Changes of Contractile Properties of Extensor Digitorum Longus in Response to Creatine-Analogue Administration and/or Hindlimb Suspension in Rats

T. WAKATSUKI^{*,†}, Y. OHIRA^{*}, K. NAKAMURA[‡],
T. ASAKURA[‡], H. OHNO[§], and M. YAMAMOTO[†]

^{*}*Department of Physiology and Biomechanics, National Institute of Fitness and Sports, Kanoya, 891–23 Japan*

[†]*Department of Biochemistry II, National Defense Medical College, Tokorozawa, 359 Japan*

[‡]*Department of Neurosurgery, Faculty of Medicine, Kagoshima University, Kagoshima, 890 Japan*

[§]*Department of Hygiene, National Defense Medical College, Tokorozawa, 359 Japan*

Abstract Changes of contractile properties of extensor digitorum longus in response to hindlimb suspension and/or altered high-energy phosphate contents were studied in rats. A reduction of high-energy phosphates, especially phosphocreatine, was seen in rats fed creatine analogue β -guanidinopropionic acid (β -GPA), but they were elevated after 10-d supplementation of creatine. The one-half relaxation time was increased by feeding β -GPA, but was normalized by creatine supply. The fatigue resistance of creatine-depleted muscle was significantly improved, but tended to decrease by suspension and creatine supply, although it was still better than that in the control diet group. It is indicated that the contractile properties of muscle are influenced by the high-energy phosphate content. It is also suggested that the endurance capacity may be influenced by the mitochondrial respiratory capacity, but not necessarily by the levels of high-energy phosphates.

Key words: extensor digitorum longus, contractile properties, high-energy phosphates, hindlimb suspension.

Spaceflight or hindlimb suspension of rats causes a predominant atrophy in soleus, slow-twitch ankle extensor [1–3]. Such changes are closely related to the passive decrease in tension caused by shortening of the muscle length because of

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Correspondence should be addressed to: Y. Ohira, Department of Physiology and Biomechanics, National Institute of Fitness and Sports, Kanoya, 891–23 Japan. Tel: +81–994–46–4931, Fax: +81–994–46–4931, 2831

plantar flexion of the ankle joint [4, 5]. A shift of contractile properties toward fast-type is associated with atrophy [6–8], but the speed-related properties of the soleus become slower as the response to chronic depletion of high-energy phosphates progresses [9–11], although the resistance to fatigue is not improved [11].

On the contrary, no atrophy relative to the pre-suspension level is induced in the ankle flexors after suspension, although those weights are less than those of the age-matched cage controls since normal muscle growth did not take place [4, 12]. The contractile properties of ankle flexors are not influenced by hindlimb suspension either [13]. However, it is not clear why the properties of flexors do not respond to suspension. The current study was carried out to investigate whether the contractile properties of the fast-twitch and fatigable extensor digitorum longus (EDL) change similarly to the slow-twitch soleus following changes in the high-energy phosphate content with or without hindlimb suspension.

MATERIALS AND METHODS

The animal care and treatment were performed in accordance with the institutional guidelines for experimental animals. Thirty-six newly weaned male Wistar rats were randomly divided into two groups ($n=18$ each). Rats in one group were fed powdered diet (CE-2, CLEA Japan, Tokyo). The same diet, but containing 1% β -guanidinopropionic acid (β -GPA), was pair-fed to the other group. The daily food supply was gradually increased with growth and each rat was fed 20 g diet daily from week 4 to the end of the experiment. Water was supplied *ad libitum*. The temperature and humidity in the animal room were maintained at approximately 23°C and 55%, respectively, with a 12:12 h light:dark cycle. After approximately 9 weeks, the diet for 6 rats from each group was switched from either control or β -GPA diet to food containing 1% creatine for 10 d. The other 6 rats from each control or β -GPA group were hindlimb-suspended for 10 d by using the method described previously [14, 15]. The remaining rats served as the cage-controls. The suspended and cage-control groups remained on the original diets.

Muscle has to be freeze-clamped for biochemical analyses of resting high-energy phosphate levels [16]. Thus, the same muscle cannot be used for determination of contractile properties. Therefore, a non-invasive determination of phosphorus compounds was performed by ^{31}P -nuclear magnetic resonance (NMR) spectroscopy (BEM 170/200, Otsuka Electronics, Osaka), although the absolute concentrations can not be measured. The validity of ^{31}P -NMR analysis for estimation of the high-energy phosphate contents in hindlimb muscles was demonstrated in our previous study [17].

Rats were anesthetized by i.p. injection of sodium pentobarbital (5 mg/100 g body weight) and the left hindlimb was shaved. The rat was placed on a probe table in a supine position. A 2-turn surface coil with 20-mm diameter was placed above the left ankle flexors and the animal's whole body was put into a 170-mm-bore-

diameter superconducting magnet (4.7 T). The analysis was performed at a resonance frequency of 81.4 MHz, a repetition time of 3.0 s, and a radio frequency pulse width of 26 μ s. The free induction decay signal was accumulated for 150 times. The Pi/PCr and $\text{PCr}/(\text{PCr} + \text{Pi})$ ratios, where Pi: inorganic phosphate and PCr: phosphocreatine, were calculated by using the peak heights of Pi and PCr spectra instead of the area [18], because it was difficult to differentiate the border between the areas of PCr and phosphorylated β -GPA.

Contractile properties of the EDL muscle were measured both *in situ* and *in vitro* as in our previous study [11]. Rats were anesthetized by I.P. injection of sodium pentobarbital. The right EDL was dissected out and wet weight was measured. The distal tendon was fixed and the proximal tendon was connected to an isometric force transducer. Muscle was immersed in Krebs-Henseleit buffer (pH 7.4, 37°C) and gassed (95% O_2 –5% CO_2) continuously. Muscle was stimulated through 2 stainless steel wires placed around the muscle. Tension production in response to the electrical stimulation at 1, 10, 20, 50, 80, 100, 140, and 200 Hz with at 3-min intervals was recorded on both tape (TEAC, RX-5000, Tokyo) and paper recorders. The peak tension, time-to-peak tension (TPT), one-half relaxation time ($1/2$ RT), and the rate of tension production (dp/dt) in a twitch curve, the summation pattern of tension, and the maximal tetanic tension were examined. After 10 min of recovery, fatigability in response to a 50-Hz train stimulation for 5 min (100-ms train duration, 60 trains/min) was also determined.

For *in situ* measurement of fatigability, the left EDL was exposed and a stimulating apparatus composed of 2 stainless steel wires was placed around the left sciatic nerves in the gluteal region. The rat was placed in a prone position on a table in a temperature-regulated chamber (37°C). The left hindlimb was fixed to the table by inserting a needle through the knee. The distal tendon of the EDL was connected to an isometric transducer. The stimulating apparatus placed around the sciatic nerve was connected to an electrical stimulator. The muscle was stimulated electrically through the sciatic nerve. The optimum muscle length and stimulus intensity with a 100- μ s pulse duration which produced the maximum twitch tension were obtained by checking the tension using an oscilloscope and paper recorder. The muscle was then stimulated at 50 Hz (100-ms train duration, 60 trains/min) and tension was recorded for 5 min. The anode and cathode in every pulse were alternated to avoid polarization at the electrodes.

All data are presented as the means $\pm$ SEM. Statistical significance was examined by analysis of variance and Student's *t*-test. Differences were considered significant at the 0.05 level of confidence.

RESULTS

Phosphorus compounds

The $\text{PCr}/(\text{PCr} + \text{Pi})$ ratio, which indicates the relative content of PCr, did not change following hindlimb suspension (Table 1), but it was decreased significantly

Table 1. Results of ^{31}P -NMR analyses in ankle flexors.

	CC	CCr	CS
PCr/(PCr+Pi) ($\times 10^{-3}$)	907 $\pm$ 15	933 $\pm$ 23	873 $\pm$ 17
Pi/PCr ($\times 10^{-3}$)	103 $\pm$ 20	65 $\pm$ 13	148 $\pm$ 22
	βC	βCr	βS
PCr/(PCr+Pi) ($\times 10^{-3}$)	704 $\pm$ 24***	909 $\pm$ 37 ^{††}	649 $\pm$ 50**
Pi/PCr ($\times 10^{-3}$)	423 $\pm$ 48***	192 $\pm$ 41 ^{††}	560 $\pm$ 55***

Mean $\pm$ SEM. $n=6$ in each group. ** $p<0.01$, and *** $p<0.001$ vs. CC and ^{††} $p<0.01$ vs. βC . CC, cage-control fed control diet; CCr, cage-control fed creatine for 10 d; CS, suspended and fed control diet; βC , cage-control fed β -GPA; βCr , cage-control fed β -GPA and then creatine for 10 d; βS , suspended and fed β -GPA.

by feeding β -GPA ($p<0.001$), and was recovered after creatine supplementation in originally creatine-depleted animals ($p<0.01$). The Pi/PCr ratio was significantly elevated by β -GPA feeding ($p<0.001$). The value in β -GPA group was lowered by creatine supplementation ($p<0.01$). A similar response to creatine supply was also seen in the control diet group ($p>0.05$). Intracellular pH was not affected by either the creatine content or hindlimb unloading (not shown in the table).

Contractile properties

The body weights of the rats with β -GPA treatment and/or hindlimb suspension were subnormal (Table 2). The absolute weights of muscles treated with β -GPA or creatine feeding and with hindlimb suspension were less than in the cage-control animals fed normal diet. However, the % weight relative to body weight was normal in the hindlimb-suspended group. The absolute peak twitch tension (Pt) and the relative tension normalized with muscle weight in β -GPA-fed rats were significantly less than normal. However, the tension was improved if the diet was switched from β -GPA to creatine ($p<0.01$). The Pt/MW was also increased by hindlimb suspension in the β -GPA group ($p<0.05$). The twitch tension of the normal diet group was not influenced by hindlimb suspension or creatine supply. The absolute maximum tetanic tension (Po) was lowered by β -GPA feeding and hindlimb suspension, but no effects of any treatments were seen in the Po/MW. The Pt/Po was not affected by diet or hindlimb unloading.

Relative to the value in normal-diet cage-controls, the dp/dt (g/s) was less in the β -GPA-fed and hindlimb-suspended groups (Table 2). In response to creatine supply, the lowered dp/dt by β -GPA feeding was significantly increased within 10 d ($p<0.01$). However, such differences did not exist if the dp/dt was normalized by the Pt, although the level in the hindlimb-suspended β -GPA group was still less than cage-controls fed normal diet ($p<0.05$). The TPT value tended to be increased by β -GPA feeding insignificantly ($p>0.05$), but it was significantly less in the hindlimb-suspended group than in the cage-control group, even though the

Table 2. Contractile properties of extensor digitorum longus.

		CC	CCr	CS
BW	(g)	338±11	307±31	289±16*
MW	(mg)	164±3	143±7*	142±6*
	(% BW × 10 ⁻²)	48.6±1.7	46.7±0.9	49.2±0.7
Pt	(g)	20.2±0.8	17.7±2.3	15.3±2.0
Pt/MW	(g g ⁻¹)	124±5	124±15	108±14
dp/dt	(g s ⁻¹)	2,741±150	2,390±300	2,032±241*
dp/dt/Pt	(g s ⁻¹ g ⁻¹)	135±4	136±2	134±9
TPT	(ms)	13.3±0.4	12.8±0.2	14.0±0.4
1/2 RT	(ms)	8.59±0.44	8.80±0.51	8.42±0.55
Po	(g)	81.9±9.1	73.5±6.1	57.1±5.4*
Po/MW	(g g ⁻¹)	502±59	517±37	398±25
Pt/Po		0.26±0.03	0.25±0.03	0.28±0.04

		βC	βCr	βS
BW	(g)	309±5**	316±17	237±14***,††
MW	(mg)	127±5***	131±4***	106±4***,††
	(% BW × 10 ⁻²)	41.0±1.2**	41.3±0.4**	44.8±2.4
Pt	(g)	12.5±0.5***	17.2±0.8*,††	14.3±1.0**
Pt/MW	(g g ⁻¹)	100±7*	133±7††	136±10†
dp/dt	(g s ⁻¹)	1,616±97***	2,273±112*,††	1,690±140***
dp/dt/Pt	(g s ⁻¹ g ⁻¹)	128±4	132±1	118±4*
TPT	(ms)	14.5±0.4	13.1±0.3	13.2±0.4†
1/2 RT	(ms)	10.43±0.47*	8.70±0.36†	8.69±0.79
Po	(g)	53.1±5.3**	60.4±6.9	42.5±3.5***
Po/MW	(g g ⁻¹)	423±49	465±56	408±41
Pt/Po		0.25±0.03	0.31±0.04	0.35±0.04

Mean±SEM. $n=6$ in each group. * $p<0.05$, ** $p<0.01$, and *** $p<0.001$ vs. CC and † $p<0.05$ and †† $p<0.01$ vs. βC. BW, body weight; MW, muscle wet weight; Pt, peak twitch tension; dp/dt, the rate of tension production; TPT, time-to-peak tension; 1/2 RT, one-half relaxation time; Po: maximum tetanic tension. See Table 1 for other abbreviations.

same β-GPA diet was supplied. The 1/2 RT was increased by β-GPA feeding ($p<0.05$) but tended to be normalized by creatine supply ($p<0.05$) and suspension ($p>0.05$).

The tensions were gradually increased initially in the control-diet group in response to stimulation at 10, 20, or 50 Hz (Fig. 1). However, sharp declines in tensions after the initial increase were noted in the β-GPA group, especially in the cage-control and hindlimb-suspended muscles. This phenomenon was minor in the β-GPA group in which creatine was supplied for 10 d. The effects of creatine feeding or hindlimb suspension in normal diet group were not clear.

Fatigue resistance in the control diet group tended to be lowered by suspension or creatine supply, but it was significantly improved by β-GPA feeding (Fig. 2).

Fig. 1. The patterns of *in vitro* isometric tension development in extensor digitorum longus. See Table 1 for the abbreviations.

Although the tension production was decreased initially in response to the train stimulation at 50 Hz, as shown in Fig. 1, it recovered again and remained above the initial tension for 5 min (Fig. 2A). Muscles in the hindlimb-suspended β -GPA group were also less fatigable than the suspended control-diet group, although they were more fatigable than β -GPA-fed cage-controls (Fig. 2B). Such beneficial effects of creatine depletion disappeared by feeding creatine (Fig. 2C). While an initial increase in the tension was noted in the control groups, this phenomenon was not seen in the β -GPA groups. The fatigue resistance of all groups measured *in vitro* (data not shown) was lower than that measured *in situ*. However, the improved fatigue resistance caused by creatine depletion, relative to other groups, was still noted.

DISCUSSION

The high-energy phosphate content in ankle flexors, as well as in extensors [17], was influenced by feeding either β -GPA or creatine. A significant reduction of PCr, especially, was induced by β -GPA feeding. However, the responses of high-energy phosphates to hindlimb suspension were different from those of ankle extensors [11]. Although the distribution of high-energy phosphates in ankle extensors was altered in response to suspension [11, 19], that in flexors was not affected by suspension with free ankle joints (Table 1). The PCr/(PCr+Pi) in

Fig. 2. Changes in isometric tension production of extensor digitorum longus in response to electrical train stimulation at 50Hz *in situ*. Values are percent relative to the first tension at the beginning of stimulation. Mean $\pm$ SEM. See Table 1 for the abbreviations.

suspended ankle extensors [11] was significantly greater than that in suspended flexors ($p < 0.05$), and the Pi/PCr ratio was less in suspended extensors [11] than flexors ($p < 0.05$).

The ankle extensors are passively shortened due to the extended ankle joints during hindlimb suspension [4, 5], but the lengths of ankle flexors is less influenced by the changes in the angle of ankle joints. However, the mass of the EDL was less if the ankle joint was fixed in a dorsi-flexed position during hindlimb suspension

than of muscles with a free joint or with a joint immobilized in the plantar-flexed position [19]. Therefore, it is suggested that the degree of loading in ankle flexors is also influenced by changes in the angle of the ankle joint. The ankle joints of cage-control rats are generally maintained in a dorsi-flexed position (approximately 30–90 degrees). Thus, hindlimb suspension may increase the loading on the ankle flexors to some degree.

The weight of the EDL in the β -GPA and hindlimb-suspended groups were less than those in cage-controls fed normal diet. Growth retardation of the EDL in response to hindlimb suspension is also reported elsewhere [4, 12]. Creatine supplementation also caused a significant reduction of absolute muscle weight, although the body weight did not change significantly. The water content of the plantaris muscle in rats fed creatine for 10 d ($75.4 \pm 0.3\%$) was less than the normal diet group ($76.2 \pm 0.3\%$, $p < 0.001$) but the dry weight was identical, suggesting that an oral creatine supply caused dehydration in the muscle. However, the water content in the muscle of rats fed creatine chronically for 9 weeks was normal. The reason for such a discrepancy is unknown.

The responses of the contractile properties in the fast-twitch EDL to creatine depletion were similar to those in the slow-twitch soleus [11]. A shift toward the slow type by feeding β -GPA was indicated by the increased 1/2 RT, although the change in TPT was not significant statistically. Such a change in the speed-related properties was associated with improvement in fatigue resistance.

Since the fatigue resistance in all groups measured *in vitro* was lower than that measured *in situ*, it is suggested that blood flow has a significant effect on fatigability. Because the oxygen supply to muscle fibers tested *in vitro* relies on only passive diffusion from the muscle surface, the availability of oxygen to fibers located at the deeper region may be limited. Thus, these data suggest that the enhanced fatigue resistance in the β -GPA group is decreased if the respiratory capacity of the muscle is inhibited by lowering oxygen transport.

However, the muscles in the β -GPA group were more fatigue-resistant than the other groups, even when the fatigability was tested *in vitro*. Therefore, it is indicated that the greater fatigue resistance in the β -GPA group may be associated with improved energy metabolism in muscle fibers, although it is decreased to some degree when the availability of oxygen is limited. The activities of mitochondrial enzyme, succinate dehydrogenase, in both slow- and fast-twitch fibers analyzed quantitatively in the same β -GPA muscles, which may affect the fatigue resistance positively, were significantly higher than those in the control-diet group [20]. Since the nerve supply, as well as the blood supply, is eliminated in the muscles tested *in vitro*, the increased fatigue resistance by feeding β -GPA is clearly not related to improved fatigability at the neuro muscular junction.

Physical work capacity at mild, but not at high, intensity both in swimming and treadmill running is also greater in rats fed β -GPA than in controls, even though the high-energy phosphates are depleted [21]. Elevated activities of mitochondrial enzymes, such as β -hydroxyacyl CoA dehydrogenase, in the EDL in

the current study may explain why the resistance to fatigue was improved in β -GPA muscle (126.0 ± 6.7 vs. $52.8 \pm 3.0 \mu\text{mol g protein}^{-1} \text{ min}^{-1}$ in control-diet group, $p < 0.001$). Increased mitochondrial enzyme activities in the fast-twitch tibialis anterior are also reported [22]. Although the activities of cytoplasmic enzymes were inhibited in these muscles, greater glucose uptake [23] and hexokinase and mitochondrial enzyme activities [24, 25] may also help to increase the oxidative capacity of muscle. Accumulated glycogen in β -GPA muscle [23, 25] might also improve the endurance capacity of muscle. It is also suggested that maintenance of high-energy phosphates at higher levels is not necessary for endurance capacity of muscle at a submaximal work intensity, if the capacity of adenosine triphosphate synthesis is improved.

The endurance capacity of muscles was decreased toward the control level following 10 d of creatine supplementation. Glycogen content and glucose uptake into muscles are decreased to the control levels following creatine loading in originally β -GPA fed rats [23], and an inhibition of β -hydroxyacyl CoA dehydrogenase activity was induced by chronic creatine supply [26] and the water content was decreased by 10-d creatine supply, as mentioned above. These phenomena could be detrimental for the endurance capacity of muscle, although the resting levels of high-energy phosphates were increased (Table 1).

In conclusion, the effects of chronic depletion of high-energy phosphates by feeding β -GPA and/or of hindlimb suspension on contractile properties were studied in rat EDL. High-energy phosphates, especially PCr, in skeletal muscles were decreased by feeding β -GPA and increased toward normal level within 10 d of creatine feeding. The 1/2 RT was increased by β -GPA feeding. These muscles also had improved resistance to fatigue compared to controls. Such alterations in the contractile properties tended to be reversed within 10 d of creatine feeding and hindlimb suspension. It is suggested that the levels of high-energy phosphates are important in the regulation of contractile properties of the EDL. The endurance capacity of muscle was unrelated to the content of high-energy phosphates. It may, however, be related to the mitochondrial respiratory capacity.

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