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Branched chain amino acids chronic treatment and muscular exercise performance in athletes: a study through plasma acetyl-carnitine levels

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Summary. In relation to energy request during physical exercise, muscular tissue Branched Chain Amino Acids (BCAA) are metabolized particularly when the oxidation rises. But in the whole-human body, it is difficult to estimate, in a quantitative sense, the role played by BCAA in sustaining exercise. During a BCAA treatment, made on a group of athletes kept under observation, it was observed, through Conconi's test, that this treatment influenced physical performance. Aim of present work is to investigate if BCAA chronic treatment effect on physiological trial is confirmed on blood circulating biochemical energy parameters and in particular on acetyl-carnitine, since acetyl-linked compounds may be an important biochemical factor.

Fourteen athletic well trained male subjects, were randomly divided into two subgroups; a first group was submitted to a chronic treatment ($n = 7$) of BCAA (oral intake was 0.2 g/Kg die) and a second group, as controls ($n = 7$), assumed oral placebo. Conconi's test demonstrated a significant difference ($p < 0.005$) in the exercise performance of the two sub-groups, comparing the measurements of ratios of deflection velocity (V_d), before and after the treatment. Therefore we studied the athletes performing a muscular exercise test (40 Km/h, cycle race, for 90 min) after one month of treatment. During this treatment period the subjects followed a well standardized diet. Samples of blood were drawn before, at the end and during the recovery (60 min) to study if traditional biochemical parameters varied and confirmed the observed differences in Conconi's test. The measurements of concentrations of FFA, KB, free carnitine, acetyl-carnitine and BCAA were performed. Plasma BCAA levels did not demonstrate variations either before or after the exercise performance, or between the two groups. The biochemical factors, substrates and hormones, KB, FFA, lactate, insulin and growth hormone plasma levels did not demonstrate significant differences from the patterns present in literature. Plasma free and acetyl-carnitine followed the well known variations, but only acetyl-carnitine levels demonstrated, at the end

of exercise, a significant ($p < 0.05$) difference between the two groups. Plasma acetyl-carnitine levels varied, before and at the end of exercise, from 3.4 ± 2.5 μM to 18.0 ± 3.1 μM in the controls ($n = 7$), and from 1.8 ± 1.6 μM to 11.5 ± 2.7 μM in the BCAA treated athletes ($n = 6$).

Present results seem to confirm that BCAA treatment might influence the exercise performance studied with training parameters, both with acetyl-carnitine as well as with a physiological parameter as in Conconi's test. A complete explanation of the hypothesis that BCAA treatment could influence the energy fuel mobilization during exercise requires further investigation.

Keywords: Amino acids – Branched chain amino acids – Conconi's test – Acetyl-carnitine – Carnitine – Muscular exercise

Introduction

Carbohydrates and lipids constitute the major energy sources for muscular exercise requirement. Von Liebig's original idea (1842), that proteins provide important contribution as muscle energy substrate, has been avoided and re-elaborated (Von Liebig, 1842; Krogh et al., 1920). In fact it is recognized that the energy sources, principally used by the organism during physical exercise, are carbohydrates, lipids, and their intermediate products. The amino acid fuel supply estimation, during prolonged exercise, ranges from 5 to 10% of total energy requirement (Brooks, 1987; Poortmans 1984). Nevertheless this fact does not reduce the important role of amino acids during muscular exercise and recovery (Blomstrand et al., 1988; Devlin et al., 1990; Einspahr et al., 1989; Lemon, 1987; Adibi, 1980). It is difficult to estimate, in a quantitative sense, the roles played by amino acids in sustaining exercise, but they seem able to influence physical performance. The very wide linkage with different metabolic pathways, in different tissues, can be one of the causes of this difficulty.

The essential nutrient branched chain amino acids (BCAA), leucine (Leu), isoleucine (Ileu) and valine (Val), unlike other indispensable amino acids, are oxidized extensively at a significant rate in skeletal muscle. Recent literature shows clearly that the BCAA metabolic pathway and the inter-organ co-operation are very complex (Abumrad et al., 1989; Harper et al., 1990; Hood et al., 1990).

Since we observed that Conconi's test, namely the measurement of the deflection velocity (V_d), demonstrated a significant difference ($p < 0.005$) in the exercise performance, comparing the individual V_d ratios after and before treatment ($V_{d-\text{after}}/V_{d-\text{before}}$), of the treated sub-group of athletes with the $V_{d-\text{after}}/V_{d-\text{before}}$ of the controls, we decided to investigate also some of the biochemical circulating variables.

In fact researches in the whole-human body usually employ sources, as well as intermediate products, as energy circulating measurement; and it also might help in studying the inter-organ substrates exchange. Then, in trying to understand the different substrate significance, some useful biochemical circulating factors might give important molecular signals. BCAA treatment might be direct energy substrate fuel, stored in muscular cells, or better might have an influence

on the metabolic pathways of energy fuels; these fuels obviously being proteins and also carbohydrates or lipids. To collect biochemical signs, we studied athletes (treated and untreated) before, during and after physical performance.

Plasma carnitine and acetyl-carnitine, in our purposes, may be one of these biochemical variables, both in the molecular inter-organ energy exchange co-operation, as well as in BCAA role comprehension. Carnitine in fact has an important function as acyl carrier across mitochondria membrane, then allowing the fatty acids entry into the mitochondria for their oxidation (Cerretelli et al., 1990; Hiatt et al., 1989; Siliprandi et al., 1989). The principal function of carnitine and acetyl-carnitine can be summarized in: a) free fatty acids carrier, for the β-oxidation, into mitochondria, b) acetyl molecular group reservoir, c) enhancing the metabolic flux in the Krebs cycle by sparing free coenzyme-A, d) affecting pyruvate dehydrogenase stimulation by acetyl-CoA/CoA ratio.

Possibly the most important single intermediate – which is shared in all categories of metabolism and which hence provides a pivotal linkage – is acetyl-coenzyme-A. The two-Carbon fragment can be derived from the catabolism of lipids, as well as glucose, and certain amino acids. Then also acetyl linked compounds, such as acetyl-carnitine, may be an important biochemical factor (Sahlin et al., 1990).

Branched chain amino acids (valine, isoleucine and leucine) in their metabolism may become a fuel source for the muscle, then an acetyl compound source, via transamination and oxidative decarboxylation (Adibi, 1980; Kasperek et al., 1985) (Fig. 1). Aim of present work is to investigate if observed influence on the physiological Conconi's test made by a branched chain amino acids chronic treatment is confirmed by blood circulating biochemical energy parameter level, such as acetyl-carnitine, variations.

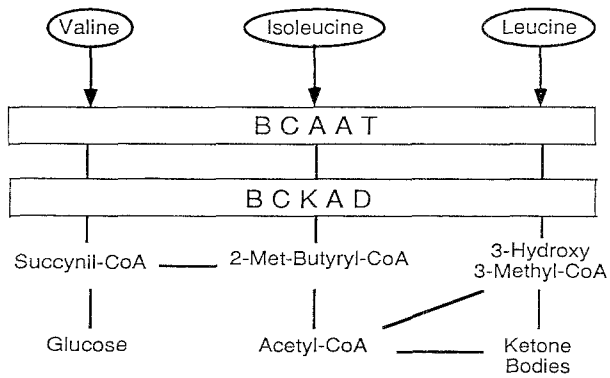

Fig. 1. Proposed schematic outline of BCAA metabolism. Enzymes: branched-chain amino acid amino trasferase (*BCAAT*), 2-keto acid dehydrogenase (*BCKAD*); Coenzyme A (*CoA*)

Material and methods

Subjects and treatment

This study was carried out on fourteen cyclists, all well trained, male subjects following the same trial agonistic programme before and during the period study, their ages ranged from

19 to 25 years (average = 21). Their average weight was 67 Kg and average height was 175 cm. All subjects volunteered to participate after being fully informed of the nature and purpose of this research.

The athlete group was randomly divided into two subgroups: first group ($n = 7$) was chronically treated with Friliver (Leu = 12%, Ileu = 6%, Val = 6% – w/w; 4 Kcal/g, kindly supplied by Bracco Industria Chimica SpA Milano, Italy) 0.2 g/die per Kg; second group ($n = 7$), controls, assumed placebo.

The one month BCAA chronic treatment was completed 2 days before the physical exercise test. During the treatment period the subjects followed a well standardized diet and for three days before the physical exercise test, all athletes assumed an iso-caloric diet: 45 Kcal/Kg die (63.5% carbohydrates, 22% lipids, 14.5% proteins).

Physical exercise test

In the morning, 3 hours before the test, all athletes assumed a 650 Kcal breakfast (74.5% carbohydrate, 15.7% fat, 9.7% protein). The muscular exercise test began with a light warm-up load (5–10 min) followed by a 40 Km/h cycling exercise for 90 min in a cycle-racing track.

Conconi's test

The test, recently developed by Conconi (Conconi et al., 1982; Droghetti et al., 1985) consisted in the measurement of the deflection velocity (V_d), called the velocity at which the linearity of the racing speed heart rate relationship (RS-HR) is lost. The relationships between the racing speeds and the heart rates in cyclists were obtained by measuring the heart rate (HR) while the cyclist under study progressively increased his racing speed (RS). All, physically fit and trained cyclists, after the usual warm-up, were submitted to the test. The HR was determined with a Heartcorder system, the speed was increased and made in 12–16 stages of 335 m (cycle-track measurement). Each athlete used his own racing cycle; the gear chosen varied from subject to subject and was kept constant throughout the test. The velocity during the test was deduced by manual timing.

Analytical methods

Samples of blood were drawn by venepuncture immediately before the warm-up period, at the end of the muscular exercise and during the recovery times, respectively at 90 and 150 min after the beginning of the test. The serum or plasma was stored at −20 °C until analysed.

Concentrations of free fatty acids (FFA), β-OH-butyrate (Ketone Bodies, KB) were determined enzymatically by conventional methods using commercial reagent kits (Boehringer-Mannheim GmbH).

Free carnitine and acetyl-carnitine levels were measured in human plasma samples using a spectrophotometric and fluorimetric enzymatic modified method (De Palo et al., 1987). Two aliquots of each plasma sample (2 × 1.0 ml) were filtered on 30 KDaltons cut-off filters (Amicon – Centricon 30), and filtrate samples (2 × 0.4 ml) were together collected and directly used for the free carnitine and acetyl-carnitine assays.

Free carnitine assay: acetyl-CoA (3.68 mM) 0.05 ml, 5,5′-dithiobis (2-nitro-benzoic) acid (DTNB – 2.45 mM) 0.01 ml, phosphate buffer (pH 7.4 0.306 M) 0.01 ml, and ethylenedinitrilo tetra-acetic acid (EDTA 12.35 mM) 0.025 ml were added to each filtrate sample (0.150 ml). After 10 min at the constant temperature of 25 °C, carnitine acetyl-transferase (CAT, EC 2.3.1.7 – 19.6 IU/ml) (0.010 ml) was added to start the reaction and absorbance was measured at 415 nm. The absorbance was measured on microplate reader mod. 450 (Biorad). Reaction equilibrium was reached after 7 min and the absorbance variations (measurements made after 30 min) were reported on a standard curve (linear throughout the concentration assayed range 10–50 μM) to measure the sample concentrations. With regard to the plasma free carnitine assay, the within batch imprecision calculated (using replicated analysis) from

duplicate estimation of a patient sample, was 2%; the between batch imprecision, similarly calculated, was 6%. The mean concentration of healthy subjects was 34 ± 1 μM (mean ± SEM, $n = 24$). The assay has a minimum detection limit of 2 μM estimated from 3 standard deviation of a standard sample without carnitine.

Acetyl-carnitine assay: malic acid (100 mM) 0.010 ml, EDTA (25 mM) 0.0125 ml, nicotinamide adenine dinucleotide (NAD, free acid, grade II, 98%, 5 mM) 0.025 ml, coenzyme-A (CoA-SH free acid, grade I, lyophilized, 5 mM) 0.005 ml, malate dehydrogenase (MDH, EC 1.1.1.37 from pig heart mitochondrial suspension, 1 mg/ml, 1.2 U/ml) 0.005 ml, citrate synthase (CS, EC 4.1.3.7 condensing crystallized 1 mg/ml, 110 U/ml) 0.005 ml, and phosphate buffer (25 mM pH 7.4) 0.03 ml, were mixed to form a medium and 0.095 ml of this medium was added directly in a cuvette to filtrate sample (0.500 ml). Fluorescence intensity was then measured and 0.010 ml carnitine acetyl-transferase (CAT 80 IU/ml, 1 mg/ml) was added to start the reaction. It was then possible to measure by fluorescence (excitation wave 337 nm; emission wave 457 nm) the newly formed NADH in the solution. Equilibrium was reached after 7 min and the fluorescence intensity variations were reported on a standard curve (linear throughout 2.5–20.0 μM) to measure the sample concentrations. With regard to the acetyl-carnitine assay, the within batch imprecision calculated from duplicate estimation of a patient sample, was 10%; the between batch imprecision, similarly calculated, was 8%. The mean concentration of healthy subjects was 4.3 ± 0.3 μM (mean ± SEM, $n = 24$). The assay has a minimum detection limit of 0.2 μM estimated from 3 standard deviation of a standard sample without acetyl-carnitine.

Plasma amino acids were assayed, following a modified version of the method of Godel (Godel et al., 1984) and the indications of Deyl's review (Deyl et al., 1986).

Equipment and reagents: the HPLC system consisted of two (Model 114 Beckman) pumps controlled by a System-Gold (mod.406) apparatus software (IBM PS/2), a Beckman valve injector (mod.340) and loop 20 μl, a 5 μm Supelcosil (LC18 4.6 × 250 mm) column, a fluorometer detector (Perkin Elmer LS5; excitation at 320 nm; emission at 445 nm). Venous blood heparinised samples were briefly centrifuged (10 min 2000 g). The plasma was deproteinised, as soon as possible, with sulphosalicylic acid (30 mg/ml) and the supernatants were removed by centrifugation and stored at −20 °C until assay. The o-phtalaldehyde (OPA) reagent was prepared by dissolving 25 mg in 2.25 ml methanol, to which 0.025 ml 2-mercaptoethanol and 0.25 ml sodium borate/boric acid (0.5 M – pH 9.5) was added; the OPA reagent can be stored up to one month at 4 °C. The OPA reaction was carried out by mixing 0.5 ml of methanol, 0.43 ml of borate buffer, 0.01 ml of deproteinised plasma sample, 0.01 ml of internal standard (norvaline 0.5 nmol/ml) and 0.05 ml of OPA reagent. The reaction mixture was incubated for 4 min at room temperature, then 0.05 ml of this solution was added to 0.45 ml of phosphate buffer (25 mM pH 6.5) before the injection (0.02 ml). The standard amino acid solution was purchased by Sigma (cat. A 9781), internal standard and freeze reference human plasma samples (stored at −20 °C), were used for quality control purpose. Chromatographic separation was carried out using phosphate buffer (0.025 mM pH 6.5) solvent A and 0.025 M sodium phosphate buffer (pH 6.5) – acetonitrile (50:50) solvent B. The solvent flow rate was 1.2 ml/min. Table 1 shows the solvent A linear gradient changes. Peak areas were used for quantification using the fluorescence response-concentration, measured for each amino acid. The plasma amino acids (Glu, Ala, Val, Ileu, Phe, Leu, Ser, Arg) reference values were in agreement with fasting measurement carried out by Godel et al. 1984, results obtained with HPLC method.

The chemicals utilized were of analytical grade and the solvents of chromatographic grade.

Statistical analysis

The concentration values are indicated in tables and figures as means ± standard error. To determine the significance of change in plasma levels on muscular exercise, t-Student and ANOVA tests were used; the level of significance was set at $p < 0.05$.

Table 1. Chromatographic linear gradient conditions for HPLC separation and analysis of amino acids. Percent of buffer A in (A + B)

Time (min)	from (% buffer A)	to (% buffer A)	duration (min)
0.0	82.5	82.5	0.5
0.5	82.5	73.0	4.5
5.0	73.0	54.5	15.0
20.0	54.5	54.5	4.0
24.0	54.5	0.0	1.0
25.0	0.0	0.0	3.0
28.0	0.0	82.5	1.0

Results

Conconi's test ($V_{d-\text{after}}/V_{d-\text{before}}$) ratios demonstrated a significant difference in the exercise performance of the two sub-groups ($p < 0.005$), as Fig. 2 shows.

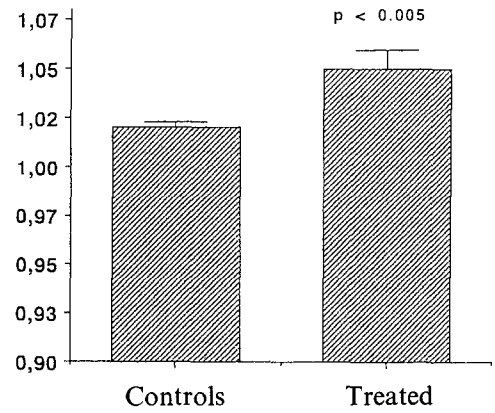

Fig. 2. Conconi's test: average ± standard error of V_d ratios after and before treatment (V_d-after/V_d-before) of the treated and control sub-groups of athletes

Table 2 shows the plasma and blood concentrations of the different measured compounds, in treated and control athletes; values are average ± standard error. Fig. 3 shows the profiles of plasma FFA and blood beta-hydroxybutyrate response in treated and untreated athletes to muscular exercise test. These biochemical traditional circulating parameters, as other substrates and hormones (blood glucose, lactic acid, alanine, growth hormone, insulin – data here not reported) do not demonstrate significant differences from the pattern usually present in literature. Fig. 4 shows the plasma BCAA profiles; plasma BCAA as well as the other traditional biochemical plasma (or blood) parameters do not seem to demonstrate variation either before or after the exercise performance, or between the two sub-groups. Fig. 5 shows the profiles of plasma carnitine and acetyl-carnitine; plasma free carnitine levels seem to decrease, though not significantly, at the end of the exercise and during the recovery in both the sub-

Table 2. Plasma FFA, BCAA, free carnitine and acetyl-carnitine, and blood β-OH-butyrate concentrations in treated and control cyclists

		basal	end	recovery
KB	Control	0.10 ± 0.06	0.12 ± 0.06	0.40 ± 0.18
	Treated	0.03 ± 0.01	0.09 ± 0.03	0.20 ± 0.06
FFA	Control	334 ± 35	906 ± 180	669 ± 109
	Treated	281 ± 34	875 ± 134	602 ± 58
Val	Control	366 ± 37	370 ± 53	383 ± 40
	Treated	365 ± 50	421 ± 60	327 ± 39
Leu	Control	169 ± 21	162 ± 19	131 ± 13
	Treated	159 ± 21	153 ± 20	136 ± 8
Ileu	Control	87 ± 11	87 ± 16	71 ± 8
	Treated	93 ± 12	91 ± 11	68 ± 9
Car	Control	34 ± 2	32 ± 1	32 ± 1
	Treated	37 ± 2	35 ± 2	32 ± 2
Ac-Car	Control	3.4 ± 1.0	18.0 ± 1.3 *	14.8 ± 3.1
	Treated	1.8 ± 0.6	11.5 ± 1.1 *	14.5 ± 1.3

Blood β-OH-butyrate (ketone bodies, *KB*, μM), plasma free fatty acids (*FFA*, μM), plasma valine (*Val*, μM), plasma leucine (*Leu*, μM), plasma isoleucine (*Ileu*, μM), plasma free carnitine (*Car*, μM) and plasma acetyl-carnitine (*Ac-Car*, μM) concentrations. Values are means ± standard errors * $p < 0.05$

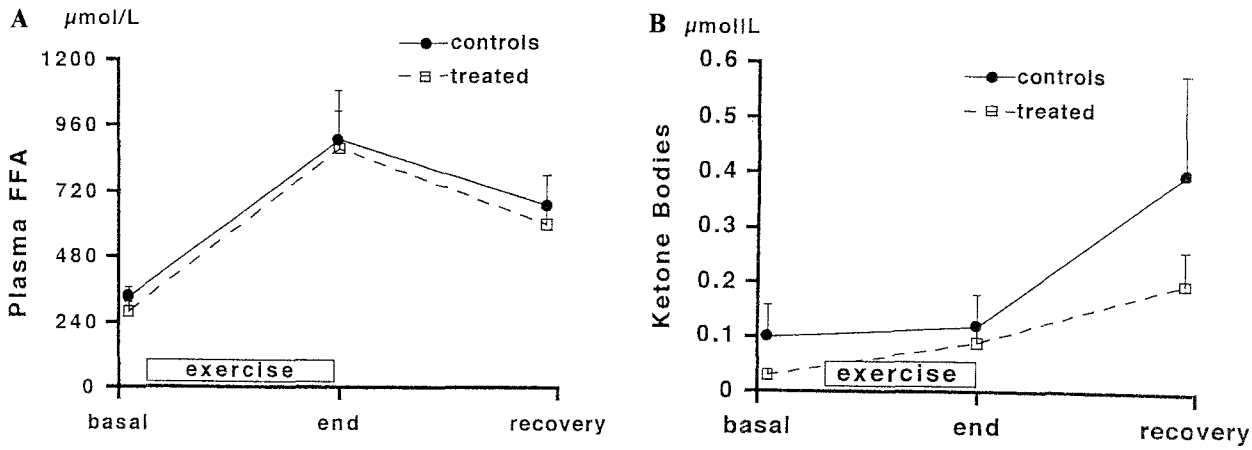

Fig. 3. Profiles of plasma FFA (**A**) and blood β-hydroxi-butyrate (**B**) concentrations in treated and control athletes at rest, at the end of the exercise (90 min) and during the recovery time (30 min post-exercise)

groups; only plasma acetyl-carnitine concentrations were significantly lower in the treated than in control athletes at the end of the exercise ($p < 0.05$).

Discussion

A BCAA treatment, made on a group of athletes kept under observation, at a Conconi's test demonstrated a slight influence on the physical performance

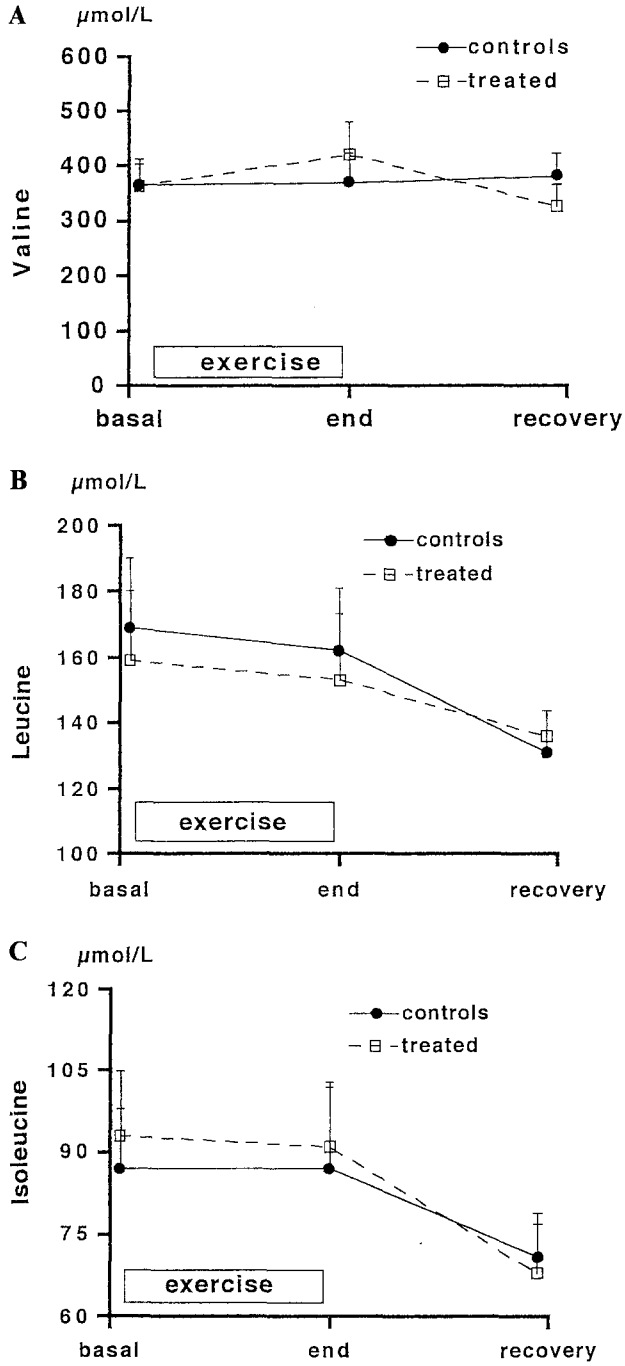

Fig. 4. Profiles of plasma valine (**A**), leucine (**B**), isoleucine (**C**) concentrations in treated and control athletes at rest, at the end of the exercise (90 min) and during the recovery time (30 min post-exercise)

(Fig. 2) so new biochemical investigation is needed. After studying this athletic group performing a muscular exercise test under BCAA treatment, in our opinion, a necessary further biochemical variable investigation might be needed to understand better this phenomenon. Present data seems to confirm only par-

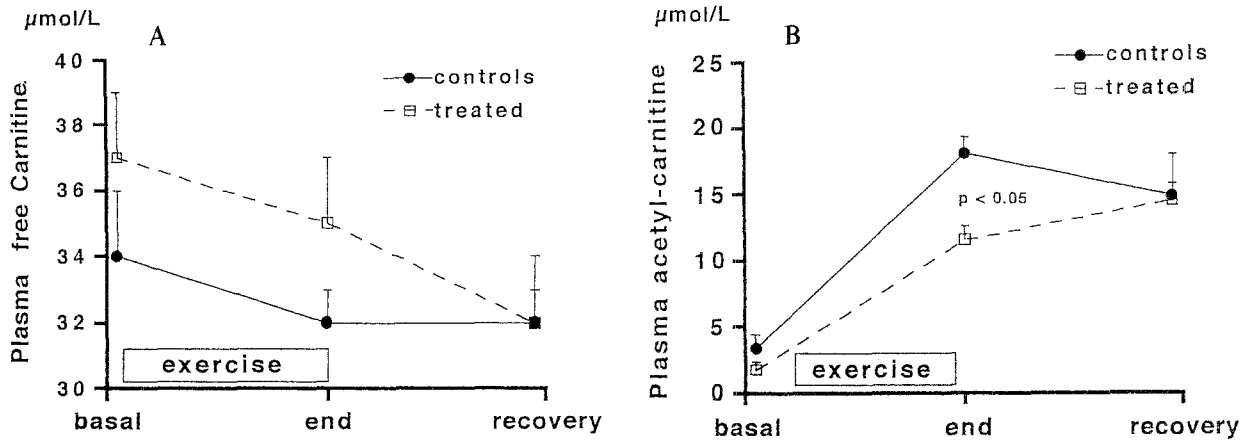

Fig. 5. Profiles of plasma free carnitine (**A**) and acetyl-carnitine (**B**) concentrations in treated and control athletes at rest, at the end of the exercise (90 min) and during the recovery time (30 min post-exercise)

tially the treatment effect observed on the physiological test. On the contrary they confirm the difficulty of indicating clearly, in vivo, in a quantitative sense, the biochemical role played by BCAA chronic treatment in sustaining physical performance.

Perhaps our new experimental observation has thrown only little light on the complexity of the metabolic BCAA and carnitine study, which in any case remains extremely difficult to "disentangle" and understand. Present study confirms an increase in FFA and carnitine acetyl ester levels after muscular exercise (Lennon et al., 1986; Oyono-Enguelle et al., 1988). Furthermore an inter-organ cooperative substrate significant variation, as might be acetyl-carnitine (Lennon et al., 1986), seems to suggest that a chronic BCAA treatment may influence the energy fuel substrate uses. The significant acetyl-carnitine level measured variations, comparing BCAA treated athletes with untreated ones, could be an interesting signal. The well known acetyl crucial role in different metabolic pathways, principally in its activated form as well as acetyl-coenzyme-A, and acetyl-carnitine, may confirm the utility of this biochemical factor as an inter-organ cooperative molecule.

We propose that after BCAA chronic treatment the acetyl-carnitine plasma levels variations, measured in present study, could be viewed either as enzyme influenced activity or/and as substrate itself supply availability. 1) *Enzyme activity*: like pyruvate dehydrogenase (PDH), also branched chain keto acid dehydrogenase (BCKAD) is a tightly regulated multi-enzyme complex and by phosphorylation it may pass from activated to inactivated form; in a fatigue state, BCKAD as PDH might be influenced by the energy ratio signals (acetyl-CoA/CoA, NADH/NAD, ATP/ADP) (May et al., 1989; May et al., 1980) and carnitine through acetyl-carnitine might help in sparing coenzyme-A. Then a smaller increase in acetyl-carnitine levels might be related to a minor fatigue situation perhaps present in BCAA treated athletes, as suggested in Fig. 5. 2) *Substrate supply*: we could suppose an increased BCAA availability, as muscular tissue pool, able to be directly used in the muscular tissue; then a smaller

increase in acetyl-carnitine levels could be related to a minor fatigue situation and to a larger energy supply availability perhaps present in BCAA treated athletes (Sahlin et al., 1990; May et al., 1989). Both mentioned hypothesis seem in concordance with a smaller acetyl-CoA substrate accumulation, or better for present study, is even more successful with athletes who give a better physical performance. In fact Conconi's test in the two sub-groups of athletes seems to suggest that BCAA treated athletes were able to give a better performance, furthermore out of curiosity we point out that the athletes treated with BCAA won more races than the untreated.

We would also like to add in conclusion that although confirming the difficulties of studies in the whole-body, our work gives an interesting clue about the possibility to use acetyl-carnitine plasma levels to understand the biochemical importance of the BCAA as substrate able to influence physical performance, but further research is needed.

The phenomenon presence might be showed better perhaps by studying untrained groups during prolonged exercise and with physical performance at exhaustion. If treatment were able to help the physical performance and to shift the fatigue, then confirmation might be a less raised plasma acetyl-carnitine level. In effect blood ammonium levels in present study did not demonstrate any variation in and between sub-groups; this latter observation could be caused by the quantity of work load, and training state of the athletes (Ji et al., 1987; Kirkendall, 1990). Moreover, as observed by Hageloch et al. (1990), the ammonia increases less during prolonged endurance exercise, and in fact the athletes of present study were all middle distance racing cyclists, and the physical performance was a prolonged endurance exercise.

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