Responses of Contractile Properties in Rat Soleus to High-Energy Phosphates and/or Unloading

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Abstract Responses of contractile properties of soleus to unloading and/or changes in high-energy phosphate contents were studied in rats. Reduction of high-energy phosphates, especially phosphocreatine, in ankle extensors was induced by feeding β -guanidinopropionic acid (β -GPA). The major finding in the study was that the fatigability and speed-related contractile properties responded to unloading and creatine supplementation in a similar manner. The high-energy phosphate contents tended to be elevated after 10-d supplementation of creatine and hindlimb suspension. The shift toward slow-type, mainly due to an increased one-half relaxation time, was seen in rats fed β -GPA. Such a shift was reversed by feeding creatine or by hindlimb suspension; however, the suspension-induced shift of contractile properties toward fast-type was not prevented completely by β -GPA feeding. Although the muscle fatigue resistance did not change by β -GPA feeding alone, the decrease in fatigue resistance following suspension and creatine supply was less in the β -GPA group. It is suggested that the levels of high-energy phosphates and tension production play important roles in the regulation of contractile properties of the soleus muscle.

Key words: soleus, contractile properties, high-energy phosphates, unloading.

It is well known that antigravity muscles atrophy when gravitational loading is removed [1–5]. Characteristic changes in these muscles include a shift from slow-

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to fast-twitch type in response to actual space flight [2] or simulation model such as hindlimb suspension [6–8]. Although the muscle activity detected by electromyography (EMG) disappears immediately when the hindlimbs are suspended, the EMG activity is gradually recovered during hindlimb suspension [9]. Such results suggest that the changes in the muscle characteristics caused by unloading are not directly related to the disuse of muscles. On the other hand, shifts of contractile properties of rat diaphragm and isomyosin of the soleus and extensor digitorum longus in mice toward slow-twitch type have been found following chronic depletion of high-energy phosphates [10, 11]. However, the mechanism responsible for the shift of contractile properties is still unclear. The current study was carried out to investigate how the contractile properties of rat soleus respond to changes in the high-energy phosphate contents induced by feeding either creatine or creatine analogue β -guanidinopropionic acid (β -GPA), or by hindlimb unloading.

METHODS

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, Nihon CLEA, Tokyo). The same diet plus 1% β -GPA was pair-fed for the other group. The daily food supply was gradually increased following the growth and each rat was fed 20 g of diet daily from week 4 to the end of the experiment. Water was supplied *ad libitum*. The temperature and humidity in the animal room with a 12:12 h light:dark cycle were maintained at approximately 23°C and 55%, respectively. 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 [12, 13]. 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 *in situ* for biochemical analyses of resting high-energy phosphate levels [14]. Thus, the same muscle can not be used for the determination of contractile properties. Therefore, a non-invasive determination of phosphorus compounds was performed by using ^{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 the estimation of high-energy phosphate contents in hindlimb muscles was demonstrated in our previous study [15].

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 prone position. A 2-turn surface coil with 20-mm diameter was placed above the left ankle extensors and whole body was put into 170-mm-bore-diameter superconducting magnet (4.7 T). The analysis was performed with a resonance

frequency of 81.4 MHz, a repetition time of 3.0 s and an rf pulse width of 26 μ s. The free induction decay signal was accumulated for 150 times. The P_i/PCr and $PCr/(PCr + P_i)$ ratios, where P_i : inorganic phosphate and PCr : phosphocreatine, were calculated using the peak heights [16], instead of the area because it was difficult to differentiate the borderline of PCr and phosphorylated β -GPA area.

Contractile properties of the soleus muscle were measured both *in vitro* and *in situ*. Rats were anesthetized by i.p. injection of sodium pentobarbital. The left soleus 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. The P_{O_2} of the buffer was maintained at approximately 700 mmHg. 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 3-min interval was recorded on both tape (TEAC, RX-5000, Tokyo) and paper recorders. The peak tension (Pt), 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 (P_o) were examined.

For *in situ* measurement of fatigability, the right soleus was exposed and a stimulating apparatus composed of 2 stainless steel wires was placed around the right 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 right hindlimb was fixed to the table by inserting a needle through the knee. The distal tendon of the soleus was connected to an isometric transducer. The stimulating apparatus placed around the sciatic nerve was connected to an electrical stimulator. The muscle was covered by gauze and kept moist by dripping a warm Krebs-Henseleit buffer. The temperature between the muscle and the wet gauze was 37°C. The muscle was stimulated electrically through the sciatic nerve. The optimum muscle length and stimulus intensity with 100- μ s pulse duration which produced the maximum twitch tension were adjusted 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

As was reported in our previous study [15], decreased peak heights of PCr and adenosine triphosphate (ATP) and appearance of a new peak for phosphorylated β -GPA were noted in the NMR spectra of rats fed β -GPA. The $PCr/(PCr + P_i)$

Table 1. Results of ^3P -NMR analyses in ankle extensors.

	CC	CCr	CS	βC	βCr	βS
PCr/(PCr+Pi) ($\times 10^{-3}$)	882 $\pm$ 1	908 $\pm$ 12	931 $\pm$ 15*	669 $\pm$ 40***	864 $\pm$ 40 ^{††}	771 $\pm$ 42*
Pi/PCr ($\times 10^{-3}$)	129 $\pm$ 4	81 $\pm$ 16*	83 $\pm$ 5***	522 $\pm$ 98**	240 $\pm$ 44* ^{††}	314 $\pm$ 70*
pH _i	6.94 $\pm$ 0.12	7.26 $\pm$ 0.20	7.02 $\pm$ 0.21	7.05 $\pm$ 0.12	6.73 $\pm$ 0.13	7.01 $\pm$ 0.01

Mean $\pm$ SEM. $n=6$ in each group. * $p<0.05$, ** $p<0.01$, 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.

Table 2. Body weight and contractile properties of soleus.

	CC	CCr	CS	βC	βCr	βS
BW (g)	338 $\pm$ 11	307 $\pm$ 31	289 $\pm$ 16*	309 $\pm$ 5**	316 $\pm$ 17	237 $\pm$ 14*** ^{††}
MW (mg)	149 $\pm$ 7	122 $\pm$ 4**	110 $\pm$ 6**	113 $\pm$ 2***	118 $\pm$ 7*	85 $\pm$ 9*** [†]
Pt (g)	17.8 $\pm$ 2.5	15.3 $\pm$ 1.4	10.5 $\pm$ 1.4*	12.7 $\pm$ 1.1*	14.5 $\pm$ 1.1	4.7 $\pm$ 0.6*** ^{†††}
Pt/MW (g/g)	119 $\pm$ 14	126 $\pm$ 12	94 $\pm$ 9	113 $\pm$ 11	126 $\pm$ 12	57 $\pm$ 7*** ^{†††}
dp/dt (g/s)	996 $\pm$ 122	954 $\pm$ 97	656 $\pm$ 64*	617 $\pm$ 53**	842 $\pm$ 64 [†]	267 $\pm$ 38*** ^{†††}
dp/dt/Pt (g/s/g)	54.2 $\pm$ 2.4	62.5 $\pm$ 2.7	64.6 $\pm$ 3.1*	48.6 $\pm$ 1.0*	58.0 $\pm$ 1.8 ^{††}	56.7 $\pm$ 1.6 ^{††}
TPT (ms)	44.8 $\pm$ 3.6	40.6 $\pm$ 1.8	37.6 $\pm$ 2.2	51.3 $\pm$ 1.9	42.5 $\pm$ 1.4 ^{††}	39.2 $\pm$ 0.8* ^{†††}
1/2 RT (ms)	52.9 $\pm$ 2.7	41.8 $\pm$ 2.3*	36.9 $\pm$ 2.7**	60.6 $\pm$ 2.0*	43.0 $\pm$ 3.3 ^{††}	44.4 $\pm$ 2.5* ^{†††}
Po (g)	75.0 $\pm$ 9.1	66.7 $\pm$ 2.9	30.5 $\pm$ 4.7***	55.4 $\pm$ 4.7*	64.6 $\pm$ 4.7	18.6 $\pm$ 2.7*** ^{†††}
Po/MW (g/g)	501 $\pm$ 49	547 $\pm$ 16	269 $\pm$ 30**	493 $\pm$ 43	559 $\pm$ 55	227 $\pm$ 37*** ^{†††}
Pt/Po	0.24 $\pm$ 0.02	0.23 $\pm$ 0.02	0.36 $\pm$ 0.03*	0.23 $\pm$ 0.01	0.23 $\pm$ 0.01	0.26 $\pm$ 0.01

Mean $\pm$ SEM. $n=6$ in each group. * $p<0.05$, ** $p<0.01$, and *** $p<0.001$ vs. CC and [†] $p<0.05$, ^{††} $p<0.01$, and ^{†††} $p<0.001$ 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.

ratio, which indicates the relative content of PCr, was decreased significantly by feeding β -GPA (Table 1, $p < 0.001$). However, it was recovered after creatine supplementation in animals that were originally creatine-depleted ($p < 0.01$). Furthermore, it was significantly increased after hindlimb suspension of normal diet group relative to the cage control ($p < 0.05$). Following the suspension of rats fed β -GPA, the PCr/(PCr+Pi) ratio also tended to increase ($p > 0.05$).

The Pi/PCr ratio was significantly elevated by β -GPA feeding ($p < 0.01$). This result may be caused by a reduction of PCr, since the Pi level analyzed biochemically does not change by β -GPA feeding [17]. The Pi/PCr value in the β -GPA group was lowered by creatine supplementation ($p < 0.01$). A significant reduction in response to creatine feeding was also seen in the control diet group ($p < 0.05$). Further, the ratio in the control diet group decreased significantly by hindlimb suspension ($p < 0.001$). Intracellular pH was not affected by either creatine content or hindlimb unloading.

Contractile properties

The body weights in rats fed β -GPA was less than in the normal control rats (Table 2, $p < 0.01$). A reduction of soleus weight, relative to that in the cage-control rats fed normal diet, was induced by feeding β -GPA or creatine and by hindlimb suspension. The absolute Pt were, in general, positively correlated with the muscle weights. But a significant reduction of the soleus Pt normalized with

Fig. 1. The patterns of *in vitro* isometric tension production in soleus. See Table 1 for the abbreviations.

Fig. 2. Fatigue resistance of soleus in response to electrical stimulation at 50 Hz (100 ms train duration, 60 trains/min) *in situ*. Mean $\pm$ SEM percent tensions relative to the initial tension produced are shown. See Table 1 for the abbreviations.

muscle weight (Pt/MW, g/g), was induced only by the combination of β -GPA feeding and hindlimb suspension ($p < 0.001$). The Po was decreased following β -GPA feeding ($p < 0.05$) and hindlimb suspension ($p < 0.001$). Although the suspension-induced decrease in the values, normalized with muscle weight (Po/MW, g/g), was still significant, the effect of β -GPA feeding on it was not statistically significant. The twitch/tetanus (Pt/Po) ratio in the unloaded muscle of normal diet group was significantly greater than that in the cage control ($p < 0.05$).

The dp/dt (g/s) in the twitch curve was also correlated with the muscle weight. Although the values, normalized with the peak twitch tension (g/s/g), were significantly lower in the β -GPA-fed cage-control than normal ($p < 0.05$), they were increased by feeding creatine and by hindlimb suspension ($p < 0.01$). A significant increase after hindlimb suspension was also seen in the normal diet group. The TPT tended to increase by β -GPA feeding, but not significantly. In response to creatine supplementation and hindlimb unloading, the values tended to decrease. These changes in β -GPA groups were statistically significant. The 1/2 RT was greater in β -GPA-fed cage-controls than normal controls ($p < 0.05$). However, it was decreased following 10 d of creatine supply ($p < 0.01$) and/or unloading ($p < 0.001$). Similar changes were also induced in the normal diet groups.

The patterns of isometric tension development in response to electrical stimulation at 10, 20, and 50 Hz are shown in Fig. 1. The tensions in β -GPA-fed cage-controls tended to fuse more than the normal controls, although the tension development was not necessarily greater because of the smaller muscle mass (Table 2). The degree of tension summation became less in response to creatine supply and hindlimb suspension.

The fatigue resistance of slow-twitch soleus was not improved by β -GPA feeding (Fig. 2). It was decreased following creatine supply and unloading. However, the muscles treated with β -GPA was less fatigable than other groups in response to suspension or creatine supply. "Sag" was observed in the fatigue curve of β -GPA diet and β -GPA + suspension groups, as was also reported by Mainwood *et al.* [10], but it was not seen in the β -GPA + creatine group.

DISCUSSION

The high-energy phosphate contents in ankle extensors were influenced by feeding either β -GPA or creatine, or by suspension unloading, although Desplanches *et al.* [18] reported that the concentration of ATP and PCr in rat soleus, which was not freeze-clamped *in situ* and homogenized in ice-cooled 5% trichloroacetic acid, did not change after 5 weeks of suspension. The responses of high-energy phosphates to creatine supply and hindlimb suspension were similar in both control and β -GPA diet groups. Even though remarkable changes were not observed in the distribution patterns of phosphorus compounds, the peak heights of PCr and ATP tended to be greater in muscles with creatine supplementation than in cage controls fed normal diet (data not shown). The PCr/(PCr + Pi) was also increased in the

β -GPA group following creatine loading. Increase in the PCr/(PCr + Pi) ratio may suggest an accumulation of PCr caused by an enhanced creatine level.

The elevation of PCr/(PCr + Pi) levels in ankle extensors following unloading may be related to the reduced metabolic rate or turnover rate of high-energy phosphates, since the ankle extensors are passively shortened due to the extended ankle joints during hindlimb suspension [19, 20]. Even though the activity of EMG is normal [9, 19], the tension production is inhibited in these muscles [19]. Estimated rate of ATP synthesis in ankle extensors by using the method of Chance *et al.* [16], $1/(1 + 0.6 \times \text{PCr}/\text{Pi})$, was significantly less in the suspended group than in cage controls ($p < 0.001$). Therefore, the unloading-related decreases in tension production may result in a reduction of the turnover rate of ATP and increase the contents of ATP and PCr.

The maximum tension production was decreased by β -GPA feeding. However, both Pt and Po per g muscle weight were normal. It is suggested that the reduction of maximum force development was due to the decreased muscle mass. Since the Pi content does not increase by β -GPA feeding [17], the change in tension level may not be related to the Pi level either.

The most striking finding of this study is the similar response of fatigability and speed-related contractile properties to suspension unloading and creatine supplementation. The β -GPA muscles tended to be slower than the control muscles, as was reported by Mainwood *et al.* [10]. However, such phenomena may not be influenced by the level of adenosine diphosphate (ADP), since the ADP content in muscle does not change following β -GPA treatment [17]. Shift of speed-related contractile properties was reversed within 10 d following creatine supplementation. The TPT ($p > 0.05$) and $1/2$ RT ($p < 0.05$) of the soleus muscle tended to decrease even in the control-diet group following creatine feeding. It is suggested that the characteristics of speed-related contractile properties are shifted toward slow-twitch type when creatine and/or high-energy phosphate contents are chronically lowered. In muscles depleted of high-energy phosphates by exposure to cold [14] as well as by feeding β -GPA [21–23], mitochondrial enzyme activities are elevated suggesting that biogenesis is stimulated.

Conversely, muscles are shifted toward fast-twitch type in response to creatine loading which elevates the contents of high-energy phosphates and reduces the mitochondrial biogenesis. For example, the activity of β -hydroxyacyl CoA dehydrogenase in soleus fed 1% creatine for 8 weeks ($84.5 \pm 1.2 \mu\text{mol/g protein/min}$) was significantly lower than the normal diet group ($120.5 \pm 2.8 \mu\text{mol/g protein/min}$, $p < 0.001$, unpublished observation). The shift of contractile properties toward slow-type by β -GPA feeding also tended to be reversed by hindlimb suspension, even though the rats remained on the same β -GPA diet. However, both TPT and $1/2$ RT in the suspended muscles of rats fed β -GPA tended to be still greater than those in the suspended normal diet group, although our study also indicated that the increase in the fast-type myosin expression following the unloading was suppressed by feeding β -GPA [24]. These results suggest that both stimulation of mitochon-

drial energy metabolism and tension production or gravitational loading are necessary for the maintenance or improvement of slow-type muscle characteristics.

Moerland *et al.* [11] found a change in the pattern of myosin isoform in hindlimb muscles of mice fed β -GPA. In the soleus, the distribution of intermediate myosin was reduced and slow myosin was increased by approximately 50% following β -GPA feeding. A shift of myosin isoform from fast V_1 to slower V_2 and V_3 in response to β -GPA feeding was also found in cardiac muscle [25]. In the current study, clear effects of creatine depletion and/or unloading on the speed-related contractile properties, especially in 1/2 RT, were found in the soleus; however, a significant change in TPT, such as that found by Mainwood *et al.* [10], was not observed. On the contrary, increased fast-type isomyosin (FM₁) expression and/or shift of contractile and histochemical properties from slow- to fast-twitch type were observed after hindlimb suspension [6-8] or space flight [2]. These results agree with the findings in the current study. The data in our study also suggest that the key factor responsible for the shift of muscle contractile properties induced by creatine supply and hindlimb unloading may be identical.

The absolute peak force production was subnormal in muscles following β -GPA feeding and unloading due to the smaller muscle mass as was mentioned above. However, the peak force per g muscle weight was normal. Furthermore, the tension level was calculated as % relative to the initial tension produced by each muscle. Therefore, it seems reasonable to compare the patterns of isometric force production between the experimental and control groups as was also done elsewhere [10, 17, 26]. The endurance capacity of muscles was decreased by creatine supplementation. Decreased glycogen content and glucose uptake into muscles [27] and inhibited mitochondrial enzyme activities following creatine supply may be the major causes for the decreased endurance capacity of muscle, although the resting levels of high-energy phosphates were increased (Table 1). The fatigue resistance of muscle was also decreased by hindlimb unloading. Although the specific activities of mitochondrial enzymes in single muscle fibers were either unchanged or even increased in unloaded muscle [2, 28, 29], most of the studies, in which whole muscle homogenates were analyzed, indicate that respiratory capacity is inhibited following hindlimb unloading [4, 18, 30, 31]. But the suspension-induced decline in fatigue resistance was prevented by feeding β -GPA; this may be due to an improved mitochondrial energy metabolism and/or a shorter diffusion distance to the center of fibers caused by muscle atrophy.

Although the rate of stimulation was different from the current study, the time course of the changes of Pi and/or pH in rat hindlimb muscle in response to 1 and/or 5 Hz stimulation were investigated by Meyer *et al.* [26]. Their results showed that the increase of Pi in the β -GPA group was less than half of controls. Further, a twofold greater acid accumulation was seen in the control compared with the β -GPA-loaded muscle, despite the higher buffering capacity by PCr hydrolysis. Such phenomena could be another factors for the different fatigability.

The sag noted in the muscles of β -GPA group with an improved fatigue

resistance may be associated with the further reduction of high-energy phosphate levels by the beginning of contractions. However, the tension production may be recovered due to an enhanced synthesis of high-energy phosphates through the activated mitochondrial metabolism, since the muscles have higher enzyme levels than normal.

In conclusion, the effects of changes in high-energy phosphate contents by feeding β -GPA or creatine and/or the effects of hindlimb suspension on contractile properties were studied in the rat soleus. High-energy phosphates, especially PCr, in ankle extensors were decreased by feeding β -GPA and increased toward a normal level within 10 d of creatine feeding. The characteristics of muscles were shifted toward slow-twitch type mainly due to the increased $1/2 RT$ by β -GPA feeding. Such alterations of contractile properties were reversed within 10 d of creatine feeding and hindlimb suspension. The suspension-induced decline of fatigue resistance was prevented by feeding β -GPA, although the shift of speed-related properties toward fast-type was not prevented completely. It is suggested that the levels of high-energy phosphates and/or tension production play important roles in the regulation of contractile properties of the soleus.

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