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Gender-related differences in carnosine, anserine and lysine content of murine skeletal muscle

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Summary. The aminoacyl-imidazole dipeptides carnosine (β-alanyl-L-histidine) and anserine (β-alanyl-1-methyl-histidine) are present in relatively high concentrations in excitable tissues, such as muscle and nervous tissue. In the present study we describe the existence of a marked sexual dimorphism of carnosine and anserine in skeletal muscles of CD1 mice. In adult animals the concentrations of anserine were higher than those of carnosine in all skeletal muscles studied, and the content of aminoacyl-imidazole dipeptides was remarkably higher in males than in females. Postnatal ontogenic studies and hormonal manipulations indicated that carnosine synthesis was up-regulated by testosterone whereas anserine synthesis increased with age. Regional variations in the concentrations of the dipeptides were observed in both sexes, skeletal muscles from hind legs having higher amounts of carnosine and anserine than those present in fore legs or in the pectoral region. The concentration of L-lysine in skeletal muscles also showed regional variations and a sexual dimorphic pattern with females having higher levels than males in all muscles studied. The results suggest that these differences may be related with the anabolic action of androgens on skeletal muscle.

Keywords: Carnosine – Anserine – Lysine – Skeletal muscle – Mice – Sex dimorphism

Introduction

Carnosine (β-alanyl-L-histidine) and anserine (β-alanyl-1-methyl-histidine) are archetypes of aminoacyl-imidazole dipeptides present in relatively high concentration in excitable tissues, such as muscle and nervous tissue (Crush, 1970; Bonfanti et al., 1999). Carnosine is synthesized by carnosine synthetase (EC 6.3.2.11), an enzyme with broad substrate specificity present in several mammalian tissues (Kalyander and Meister, 1959; Winnick and Winnick, 1959; Horinishi et al., 1978; Boldyrev and Severin, 1990; Bauer and Schulz, 1994), while anserine is formed from carnosine by the enzyme S-adenosylmethionine:carnosine N methyl transfeprase (EC 2.1.1.22) (McManus, 1962). In mammals, carnosine and carnosine-related peptides have been found in different cell populations within the nervous system like in neurons of the olfactory system and in other cell types such as glial and ependymal cells (Bonfanti et al., 1999). Skeletal muscles contain very high levels of these peptides, although the occurrence of each particular dipeptide in skeletal muscles is highly variable among species (Tamaki et al., 1976). Carnosine is present in human skeletal muscles but not in cardiac muscles, while anserine is normally absent in human tissues and body fluids (Scriver and Gibson, 1995). Skeletal muscles from different mammalian species, including rabbit and rat, contain both carnosine and anserine (Crush, 1970; O'Dowd et al., 1988).

The biological function(s) of imidazole dipeptides still remain enigmatic, although several hypotheses have been proposed. Thus it has been suggested that they may act as buffers to prevent pH decrease produced by lactic acid formed during muscle contraction under anaerobic conditions (Davey, 1960; Parkhouse and McKenzie, 1984). In addition, they show antioxidant properties in brain and muscle (Kohen et al., 1988; Stvolinsky et al., 1999) as well as divalent-metal chelating activity (Kohen et al., 1991). Other possible functions of these compounds have also been proposed, including their role as putative neurotransmitter or neuromodulator in the mammalian olfactory pathway (Margolis, 1974; Snyder, 1980; Bonfanti et al., 1999), as effectors of different enzymes such as myosinATPase (Avena and Bowen, 1969; Parker and Ring, 1970), muscle calpains (Johnson and Hammer, 1989), phosphorylases. a and b (Johnson et al., 1982)

and also as histidine reservoir for histamine synthesis (Flancbaum et al., 1990). In addition, N-acetyl forms of carnosine and anserine have been found in the heart of amphibian and mammals at concentrations sufficient to alter the sensitivity of their contractile apparatus to calcium ions (O'Dowd et al., 1988).

Aminoacyl-imidazole dipeptide content has been measured in different species, but mainly in those involved in competitive racing, including humans, greyhounds, horses and camels (Harris et al., 1990; Dunnet and Harris, 1997) but little and controversial information exists about carnosine and carnosine-related peptides in mice (Crush, 1970; Parker et al., 1985; Fisher and Margolish, 1986). In the latter there is a very well known extragenital sexual dimorphism affecting biochemical processes and cellular structure of kidney, liver and muscle (Bardin and Catterall, 1981). In this regard, we recently found that in CD1 mice arginine levels in plasma and skeletal muscle were higher in female than in male and that this dimorphism was dependent on dietary arginine (Ruzafa et al., 2003). In the present work we show that there are marked gender differences in carnosine and anserine content in several murine skeletal muscles and that testosterone increases the levels of these two dipeptides in the mouse skeletal muscle.

Materials and methods

Animals and treatments

Male and female Swiss CD1 mice (Harlan Interfauna Ibérica, Barcelona, Spain) of different ages (25-, 30-, 42-, 60-days old and adults 8–10 weeks old and weighing 35–38 g males and 26–30 females) and adult Sprague-Dawley rats weighing 250–300 g (Harlan Interfauna Ibérica, Barcelona, Spain) were bred in our animal facilities. They were housed at 22 ± 1°C ambient temperature and 50% relative humidity under a 12 h light-dark cycle (lights on at 0800 h) in stainless-steel cages. They received a commercial rodent solid diet and water ad libitum. The standard chow (A04, Panlab, Barcelona, Spain) contained: 17% proteins, 3% fat, 59% carbohydrate, 12% moisture, 4% fiber, 1% vitamins, and 4% minerals, with a caloric content of about 2,900 kcal/kg. The L-arginine, L-lysine and L-methionine contents of the diet were 0.98%, 0.85% and 0.31%, respectively. In some experiments, adult mice were fed with a synthetic diet (ICN Biomedicals Inc, Aurora, OH) that contained a total of 17% by total weight amino acids, for at least two weeks. Gonadectomy was performed under ether anaesthesia, and mice were killed 2 weeks after surgery. Adult female mice were treated with testosterone propionate (100 mg/kg) and killed after 2 weeks of treatment. Testosterone propionate, purchased from Sigma Chemical Co (St Louis, MO), was dissolved in olive oil and injected subcutaneously every other day. Animals were killed by cervical dislocation under ether anaesthesia and skeletal muscle and other tissues were quickly removed, weighed and processed. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national (RD 223/1988) and international laws and policies (EEC Council Directive 86/009; NIH Guide for the Care and Use of Laboratory Animals, NIH publication No 85-23, 1985).

Amino acid analysis

Muscle sample from the pelvic limbs included gastrocnemius, semitendinous, straight femoral and superficial gluteal muscles. Thoracic limb samples included brachial triceps muscle, lateral head muscle, long radial extensor muscle of digits and lateral extensor muscle of digits. The pectoral muscle sample was composed mainly of pars abdominalis muscle pectoralis profundus and serratus ventralis.

For amino acid analysis tissues were extracted with 5% trichloroacetic acid (1:5 w/v) using a Polytron homogenizer, and after centrifugation at 10,000 × g for 10 min the supernatants were analyzed. Amino acids were isolated by ion exchange chromatography, using an amino acid autoanalyzer (Chromaspeck, Rank Hilger Analytical, UK) and detected by fluorometry after reaction with o-phthaldialdehyde (Sigma Chemical Co, St Louis, MO). Nor-leucine was used as internal standard. The system was calibrated using 0.01 mM amino acid standard solution, including physiological acidic, neutral and basic amino acids (Sigma Chemical Co, St Louis, MO), supplemented with 0.01 mM of L-carnosine, L-anserine, 1-methyl-L-histidine and 3-methyl-L-histidine (Sigma Chemical Co, St Louis, MO). Amino acid and dipeptide concentrations were calculated and expressed as nmoles per g of wet tissue.

Statistical analysis

Results are given as means ± SD. Statistical comparisons were calculated by ANOVA followed by the post hoc Newman-Keuls multiple range test. The results were considered statistically significant when P values were <0.05.

Results

Free amino acids were measured in brain, liver, kidney, heart and skeletal muscles of adult CD1 mice. Among these tissues carnosine and anserine were only detected in skeletal muscles, where the concentrations of carnosine and anserine were higher than those of free histidine or other cationic amino acids such as arginine, lysine and ornithine (Table 1). Interestingly, the levels of both dipeptides were considerably higher in the skeletal muscle of males than in females. On the contrary, the concentrations of the basic amino acids lysine and arginine were 2–3 fold higher in the females. In consequence, in the female mice the concentrations of dipeptides and basic amino acids were almost similar, while in the male mice the dipeptide content was about 6-fold higher than the sum of lysine, arginine and ornithine. Muscle histidine concentration was almost similar in the two sexes. It must be noted that anserine almost duplicated carnosine values in both male and female mice muscle, and that in the two sexes total dipeptide concentration was about 20–50 fold higher than that found for histidine. This dimorphism was also observed when animals were fed with a synthetic diet devoid of carnosine and anserine (data not shown).

Figure 1 compares carnosine, anserine and lysine content between skeletal muscles from rats and mice. In rats,

Table 1. Free amino acid and dipeptide content in the skeletal muscles from mouse hind legs

Amino acid (nmol/g)	Male	Female
Taurine	29,510 ± 4,120	25,431 ± 2,846
Aspartic acid	253 ± 76	387 ± 126
Threonine	677 ± 101	615 ± 92
Serine	568 ± 75	587 ± 101
Glutamic acid	963 ± 72	940 ± 123
Glutamine	979 ± 103	1,156 ± 244
Glycine	2,210 ± 234	1,935 ± 212
Alanine	2,386 ± 197	1,343 ± 141[a]
Valine	234 ± 24	249 ± 52
Methionine	110 ± 13	107 ± 9
Isoleucine	137 ± 26	146 ± 27
Leucine	177 ± 11	187 ± 18
Tyrosine	168 ± 13	124 ± 19
Phenylalanine	117 ± 12	92 ± 8
Histidine	212 ± 21	228 ± 33
Carnosine	1,947 ± 244	547 ± 140[a]
Anserine	3,390 ± 693	1,537 ± 544[a]
Ornithine	77 ± 10	88 ± 9
Lysine	442 ± 66	1,115 ± 311[a]
Arginine	376 ± 61	747 ± 224[a]

Results are the means ± SD from 5–6 animals per group. a) $P < 0.001$ vs male

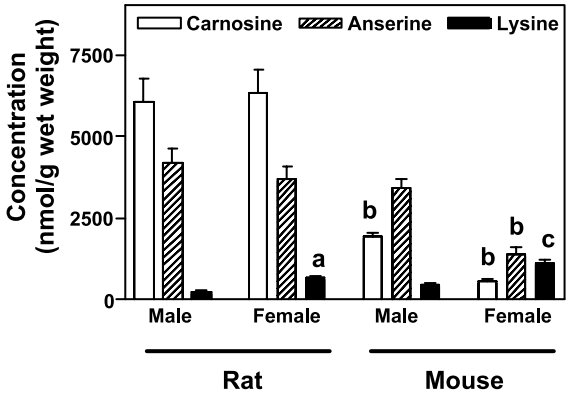

Fig. 1. Influence of gender on skeletal muscle carnosine, anserine and lysine content in adult mice and rats. Results are the means ± SD from 5–6 animals per group. Statistical significance: a) $P < 0.01$ vs male; b) $P < 0.001$ vs rat; c) $P < 0.01$ vs rat

dipeptide levels were higher than in mice, the concentration of carnosine being also higher than that anserine, in contrast to that found in mice. Moreover, no sex dimorphism in these dipeptides was evident. Lysine concentration in the muscle from female rats was higher than in male rats although the difference was less marked than that found in mice.

In order to determine the influence of sex steroid hormones on muscle dipeptide concentrations two groups of mice (one male and another female) were castrated to decrease circulating levels of androgens or estrogens while in a third group female mice were given exogenous administration of testosterone propionate (100 mg/kg, sc, 15 days). Table 2 shows that gonadectomy decreased carnosine and anserine concentrations in the skeletal muscle, the effect being more marked for carnosine (about 40% versus 15% for anserine). Testosterone administration to female mice produced a remarkably increase in both muscle carnosine and anserine concentrations (about 268% and 45%, respectively). The effect of sex hormones on muscle dipeptide content is also illustrated by the changes observed in carnosine and anserine levels along postnatal life. Figure 2 shows that in the skeletal muscle of immature animals carnosine and anserine concentrations were similar in male and female mice. In the males, both carnosine and anserine concentrations increased markedly with age reaching in the adult period values about 3 and 7 fold higher, respectively, than in the prepuberal period ($P < 0.001$, Fig. 2A and B). In the female mice, muscle dipeptide ontogeny was different from males since no significant elevation was observed in carnosine concentration (Fig. 2C) while anserine concentration increased about 2–3 fold ($P < 0.001$, Fig. 2D).

A sexual dimorphic pattern of carnosine and anserine was observed not only in muscles from the hind legs but also in other skeletal muscles from the fore legs and pectoral region. Figure 3 compares carnosine and anserine concentrations in different skeletal muscles from male and female mice. In all cases anserine was more abundant than carnosine and the concentrations of both dipeptides were higher in males than in females ($P < 0.001$). Moreover, the levels of dipeptides were highest in the hind leg muscles and lowest in pectoralis. Interestingly, muscle lysine concentration was also gender dependent, females having

Table 2. Effect of sex hormones in lower limb skeletal muscle dipeptide content

	Carnosine (nmol/g)	Anserine (nmol/g)
Male	1,944 ± 243	3,394 ± 694
Female	544 ± 142[a]	1,374 ± 174[a]
Castrated male	1,206 ± 276[a]	3,018 ± 307
Castrated female	320 ± 81	1,120 ± 104
Female + TP	2,004 ± 394[b]	2,001 ± 143[c]

Results are the means ± SD from 5 animals per group. Animals were killed 15 days after gonadectomy or treatment with testosterone propionate (TP) (100 mg/kg, every other day). Statistical significance: a) $P < 0.001$ vs male; b) $P < 0.001$ vs female; c) $P < 0.05$ vs female

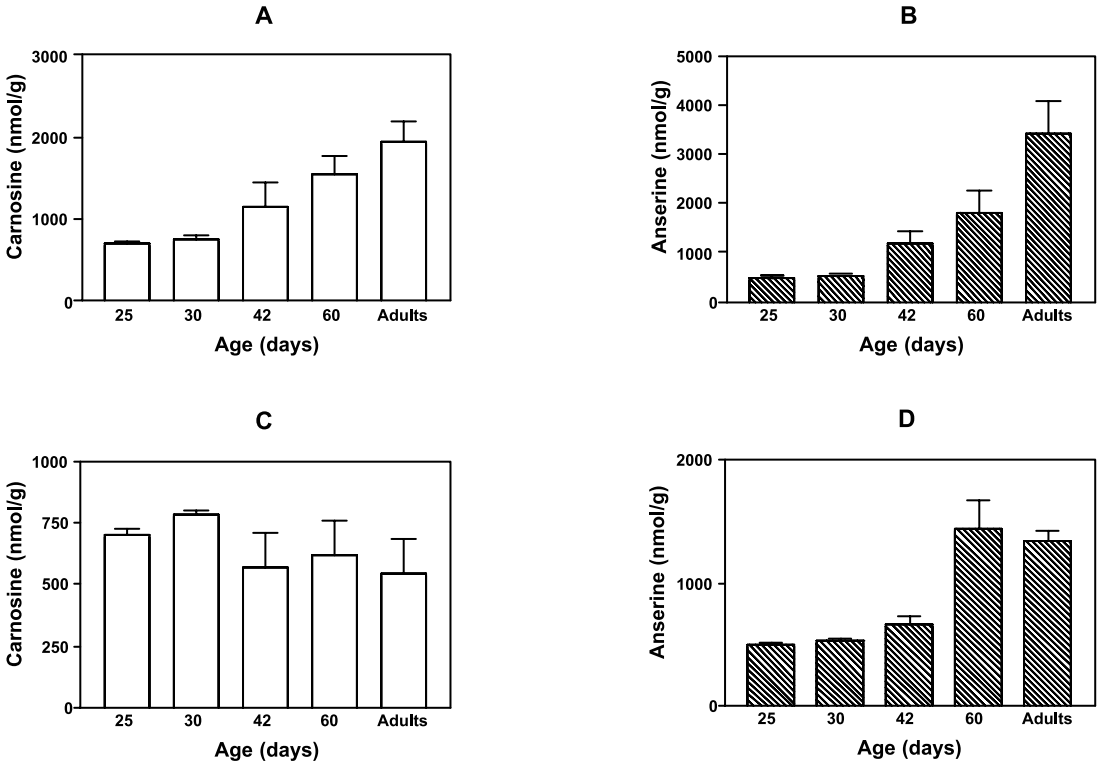

Fig. 2. Postnatal changes in carnosine and anserine content of skeletal muscles from male (A, B) and female (C, D) mice. Results are the means ± SD from 4–6 animals per group

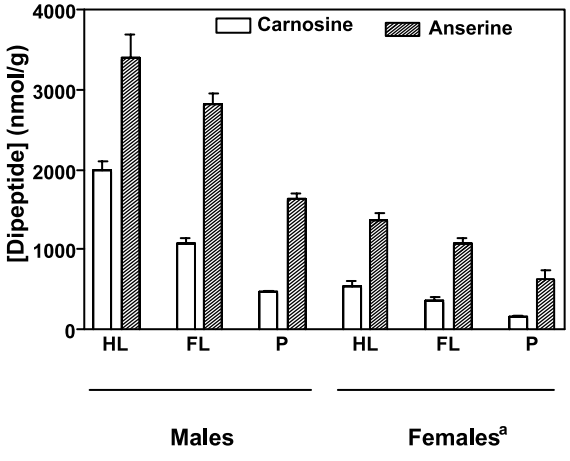

Fig. 3. Carnosine and anserine content of different skeletal muscles in adult mice. Results are the means ± SD from 5–6 animals per group. HL: hind leg muscles; FL: fore leg muscles; P: pectoral muscles. Statistical significance: a) $P < 0.001$ vs males

higher concentration than males in all skeletal muscles examined (Table 3). Contrary to that found with carnosine and anserine, lysine concentration in the abdominal muscle was considerably higher than in leg muscles. Moreover, a clear decrease of lysine concentration with age was observed, mainly in the male mice (Table 3). Male gonadectomy increased lysine concentration in the skeletal muscle of hind legs (1564 ± 284 nmol/g wet weight) while testosterone treatment of female mice (100 mg/kg, 15 days) decreased lysine concentration till 694 ± 98 nmol/g wet weight.

Table 3. Effects of sex and age on the lysine content (nmol/g) of different skeletal muscles

	Hind leg	Fore leg	Pectoralis
Adult male	440 ± 60	662 ± 151	1,725 ± 342[b]
Adult female	1,110 ± 211	978 ± 253	2,811 ± 275[b]
25-days-old	2,976 ± 299	1,599 ± 250[a]	3,440 ± 565[c]

Results are the means ± SD from 5–6 animals per group. Statistical significance: a) $P < 0.001$ vs hind leg; b) $P < 0.001$ vs hind or fore leg; c) $P < 0.001$ vs fore leg

Discussion

Our results indicate that in CD1 mice the two β-alanyl-imidazole dipeptides carnosine and anserine are present in relatively high amounts in the different types of skeletal muscles examined in comparison with other mouse tissues where these compound were almost undetectable.

Moreover, their levels are dependent on sex, age and anatomical localization of the muscles. Among all free amino acids examined only the basic or cationic amino acids and especially L-lysine exhibited regional, temporal and sexual differences comparable to those found in carnosine and anserine. Moreover, the levels of imidazole dipeptides and lysine were inversely related.

Whereas the main biochemical role of L-lysine is its participation in protein biosynthesis, the biological function of imidazole dipeptides still remains enigmatic. The quantitative differences described in the present study reveal that a multifactorial regulatory system appears to control the levels of these dipeptides in the murine skeletal muscles. This suggests that these compounds might exert some physiological functions in the muscle rather than being mere byproducts. In fact, studies with skeletal muscle cells in primary culture have demonstrated that carnosine and related peptides are not merely deposited in the skeletal muscle but that they are actively synthesized by muscle cells in culture (Bauer and Schulz, 1994). Our results reveal, for the first time, that sex steroid hormones regulate muscle dipeptide content, androgens being more effective than estrogens. The increase in muscle carnosine content observed after treatment of female mice with testosterone propionate together with the significant rise of this dipeptide with sexual development in males but not in females suggest that carnosine synthetase may be up regulated by androgens. On the other hand, the synthesis of anserine seems to be dependent on age. This could be explained as the consequence of developmental changes in S-adenosyl methionine:carnosine methyl transferase activity and/or as the result of changes in the availability of the substrates S-adenosylmethionine or carnosine. However, the influence of sex hormones in β-alanine uptake by affecting muscle β-amino acid transporter (Bakardjiev and Bauer, 1994) cannot be excluded.

The sexual dimorphism in carnosine and anserine content in skeletal muscle found in the present work was not surprising since it is well known that in mice there is a marked extragenital sex dimorphism affecting the liver, kidney and skeletal muscle among other tissues, and where testosterone seems to be the major hormone responsible for this dimorphism (Bardin and Catterall, 1981). There is not convincing evidence about the reasons for the existence of this extragenital dimorphism. In the case of carnosine and anserine, if one consider that muscular imidazole dipeptides may act as buffers of the protons derived from lactic acid produced by muscle contraction under anaerobic conditions (Abe, 2000) the higher concentration of these substances in the skeletal muscle of males would enable them to perform greater muscular activity than females. The fact that carnosine and anserine levels increase in muscles from pectorals to fore legs and hind legs also support the hypothesis that these dipeptides may be related with a high throughput of muscular activity. This regional distribution may be related to differences in muscle androgen receptor concentration as it has been reported in bovine skeletal muscle (Branstetter et al., 2000). However, these results differ from those described in horses where no significant sex difference in muscle carnosine content was found, whereas a trend towards lower muscle carnosine content with age was observed (Marlin et al., 1989).

Our results indicate that, in mice, muscular sex dimorphism affects not only carnosine and anserine but also arginine and lysine. The physiological consequences of this difference are presently unknown. It is also uncertain the meaning of the inverse relationship between basic amino acid and dipeptide content observed in skeletal muscle. While sex differences in dipeptide concentrations can be mainly explained by changes in the activities of synthetic enzymes, those found for arginine and lysine could be related to differences in amino acid uptake, protein turnover or both. Taking into consideration the role that androgens have in the control of muscle protein synthesis (Sheffield-Moore, 2000) and our results in mice, it is tempting to speculate that the gender differences observed in lysine and imidazole dipeptides in the skeletal muscles of mice could be related to a higher consumption of lysine due to the myotrophic effect of testosterone in the skeletal muscle and to the need to improve the muscle buffering capacity in response to the increase of skeletal muscle mass, respectively.

In conclusion, our results reveal that there is a marked sexual and regional dimorphism in the content of β-alanyl-imidazole dipeptides in the skeletal muscles of mice, the physiological significance of this finding remains to be established.

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