Differential effects of endurance training and creatine depletion on regional mitochondrial adaptations in rat skeletal muscle

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To examine the combined effects of 2-week endurance training and 3-week feeding with β -guanidinopropionic acid (GPA) on regional adaptability of skeletal muscle mitochondria, intermyofibrillar mitochondria (IFM) and subsarcolemmal mitochondria (SSM) were isolated from quadriceps muscles of sedentary control, trained control, sedentary GPA-fed and trained GPA-fed rats. Mitochondrial oxidative phosphorylation was assessed polarographically by using pyruvate plus malate, succinate (plus rotenone), and ascorbate plus N,N,N',N' -tetramethyl-*p*-phenylenediamine (TMPD) (plus antimycin) as respiratory substrates. Assays of cytochrome *c* oxidase and F_1F_0 -ATPase activities were also performed. In sedentary control rats, IFM exhibited a higher oxidative capacity than SSM, whereas F_1F_0 -ATPase activities were similar. Training increased the oxidative

phosphorylation capacity of mitochondria with both pyruvate plus malate and ascorbate plus TMPD as substrates, with no differences between IFM and SSM. In contrast, the GPA diet mainly improved the overall SSM oxidative phosphorylation capacity, irrespective of the substrate used. Finally, the superimposition of training to feeding with GPA strongly increased both oxidase and enzymic activities in SSM, whereas no cumulative effects were found in IFM mitochondria. It therefore seems that endurance training and feeding with GPA, which are both known to alter the energetic status of the muscle cell, might mediate distinct biochemical adaptations in regional skeletal muscle mitochondria.

Key words: β -guanidinopropionic acid, intermyofibrillar, mitochondrial respiration, oxidative capacity, subsarcolemmal.

INTRODUCTION

Skeletal muscles adapt to regularly performed endurance exercise by increasing mitochondrial density and oxidative capacities [1]. Among numerous factors proposed as possible triggering signals for mitochondrial biogenesis [2], the small but repetitive changes in ATP homeostasis during contractile activity represent an attractive candidate [3,4]. Indeed, high-energy phosphate depletion induced by supplementation with β -guanidinopropionic acid (GPA) decreases skeletal muscle ATP and phosphocreatine levels to 50% and 90% respectively [5] but enhances citrate synthase activity, as well as other mitochondrial enzyme activities, in non-exercised fast-twitch skeletal muscles [6]. Such an increase in oxidative capacity is similar to that reported after an intensive endurance training programme [1,2]. Even though these mitochondrial adaptations usually take 10–12 weeks to reach full expression, it has been confirmed that daily running for only 1–2 weeks [7–9] or GPA supplementation for only 3 weeks [10] are sufficient to significantly improve the oxidative capacity of muscles. In the present study we wished to determine whether a combination of training and energy depletion for a short-term period (2 weeks of training and 3 weeks of GPA diet) resulted in cumulative effects on skeletal muscle properties. By using such mild conditioning protocols, we expected (1) to decrease the energy phosphate pool substantially, by 70% [11], but prevent crystalline mitochondrial inclusion caused by prolonged administration of GPA [6] and (2) to allow the mitochondrial responses to combined treatment (training plus diet) to develop fully within skeletal muscle, because mitochondrial adaptations are unfulfilled under conditions of these two short-term treatments.

Another feature of skeletal muscle is that two mitochondrial populations located in spatially distinct regions exhibit distinct

biochemical and functional properties [12–15]. On the basis of their location within the myocyte, they have been termed subsarcolemmal mitochondria (SSM) and intermyofibrillar mitochondria (IFM): the SSM reside beneath the sarcolemma, whereas the IFM lie between myofibrils. However, to the best of our knowledge, no information is available on the responses to short-term endurance training or creatine depletion in the biochemical properties of IFM and SSM. Thus the first goals of the present study were to examine the oxidative phosphorylation capacity of SSM and IFM in response to (1) 2 weeks of endurance training and (2) 3 weeks of feeding with GPA. In addition, because earlier studies had illustrated that the two populations adapt differently to change after long-term endurance training [15,16], we wished to determine whether adaptations in oxidative phosphorylation after short-term endurance training, as well as GPA supplementation alone or in combination with endurance training, occurred in parallel in these two mitochondrial populations.

EXPERIMENTAL

Animals

The present investigation was performed in accordance with the guiding principles in the care and use of animals. Male Wistar rats, obtained at 5 weeks of age (100–120 g) from IFFA-CREDO (Centre de Recherche et d'Élevage du Domaine des Oncins de l'Institut Français Fièvre Aphteuse, Saint-Germain sur l'Arbresle, France) were housed two per cage in a temperature-controlled room (23 °C) with a dark/light cycle of 12 h:12 h. They were provided with a standard rat chow (A03; UAR, Villemoisson, France) and tap water *ad libitum* and were

Abbreviations used: CK, creatine kinase; COX, cytochrome *c* oxidase; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; GPA, β -guanidinopropionic acid; IFM, intermyofibrillar mitochondria; M-CK, muscle cytosolic creatine kinase; OPR, oxidative phosphorylation rate; RCR, respiratory control ratio; SSM, subsarcolemmal mitochondria; TMPD, N,N,N',N' -tetramethyl-*p*-phenylenediamine.

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acclimatized to their new environment for 1 week. Thereafter, half of the rats were treated once daily by force-feeding with the creatine analogue GPA (100 mg/ml) dissolved in distilled water. The other half group (control) received distilled water under the same conditions (1 ml/100 g body weight). Treatment with GPA was continued for 3 weeks.

Exercise training

Each group (control and GPA) was subdivided into two groups, trained and sedentary. At 1 week after the initiation of treatment, trained rats were subjected to endurance training on a motor-driven treadmill (TecMachine, Andrézieux-Bouthéon, France) as described previously [16]. Initially the rats ran for 30 min at 30 m/min on 0% slope. The duration of exercise was then increased progressively so that the animals were running for 50 and 70 min after 1 and 2 weeks of training respectively. Sedentary animals were exercised once a week for 10 min at 20 m/min on 0% slope. Although rats were exercised in a relatively high-speed programme, the GPA-fed rats exhibited no obvious exercise intolerance at this speed.

Rat skeletal muscle mitochondria

The animals were anaesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg). Samples were taken from the trained animals 48 h after the last bout of running, to exclude any acute effect of exercise on mitochondrial properties. The deep (red) portion of the quadriceps was removed, weighed and immediately placed into ice-cold buffer for the isolation of mitochondria. The animals were then killed by cervical dislocation. IFM and SSM were isolated from the quadriceps muscle by a modification of the differential centrifugation procedure of Palmer et al. [17]. Quadriceps muscles were quickly removed and placed in ice-cold isolation medium consisting of 100 mM sucrose, 50 mM KCl, 5 mM EGTA and 50 mM Tris/HCl, pH 7.4. Muscles were minced with scissors and homogenized with a Potter–Elvehjem homogenizer (five passages). The homogenate was centrifuged at 800 *g* for 10 min. The supernatant containing the SSM was centrifuged at 1000 *g* for 10 min and the pellet containing most of the IFM was resuspended in 40 ml of isolation medium. It was then treated with Nagarse (1 mg/g muscle wet weight) for 5 min in an ice bath. The mixture was diluted 1:2, homogenized and then centrifuged at 1000 *g* for 10 min. The resulting supernatants were filtered through cheesecloth and centrifuged at 10000 *g* for 10 min to obtain the IFM and SSM pellets. These pellets were washed twice by resuspension in the isolation medium and were re-centrifuged at 10000 *g* for 10 min. Finally, IFM and SSM pellets were stored in ice-cold medium consisting of 250 mM sucrose, 1 mM EGTA and 20 mM Tris/HCl, pH 7.4. Protein was determined by a biuret method with BSA as standard. All centrifugations were performed at 4 °C.

Mitochondrial enzyme activities

Cytochrome *c* oxidase (COX) was used as a marker enzyme for the mitochondrial oxidative capacity; F_1 -ATPase was used as a marker enzyme for the phosphorylative capacity of mitochondria. Muscle mitochondria were diluted in a medium containing 1 mM ATP, 100 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 5 mM EGTA and 50 mM Hepes/KOH, pH 7.4. Lubrol (100 mg/g of mitochondrial protein) was used to unmask enzyme activity in mitochondria that had been standing in ice for 30 min. COX activity was determined polarographically at 25 °C, with a Clark

oxygen electrode, in 1.5 ml of reaction medium containing 30 μ M cytochrome *c*, 4 μ M rotenone, 0.5 mM dinitrophenol, 10 mM sodium malonate and 75 mM Hepes/NaOH, pH 7.4 [18]. COX activity was the difference between the rate of oxygen consumption measured after the addition of mitochondria (0.15 mg of mitochondrial protein) plus substrates [8 mM ascorbate and 0.6 mM *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD)] and the rate of oxygen consumption measured after the addition of substrate alone to take the autooxidation of ascorbate into account. F_1 -ATPase activity was determined at 25 °C, with a spectrophotometric procedure in which the ADP formed was coupled to the pyruvate kinase and lactate dehydrogenase reactions [19]. The reaction mixture contained the following in a volume of 1 ml: 100 mM KCl, 5 mM $MgCl_2$, 0.1 mM EGTA, 5 mM ATP, 1 mM phosphoenolpyruvate, 0.2 mM NADH, 20 units of lactate dehydrogenase, 15 units of pyruvate kinase and 20 mM Hepes/KOH, pH 7.4. The reaction was initiated by the addition of mitochondria (20 μ g/ml mitochondrial protein). ATPase activity was monitored by a continuously recording spectrophotometer after the oxidation of NADH at 340 nm. Then 10 μ l of oligomycin (100 μ g/ml) was added to measure oligomycin-insensitive ATPase activity. The F_1 -ATPase activity was calculated as the total ATPase activity minus the oligomycin-insensitive ATPase activity. The amount of oligomycin-insensitive ATPase was less than 10% of the total in all groups.

Mitochondrial respiration

Oxygen was measured with a Clark oxygen electrode (oxygraph Gilson 5/6H), in a glass cell 1.5 ml in volume, thermostatically controlled at 25 °C, with constant stirring. The respiratory reaction medium, saturated with room air, contained 200 mM sucrose, 10 mM potassium phosphate tribasic and 20 mM Tris/HCl, pH 7.4, with a final fatty-acid-free BSA concentration of 2 mg/ml (0.2% w/v). Respiratory substrates (sodium salts) were succinate (10 mM), pyruvate (5 mM) plus malate (5 mM) and ascorbate (8 mM) plus TMPD (0.6 mM). Inhibitors of the electron transport chain were used to permit oxidative phosphorylation parameters of specific coupling sites to be measured. Thus the addition of 5 μ M rotenone to inhibit site 1 allowed sites 2 and 3 to be measured in the presence of 10 mM succinate. Antimycin (5 μ M) inhibited electron flow beyond sites 1 and 2 and permitted site 3 to be evaluated in the presence of 8 mM ascorbate plus 0.6 mM TMPD. The concentration of mitochondrial protein was 0.5 mg/ml. The controlled state of respiration (state 4) was initiated by the addition of 10 μ l of substrate; the active state of respiration (state 3) was initiated by the addition of 100 μ M ADP. The method of Estabrook [20] was used for the calculation of state 4, state 3 and the respiratory control ratio (RCR). The ADP-to-O ratio was calculated by measuring the total oxygen consumed during the phosphorylation of a pulse of ADP [20].

Assessment of oxidative phosphorylation

The efficiency of the mitochondrial oxidative phosphorylation process was assessed by the following parameters: (1) the ADP-to-O ratio, which measures the efficiency of the phosphorylating system; (2) the RCR, the ratio of oxygen consumed in the presence of ADP to that after phosphorylation of ADP, which measures the degree of control imposed on oxidation by phosphorylation; (3) the oxidative phosphorylation rate (OPR), calculated as the product of the ADP-to-O ratio and the oxygen consumed during state 3 respiration and expressed as nmol of ATP produced during state 3/min per mg of mitochondrial

protein [21]. It must be emphasized that the rate of ATP production derived from mitochondrial respiration, i.e. the OPR, has been previously demonstrated to be significantly correlated with the rate of mitochondrial ATP production derived from bioluminescent techniques [22].

Statistical analysis

Differences occurring within groups during the time course of the study were compared by a two-way analysis of variance with post-hoc comparisons performed with the Student–Newman–Keuls test of multiple comparisons. Differences between IFM and SSM derived from the same muscle were compared by paired *t* test. A difference of $P < 0.05$ was considered significant. Results are expressed as means $\pm$ S.E.M.

RESULTS

Animals

GPA-fed and control rats did not differ with respect to body weight at the beginning of our experimental protocol. Before being killed, GPA-fed rats weighed approx. 10% less than control rats (277 ± 11 g compared with 303 ± 7 g in the sedentary group, and 286 ± 6 g compared with 315 ± 6 g in the trained group respectively), with no differences between body weights of sedentary and trained rats in the same diet group.

Mitochondrial enzyme activities

In sedentary control rats, IFM exhibited a COX activity 1.3-fold than that in SSM, whereas F_1 -ATPase activities were similar in both mitochondrial populations (Table 1).

The COX and F_1 -ATPase activities were significantly affected by the training programme in both mitochondrial populations. Considering results in the control group, IFM COX and F_1 -ATPase activities were increased significantly, by 31% and 24% respectively, whereas a slight but not statistically significant 16% increase was noted in both enzymic activities in SSM. Regardless of the exercising group, these two enzymic activities were

significantly increased by GPA feeding in SSM, whereas they remained virtually unchanged in IFM. However, in the sedentary group, these slightly higher values in the GPA-fed SSM enzyme activities were not significantly different from the control. Interestingly, it is also noteworthy that the GPA-fed SSM displayed a higher F_1 -ATPase activity than their corresponding IFM population. This mainly resulted from the slight (16%), non-significant increase in SSM enzyme activity. When GPA feeding was superimposed on training, there was a significantly greater enhancement in both COX and F_1 -ATPase activities (45–50%) in SSM in comparison with the control rats. Furthermore, COX activity in SSM was so sharply increased by combining these two treatments that COX activity in the two mitochondrial populations from trained GPA muscles did not differ significantly. In contrast, COX and F_1 -ATPase were similarly increased by training alone or in combination with the GPA diet in IFM.

Mitochondrial oxidative phosphorylation

Tables 2 and 3 report the respiratory parameters of IFM (Table 2) and SSM (Table 3) isolated from quadriceps muscles of sedentary control, sedentary GPA-fed, trained control and trained GPA-fed rats. In comparison with the enzyme activities, the respiratory rates [state 3 and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP)-induced uncoupled state] and the phosphorylation rates (OPR values) in IFM were much higher than in SSM with any given substrate, regardless of experimental group. In sedentary control rats, IFM oxidized substrates at a level approx. 2.5-fold that of SSM. These differences might indicate a higher metabolite translocation in IFM (e.g. respiratory substrate or adenine nucleotide), which could be attributed to the higher rates of protein synthesis and protein import reported in IFM than in SSM [12,13].

All results considered, the two-way analysis of variance indicates that endurance training elicited significant increases in oxidative phosphorylation capacity in both mitochondrial populations by using pyruvate/malate and ascorbate/TMPD as oxidative substrates, with no differences between IFM and SSM. Training did not modify the rate of oxidative phosphorylation (state 3 respiration and OPR values) in mitochondria respiring

Table 1 Effects of endurance training and GPA feeding on mitochondrial enzyme activities

Both enzymic activities were determined at 25 °C in IFM and SSM as described in the Experimental section. Values are means $\pm$ S.E.M. for six to eight animals. Symbols indicate significant differences ($P < 0.05$) as follows: *difference from sedentary within the same diet group; †difference from control within the same exercising group; ‡difference from IFM. Abbreviation: n.s., not significant.

Mitochondria	Cytochrome <i>c</i> oxidase activity (nmol of oxygen/min per mg of protein)						
	Sedentary rats		Endurance-trained rats		Statistical effects of treatments		
	Control	β -GPA-fed	Control	β -GPA-fed	Training	Diet	Interaction
IFM	906 $\pm$ 85	973 $\pm$ 51	1188 $\pm$ 41*	1140 $\pm$ 61*	$P < 0.01$	n.s.	n.s.
SSM	682 $\pm$ 35‡	758 $\pm$ 37‡	796 $\pm$ 52‡	1029 $\pm$ 51*†	$P < 0.001$	$P < 0.01$	n.s.
Mitochondria	F_1 -ATPase activity (nmol of phosphate/min per mg of protein)						
	Sedentary rats		Endurance-trained rats		Statistical effects of treatments		
	Control	β -GPA-fed	Control	β -GPA-fed	Training	Diet	Interaction
IFM	709 $\pm$ 59	652 $\pm$ 57	880 $\pm$ 33*	878 $\pm$ 39*	$P < 0.01$	n.s.	n.s.
SSM	833 $\pm$ 31	973 $\pm$ 57‡	964 $\pm$ 49‡	1202 $\pm$ 26*†‡	$P < 0.05$	$P < 0.01$	n.s.

Table 2 Effects of endurance training and GPA feeding on respiratory parameters of IFM

Mitochondria were isolated from quadriceps muscles as described in the Experimental section. Respiration rates were determined at 25 °C in IFM with pyruvate/malate, succinate, and ascorbate/TMPD as respiratory substrates. The mitochondrial protein concentration was 0.5 mg/ml. Values are means $\pm$ S.E.M. for seven or eight animals. State 3, ADP-stimulated respiration (nmol of oxygen/min per mg of protein); OPR, oxidative phosphorylation rate (nmol of ATP/min per mg of protein); state 3unc, uncoupled respiration measured with 2 μ M FCCP (nmol of oxygen/min per mg of protein). Symbols indicate significant differences ($P < 0.05$) as follows: *difference from sedentary within the same diet group; †difference from control within the same exercising group. Abbreviation: n.s., not significant.

Respiratory substrates	Parameter	Sedentary rats		Endurance-trained rats		Statistical effects of treatments		
		Control	β -GPA-fed	Control	β -GPA-fed	Training	Diet	Interaction
Pyruvate/malate	State 3	144 $\pm$ 8	128 $\pm$ 9	163 $\pm$ 3*	153 $\pm$ 5*	$P < 0.01$	$P < 0.05$	n.s.
	RCR	6.0 $\pm$ 0.2	8.2 $\pm$ 1.2	6.8 $\pm$ 0.4	6.8 $\pm$ 0.5	n.s.	n.s.	n.s.
	ADP-to-O	2.62 $\pm$ 0.03	2.77 $\pm$ 0.09	2.64 $\pm$ 0.02	2.55 $\pm$ 0.08	n.s.	n.s.	n.s.
	OPR	377 $\pm$ 24	350 $\pm$ 14	430 $\pm$ 9*	389 $\pm$ 11*†	$P < 0.01$	$P < 0.05$	n.s.
	State 3unc	138 $\pm$ 10	133 $\pm$ 8	172 $\pm$ 4*	166 $\pm$ 6*	$P < 0.001$	n.s.	n.s.
Succinate + rotenone	State 3	184 $\pm$ 8	172 $\pm$ 11	197 $\pm$ 7	180 $\pm$ 10	n.s.	n.s.	n.s.
	RCR	3.6 $\pm$ 0.1	4.1 $\pm$ 0.3	3.5 $\pm$ 0.1	3.6 $\pm$ 0.3	n.s.	n.s.	n.s.
	ADP-to-O	1.47 $\pm$ 0.03	1.52 $\pm$ 0.06	1.48 $\pm$ 0.01	1.45 $\pm$ 0.05	n.s.	n.s.	n.s.
	OPR	270 $\pm$ 14	260 $\pm$ 11	291 $\pm$ 11	258 $\pm$ 12	n.s.	n.s.	n.s.
	State 3unc	221 $\pm$ 13	—	232 $\pm$ 6	—	—	—	—
Ascorbate/TMPD + antimycin	State 3	294 $\pm$ 18	285 $\pm$ 22	319 $\pm$ 9	335 $\pm$ 14	$P < 0.05$	n.s.	n.s.
	RCR	1.28 $\pm$ 0.02	1.33 $\pm$ 0.03	1.30 $\pm$ 0.02	1.23 $\pm$ 0.02	n.s.	n.s.	n.s.
	ADP-to-O	0.85 $\pm$ 0.01	0.83 $\pm$ 0.02	0.87 $\pm$ 0.01	0.77 $\pm$ 0.03†	n.s.	$P < 0.01$	n.s.
	OPR	250 $\pm$ 17	234 $\pm$ 14	277 $\pm$ 10	263 $\pm$ 11	$P < 0.05$	n.s.	n.s.
	State 3unc	339 $\pm$ 25	—	356 $\pm$ 19	—	—	—	—

Table 3 Effects of endurance training and GPA-feeding on respiratory parameters of SSM

Mitochondria were isolated from quadriceps muscles as described in the Experimental section. Respiration rates were determined at 25 °C in SSM with pyruvate/malate, succinate, and ascorbate/TMPD as respiratory substrates. The mitochondrial protein concentration was 0.5 mg/ml. Values are means $\pm$ S.E.M. for six or seven animals. State 3, ADP-stimulated respiration (nmol of oxygen/min per mg of protein); ADP-to-O ratio, phosphorylation efficiency; OPR, oxidative phosphorylation rate (nmol of ATP/min per mg of protein); state 3unc, uncoupled respiration measured with 2 μ M FCCP (nmol of oxygen/min per mg of protein). Symbols indicate significant differences ($P < 0.05$) as follows: *differences from sedentary within the same diet group; †differences from control within the same exercising group. Abbreviation: n.s., not significant.

Respiratory substrates	Parameter	Sedentary rats		Endurance-trained rats		Statistical effects of treatments		
		Control	β -GPA-fed	Control	β -GPA-fed	Training	Diet	Interaction
Pyruvate/malate	State 3	50 $\pm$ 4	57 $\pm$ 7	56 $\pm$ 3	74 $\pm$ 3*†	$P < 0.05$	$P < 0.01$	n.s.
	RCR	3.6 $\pm$ 0.3	6.6 $\pm$ 0.9†	4.1 $\pm$ 0.3	4.5 $\pm$ 0.2*	n.s.	$P < 0.001$	$P < 0.01$
	ADP-to-O	2.37 $\pm$ 0.07	2.66 $\pm$ 0.14	2.41 $\pm$ 0.03	2.39 $\pm$ 0.10	n.s.	n.s.	n.s.
	OPR	120 $\pm$ 14	147 $\pm$ 11	135 $\pm$ 8	181 $\pm$ 16†	$P \leq 0.05$	$P < 0.01$	n.s.
	State 3unc	47 $\pm$ 5	58 $\pm$ 6	57 $\pm$ 3	74 $\pm$ 3*†	$P < 0.01$	$P < 0.01$	n.s.
Succinate + rotenone	State 3	79 $\pm$ 7	84 $\pm$ 8	81 $\pm$ 4	99 $\pm$ 4†	n.s.	$P \leq 0.05$	n.s.
	RCR	2.1 $\pm$ 0.1	3.4 $\pm$ 0.7	2.2 $\pm$ 0.1	2.5 $\pm$ 0.2†	n.s.	$P < 0.05$	n.s.
	ADP-to-O	1.11 $\pm$ 0.02	1.33 $\pm$ 0.07†	1.15 $\pm$ 0.02	1.19 $\pm$ 0.02	n.s.	$P < 0.01$	$P < 0.05$
	OPR	88 $\pm$ 8	109 $\pm$ 5†	93 $\pm$ 4	117 $\pm$ 6†	n.s.	$P < 0.001$	n.s.
	State 3unc	83 $\pm$ 7	84 $\pm$ 8	91 $\pm$ 5	103 $\pm$ 5*	$P < 0.05$	n.s.	n.s.
Ascorbate/TMPD + antimycin	State 3	120 $\pm$ 10	130 $\pm$ 14	132 $\pm$ 4	167 $\pm$ 10*†	$P < 0.05$	$P < 0.05$	n.s.
	RCR	1.13 $\pm$ 0.03	1.38 $\pm$ 0.14	1.15 $\pm$ 0.03	1.17 $\pm$ 0.03	n.s.	n.s.	n.s.
	ADP-to-O	0.63 $\pm$ 0.05	0.68 $\pm$ 0.02	0.65 $\pm$ 0.01	0.63 $\pm$ 0.02	n.s.	n.s.	n.s.
	OPR	78 $\pm$ 11	88 $\pm$ 9	85 $\pm$ 3	107 $\pm$ 9†	n.s.	$P \leq 0.05$	n.s.
	State 3unc‡	161 $\pm$ 9	—	139 $\pm$ 4	—	—	—	—

‡ Values are means $\pm$ S.E.M. for two animals.

on succinate. Regardless of diet, 2 weeks of treadmill running was long enough to increase by 13 %, 14 % and 25 % the state 3 respiration, OPR values and uncoupled state respiration respectively in IFM respiring on pyruvate/malate, whereas the slightly higher values (+5–11 %) noted in IFM respiring on ascorbate/TMPD were not statistically significant for any of these three parameters (Table 2). Exercise training also increased the oxidative phosphorylation capacity of SSM, which was in the same order of magnitude as that in IFM (Table 3). There were no

training effects on ADP-to-O ratio or RCR in either mitochondrial population by using either pyruvate/malate or ascorbate/TMPD.

The present results also demonstrate that there was a significant effect of GPA diet in mitochondrial oxidative phosphorylation capacity (state 3 respiration and OPR values), that was mitochondria specific. Thus, regardless of exercising groups, SSM from GPA muscles exhibited greater state 3 respiration and OPR than those from controls (Table 3), whereas the rates of oxygen

consumption, and also the OPR, were in contrast lowered in IFM (Table 2). These results therefore did not depend on the substrates used. However, when one considers the results in the sedentary group only, there were no significant effects of diet on respiration in both mitochondrial populations (Tables 2 and 3). Table 3 also shows that RCR values were significantly increased by diet in SSM with any given substrate. This resulted in a smaller state 4 respiration rate (results not shown) concomitantly with a greater state 3 respiration rate. In contrast, the diet did not modify the RCR in IFM (Table 2).

Effects on mitochondrial respiratory parameters of the superimposition of exercise training on feeding with GPA

There was a marked difference in the respiratory responses of IFM and SSM when feeding with GPA was superimposed on training (Tables 2 and 3 respectively). In IFM, ADP-stimulated respiration (state 3), OPR values and the FCCP-induced uncoupled state of GPA-trained mitochondria were of the same order of magnitude as those of corresponding sedentary control mitochondria in all substrates tested. In contrast, the combination of training and feeding with GPA significantly increased these two first respiratory parameters by approx. 50%, 29% and 38% in SSM respiring on pyruvate/malate, succinate and ascorbate/TMPD respectively, becoming significantly greater than those measured in SSM from trained control muscles (Table 3). Similarly, the FCCP-induced uncoupled state was increased by 57% and 24% in GPA-trained SSM respiring on pyruvate/malate and succinate respectively. As a result of the great improvement of state 3 respiration in SSM by the superimposition of 2 weeks of exercise training on 3 weeks of feeding with GPA, RCR values in SSM remained significantly greater than those in the sedentary control group for any substrate used except ascorbate/TMPD (Table 3). The ADP-to-O ratios were not significantly different from the sedentary control in either of these two mitochondrial populations.

DISCUSSION

The observation that IFM displayed a higher oxidative phosphorylation capacity than SSM is in agreement with previous reports [12,14,15]. The difference in the maximal oxidation rate of ascorbate/TMPD between IFM and SSM was markedly higher than that in COX activity. This might indicate a higher cytochrome *c* content in IFM than in SSM. Although the possibility cannot be excluded that cytochrome *c* is preferentially lost from SSM during isolation [12], the differential adaptation between IFM and SSM elicited in the present study (see also [14,15]) suggests the existence of specific functional properties for these populations.

We report that 2 weeks of exercise training was long enough to increase significantly the mitochondrial COX and F_1 -ATPase activities of control quadriceps muscle. These results are in line with either the increased COX and citrate synthase activities previously observed in mammal skeletal muscle undergoing daily running for 1–2 weeks [7–9,23–27] and the half-life of 5–10 days reported for many muscle mitochondrial proteins [3,28]. This increase in mitochondrial enzyme activity is currently described as the second phase of mitochondrial biogenesis, namely a slow co-ordinated increase in the synthesis of mitochondrial proteins involved in ATP generation [3,29–31]. We also observed an increase in oxidative phosphorylation capacity of control mitochondria respiring on pyruvate/malate, and to a smaller extent on ascorbate/TMPD, but not on succinate. This means that short-term endurance training might act on complex I or prior

sites, or complex IV, but not on complex II of the electron transport chain. Because Benzi et al. [27] have shown that complex I activity was not improved after 2 weeks of daily training with running, the increased pyruvate/malate oxidation rate therein would be a consequence of an increased capacity of the citric acid cycle to provide NADH from pyruvate to the respiratory chain. In agreement with these results, it has previously been shown that citrate synthase [7–9] and COX activities [24], as well as cytochrome *c* content [28–30], increase significantly in skeletal muscle during the first week of training, whereas the activities of complex II and complex I of the electron transport chain increase after 4 weeks [27] and 6 weeks [32] of training respectively. Finally, no differences in these adaptive responses were found between IFM and SSM isolated after 2 weeks of endurance training. However, it is noteworthy that, beyond this duration, several studies have shown differential adaptations in mitochondrial enzyme activities between IFM and SSM in response to long-term endurance training, including a substrate-specific enhancement of their maximal respiratory capacity [14,15]. All findings considered, it therefore seems that there is a sequential assembly of the mitochondrial enzymes into the respiratory chain, as well as a regional adaptation of the mitochondrial metabolism, during the course of endurance training in skeletal muscle [3].

Importantly, we also observed that the relative change in mitochondrial oxidative phosphorylation capacities was lower than that in COX and F_1 -ATPase activities. This probably results from the small flux control exerted by these enzymes on oxidative phosphorylation in isolated skeletal muscle mitochondria [33]. The question therefore arises whether the changes in these enzyme activities have any physiological significance for the overall capacity of mitochondrial oxidative phosphorylation in muscle. In this regard, there is recent evidence that the flux control exerted by COX on mitochondrial oxidative phosphorylation becomes strongly significant at physiological oxygen concentrations in the muscle tissue [34], including its regulation by adenine nucleotides [35]. From these findings it follows that under physiological conditions oxygen delivery and/or intra-mitochondrial ATP-to-ADP ratios could affect the mitochondrial ATP production rate. In this regard, our findings suggest that exercise induced an increase in the excess capacity of COX and F_1 -ATPase relative to the mitochondrial oxidative phosphorylation flux, decreasing the contribution of these enzymes to the control of mitochondrial metabolic flux in the cell. This hypothesis needs further experimental testing to be demonstrated clearly.

Numerous factors have been proposed as possible signals triggering mitochondrial biogenesis in response to repeated bouts of aerobic exercise, among them levels of high-energy phosphates [2,4]. In this regard, the perturbation induced by chronic feeding of GPA, a competitive inhibitor of creatine import through the sarcolemma [5], provides evidence that the depletion of tissue ATP and creatine phosphate might mediate mitochondrial adaptations in skeletal muscle [4,10,36–39]. To extend this hypothesis further to the endurance-training-induced regional mitochondrial adaptations, we have examined biochemical adaptations in IFM and SSM to a diet of 1% GPA fed to rats for 3 weeks, alone or in combination with 2 weeks of endurance training. Data therein indicated (1) an increase in oxidative phosphorylation capacity in SSM, but not in IFM, in GPA-fed rats, and (2) a further improvement in the oxidative phosphorylation capacity in SSM from GPA-fed rats undergoing 2 weeks of exercise, whereas the capacity of IFM was similarly affected by training alone or in combination with a GPA diet. None of these mitochondrial adaptations to short-term creatine

depletion were dependent on the substrates used. Therefore these findings do not sustain the hypothesis that high-energy phosphate levels in themselves might serve as a stimulus to induce regional mitochondrial changes in rat skeletal muscle. Indeed, if the observed SSM adaptations to combined treatment could be explained by the cumulative action of these two treatments to decrease high-energy phosphate levels in skeletal muscle [11], it therefore appears that IFM adaptations are not directly initiated by this signal; otherwise, results similar to those in SSM would be expected. To explain these results, it has to be emphasized that exercise, but not GPA treatment, is an ATP-requiring process, resulting in an increased ATP metabolic turnover through the net phosphoryl transfer catalysed by the creatine kinase (CK) system [40] and probably in interrelation with the adenylate kinase system [39,41]. Interestingly, the earliest studies on transgenic mice deficient in muscle cytosolic creatine kinase (M-CK) are fully in line with our results [42,43]. In resting M-CK-deficient muscle, the high-energy phosphate state is normal but the rate of high-energy phosphate exchange between phosphocreatine and ATP is drastically reduced [42]. This model led to an enhancement in the aerobic capacity of muscles and an enlargement of the intermyofibrillar mitochondrial network [42]. Another report investigating mutant mice fed with GPA has shown that in GPA-treated M-CK-deficient muscles (with no M-CK activity and decreased substrate levels), increased muscle aerobic capacity is associated with a high proportion of enlarged mitochondria in both the intermyofibrillar and subsarcolemmal spaces. Whereas in GPA-treated wild-type muscles (i.e. muscles with normal M-CK activity but decreased substrate levels), the increased aerobic capacity of muscle is mostly related to an alteration in shape and volume, as well as in the oxidative capacity of mitochondria located in the subsarcolemmal region ([43]; see also [36,39]).

All these findings considered, we speculate that the mitochondrial biogenesis of the IFM population is sensitive to a change in ATP turnover through the CK system rather than in the high-energy phosphate levels of the muscle, whereas the SSM biogenesis is sensitive to a change in high-energy phosphate levels or, alternatively, in metabolite levels (such as AMP [44] or Ca^{2+} [45,46]). Indeed, the Ca^{2+} level within muscle was recently found to increase mostly in the subsarcolemmal region in response to electrical stimulation [46]. We therefore propose that the observed regional differences in the mitochondrial adaptations are because of biochemical and functional differences in local energy-consuming mechanisms, namely muscle contraction for IFM and the active transport of metabolites through the sarcolemma for SSM [47]. Although the functional significance of the observed differences between IFM and SSM with regard to energy flux in muscle cells is currently not clear, the proposed hypothesis deserves further attention.

We thank Mrs A. Gaunt for reviewing the English manuscript; Professor J. O. Holloszy for helpful comments on the manuscript; and Mrs J. Buckingham and Mr S. Roebuck for reviewing the revised manuscript. This work was supported by a grant from the Université Claude Bernard, and the Centre National de la Recherche Scientifique (CNRS).

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Received 28 April 2000/2 June 2000; accepted 23 June 2000