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Energetic status and mitochondrial oxidative capacity of rat skeletal muscle in response to creatine analogue ingestion

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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 for 6 weeks, while 8 control rats received a standard diet. Mitochondrial oxidative capacity and cytosolic modulators of mitochondrial oxidative phosphorylation (free ADP, ATP-to-free ADP ratio) were evaluated in the soleus and extensor digitorum longus (EDL) muscles. Mitochondrial adaptation to the diet was significantly different between muscles. Citrate synthase activity and mitochondrial ATP synthesis rate were 35 and 45% higher in EDL muscle, respectively, whereas they were virtually unchanged in the soleus muscle. In both muscles, 3-hydroxyacyl-CoA dehydrogenase activity remained unaffected. Regardless of muscle type, creatine, phosphocreatine and ATP concentrations, as well as the total adenine nucleotide content (ATP + ADP + AMP), were significantly lower in β-GPA fed rats. Whereas free ADP concentration remained unchanged, a significantly greater decrease in ATP-to-free ADP ratio was observed in EDL than in the soleus muscle. It is suggested that regulation of mitochondrial oxidative phosphorylation, through changes in metabolite concentrations, could be an important factor to consider for mitochondrial adaptation induced by β-GPA feeding.

Keywords: β-Guanidinopropionic acid; Skeletal muscle; Muscle contraction; Mitochondrion; Mitochondrial biogenesis; Oxidative phosphorylation

1. Introduction

Biochemical and physiological characteristics of animal and human skeletal muscle are capable of profound modification according to imposed functional demand. Thus, an increased muscle contractile activity, as initiated by endurance training or chronic electrical stimulation, leads to an increase in mass and volume of mitochondria associated with a parallel increase in most of the mitochondrial enzyme activities [1,2].

Chronic feeding of β-guanidinopropionic acid (β-GPA), a structural analogue of creatine which decreases phosphocreatine (PCr) and ATP concentrations in rat skeletal muscle [3], also increases mitochondrial oxidative potential. Using this experimental model, Shoubridge et al. [4] reported a 30–40% increase in the oxidative capacity of fast-twitch muscles, as reflected by the increased citrate synthase (CS) activity (per unit of muscle weight) in the rat gastrocnemius and plantaris muscles. However, the oxidative capacity of slow-twitch muscles (soleus and heart muscles) remained unchanged. It was proposed that the mechanism by which β-GPA ingestion could alter the phenotypic expression of aerobic enzymes in a tissue-specific manner could be mediated by the β-GPA inducing changes in the energetic status of the muscle [5,6]. Moreover, other studies have shown that changes in adenine nucleotide concentrations stimulate mitochondrial biogenesis through an increase in mitochondrial oxidative phosphorylation [7,8]. Thus, the putative modulators of mitochondrial respiration, i.e. the cytosolic free ADP concentration, the cytosolic ATP-to-free ADP ratio and the cytosolic phosphorylation potential (ATP/(free ADP · free P_i)), could postulate as cellular signals leading to the phenotypic changes observed after β-GPA feeding.

Our objective here was to examine the changes in mitochondrial oxidative capacity in relation to modulators of mitochondrial respiration in the soleus and EDL muscles of rats fed with β-GPA for 6 weeks.

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2. Materials and methods

Animal care and feeding. Animal protocol was approved by the Ministère de l'Agriculture et de la Forêt. Sixteen male Sprague Dawley rats (10 weeks old) weighing initially 235–265 g were individually caged in a temperature controlled room (21°C). 1 week later, animals were randomly assigned to two groups of 8 rats, fed on for 6 weeks either with a standard rat chow diet or with 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 intake and body weight were daily monitored throughout the diet period.

Muscle samples. At the end of the diet period, the soleus (fibre I predominant) and EDL (fibre IIb predominant) muscles [9] were removed under halothane anesthesia (3%, in N_2/O_2 1:3). After weighing, one part of the muscle was frozen in liquid nitrogen to be later used 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.

Metabolites measurement. Freeze-dried samples were dissected from connective tissue and extracted with 0.5 M perchloric acid. Creatine and PCr concentrations were measured enzymatically from neutralised extracts [10]. ATP and ADP concentrations were measured by bioluminescence [11,12]. Determination of AMP concentration was done from the same neutralised extracts with the use of ion-pair reversed-phase high-performance liquid chromatography, as detailed in previous publications [13,14]. Assays were done in triplicate at 25°C. Results were expressed per μmol g^{-1} of dry muscle.

Calculations. The cytosolic free ADP concentration was estimated on the basis of the near-equilibrium properties of the creatine kinase reaction, using the measured concentrations of creatine, PCr and ATP [15]. 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 [15]. For this calculation, it was assumed that the total creatine pool was in a near equilibrium state and that the intracellular pH was similar in control and β-GPA-fed rat muscles [16,17]. Free ADP concentration was expressed as μmol g^{-1} of dry muscle. The cytosolic ATP-to-free ADP ratio, was calculated using the measured ATP and estimated free ADP concentrations.

Enzyme assays in the whole muscle. Frozen tissue was used for all assays. For CS (EC 4.1.3.7), 3-hydroxyacyl-CoA dehydrogenase (HAD, EC 1.1.1.35) and hexokinase (HK, EC 2.7.1.1) assays, muscle samples (~ 20 mg) were homogenised in 15 volumes of 100 mM phosphate buffer (pH 7.5), consisting of 2 mM EDTA, 5 mM $MgCl_2$ and 20 mM β-mercaptoethanol. Homogenates were frozen and thawed three times to disrupt the mitochondrial membrane. CS and HAD activities were determined after adding 1% Triton X-100. Fluorometric measurements were done in triplicate at 25°C in the crude homogenate [18,19]. Results were expressed as μmol min^{-1} g^{-1} of wet muscle. Total muscle CS activity (μmol min^{-1}) was obtained by multiplying CS activity by muscle weight.

Proteins measurement. Homogenate protein concentrations of the soleus and EDL muscles (mg per g of wet muscle) were determined according to Lowry et al. [20]. Total muscle protein concentration (mg per whole muscle) was obtained by multiplying homogenate protein concentration by muscle weight.

Enzyme assays in the mitochondrial suspension. Buffer solutions have been previously described [21]. Soleus and EDL muscle samples were rapidly weighed and homogenised in Tris buffer. Then, after Nagarse digestion, mitochondria were extracted [22] and suspended in an isotonic solution.

Mitochondrial ATP synthesis rate was determined by bioluminescence using pyruvate-malate as substrate [21,23]. Assays were done in duplicate at 25°C (pH 7.5) using a LKB 1251 luminometer (LKB Wallac, Pharmacia, Finland). Expression of mitochondrial ATP synthesis rate is described below.

CS activity was measured [18] in the mitochondrial suspension (CS_{ms}), after disruption of the mitochondrial membrane by 1% Triton X-100 and three successive freeze-thawings.

Expression of mitochondrial ATP synthesis rate. In order to refer mitochondrial ATP synthesis rate to the whole muscle (expressed as μmol 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. An analysis of variance with two factors, muscle (soleus and EDL) and diet (con-

Table 1
Muscle weights and protein concentrations in the soleus and extensor digitorum longus (EDL) muscles of control and β-GPA fed rats

Parameter	Soleus muscle		EDL muscle		Effects		
	control ($n = 8$)	β-GPA fed ($n = 7$)	control ($n = 8$)	β-GPA fed ($n = 7$)	muscle	diet	interaction
Muscle weight mg	116.7 ± 12.7	105.4 ± 10.4	134.5 ± 12.1	117.6 ± 7.8	$P < 0.001$	$P < 0.01$	n.s.
Muscle protein							
mg prot/g muscle	145.7 ± 10.5	142.1 ± 16.2	154.3 ± 21.7	141.9 ± 12.8	n.s.	n.s.	n.s.
mg prot per whole muscle	16.9 ± 1.9	15.0 ± 2.2	20.7 ± 3.3	16.7 ± 1.9	$P < 0.01$	$P < 0.01$	n.s.

Protein concentrations were measured according to Lowry et al. [20]. Number of animals is shown in parentheses. Values are means ± S.D. n.s., non-significant.

Table 2
Enzymatic activities in the soleus and extensor digitorum longus (EDL) muscles of control and β-GPA fed rats

Enzymatic activities	Soleus muscle		EDL muscle		Effects		
	control ($n = 8$)	β-GPA fed ($n = 7$)	control ($n = 8$)	β-GPA fed ($n = 7$)	muscle	diet	interaction
CS	9.8 ± 0.7	9.6 ± 1.3	9.0 ± 1.7	12.2 ± 2.1	n.s.	$P < 0.05$	$P < 0.01$
Mitochondrial ATP synthesis rate	7.4 ± 1.2	7.5 ± 1.8	6.1 ± 1.9	8.9 ± 2.2	n.s.	$P < 0.05$	$P < 0.05$
HAD	3.00 ± 0.59	3.17 ± 0.82	1.48 ± 0.27	1.90 ± 0.38	$P < 0.001$	n.s.	n.s.
HK	1.61 ± 0.26	1.95 ± 0.44	1.23 ± 0.18	1.69 ± 0.20	$P < 0.01$	$P < 0.001$	n.s.
Total muscle CS activity	1.14 ± 0.17	1.01 ± 0.20	1.21 ± 0.26	1.42 ± 0.20	$P < 0.01$	n.s.	$P < 0.05$

Enzymatic activities were measured in the homogenates of frozen muscles. Mitochondrial ATP synthesis rate was measured in the mitochondrial suspension and recalculated to muscle mass. For experimental details see Materials and Methods. Results are given in μmol min^{-1} g^{-1} of wet muscle. Total muscle citrate synthase (CS) activity is given per μmol min^{-1} for the whole muscle. HAD, 3-hydroxyacyl-CoA dehydrogenase; HK, hexokinase. Number of animals is shown in parentheses. Values are means ± S.D. n.s., non-significant.

trol and β-GPA fed), was used. The reported P values refer to significant main effects for muscle and diet, as well as interaction effects. Mean body weights were compared using unpaired t-test. All results are presented as means ± S.D. Significant level was defined as $P < 0.05$.

3. Results

3.1. Body and muscle weights, protein concentrations (Table 1)

Body weights of control (269 ± 10 g) and β-GPA fed rats (260 ± 17 g) were not significantly different. Muscle weight was higher in EDL than in the soleus muscle ($P < 0.001$) and lower in β-GPA fed than in the control rats ($P < 0.01$). Muscle type and diet status did not affect muscle homogenate protein concentration (expressed per gram of muscle). Therefore, variations in total muscle protein concentration (expressed on a per muscle basis) reflected those of muscle weight.

3.2. Enzymatic activities (Table 2)

CS activity and mitochondrial ATP synthesis rate were significantly affected by the diet (both $P < 0.05$). As reflected by the significant interaction effects, CS activity and mitochondrial ATP synthesis rate did not respond in a similar way to the diet in the soleus and EDL muscles. In EDL muscle, CS activity and mitochondrial ATP synthesis rate increased by 35 and 45%, respectively, whereas in the soleus muscle they remained virtually unchanged. HAD activity was not affected by the diet, but was significantly higher in the soleus than in EDL muscle ($P < 0.001$). HK activity was higher in the soleus than in EDL muscle ($P < 0.01$) and higher in β-GPA fed than in the control rats ($P < 0.001$).

The diet did not modify total muscle CS activity. This is probably due to an opposite evolution in the soleus (11% decrease) and EDL (17% increase) muscles. This was reflected by a significant interaction effect ($P < 0.05$).

3.3. Metabolite analysis (Table 3)

A clear pattern emerges from the movement of high energy phosphates in the soleus and EDL muscles of rats receiving β-GPA. Regardless of muscle type, creatine, PCr and ATP concentrations were significantly decreased by the diet, whereas the measured ADP concentration was significantly increased. The measured AMP concentration remained unchanged. The total adenine nucleotide content (ATP + ADP + AMP) was significantly decreased by the

Table 3
Metabolite concentrations in the soleus and extensor digitorum longus (EDL) muscles of control and β-GPA fed rats

Metabolic parameters	Soleus muscle		EDL muscle		Effects		
	control ($n = 8$)	β-GPA fed ($n = 7$)	control ($n = 8$)	β-GPA fed ($n = 7$)	muscle	diet	interaction
Creatine	38.8 ± 7.2	8.0 ± 3.8	36.2 ± 6.7	17.8 ± 2.7	n.s.	$P < 0.001$	$P < 0.01$
PCr	55.8 ± 5.6	7.1 ± 2.1	88.2 ± 6.7	22.5 ± 3.1	$P < 0.001$	$P < 0.001$	$P < 0.001$
ATP	22.9 ± 1.4	15.0 ± 2.1	30.2 ± 1.8	22.4 ± 0.8	$P < 0.001$	$P < 0.001$	n.s.
ADP	3.6 ± 0.1	4.1 ± 0.3	3.4 ± 0.1	3.7 ± 0.1	$P < 0.001$	$P < 0.001$	n.s.
AMP	0.120 ± 0.025	0.155 ± 0.041	0.134 ± 0.046	0.122 ± 0.034	n.s.	n.s.	n.s.
ATP + ADP + AMP	26.3 ± 1.3	18.9 ± 2.4	34.2 ± 1.6	26.2 ± 0.9	$P < 0.001$	$P < 0.001$	n.s.
Free ADP	0.098 ± 0.030	0.109 ± 0.062	0.075 ± 0.015	0.108 ± 0.024	n.s.	n.s.	n.s.
ATP/free ADP	249.4 ± 65.4	171.4 ± 77.1	417.5 ± 81.6	216.7 ± 54.1	$P < 0.001$	$P < 0.001$	$P < 0.05$

Metabolite concentrations were measured as outlined in Materials and Methods. Free ADP concentration was calculated on the basis of the near-equilibrium properties of the creatine kinase reaction [15,23]. All metabolite concentrations are given in μmol per g of dry muscle. Number of animals is shown in parentheses. Values are mean ± S.D. n.s., non-significant.

diet ($P < 0.001$) and was higher in EDL than in the soleus muscle ($P < 0.001$). The diet determined a significant greater creatine decrease ($P < 0.01$) and a lower PCr decrease ($P < 0.001$) in the soleus than in EDL muscle.

Whereas the diet did not change the calculated free ADP concentration in both muscles, the cytosolic ATP-to-free ADP ratio was significantly decreased by the diet in the soleus and EDL muscles ($P < 0.001$). However, a significant greater decrease in the cytosolic ATP-to-free ADP ratio was observed ($P < 0.05$) in EDL than in the soleus muscle.

4. Discussion

In agreement with previous studies regarding the effects of β-GPA on skeletal muscle enzyme activities [4,24], the adaptations of enzymes reported in the present study were muscle specific. In EDL muscle, the diet had a pronounced effect on the level of aerobic enzymes which, except for HAD, were all significantly increased. HK activity also increased in a similar way, suggesting, in agreement with other authors [4], that a coordinate regulation of enzymes involved in aerobic metabolic pathway occurs in fast-twitch muscles of β-GPA fed rats. Taking into account the reported decreases in some key glycogenolytic enzyme activities [4], these data suggest a marked shift of the ratio between the capacities of glycolytic and oxidative pathways in EDL muscle towards an increased aerobic capacity. In contrast to EDL muscle, treatment with β-GPA had no apparent effect on the soleus muscle oxidative capacity, as reflected by the unchanged mitochondrial enzyme activities.

The increased aerobic enzyme activities in EDL muscle of β-GPA fed rats might involve an elaboration of the mitochondrial reticulum, or a decrease in fibre size (especially type IIb fibre) in absence of mitochondrial proliferation, or both [4]. Although a relative increase in mitochondrial content due to reduction in type IIb fibre size [25] cannot be excluded, our data suggest that the large increase in the aerobic enzyme activities would be also partly due to a mitochondrial biogenesis. Indeed, whereas weight loss and decrease in total protein concentration were similar in both muscles, total muscle CS activity increased in EDL muscle but decreased in the soleus muscle. Furthermore, evidences for an altered transcriptional level of mitochondrial proteins were previously reported in type IIb muscle of rats fed with β-GPA [26]. A mechanism by which β-GPA feeding could alter the phenotypic expression of aerobic enzymes in a tissue-specific manner is discussed below.

As previously described [25], one of the main effect of β-GPA feeding on high energy phosphate concentrations was the reduction in the total adenine nucleotide content (ATP + ADP + AMP) of the soleus and EDL muscles. It has been suggested [16] that the chronic decrease in creatine and PCr concentrations might lead to chronically elevated ADP and AMP concentrations, resulting in a greater basal rate of AMP deamination, and therefore to a reduced total adenine nucleotide content. In contrast to this assumption, lowered AMP deaminase activities were reported in slow- and fast-twitch muscles of rats fed with β-GPA [27]. However, these lowered activities did not change the level of IMP accumulation in response to contractil activity [27]. The significance of such a result in the regulation of the adenine nucleotide content in muscles of rats fed with β-GPA is unclear and remains to be elucidated.

We have hypothesised that the mitochondrial adaptation observed in the muscles of β-GPA fed rats could be mediated by the creatine analogue inducing changes in the cytosolic modulators of mitochondrial respiration. Therefore, the free ADP concentration and the cytosolic ATP-to-free ADP ratio were calculated [15,23,28]. In contrast to free ADP concentration, which was unchanged in both muscles, a significantly greater decrease in the ATP-to-free ADP ratio was observed in EDL than in the soleus muscle after β-GPA feeding. Furthermore, assuming that the muscle resting free P_i concentrations remain unchanged after β-GPA feeding [16,17,24], the response of cytosolic phosphorylation potential (ATP/(free ADP · free P_i)) to β-GPA feeding should be similar to that of ATP-to-free ADP ratio. Thus, in contrast to the soleus muscle, the increase in mitochondrial content observed in EDL muscle of β-GPA fed rats was associated with a dramatic decrease in the cytosolic energetic status. Since early changes in the phosphagen pool are observed as early as the 12th day of β-GPA ingestion before the adaptations in muscle enzyme activities take place [29], it is possible that the present dramatic decrease in the cytosolic energetic status in EDL muscle of β-GPA fed rats might trigger the changes in mitochondrial oxidative capacity. A time-course study would be necessary to clearly evaluate the differences in muscle types regarding the effects of β-GPA on metabolite concentrations. A mechanism which stimulates mitochondrial biogenesis through changes in the cytosolic concentrations of metabolites has already been observed. Transcription of the human mitochondrial genome is directly influenced by ATP and ADP concentrations and further enhanced in presence of an oxidizable substrate [7]. Mitochondrial protein translation has also been shown to be tightly coupled through substrate oxidation [8]. Thus, in the present study, an increased mitochondrial oxidative phosphorylation, resulting from a decrease in cytosolic potential, might enhance mitochondrial biogenesis.

Our results show that the increased mitochondrial oxidative capacity observed in EDL muscle after 6 weeks of β-GPA feeding was associated with a dramatic decrease in some cytosolic modulators of mitochondrial respiration. Such a response was not observed in the soleus muscle. This could suggest that the regulation of mitochondrial oxidative phosphorylation, through changes in cytosolic

metabolite concentrations, is important to consider for mitochondrial adaptation induced by β-GPA feeding.

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