark cycle (lights on from 08:00–20:00 h). Mice had free access to food and drinking water and were acclimatized with a non-purified commercial rodent diet (MF, Oriental Yeast, Tokyo, Japan) for 7 days before being subjected to experiments. After that, a total of 32 mice were randomly divided into four groups ($n = 8$/group) for receiving a basal diet (Kumrungsee et al. 2019) mixed with 0 (control), 5 (0.5%), 20 (2%), or 50 (5%) g GABA/kg diet for 6 weeks. To assess the effect of feeding period and age of mice on homocarnosine levels, mice at 6 or 10 weeks of age (at the beginning of the treatment) were randomly divided into two groups ($n = 5$/group) for receiving the basal diet mixed with 0 or 50 (5%) g GABA/kg diet for 2 weeks. The results of these 2 weeks feeding period were compared with those 6 weeks feeding period mentioned above.

To determine if the GABA degradation pathway is involved in homocarnosine synthesis, the GABA-degrading enzyme inhibitor vigabatrin (Sabril®; Sanofi Aventis, Patheon, France) at a dose of 250 mg/kg body weight was administered daily by subcutaneous (SC) injections for 2 weeks to mice (6 weeks of age at the beginning of the treatment) receiving a basal diet mixed with 5 (0.5%) or 20 (2%) g GABA/kg diet. The same volume was used for saline injections in control groups. A control group received a basal diet without GABA mixture and injection of saline or vigabatrin ($n = 6$/group). At the end of each experiment, all mice were sacrificed under isoflurane anesthesia (between 13:00 and 15:00 h) after removal of food (06:00 h). Blood was collected from abdominal vein into tubes containing heparin as an anticoagulant on ice. Then, plasma was obtained by centrifugation at 3500 rpm for 20 min and stored at − 80 °C. Gastrocnemius muscles and other organs were harvested immediately, weighed, snapped frozen in liquid nitrogen, and stored at − 80 °C until analyzed.

Enzyme activity analysis

Endogenous activity of GABA-T in the liver was examined according to previous protocols (De Ita-Pérez et al. 2014; Blancquaert et al. 2016) with some modifications. Briefly, the liver tissue ($n = 6$) was homogenized in 10 volumes of 40 mM phosphate buffer (pH 7.0) at 13,000 rpm for 30 s on ice, followed by centrifugation at 10,000 rpm for 15 min at 4 °C to collect supernatant. Then, 10 µL of supernatant was incubated with 190 µL of 100 mM phosphate buffer (pH 8.6) containing 1 mM NAD^+ (Nacalai Tesque, Kyoto, Japan), 5 mM α-ketoglutarate (Nacalai Tesque), 3.5 mM mercaptoethanol (Nacalai Tesque), and 6 mM GABA (Nacalai Tesque) for 30 min at 37 °C. The same mixture without GABA was run in parallel and used as a blank. Since NAD^+ is reduced to NADH in the course of the reaction, the rate of the enzymatic reaction was determined by measuring NADH production using a spectrophotometer (Jasco, Tokyo, Japan) at 340 nm.

Analysis of imidazole peptides, GABA, and β-alanine content

Tissues and plasma were homogenized in methanol containing an internal standard (20 µM methionine sulfone) as previously reported (Kumrungsee et al. 2019; Soga et al. 2003). Then, supernatants were concentrated by evaporation to dryness and were resuspended in methanol before analysis. Anserine (Wako Pure Chemical Industries, Osaka, Japan), carnosine (Wako), homocarnosine (Cosmo Bio International, Tokyo, Japan), and GABA (Nacalai Tesque) were detected by ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) (Waters, Milford, MA) as described previously (Waterval et al. 2009) with some modifications. Briefly, liquid chromatography was performed at 30 °C using an Acquity UPLC BEH C18 (1.7 µm, 2.1 × 50 mm) column (Waters) and a gradient system with the mobile phase consisting of buffer A; 5 mM perfluoroheptanoic acid (PFHpA; Sigma-Aldrich, Louis, MO) in Milli-Q water and buffer B; 5 mM PFHpA in methanol at a flow rate of 400 µL/min. The gradient program was started with an initial condition of 95% A and 5% B. Then, a linear gradient from 40 to 50% B was reached in 10 min, followed by 50% to 100% B in 0.5 min with a holding period for 1 min. The gradient was finally reinstated to the initial conditions in 0.5 min with equilibration for 5 min before the next injection. Run-to-run time was 17.5 min. The injected volume was 5 µL. Mass spectrometric analysis was performed by multiple reaction monitoring (MRM) in the ESI-positive mode. The desolvation temperature was 400 °C, and the source temperature was 120 °C. The capillary voltage, cone voltage, and MRM for each of compounds were set as shown in Table 1. Nitrogen gas was used in both desolvation and cone gas flows. MRM and daughter-ion scans were performed using argon as the collision gas.

As β-alanine (Nacalai Tesque) could not be detected using the UPLC-MS/MS method, it was tracked by an

Table 1 Conditions of UPLC-MS/MS analysis of compounds

Compounds	MRM (*m/z*)	Capillary voltage (kV)	Cone voltage (V)	Collision energy (V)	Retention time (min)
Homocarnosine	241 > 155.9	3	7	10	7.53
Carnosine	227.1 > 110.1	3	10	20	7.41
Anserine	241 > 109.2	3	15	20	7.06
GABA	103.9 > 86.9	3	10	10	1.56

o-phthalaldehyde (OPA) based high-performance liquid chromatography (HPLC) method (Kamisaki et al. 1990) as described previously with some modifications. A Cosmosil $5C_{18}$-MS-II column (4.6 × 250 mm; Nacalai Tesque) with an isocratic elution of 0.1 mol/L of a mixture containing sodium citrate (pH 3.5)/acetonitrile/methanol (60:30:10 (v/v). A fluorescence detector set at an excitation wavelength of 350 nm and emission wavelength of 440 nm was used to monitor the compound.

Statistical analysis

Data are expressed as means ± SD. Statistical analysis was performed by unpaired Student's *t* test or by one-way ANOVA, followed by Tukey's multiple comparisons test using GraphPad Prism8 (GraphPad Software, CA, USA). For all tests, $P < 0.05$ was considered statistically significant.

Results

Food intake and body weight

As compared with the control group (0% GABA), the 5% GABA intake group exhibited significantly lower food intake and final body weight ($P = 0.0007$ and $P = 0.0006$, respectively, Table 2), but no significant difference in these parameters between the control, 0.5%, and 2% GABA intake groups. Although lower food intake was observed in the 5% GABA intake group, all GABA intake groups significantly increased the estimated amounts of dietary GABA (0.0 ± 0.0 (cont) vs. 1.7 ± 0.1 ($P = 0.003$), 5.9 ± 0.8 ($P < 0.0001$), and 10.7 ± 0.8 ($P < 0.0001$) g/6 weeks in the 0.5%, 2%, and 5% GABA intake groups, respectively, Table 2).

Effect of dietary GABA levels on homocarnosine and other imidazole peptides in skeletal muscles

To determine if dietary GABA can induce endogenous synthesis of homocarnosine in skeletal muscles, mice were separated into four groups receiving a basal diet mixed with 0, 0.5, 2, or 5% GABA for 6 weeks. As expected, in the control group without GABA intake, both homocarnosine and GABA were present in very low concentrations (0.02 µmol/g and 2.04 nmol/g, respectively, Fig. 2a and b). Unlike the control group, the 0.5% GABA intake group showed slight but significant increase in homocarnosine concentrations (0.02 ± 0.002 vs. 0.03 ± 0.01 µmol/g, $P = 0.043$), whereas the 2% and 5% GABA intake groups showed a marked increase in homocarnosine concentrations (0.02 ± 0.002 vs. 0.22 ± 0.07 and 1.18 ± 0.72 µmol/g, $P < 0.0001$ and $P = 0.001$, respectively, Fig. 2a). The 2% and 5% GABA intake groups also had significantly elevated GABA concentrations in skeletal muscles (2.0 ± 0.6 vs. 5.1 ± 0.9 and 35.9 ± 9.4 nmol/g, respectively, $P < 0.0001$), whereas the 0.5% GABA intake group showed no significant difference (Fig. 2b) compared to the control group concentrations. Notably, dietary GABA levels had no significant effect on levels of carnosine (4.2 ± 1.7 (cont) vs. 5.1 ± 1.4 (5% GABA) µmol/g, $P = 0.277$), anserine (7.5 ± 2.3 (cont) vs. 7.8 ± 2.3 (5% GABA) µmol/g, $P = 0.811$), and the substrate β-alanine (227.2 ± 61.4 (cont) vs. 299.4 ± 79.8 (5% GABA) nmol/g,

Table 2 Food intake and body weight

	GABA (%)				One-way ANOVA (*P* value)
	0	0.5	2	5	
Food intake (g/6 wk)	304.8 ± 14.9[a]	334.6 ± 15.9[a]	296.8 ± 38.6[a]	213.6 ± 15.2[b]	< 0.0001
Estimated GABA intake (g/6 wk)	0.0 ± 0.0[a]	1.7 ± 0.1[b]	5.9 ± 0.8[c]	10.7 ± 0.8[d]	< 0.0001
Initial body weight (g)	31.9 ± 1.4	32.3 ± 2.4	31.7 ± 2.5	32.1 ± 0.5	0.913
Final body weight (g)	47.1 ± 6.0[a]	47.2 ± 6.2[a]	46.1 ± 5.8[a]	35.1 ± 2.5[b]	0.0002
Body weight gain (g/6 wk)	15.2 ± 5.2[a]	14.9 ± 5.4[a]	14.4 ± 3.9[a]	3.0 ± 2.5[b]	< 0.0001

Values are expressed as means ± SD (n = 8). Values without a common superscript letter are significantly different, $P < 0.01$, analyzed by one-way ANOVA followed by Tukey's multiple comparisons test

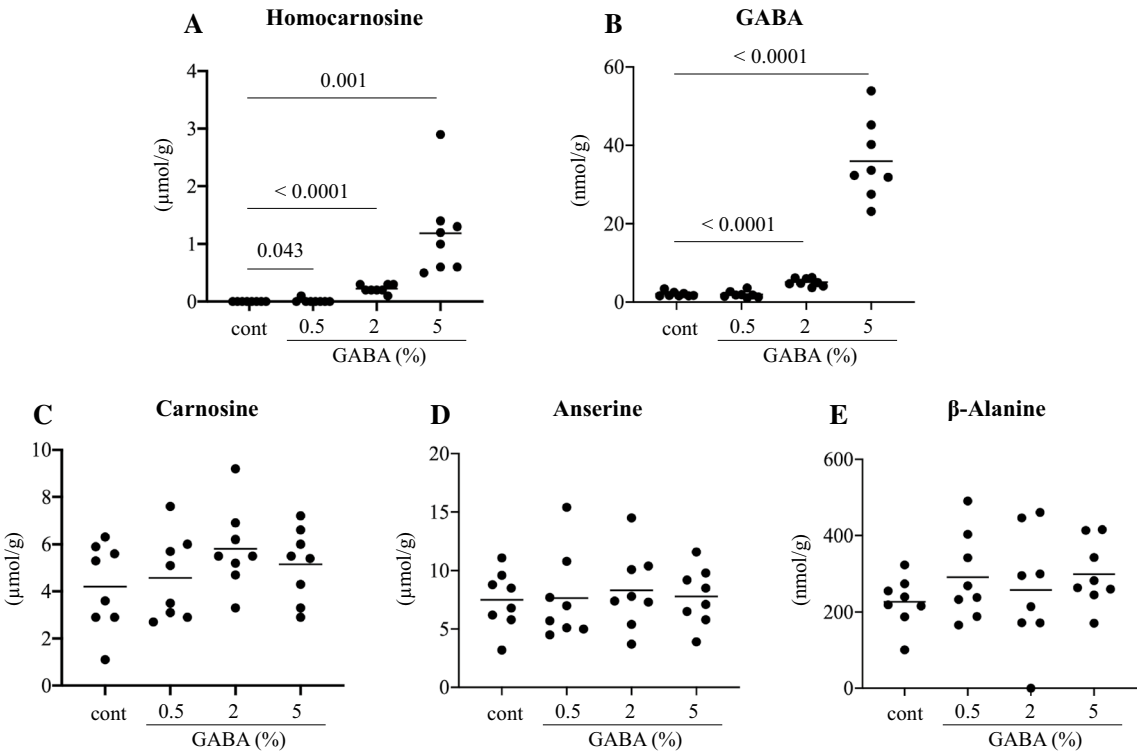

Fig. 2 Effect of dietary GABA levels on homocarnosine, GABA, and other imidazole peptides in skeletal muscles. Levels of homocarnosine (**a**), GABA (**b**), carnosine (**c**), anserine (**d**), and β-alanine (**e**) were analyzed by UPLC-MS/MS and OPA-HPLC methods as described in "Materials and methods". Values are expresses as the individual data points (dots) with the mean (dash line) and per wet weight of tissues ($n=8$). Statistical significance was determined by Student's t test, a P value <0.05 was considered as significant

$P=0.079$) (Fig. 2c–e) across all GABA intake groups. The levels of carnosine and anserine reported here are in the similar concentration range with previous works (Blancquaert et al. 2016; Penafiel et al. 2004), which also demonstrated that carnosine and anserine are the predominant imidazole peptides in rodent skeletal muscles.

Effect of dietary GABA levels on homocarnosine and other imidazole peptides in other tissues and plasma

Overall, dietary GABA had little or no effect on the levels of homocarnosine, GABA, carnosine, and anserine in the plasma, heart, brain, liver, and kidney (Table 3). In the plasma, 2% and 5% GABA intake significantly elevated circulating GABA concentrations, but exhibited lesser effect on homocarnosine levels and no effect on carnosine and anserine levels (Table 3). In the heart and liver extracts, 5% GABA intake slightly but significantly increased levels of homocarnosine as well as carnosine, but exhibited no changes in GABA and anserine levels. In the kidney extracts, 5% GABA intake showed a trend toward an increase in GABA concentrations compared to those of the control group (21.6 ± 24.7 vs. 41.4 ± 14.8 nmol/g, $P=0.071$), with the magnitude of increase in concentration similar to that in skeletal muscles (35.9 ± 9.4 nmol/g, Fig. 2b (5% GABA)). However, 5% GABA intake induced only a slight increase in kidney homocarnosine levels (11.5 ± 0.1 (cont) vs. 13.4 ± 0.9 nmol/g, $P=0.0001$) compared to the magnitude of increase in skeletal muscles (0.02 ± 0.002 (cont) vs. 1.18 ± 0.72 μmol/g, $P=0.001$). In brain extracts, GABA was present in very high (micromolar) concentration in both the hippocampus and the cortex, which is consistent with the previous work reporting the similar GABA concentrations in whole brain (Blancquaert et al. 2016). However, homocarnosine concentrations were slightly higher in the hippocampus than in the cortex. Concentrations of carnosine and anserine in both the hippocampus and the cortex were relatively low compared to those of homocarnosine. Dietary GABA levels had no effect on levels of homocarnosine, GABA, and carnosine, but significantly increased anserine levels (61.5 ± 5.4 (cont) vs. 74.2 ± 0.4 nmol/g, $P<0.0001$) in the cortex. However, in the hippocampus, the 5% GABA intake group exhibited a trend toward an increase in GABA levels (3.89 ± 0.52 (cont) vs. 4.96 ± 0.98 μmol/g, $P=0.061$) and a slight increase in carnosine and anserine levels (170.4 ± 22.4 (cont) vs. 210.2 ± 33.7 nmol/g, $P=0.015$; 85.4 ± 7.0 (cont) vs. 102.6 ± 1.3 nmol/g, $P<0.0001$, respectively); but no significant changes in homocarnosine levels (469.5 ± 134.2 (cont)

Table 3 Effect of dietary GABA levels on homocarnosine and other imidazole peptides in plasma and other tissues

	GABA (%)				One-way ANOVA (*P* value)
	0	0.5	2	5	
Plasma					
Homocarnosine (μM)	9.58 ± 0.01	9.58 ± 0.02	9.59 ± 0.02	9.78 ± 0.15$^{0.002}$	< 0.0001
Carnosine (μM)	18.1 ± 0.2	15.3 ± 0.2	15.3 ± 0.2	15.5 ± 0.5	0.175
Anserine (μM)	18.6 ± 0.3	19.2 ± 0.9	18.8 ± 0.44	19.1 ± 0.8	0.227
GABA (μM)	0.05 ± 0.04	0.05 ± 0.05	0.15 ± 0.11$^{0.026}$	0.80 ± 0.41$^{0.0001}$	< 0.0001
Heart					
Homocarnosine (nmol/g)	7.80 ± 0.18	7.65 ± 0.10	8.32 ± 0.50$^{0.041}$	14.57 ± 4.93$^{0.007}$	0.0002
Carnosine (nmol/g)	34.9 ± 10.8	32.1 ± 9.2	43.3 ± 19.8	67.4 ± 33.2$^{0.043}$	0.026
Anserine (nmol/g)	21.0 ± 4.5	18.4 ± 2.5	23.2 ± 7.1	32.9 ± 15.9	0.058
GABA (nmol/g)	3.50 ± 4.42	1.96 ± 0.93	3.40 ± 6.89	3.51 ± 2.27	0.897
Hippocampus					
Homocarnosine (nmol/g)	469.5 ± 134.2	463.4 ± 266.7	629.1 ± 190.9	568.6 ± 136.7	0.306
Carnosine (nmol/g)	170.4 ± 22.4	178.9 ± 33.4	199.4 ± 29.3$^{0.043}$	210.2 ± 33.7$^{0.015}$	0.051
Anserine (nmol/g)	85.4 ± 7.0	85.3 ± 8.0	84.7 ± 7.1	102.6 ± 1.3$^{<0.0001}$	< 0.0001
GABA (μmol/g)	3.89 ± 0.52	–[a]	–	4.96 ± 0.98$^{0.061}$	NA
Cortex					
Homocarnosine (nmol/g)	115.1 ± 46.1	135.8 ± 128.0	150.0 ± 109.0	117.6 ± 40.1	0.625
Carnosine (nmol/g)	133.0 ± 42.9	124.0 ± 36.2	140.4 ± 42.0	139.7 ± 33.9	0.847
Anserine (nmol/g)	61.5 ± 5.4	61.5 ± 5.8	61.5 ± 5.6	74.2 ± 0.4$^{<0.0001}$	< 0.0001
GABA (μmol/g)	3.61 ± 1.11	4.32 ± 1.35	3.96 ± 1.80	4.00 ± 1.43	0.821
Liver					
Homocarnosine (nmol/g)	11.8 ± 0.05	11.8 ± 0.04	11.8 ± 0.05	11.9 ± 0.14$^{0.026}$	0.007
Carnosine (nmol/g)	14.4 ± 0.3	14.2 ± 0.1	14.7 ± 0.7	15.1 ± 0.7$^{0.029}$	0.013
Anserine (nmol/g)	21.4 ± 0.2	21.3 ± 0.2	21.7 ± 0.5	21.5 ± 0.3	0.244
GABA (nmol/g)	10.5 ± 12.1	–	–	8.7 ± 7.5	NA
Kidney					
Homocarnosine (nmol/g)	11.5 ± 0.1	11.5 ± 0.1	11.9 ± 0.3$^{0.0004}$	13.4 ± 0.9$^{0.0001}$	< 0.0001
Carnosine (nmol/g)	18.5 ± 3.7	17.1 ± 0.5	19.1 ± 4.2	18.6 ± 4.7	0.714
Anserine (nmol/g)	24.9 ± 6.3	22.3 ± 1.1	28.7 ± 11.9	27.5 ± 6.8	0.345
GABA (nmol/g)	21.6 ± 24.7	13.8 ± 5.6	25.8 ± 15.2	41.4 ± 14.8$^{0.071}$	0.018

Values are expressed as means ± SD and per wet weight of tissues ($n = 8$)

P values vs. 0% GABA were analyzed by Student's *t* test. *NA* no analysis

[a] Not measured

vs. 568.6 ± 136.7 nmol/g) (Table 3). It is noteworthy that the homocarnosine synthesized in skeletal muscles due to 2% and 5% GABA intake (0.22 and 1.18 μmol/g, respectively, Fig. 2a) is similar to the basal levels of brain homocarnosine (115.1 and 469.5 nmol/g in the cortex and hippocampus, respectively, Table 3).

Effect of vigabatrin on GABA-T activity in the liver and circulating GABA and skeletal muscle homocarnosine levels

To examine if the GABA-degrading enzyme GABA-T regulates intramuscular GABA levels and affects the synthesis of homocarnosine in skeletal muscles, the enzyme inhibitor vigabatrin was injected into mice. Since GABA-T has been reported to be highly expressed in the liver, we examined GABA-T activity in the liver extracts. Vigabatrin significantly inhibited GABA-T activity (0.1 ± 0.1 vs. 0.6 ± 0.2 and 0.5 ± 0.1 nmol min^{-1} mg $liver^{-1}$ in the 2% GABA intake group with vigabatrin and saline injection, and basal diet group with saline injection (cont), respectively, $P = 0.004$, Fig. 3a). The inhibition of GABA-T by vigabatrin protects GABA from the degradation pathway, resulting in a significant increase in GABA concentrations in the liver, plasma, and skeletal muscles (Fig. 3b–d). This contributes to a trend toward an increase in skeletal muscle homocarnosine

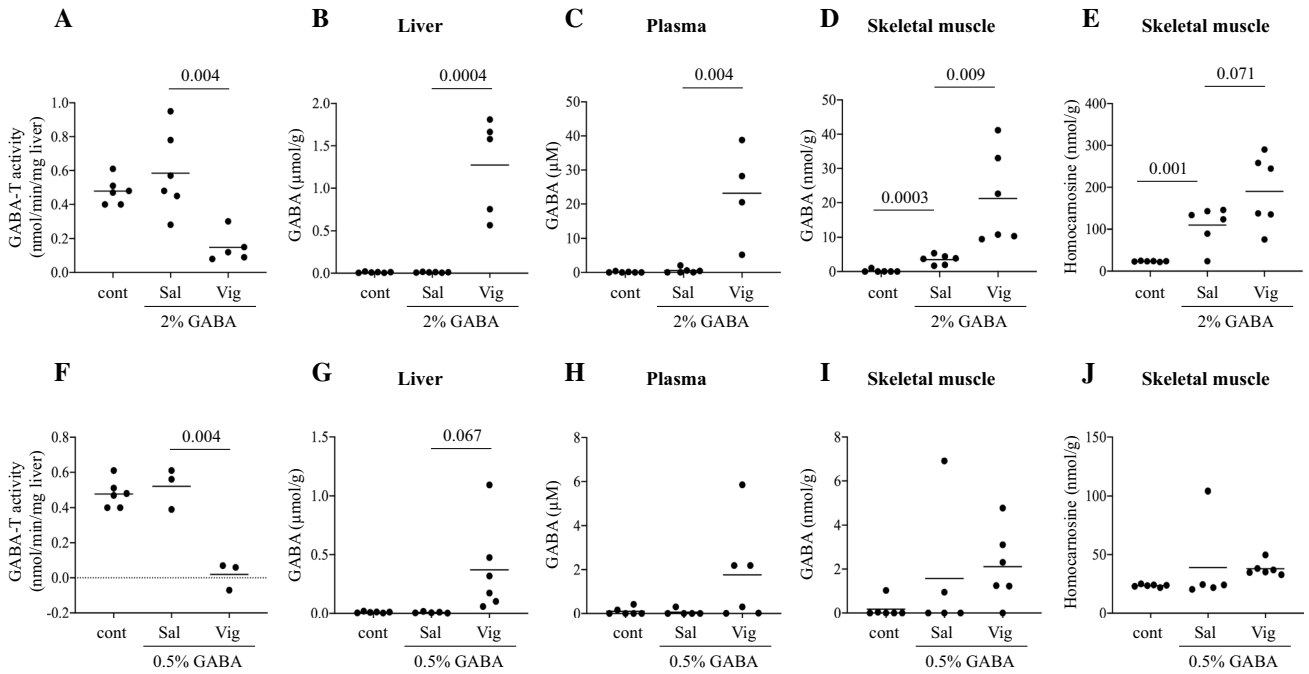

Fig. 3 Effect of vigabatrin on GABA-T activity in the liver extracts (**a**, **f**); GABA levels in the liver (**b**, **g**), plasma (**c**, **h**), and skeletal muscle (**d**, **i**); and homocarnosine levels in the skeletal muscle (**e**, **j**) of mice fed with 2% or 0.5% GABA, respectively. Activity of GABA-T in liver tissue extracts. Reaction mixtures contained 6 mM GABA and 10 µL liver tissue extract in 100 mM phosphate buffer (pH 8.6). After incubation for 30 min at 37 °C, NADH concentrations were measured using a spectrophotometer at 340 nm. Values are expresses as the individual data points (dots) with the mean (dash line) and per wet weight of tissues ($n = 3$–6). Statistical significance was determined by Student's t test, a P value < 0.05 was considered as significant

concentration (109.8 ± 46.7 vs. 190.1 ± 85.4 nmol/g in the 2% GABA intake group injected with saline and vigabatrin, respectively, $P = 0.071$, Fig. 3e). We also tested for enhanced skeletal muscle homocarnosine synthesis in mice fed with lower dietary GABA level (0.5%) and injected with vigabatrin. As shown in Fig. 3f, vigabatrin significantly inhibited GABA-T activity in the liver (0.02 ± 0.08 vs. 0.52 ± 0.12 and 0.48 ± 0.08 nmol min^{-1} mg $liver^{-1}$ in the 0.5% GABA intake group with vigabatrin and saline injection, and the basal diet group with saline injection (cont), respectively, $P = 0.004$). This led to a trend toward an increase in GABA concentration in the liver (0.01 ± 0.01 vs. 0.37 ± 0.38 µmol/g in the 0.5% GABA intake groups with saline and vigabatrin injection, respectively, $P = 0.067$, Fig. 3g). However, this increased GABA levels in the liver due to vigabatrin injection could not significantly elevate circulating GABA levels (Fig. 3h). Therefore, no significant changes in GABA and homocarnosine levels in skeletal muscle were observed (Fig. 3i and j).

Effect of feeding period and age of mice on homocarnosine status in skeletal muscle

To examine if feeding period and age of mice affect homocarnosine synthesis, mice at 6 or 10 weeks of age at the beginning of experiment were subjected to the 0% or 5% GABA-containing diet for 2 or 6 weeks. At the end of experiment, 6-week-old mice with 2 and 6 weeks of feeding, and 10-week-old mice with 2 weeks of feeding became 8, 12, and 12 weeks old. As shown in Fig. 4a and b (white bars), GABA and homocarnosine concentrations were not significantly different between these three groups, indicating that both feeding period and age do not have an influence on the amount of GABA and homocarnosine.

Discussion

In previous work from our laboratory, the homeostasis of a GABA-containing imidazole peptide homocarnosine present in rat heart muscles was found to depend on GABA levels (Kumrungsee et al. 2019). This led us to hypothesize that skeletal muscle homocarnosine synthesis might be induced by dietary supply of GABA. Therefore, we fed mice with a basal diet mixed with GABA and found that circulating and intramuscular GABA levels were elevated, consequently increasing homocarnosine levels in skeletal muscle. These results confirmed that the absence of homocarnosine in skeletal muscles in animals, including humans, is due to absence of the precursor GABA, and not because of the lack

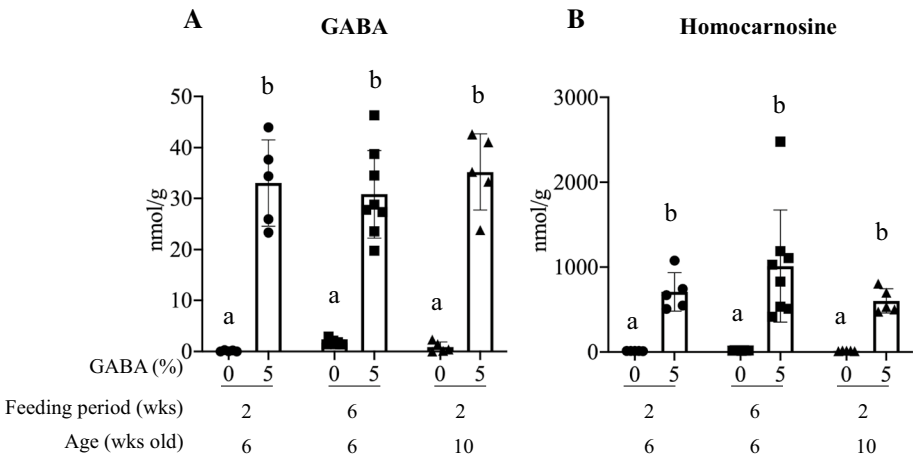

Fig. 4 Effect of feeding period and age of mice on GABA levels (**a**) and homocarnosine synthesis (**b**) in skeletal muscles. Values are expressed as the individual data points (dots) with the mean (dash line) ± SD and per wet weight of tissues ($n = 5–8$). Different letters (**a** and **b**) above the bars indicate significant differences between groups; $P < 0.05$ (Tukey's multiple comparisons test)

of biosynthesis machinery particular to this peptide in muscles. To the best of our knowledge, this is the first report demonstrating that, in addition to carnosine and anserine, other imidazole peptides can be synthesized and retained in skeletal muscles by providing their substrates or precursors. This was supported by the fact that high and low abundance of GABA and β-alanine in brains translates high and low concentrations of homocarnosine and carnosine, respectively (Bauer 2005). In this study, we also found that GABA supplementation, even at a high dose of 5% in the diet, had limited effects on carnosine, anserine, and β-alanine levels in skeletal muscle, implying that CARNS in muscles remain unperturbed even at high levels of GABA supplementation. This may be explained by the fact that CARNS has a 17–25 times higher affinity for β-alanine than to GABA (Drozak et al. 2010; Nagai et al. 2012). Although homocarnosine synthesized in skeletal muscle due to GABA intake is only about 25% of total muscle carnosine, it is similar to normal levels of brain homocarnosine.

Dietary intake of GABA had negligible effect on GABA and homocarnosine levels in the brain, thus suggesting the possibility that dietary GABA may not likely to penetrate the blood–brain barrier or, on the other hand, GABA homeostasis is important in the brain so that although it might cross the blood–brain barrier, its levels might be tightly controlled by the GABA-degrading enzyme (GABA-T) in the brain. This speculation could be supported by a previous report suggesting that the mechanism of action of GABA food supplements in brain, including its possibility to cross the blood–brain barrier, is controversial, and further studies are needed (Boonstra et al. 2015). Notably, dietary GABA induced slight increase in GABA and homocarnosine levels in the hippocampus, with higher homocarnosine levels in the hippocampus than in the cortex. This may be explained by the greater permeability of the blood–brain barrier (Vorbrodt et al. 1995; Liu et al. 2012b) and higher expression of CARNS (The Human Protein Atlas; Drozak et al. 2010) in the hippocampus than in the cortex. The higher hippocampal levels of homocarnosine might also be associated with some functional consequences. It is noteworthy that dietary GABA induces a slight increase in homocarnosine and carnosine levels in heart. This finding is consistent with our previous report in rat heart showing that homocarnosine and carnosine homeostasis depends on GABA and β-alanine levels and that GABA is possibly exchanged to β-alanine through amino acid metabolism (Kumrungsee et al. 2019). It seems likely that GABA-T might have less effects on regulating GABA and β-alanine levels, since it appears that GABA-T had similar activity toward both GABA and β-alanine (Blancquaert et al. 2016; Jeremiah and Povey 1981). In the liver and kidney, dietary GABA exhibited no effect on homocarnosine levels, which could be explained by the high, moderate, and negligible expression levels of CARNS in the skeletal muscles and brain; the heart; and the liver and kidney, respectively (The Human Protein Atlas; Blancquaert et al. 2016). In addition, the carnosine/homocarnosine-degrading enzyme carnosinase 1 (CN1) is abundant in the liver (humans) and kidney (rodents) (Teufel et al. 2003).

Interestingly, the increase in muscle homocarnosine by 2% GABA intake accounts for only 18% of the increase in muscle homocarnosine in the 5% GABA intake group. Based on this, we hypothesized that dietary GABA might be routed to other pathways after absorption, rather than entering the homocarnosine synthesis pathway. We speculated that direct GABA degradation by GABA-T could be the pathway of choice. In this, GABA is transaminated into succinate semialdehyde (SSA) by GABA-T, followed by conversion of SSA into succinate, which is finally fed into the tricarboxylic acid cycle by succinic semialdehyde dehydrogenase (Bouché et al. 2003). By injection of vigabatrin, the specific GABA-T inhibitor, mice on the 2% GABA diet exhibited an increase in circulating and intramuscular GABA levels, thereby increasing muscle homocarnosine. On the other hand, mice on the 0.5% GABA diet did not

show any increase in circulating and intramuscular GABA and muscle homocarnosine levels. These results suggest that the homocarnosine synthesis in skeletal muscle was stringently dependent on the availability of exogenous dietary GABA, whose homeostasis is, in turn, highly regulated by GABA-T in the liver. Our finding is consistent with previous animal and human studies showing that the administration of vigabatrin led to an increase in GABA and homocarnosine levels in the brain, where GABA-T is also highly expressed (Petroff et al. 1998a, b; Blancquaert et al. 2016). Taken together with the fact that GABA has higher affinity to GABA-T ($K_m = 1.02$ mM) than to CARNS ($K_m = 6.44$ mM) (Armijo 1989; Drozak et al. 2010), our results indicated that a large fraction of exogenous GABA is being used as a GABA-T substrate in the liver and only a small fraction is used for homocarnosine synthesis. Although the present study demonstrates that GABA supplementation for increasing muscle homocarnosine is less effective than β-alanine supplementation for increasing muscle carnosine, it suggests that GABA-T inhibitors may be a novel approach for increasing homocarnosine and carnosine levels in skeletal muscles, since β-alanine is also a substrate for this enzyme.

Homocarnosine is known to be exclusively present in brain and cerebrospinal fluid (Bauer 2005) and acts as an endogenous neuroprotective agent in a broad range of disease conditions. This includes protection of brain endothelial cells from β-amyloid peptide induced toxicity (Preston et al. 1998), anti-inflammatory action in brain ischemic injuries (Huang et al. 2018), and inhibition of brain cell death (Tabakman et al. 2002). Clinical studies have suggested that homocarnosine homeostasis in brain plays a crucial role in neurological diseases such as Alzheimer's and epilepsy, in which higher brain homocarnosine levels correlated with a better control of epilepsy (Perry et al. 1987; O.A.C. et al. 2001; Petroff et al. 2006; Bai et al. 2015). However, little is known about its occurrence and function in skeletal muscles. Homocarnosine, like carnosine, may emerge as a promising imidazole peptide for skeletal muscle therapy. Homocarnosine has been reported to exert antioxidant activity and prevent DNA oxidative damage to a similar extent as carnosine (Kang 2005; Kohen et al. 2006). In addition, homocarnosine inhibits the formation of advanced glycation end products in peritoneal dialysis fluid to a greater extent than carnosine (Alhamdani et al. 2007). The work of Yuneva et al. (2002) suggests that homocarnosine might play a role in the reported effect of carnosine supplementation in increasing lifespan in flies. This is because tissue concentrations of homocarnosine, but not of carnosine, increased in a dose-dependent manner with carnosine supplementation. Kang et al. (2002) demonstrated that homocarnosine was similar to carnosine in protecting Cu, Zn superoxide dismutase from oxidative damage through a combination of copper chelation and peroxyl radical scavenging, suggesting its possible role in the maintenance of the antioxidant system in skeletal muscle. In addition to these carnosine-like biological functions, homocarnosine has a significant advantage over carnosine due to greater resistance to carnosinase hydrolysis over carnosine and nonspecific histidine-containing dipeptides. It has been reported that amino acids can be covalently linked to histidine to form nonspecific histidine-containing dipeptides (X-histidine dipeptide) in skeletal muscles (Teufel et al. 2003; Veiga-da-Cunha et al. 2014). However, compared to carnosine, X-histidine dipeptides such as leucine-, serine-, tyrosine-, alanine-, valine-, isoleucine-, methionine-, and glycine-histidine dipeptides are highly hydrolysable by the cytosolic nonspecific dipeptidase carnosinase 2 (CN2), whereas homocarnosine is resistant to CN2-mediated hydrolysis (Teufel et al. 2003; Veiga-da-Cunha et al. 2014). In addition, homocarnosine is somewhat resistant to hydrolysis by the specific carnosine/homocarnosine-hydrolyzing enzyme CN1 (also known as human serum carnosinase) than carnosine (Teufel et al. 2003; Peters et al. 2010). Taken together with the fact that homocarnosine is a natural occurring imidazole peptide found in our body and it is more stable with possibly longer half-life than carnosine and other nonspecific histidine-containing dipeptides, this peptide may emerge as a novel promising imidazole peptide for skeletal muscles. Our findings suggest that supplementation of homocarnosine with carnosine or GABA with β-alanine may be an alternative intervention to enhance the efficiency of increase or replenishment of imidazole peptides in skeletal muscles.

Conclusion

The present study demonstrated that the imidazole peptide homocarnosine, predominantly present in the brain, can be synthesized in skeletal muscles if GABA is introduced into the tissue. It is of great interest to explore the potential physiological functions of homocarnosine in skeletal muscles in the future.

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Author contribution All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Takeshi Arima, Kanako Sato, and Takumi Komaru. The first draft of the manuscript was written by Thanutchporn Kumrungsee. Mikako Sato, Yasuyuki Oishi, Ai Egusa, and Noriyuki Yanaka critically read the manuscript and made valuable suggestions for its improvement. All authors read and approved the final manuscript. Thanutchaporn Kumrungsee and Takeshi Arima contributed equally to this work.

Compliance with ethical standards

Conflict of interest Mikako Sato is a patent holder (agent containing imidazole dipeptide-Publication number: 20180140655) and works for NH food Ltd. Although this study was partially supported by the grant from NH Foods Ltd. and some authors work at the company, the funding organization and those authors have no influence on the submitted work and all authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. The experiments were approved by the local ethics committee (Ethical Approval No. C17-19, granted by the Ethics Committee, Hiroshima University).

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