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Muscle Carnosine Content Is Unchanged during Maximal Intermittent Exercise

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ABSTRACT. Carnosine contributes 30% of the total physico-chemical buffering in equine skeletal muscle. However, the recent finding of a fall in carnosine content with exercise suggests that it may have other functions such as a reservoir of histidine for use as an energy substrate. To further investigate this, the carnosine content (mmol kg^{-1} d.m.) was measured by HPLC in freeze-dried biopsy samples from the middle gluteal muscle of 5 horses before, immediately after, and, 1 and 6 hours after performing four 700 m maximal intermittent gallops. We consider this exercise to be more severe than normally encountered during racing. No significant changes occurred in the muscle carnosine content. Previous findings of a fall may possibly have been the result of an increase in the blood content of muscle samples obtained after exercise.

Key words HPLC; middle gluteal; horses; buffering

INTRODUCTION

The imidazole dipeptide carnosine (β-alanyl-L-histidine) and its methylated homologues anserine (β-alanyl-L-1-methylhistidine) and ophidine (β-alanyl-L-3-methylhistidine) are found in the skeletal muscles of a large number of species.[4] In the horse carnosine is the predominant dipeptide. With a pKa value of 6.83 at 37°C,[15] carnosine will contribute significantly to intracellular physicochemical buffering in muscle, moderating H^+ increase arising from lactate production during exercise.[1,5] Recently, it has been shown that carnosine contributes 30% of the nonbicarbonate intracellular physico-chemical buffering in the Thoroughbred horse.[6] Although several other physiological roles have been proposed for carnosine in muscle, such as an activator of muscle calpains,[10] an antioxidant[12] and an activator of muscle phosphorylase,[11] its principal function appears to be as a buffer. High mean muscle carnosine concentrations of 108.6 mmol kg^{-1} dry muscle (d.m.) in the Thoroughbred horse[13] and approximately 156.8 mmol kg^{-1} d.m. in the Quarterhorse[2] are consistent with such a role.

Recently, it was reported that a reduction in the carnosine content of muscle occurred in exercising Quarterhorses.[3] It was suggested that this may have been due to hydrolysis of carnosine and utilisation of the histidine released for the production of metabolic energy through the TCA cycle. In this study, samples obtained during a further study in which Thoroughbred horses undertook repeated bouts of maximal exercise, were investigated for changes in carnosine content.

MATERIALS AND METHODS

Exercise protocol

For this study 5 trained Thoroughbred horses (4 geldings and 1 mare) performed four 700 m intermittent gallops. Horses were given an initial canter of 200 m before the start of the first gallop. Twenty min recovery was allowed between gallops, during which the horses were walked continuously. Three

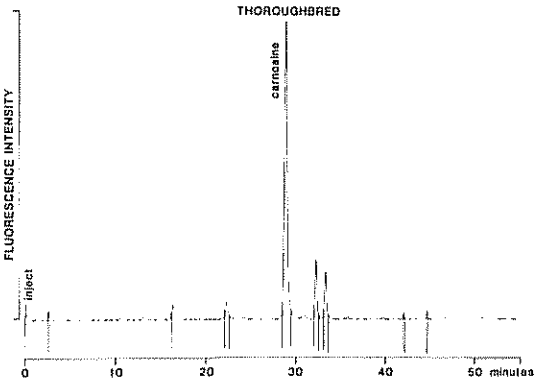

Fig 1 HPLC chromatogram of a neutralized perchloric acid extract of a biopsy taken from the middle gluteal muscle of a Thoroughbred horse.

of the 5 horses completed the investigation on two occasions.

Muscle biopsies

Biopsy samples from the middle gluteal muscle were obtained from a depth of 6 cm using 5 mm Bergstrom-Stille biopsy needles and at a fixed site one third of the distance along a line running from the tuber coxae to the head of the tail.[14] This procedure was adopted in an attempt to ensure an identical relative sampling site in each horse. Biopsy samples were taken before the first gallop, at 1–2 min of completion of the final gallop, 1 hour and 6 hours after the final gallop (horses had returned to the stables before the 1 and 6 hour biopsies were taken). Muscle tissue was immediately frozen in liquid nitrogen and the samples subsequently freeze-dried. Particular care was taken to ensure that the freeze-dried muscle was rendered as free as possible from blood and connective tissue. This was achieved by rolling of the sample on tissue paper, causing contaminating blood to fracture off. The muscle was then powdered and extracted with 0.5 mol l^{-1} perchloric acid[7] and neutralised with 2.1 mol l^{-1} potassium bicarbonate solution. One ml of neutralised extract was equivalent to 10 mg of muscle powder.

Blood samples

Three ml blood samples were collected from the jugular vein via an indwelling catheter (Becton-Dickinson, 14 gauge). Samples were deproteinised with 5 ml of 1 mol l^{-1} perchloric acid and analysed for lactate using lactate dehydrogenase.[9] Blood samples were collected after the warm-up canter (Pre) and 10 min after each gallop.

High performance liquid chromatography (HPLC)

Neutralised muscle extracts (20 µl) were mixed with 0.4 mol l^{-1} boric acid buffer, pH 9.65 (100 µl), in autosampler vials. Automated pre-column derivatisation of the buffered extracts was carried out using *o*-phthaldialdehyde/mercaptoethanol (OPT-Thiol) reagent (reaction time 60 seconds, 20 µl extract, 10 µl reagent). The reaction was performed at room temperature. The reagent was replaced at 12 hourly intervals. In this procedure, primary amino acids and dipeptides in the extracts are converted to isoindoles. Chromatography of the derivatives was performed on a 3 µm Hypersil ODS column (150 mm×4.6 mm) protected by a 40 µm Sepralyte ODS guard column (20 mm×2.0 mm). The mobile phase was a binary mixture. Solvent A consisted of 0.1 mol l^{-1} sodium acetate buffer (pH 7.2), methanol and tetrahydrofuran (THF) in the ratio 900:95:5. Solvent B was methanol. Both solvents were filtered to 0.45 µm and degassed with helium before use and periodically throughout the analysis. High pressure dynamic mixing of the solvents was employed. A gradient was formed by increasing the proportion of methanol from 10% to 90% over 50 min, followed by re-equilibration at 10% methanol between samples. The flow rate was 1.2 ml min^{-1}. The individual derivatives were detected and quantified by their fluorescence response (excitation wavelength 340 nm, emission wavelength 465 nm). Quantitation was based on comparison of the integrated peak areas with those from a range of known standards.

Table 1. *Mean concentrations of lactate in venous whole blood over the four gallops and mean gallop times*

Blood samples were drawn 10 min after completion of each gallop

Lactate (mmol l^{-1})		10 min post-exercise			
Pre-exercise		Gallop 1	Gallop 2	Gallop 3	Gallop 4
$\bar{X}$	1.7	17.5	21.6	24.3	26.8
SD	0.8	3.2	4.5	4.7	4.1
n	8	8	8	8	8
Gallop times (seconds)					
$\bar{X}$		45.5	46.9	48.0	51.3
SD		2.1	1.7	2.5	5.2
n		8	8	8	8

The HPLC system comprised LDC/Milton Roy Constametric I and 3000 pumps, a LDC/Milton Roy dynamic mixer, Stone, Staffs., U.K., a Waters Wisp 712 autosampler (Auto-addition facility) Watford, Middlesex U.K., a Spectrovision FD300 dual monochromator fluorescence detector, Shefford, Bedfordshire, U.K., a LDC/Milton Roy MP 3000 HPLC Controller and a Sekonics printer-plotter. OPT-Thiol reagent was stored at 4°C under nitrogen. Water was purified by reverse-osmosis followed by passage through two beds of nuclear grade ion-exchange resin and one bed of activated charcoal.

RESULTS

The main changes in whole blood lactate for the 5 horses (3 ran twice) are shown in Table 1. Lactate concentration increased progressively throughout the study and attests to the severity of the exercise undertaken. Times for the 700 m gallops ranged from 42 to 47 seconds over the first gallop, increasing to 45 to 59 seconds over the fourth gallop (Table 1).

The change in the carnosine content of the middle gluteal muscle with exercise, expressed as a mean value for all the horses, is shown in Table 2. No significant change was

Table 2. *Change in carnosine content of the middle gluteal muscle with exercise*

Values are mmol kg^{-1} d.m.

		Change from pre-exercise		
	Pre-exercise	1 min post Gallop 4	1 hour post Gallop 4	6 hours post Gallop 4
$\bar{X}$	95.6	0.7^{NS}	-0.2^{NS}	-2.7^{NS}
SD	16.3	18.2	12.6	17.9
n	5	4	4	5
Horses	5	5	5	5

NS = not significant (Student's *t*-test for paired data).

found between the pre-exercise and the post-exercise content.

A typical chromatogram of the dipeptide and amino acid content of a neutralised muscle extract is shown in Fig. 1. The lower limit of detection was 0.6 mmol kg^{-1} d.m.

DISCUSSION

The present study was undertaken in response to a recent publication[3] which described a reduction in the muscle carnosine content with exercise. A fall of approximately 20% (i.e. -8 mmol kg^{-1} wet muscle) was indicated with a concurrent increase in blood lactate concentration to 9–10 mmol l^{-1}, following 13 min of submaximal exercise. It was proposed that histidine released from carnosine hydrolysis may have been utilised in ATP production via the formation of α-ketoglutarate and entry into the TCA cycle. A small increase in the free histidine content of the muscle was also reported. The present results clearly do not support these findings and conclusions.

This study comprised periods of maximal and submaximal exercise. Although the work model exceeded that employed by Bump et al.[3] in both intensity and duration, and produced a total work output probably in excess of that encountered during normal flat racing, no reduction in muscle carnosine content with exercise or during the first six hours of recovery was found.

The present results on carnosine were derived from the analysis of freeze-dried muscle biopsies. During the preparation of samples considerable care was taken to maximise the removal of contaminating blood. It has been demonstrated that freeze-dried blood may, on occasion, account for as much as 50% of the dry biopsy weight.[8] Blood was easily removed by gentle rolling of freeze-dried biopsies on tissue paper. This causes the blood to fracture into a fine powder which then remains on the paper.

In the study of Bump et al. analysis was performed on wet muscle which had been frozen beforehand. Simple calculation illustrates that errors arising from an increase in the blood content of post-exercise samples are further exaggerated in wet tissue compared to samples which are freeze-dried (even without the attempted removal of contaminating blood). Increased water content of muscle cells after exercise will introduce further error when analysing wet muscle. It is our contention that the findings of Bump et al. may have resulted from such changes, which could easily have produced the apparent 20% decline in the measured carnosine concentration.

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REFERENCES

1 Bate-Smith, E C (1983) The buffering of muscle in rigor: protein, phosphate and carnosine J Physiol 92, 336.

2 Bump, K. D., Lawrence, L M, Moser, L R., Miller-Graber, P A and Kurcz, E V (1989) Effect of breed type on muscle carnosine. Proc 11th Eq Nutr and Physiol Symp, pp 252–256

3 Bump, K D, Lawrence, L M, Moser, L R, La-Rocca, G L, Miller-Graber, P A, Kurcz, E V and Fisher, M G (1989) Muscle carnosine levels during training and exercise. Proc 11th Eq Nutr and Physiol Symp, pp 35–40

4 Crush, K G (1970) Carnosine and related substances in animal tissues Comp Biochem Physiol 34, 3–30.

5 Davey, C L (1960) The effects of carnosine and anserine on glycolytic reactions in skeletal muscle Arch. Biochem Biophys 98, 296–302

6 Harris, R C, Marlin, D J, Dunnett, M, Snow, D H and Hultman, E (1990) Muscle buffering capacity and dipeptide content in the Thoroughbred horse, greyhound dog and man Comp Biochem Physiol 97A, 249–251

7 Harris, R C, Hultman, E and Nordesjö, L.-O. (1974) Glycogen, glycolytic intermediates and high energy phosphates determined in biopsy samples of musculus quadriceps femoris in man at rest Methods and variance of values Scand J Clin Lab Invest 33, 109–120

8 Harris, R. C., Harman, J C, Marlin, D J and Snow, D H (1987) Acute changes in the water content and density of blood and plasma in the Thor-

oughbred during maximal exercise *In* Gillespie, J. R. and Robinson, N. E. (eds.): *Equine Exercise Physiology 2*. ICEEP Publications, Davis, CA, pp. 464–475.

9 Hohorst, H. J. (1963) L-(+)-Lactate determination with lactate dehydrogenase *In* Bergmeyer, H. U. (ed.): *Methods of Enzymatic Analysis*. Academic Press, New York and London, pp. 266–270.

10 Johnson, P. and Hammer, J. L. (1989) Effects of L-1-methylhistidine and the muscle dipeptides carnosine and anserine on the activities of muscle calpains. Comp. Biochem. Physiol. 94, 45–48.

11 Johnson, P. and Aldstadt, J. (1984). Effects of carnosine and anserine on muscle and non-muscle phosphorylases. Comp. Biochem. Physiol. 78, 331–333.

12. Kohen, R., Yamamoto, Y., Cundy, K. C. and Ames, B. N. (1988) Antioxidant activity of carnosine, homocarnosine and anserine present in muscle and brain. Proc. Nat. Acad. Sci., USA. 85 (a), 3175–3179.

13. Marlin, D. J., Harris, R. C., Gash, S. P. and Snow, D. H. (1989). Carnosine content of the middle gluteal muscle in Thoroughbred horses with relation to age, sex and training. Comp. Biochem. Physiol. 93A, 629–632.

14. Snow, D. H. and Guy, P. S. (1976). Percutaneous needle muscle biopsy in the horse. Equine Vet. J. 8, 150–155.

15 Tanokura, M., Tasumi, M. and Miyazawa, T. (1976) 1H nuclear magnetic resonance studies of histidine containing di- and tri-peptides. Estimation of the effects of charged groups on the pKa value of the imidazole ring. Biopolymers 15, 393–401.

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Fibre Composition and Tubulin Localization in Muscle of Thoroughbred Sprinters and Stayers

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ABSTRACT Fibre type composition and tubulin distribution were studied in the gluteus medius muscle of Thoroughbreds. Needle biopsies were taken from 5 sprinters and 5 stayers after completion of their racing career. Histochemical analysis was performed on cryostat sections using a new inhibition-reactivation myofibrillar ATPase technique. No significant differences were found between sprinters and stayers in mean fibre type frequency, diameter and relative fibre type area. Immunocytochemical techniques with monoclonal antibodies TU-01 and TU-02 against the alpha-subunit of tubulin revealed its heterogeneous distribution in different fibre types. Type II fibres reacted strongly and type I fibres weakly with antibody TU-01, whilst the reverse staining pattern was found with antibody TU-02.

Key words Horse muscle; histochemical analysis; tubulin.

INTRODUCTION

Mammalian skeletal muscles are composed of a mixture of different fibre types. Inheritance of the fibre type composition and the influence of training on this have been extensively studied in locomotory muscles of laboratory animals and man.[1,16,21] The proportion of slow-twitch (type I) and fast-twitch (type II) fibres shows a high heritability value implying a marked genetic influence. In man, a high correlation has been found between fibre type proportion and athletic discipline. Long distance runners demonstrate increased frequency of type I fibres whilst a high proportion of type II fibres is characteristic of sprinters.[4] Similarly, various equine breeds differ in the proportion of type I fibres which reflects probably long-lasting selection for distinct muscle performance.[23]

Numerous studies have been published which describe muscular adaptations in response to increased physical activity. The oxidative capacity of muscle fibres and the whole fibre number are mainly increased, but the type I/type II fibre ratio can also be changed depending on training intensity and animal age.[1,14,21,22] Such studies suggest that not only hereditary factors are significant determinants but that certain environmental stimuli are also important in the establishment of the metabolic profile and fibre type composition of skeletal muscle.

Monoclonal antibodies against various contractile and regulatory myofibrillar proteins provide a precise tool for studying their isoform distribution in different fibre types of adult animals. Structural changes of myofibrillar proteins during ontogenetic development and training have also been revealed by immunocytochemical methods.[8,22] Differential localization of tubulin, one of the microtubular proteins, has been demonstrated in various animal tissues using monoclonal antibodies against alpha- or beta-tubulin subunits.[5,6] However, data about tubulin distribution in skeletal muscle and its relation to fibre types are unknown up to now.