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ATP synthesis kinetic properties of mitochondria isolated from the rat extensor digitorum longus muscle depleted of creatine with β-guanidinopropionic acid

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Abstract

A creatine analogue, β-guanidinopropionic acid (β-GPA), was administered in the food (1% w/w) of 8 male rats while 8 control rats received a standard diet. Mitochondrial oxidative capacity and kinetic parameters of mitochondrial ATP synthesis, apparent maximal ATP synthesis rate (V_{max}) and apparent Michaelis constant for free ADP (K_m), were investigated in the extensor digitorum longus (EDL) muscle. Mitochondrial ATP synthesis rate was measured by a bioluminescent method over a large range of ADP concentration (2–30 μM). As a result of the diet, V_{max} was significantly increased ($P < 0.05$) while K_m remained unchanged at around 20 μM. Citrate synthase (CS) and 3-hydroxyacyl-CoA dehydrogenase activities were significantly increased (both $P < 0.05$). V_{max} was tightly correlated with CS activity ($P < 0.001$; $r = 0.84$). It was concluded that the increase in maximal mitochondrial ATP synthesis rate after β-GPA feeding in EDL muscle was essentially due to a general increase in mitochondrial enzyme concentrations.

Key words: Fast-twitch skeletal muscle; Muscle, skeletal; β-Guanidinopropionic acid; Mitochondron; ATP synthesis rate; Kinetic parameter

1. Introduction

ATP hydrolysis is the main source of energy used by muscle cells in order to maintain homeostasis and perform muscular work. The major metabolic pathways whereby ATP is produced are glycolysis and mitochondrial oxidative phosphorylation. This latter process involves oxidation of reducing equivalents through an intramitochondrial multistep enzymatic process. ATP is made from ADP and P_i by the inner mitochondrial membrane bound F_1F_0-ATPase (for recent review see [1]). The precise control of mitochondrial oxidative phosphorylation in skeletal muscle depends on the energetic state of the cell, through ADP concentration, [ATP]/[ADP] and [ATP]/[ADP]·[P_i] ratios (for review see [2]).

An alteration in the energetic state of the cell can easily be obtained by feeding rats with β-guanidinopropionic acid (β-GPA), a competitive inhibitor of the creatine delivery across the sarcolemmal membrane [3]. This diet reduces the muscular store of phosphocreatine (PCr) and ATP by 90% and 50%, respectively [4], and leads to an accumulation of the phosphorylated form of the analogue in the muscle [5,6]. However, this phosphorylated β-GPA cannot substitute for PCr, since it is a poor substrate for creatine kinase [7], determining an impaired capacity of skeletal muscle to provide energy from PCr breakdown [7]. Surprisingly, the isometric twitch characteristics of fast-twitch muscles are unchanged: twitch amplitude, rise and half relaxation times are virtually the same in the plantaris muscle of control and β-GPA-fed rats [8]. A decrease in O_2 diffusion distance from capillary to tissue, a decrease in glycolytic capacity and an increase in mitochondrial aerobic capacity could explain the unaltered fast-twitch muscle contractile characteristics [9]. However, the effects of a β-GPA diet on mitochondrial ATP synthesis capacity have not been studied.

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To what extent can the β-GPA diet affect the kinetic properties of mitochondrial ATP synthesis in order to provide a greater amount of ATP from mitochondrial oxidative phosphorylation? This was investigated by analyzing the effects of a 6-week 1% β-GPA diet on the kinetic parameters of mitochondrial ATP synthesis, apparent maximal ATP synthesis rate (V_{max}) and apparent Michaelis constant for free ADP (K_m), in the rat extensor digitorum longus (EDL) muscle.

2. Materials and methods

Animal care and feeding. Animal protocol was approved by the Ministère de l'Agriculture et de la Forêt. Sixteen 10-week-old Sprague Dawley male rats weighing initially 235–265 g were caged individually in a temperature controlled room (21°C). One week later, animals were randomly assigned to two groups of 8 rats, fed for 6 weeks, either by a standard rat chow diet, or by the same diet containing 1% (w/w) of the creatine analogue, β-GPA (Sigma, St. Louis, MO). Animals were allowed food and water ad libitum. Food intakes and body weights were monitored daily throughout the diet period.

Muscle samples. At the end of the diet period, EDL muscles were removed under halothane anesthesia (3% in N_2/O_2 1:3) and weighed. Then, one part of the muscle was frozen in liquid nitrogen to be used later for the analysis of metabolite concentrations and enzyme assays in the whole muscle, another part being directly used for enzyme assays in the mitochondrial suspension after extraction of mitochondria.

Metabolite measurements. Freeze-dried samples were dissected from connective tissue and extracted with 0.5 M perchloric acid. The neutralized extracts were measured fluorometrically for creatine and PCr concentrations [10]. ATP concentration was measured by a bioluminescent method [11]. Assays were done in triplicate at 25°C and results reported as $\mu mol\ g^{-1}$ of dry muscle.

Free ADP concentration was calculated on the basis of the near-equilibrium properties of the creatine kinase reaction, using the measured values of creatine, PCr and ATP concentrations. The equilibrium constant used for the creatine kinase reaction at 38°C was $1.66 \cdot 10^9\ M^{-1}$ with a free Mg^{2+} concentration of 1 mM and a pH of 7 [12]. For these calculations, it was assumed that the total creatine pool was in a near equilibrium state and the intracellular pH similar in the muscles of control and β-GPA-fed rats [6,13]. Free ADP concentrations were reported as μM in order to make them comparable to the K_m values. The dry to fresh muscle weight ratio was assumed to be 27.5% and 28.6% for control and β-GPA-fed rats, respectively [14].

Enzyme assays in the whole muscle. Frozen tissue was used for all assays. Muscle samples (~ 20 mg) were homogenized in 15 volumes of 100 mM phosphate buffer solution (pH 7.5), consisting of 5 mM $MgCl_2$, 20 mM β-mercaptoethanol and 2 mM EDTA. Homogenates were frozen and thawed three times to disrupt the mitochondrial membrane. Fluorometric measurements of citrate synthase (CS, EC 4.1.3.7) and 3-hydroxyacyl-CoA dehydrogenase (HAD, EC 1.1.1.35) activities were made in triplicate at 25°C in the crude homogenate, using 1% Triton X-100 [15]. Results were expressed as $\mu mol\ min^{-1}\ g^{-1}$ of wet muscle.

Enzyme assays in the mitochondrial suspension. Buffer solutions used have been previously described [16]. EDL muscle samples were rapidly weighed (30–40 mg) and homogenized in Tris buffer. Then, after nagarse digestion, mitochondria were extracted [17] and suspended in an isotonic solution.

Mitochondrial ATP synthesis rate was measured in the mitochondrial suspension by a bioluminescent method as follows (see also [16]): 5 μl of mitochondrial suspension at a suitable dilution (1:500 w/v) was added to a cuvette containing 400 μl of an ATP monitoring reagent (LKB-Wallac, Pharmacia, Finland), an excess of inorganic phosphate (35 mM) and ADP at the concentrations of 2.43, 4.86, 7.29, 9.69, 14.55, 19.41 and 29.10 μM, successively. The reaction was started by adding 15 μl of pyruvate-malate at a final concentration of 1.0 mM for both substrates. Due to a residual activity of adenylate kinase in the ATP monitoring reagent [16] and in the mitochondrial suspension, a measurement without pyruvate-malate was done before each assay. This blank was then subtracted from the global rate, to give mitochondrial ATP synthesis rate. Assays were done in duplicate at 25°C (pH 7.5), using an LKB 1251 luminometer (LKB-Wallac, Pharmacia, Finland). Expression of mitochondrial ATP synthesis rate is described below.

CS activity was measured in the mitochondrial suspension (CS_{ms}), after disruption of the mitochondrial membrane by 1% Triton X-100 and three successive freeze-thawings [15]. CS_{ms} activity was expressed as $nmol\ min^{-1}\ \mu l^{-1}$ of mitochondrial suspension.

Expression of mitochondrial ATP synthesis rate. Mitochondrial ATP synthesis rate measured in the mitochondrial suspension was expressed as nmol ATP $min^{-1}\ \mu l^{-1}$ of mitochondrial suspension. In order to refer mitochondrial ATP synthesis rate to the whole muscle (expressed as μmol ATP $min^{-1}\ g^{-1}$ of wet muscle), the rate of ATP synthesis measured in the mitochondrial suspension was divided by the CS_{ms}/CS ratio, which defines the mitochondrial extraction efficiency of the isolation procedure.

Data analysis and statistics. For technical reasons, data of 14 rats were used in the present study (7 control rats and 7 β-GPA-fed rats). V_{max} and K_m

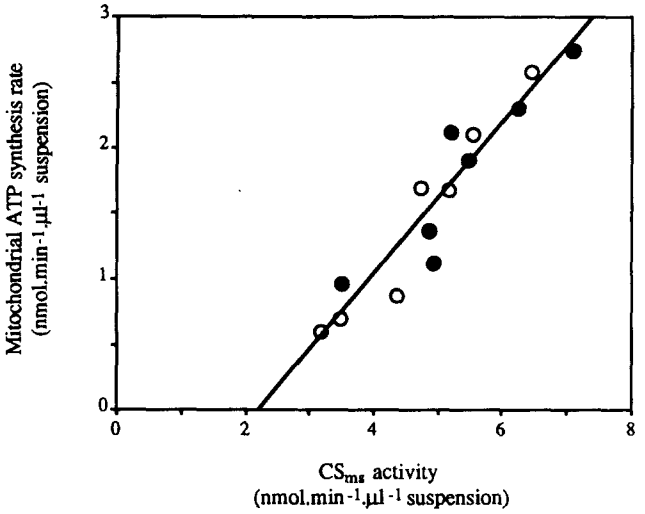

Fig. 1. A plot of mitochondrial ATP synthesis rate vs. citrate synthase (CS_{ms}) activity in the extensor digitorum longus muscle of control (○) and β-GPA-fed rats (●), for an ADP concentration of 7.29 μM. Measurements were made in the suspensions of isolated mitochondria, as described in Section 2. Data are representative of individual animals. The solid line is the best least squares fit to the experimental points ($P < 0.001$; $r = 0.94$).

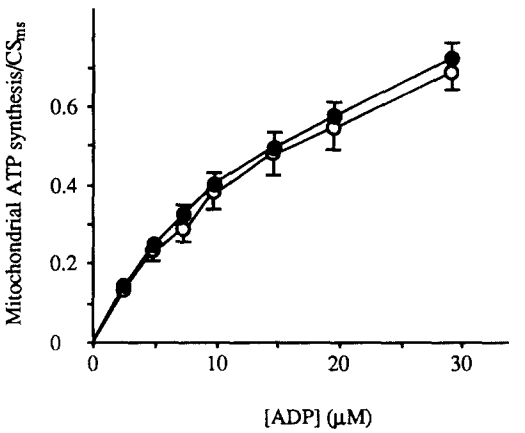

Fig. 2. Effect of ADP concentration on mitochondrial ATP synthesis rate related to citrate synthase activity (mitochondrial ATP synthesis/CS_{ms}) in the extensor digitorum longus muscle of control (○) and β-GPA-fed rats (●). Measurements were made in the mitochondrial suspensions, as described in Section 2. Note that the ratio was not significantly different between control and β-GPA-fed rats, whatever the ADP concentration used. Values are means ± S.E. of 7 rats. Standard errors not shown are within symbol size.

values were inferred for each rat from a Lineweaver-Burk plot, where 1/mitochondrial ATP synthesis rate (expressed as μmol ATP min^{-1} g^{-1} of wet muscle) was plotted against 1/ADP concentration. The best curve fit to the experimental points was obtained by linear regression analysis (Figs. 1, 3 and 4).

Mean values were compared using unpaired Student's *t*-test. Significant level was defined as $P < 0.05$.

3. Results

3.1. Body and muscle weights

Body weights were not significantly different in control rats (269 ± 10 g) and in β-GPA-fed rats (260 ± 17 g). A significant decrease ($P < 0.01$) in the wet EDL muscle weight of β-GPA-fed rats was observed (132.4 ± 11.4 vs. 117.6 ± 7.8 mg for control and β-GPA-fed rats, respectively). This atrophy is generally reported in fast-twitch muscles after β-GPA feeding [9,18].

3.2. Metabolite concentrations, CS and HAD activities (Table 1)

As a result of the diet, creatine, PCr and ATP concentrations were reduced by 51% ($P < 0.001$), 74% ($P < 0.001$) and 26% ($P < 0.001$) of their control values, respectively, whereas calculated free ADP concentration was significantly increased ($P < 0.01$).

Both CS and HAD activities increased significantly ($P < 0.05$).

3.3. Mitochondrial ATP synthesis kinetic parameters (Table 1; Figs. 1–4)

Mitochondrial ATP synthesis rate (expressed per μl of mitochondrial suspension) and CS_{ms} activity were

Table 1
Metabolite concentrations, enzymatic activities and mitochondrial ATP synthesis kinetic parameters in the extensor digitorum longus muscle of control and β-GPA-fed rats

	Metabolite concentrations				Enzymatic activities		Kinetic parameters	
	Creatine	PCr	ATP	Free ADP	Citrate synthase	3-Hydroxyacyl-CoA dehydrogenase	V_{max}	K_m
Control	36.3 ± 7.3	87.9 ± 7.2	30.3 ± 1.9	15.1 ± 3.3	9.3 ± 1.6	1.49 ± 0.29	10.1 ± 2.9	19.5 ± 6.8
β-GPA-fed	17.8 ± 2.7 ***	22.5 ± 3.1 ***	22.4 ± 0.8 ***	22.1 ± 4.9 **	12.2 ± 2.1 *	1.90 ± 0.38 *	14.2 ± 4.0 *	18.9 ± 4.6

Metabolite concentrations and enzyme activities were measured as outlined in Section 2. Free ADP concentration was calculated on the basis of the creatine kinase reaction equilibrium properties, assuming $K_{eq} = 1.66 \cdot 10^9$ M^{-1}, a free Mg^{2+} concentration of 1 mM and a pH of 7 [12]. V_{max} and K_m values were inferred from Lineweaver-Burk plots (for experimental details, see Section 2). Measured metabolite concentrations are given in μmol g^{-1} of dry muscle, free ADP concentration and K_m in μM. Enzymatic activities and V_{max} are expressed in μmol min^{-1} g^{-1} of wet muscle. PCr, phosphocreatine; ATP, adenosine triphosphate; ADP, adenosine diphosphate; V_{max}, apparent maximal ATP synthesis rate; K_m, apparent Michaelis constant for free ADP. Values are means ± S.D. of 7 rats. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$: β-GPA effect.

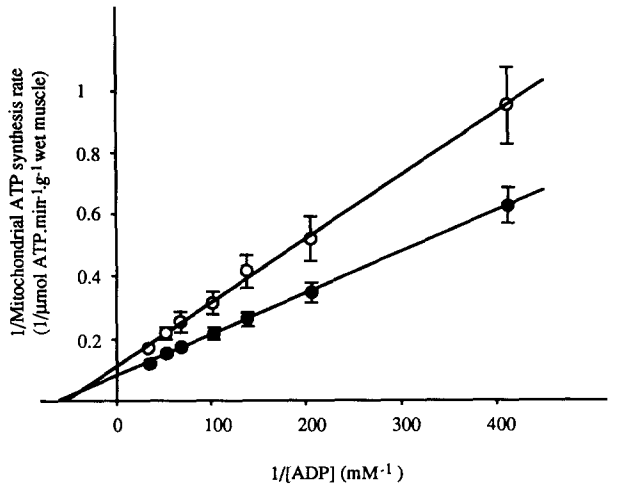

Fig. 3. Lineweaver-Burk plots showing in vitro the dependence of mitochondrial ATP synthesis rate towards ADP concentration, in the extensor digitorum longus muscle of control (○) and β-GPA-fed rats (●). Mitochondrial ATP synthesis rate was measured in the mitochondrial suspensions and was recalculated to muscle mass (see Section 2). The solid lines are the best least squares fit to the experimental points ($P < 0.001$; $r = 0.999$). Values are means ± S.E. of 7 rats. Error bars not shown are within symbol size.

tightly correlated over the whole range of ADP concentrations. Fig. 1 illustrates the correlation obtained with an ADP concentration of 7.29 µM. When mitochondrial ATP synthesis rate, standardized for each suspension by its own CS_{ms} activity (mitochondrial ATP synthesis-to-CS_{ms} ratio), was plotted against ADP concentration, the relationship then described a rectangular hyperbola (Fig. 2). Whatever the ADP concentration used, the mitochondrial ATP synthesis-to-CS_{ms} ratio was not significantly different between control and β-GPA-fed rats.

V_{max} and K_m values were inferred from individual Lineweaver-Burk plots. All individual correlation coefficients were at least 0.993. As a result of the diet, V_{max} was significantly increased ($P < 0.05$), whereas K_m remained unchanged at around 20 µM (Table 1). Lineweaver-Burk plots illustrating the dependence of the mitochondrial ATP synthesis rate towards the added ADP concentration are shown in Fig. 3, for control and β-GPA-fed rats. V_{max} and CS activity were significantly correlated ($P < 0.001$; $r = 0.84$) (Fig. 4).

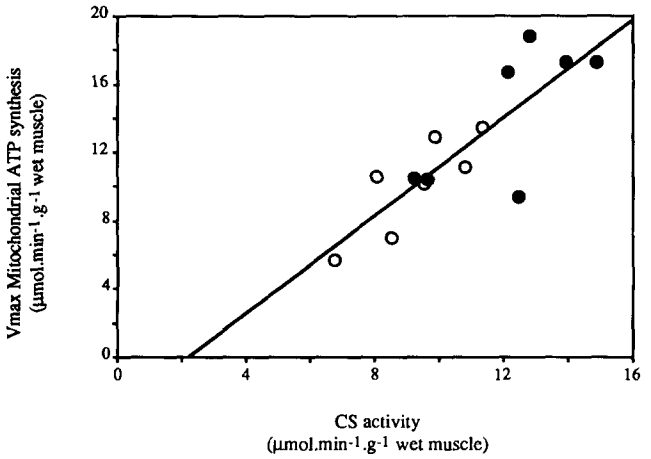

Fig. 4. A plot of maximal mitochondrial ATP synthesis rate (V_{max} mitochondrial ATP synthesis) vs. citrate synthase (CS) activity in the extensor digitorum longus muscle of control (○) and β-GPA-fed rats (●). V_{max} was inferred from Lineweaver-Burk plots and CS activity was measured in homogenates of frozen muscles (see Section 2). Data are representative of individual animals. The solid line is the best least squares fit to the experimental points ($P < 0.001$; $r = 0.84$).

4. Discussion

As a result of the diet, the whole muscle maximal capacity for mitochondrial ATP synthesis, measured using isolated mitochondria with pyruvate-malate as substrate, was greatly increased (+40%), whereas affinity of mitochondria towards ADP remained unchanged.

These kinetic parameters were inferred from Lineweaver-Burk plots, assuming a Michaelis-Menten kinetic for the response of mitochondria. This kinetic behavior was observed by other authors [19,20] who analyzed the relationship between ADP concentration and oxygen consumption in isolated mitochondria. However, deviation from this kinetic behavior was reported on bovine phosphorylating submitochondrial particles for which curvilinear Eadie-Hofstee plots ($v/[S]$ vs. v) were obtained, over a large range of ADP concentration (1–1200 µM) [21,22]. Our data do not fit curvilinear Eadie-Hofstee plots in control as well as in β-GPA-fed rats. This fact may be explained, in addition to the smaller variable range of ADP concentration used here (2–30 µM vs. 1–1200 µM), by the different kinetic response of isolated mitochondria and phosphorylating submitochondrial particles towards ADP. In isolated mitochondria, other factors such as NADH supply or the kinetic properties of the adenine nucleotide translocase could be dominating in the activation of mitochondrial ATP synthesis rate [2,20,23]. Therefore, the kinetic parameters reported here cannot be identified as those of the mitochondrial F_1F_0-ATPase alone. In such conditions, it was suggested that K_m would probably be a property of the adenine nucleotide translocase [20], whereas V_{max} should reflect maximal ATP flux through either the translocase or the F_1F_0-ATPase.

The K_m of control rats, 19.4 µM, is within the range of values measured using polarographic procedure [19,20] in suspensions of isolated mitochondria. The unchanged K_m of β-GPA-fed rats, 18.9 µM, indicates that the affinity of isolated mitochondria towards ADP remains unchanged. Since free ADP concentration was

significantly increased from 15.1 ± 3.3 μM in the control rats to 22.1 ± 4.9 μM in the β-GPA-fed rats, the strict implication of these data is that the mitochondrion is 38% (control) and 55% (β-GPA-fed) saturated with ADP at rest. To play a regulatory role on enzyme activity, free ADP concentration must be below its K_m value. This is only observed for control rats, allowing in principle a greater scope for an increase in free ADP concentration to increase mitochondrial ATP synthesis rate than in β-GPA-fed rats. Thus, in EDL muscle of β-GPA-fed rats, a change in free ADP concentration should not play a significant role in the regulation of mitochondrial oxidative phosphorylation.

The increase in V_{max} was associated with a rise in CS and HAD activities. Changes in CS and HAD activities generally parallel changes in the content of cytochrome *c* and other mitochondrial enzymes [24] and changes in the mitochondrial volume [25,26]. Thus, in the present study, the increase in CS and HAD activities is probably part of a more general increase in mitochondrial enzyme content and in mitochondrial volume. Besides, ATP synthesis rate measured in the mitochondrial suspension at any ADP concentration was correlated with CS_{ms} activity and when expressed relative to CS_{ms} activity, the hyperbolic relationship was the same in control and β-GPA-fed rats. In the whole muscle, the inferred V_{max} of mitochondrial ATP synthesis was also correlated with CS activity, both being increased after β-GPA feeding. Taken together, our data suggest that a quantitative change, i.e., a general increase in mitochondrial enzyme concentrations, mainly occurs in EDL muscle of β-GPA-fed rats to increase mitochondrial ATP synthesis rate. A stimulation of mitochondrial biogenesis has been proposed to explain such variations in enzyme concentrations [9,27]. Changes in the effective level of mitochondrial enzymes, by changing enzyme concentrations, could also modify other parameters which act on mitochondrial ATP synthesis rate. For example, the mitochondrial membrane potential at maximum ADP concentration could be higher in EDL muscle of β-GPA-fed rats due to an increase in NADH supply and in the content of cytochrome elements. Quantification of the control strength, in the terminology of metabolic control theory [28,29], which exerts a particular step on mitochondrial ATP synthesis flux, would be necessary to further determine the source of the observed increase in mitochondrial ATP synthesis rate.

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