Metabolic Modulation of Muscle Fiber Properties Unrelated to Mechanical Stimuli

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Abstract: The effects of chronically increasing (creatine-fed) or decreasing (β -guanidinopropionic acid [β -GPA]-fed) high-energy phosphates for up to 8 weeks on daily voluntary activity levels, swimming endurance capacity, electromyogram (EMG) activity, and the morphological and metabolic properties of single fibers in the soleus and extensor digitorum longus (EDL) muscles in young rats were determined. High-energy phosphate, voluntary activity, and soleus-integrated EMG levels were lower in β -GPA-fed rats than in control rats. Endurance capacity was higher at a relatively low intensity of swimming and lower at a relatively high intensity in β -GPA-fed rats than in control rats. Muscle mass and fiber size were smaller, and the percentage of slow fibers was

higher in the soleus and EDL of β -GPA-fed rats than in control rats. Succinate dehydrogenase activity was higher in both the fast and slow fibers of the EDL of β -GPA-fed rats than in control rats. Thus, a reduction in high-energy phosphates transformed some fast fibers toward a slow phenotype. Creatine supplementation had minimal effects: The only significant change was an increase in α -glycerophosphate dehydrogenase activity in the fast fibers of the EDL. These results indicate that the metabolic environment of a muscle fiber can influence the prominence of a given muscle fiber independent of the activity level of muscle. [The Japanese Journal of Physiology 53: 389–400, 2003]

Key words: creatine, β -guanidinopropionic acid, rat muscle fibers, daily activity, endurance performance.

There are two aspects of the findings from previous experiments involving creatine depletion via dietary manipulation of β -guanidinopropionic acid (β -GPA) that seem unusual. First, there is an increase in the proportion of fibers expressing slow myosin [1–6], a shift in the contractile properties toward those observed in a slow muscle phenotype [7–9], and an increase in the resistance to fatigue during an endurance test [10, 11]. All of these adaptations are usually associated with increased levels of activity of skeletal muscles [12, 13]. However, the daily voluntary activity levels of β -GPA fed animals are not necessarily increased [5, 10], and muscle growth may even be inhibited [8, 9, 11]. A second factor that appears to be discordant is the reduction in the content of phosphocreatine (PCr) and adenosine triphosphate (ATP) in

muscles of β -GPA-fed rats [6, 11, 14–18]. These adaptations would be expected only in highly active muscles or muscle fibers. Adaptations in the metabolic properties of skeletal muscle also are observed with exposure to cold, a condition that results in a decrease in voluntary movement and a significant depletion of muscle high-energy phosphate levels [19].

Combined, these results suggest that metabolic stimuli associated with the depletion of high-energy phosphates may have a significant impact on muscle fiber phenotype and endurance capacity independent of neuromuscular activity levels. Kushmerick and colleagues [5, 20] suggested the following scenario: With β -GPA administration there is a reduction in the normal buffering capacity when ATP demands exceed supply because β -GPA is a much poorer substrate for

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creatine kinase than PCr. The result is that the muscle has a decrease in chemical potential and then adapts by increasing its oxidative capacity [21] and shifting to the slower myosin heavy chain (MHC) isoforms, which have lower ATPase activities. The purposes of the present study were (1) to identify the phenotypic and metabolic adaptations to a chronic elevation or reduction of creatine at the single muscle fiber level; (2) to quantify the impact of altered creatine levels on the daily activity levels of individual muscles as measured by electromyography (EMG); and (3) to measure the *in vivo* work capacity at relatively high and low work intensities of rats having altered creatine levels.

MATERIALS AND METHODS

Experimental design. All experimental procedures were conducted in accordance with the Japanese Physiological Society Guide for the Care and Use of Laboratory Animals, approved by the Committee on Animal Care and Use at the university and at the National Space Development Agency of Japan. Newly weaned male Wistar rats with a mean ($\pm$ SEM) body weight of 36 ± 1 g (Kyudo, Kumamoto, Japan) were divided randomly into three groups ($n=5$ /group). The rats were housed individually in stainless steel cages (35 cm $\times$ 24 cm $\times$ 20 cm) and pair-fed. One group of rats (control group) was fed commercial powdered food (CE-2, Nihon CLEA, Tokyo, Japan). The other groups were fed the same diet, but containing either 1% β -GPA (β -GPA-fed group) or 1% creatine (creatine monohydrate, Nacalai Tesque, Tokyo, Japan) (creatine-fed group). The β -GPA was synthesized as reported previously [22, 23]. The amount of food provided during the first 3 weeks of the experiment was increased gradually. From week 4 to the end of the experiment, each rat was fed 20 g of food per day, i.e., 0.2 g of β -GPA or creatine per day in the treatment groups. Water was given *ad libitum*. Temperature and humidity in the animal room with a 12:12-h light:dark cycle were maintained at $\sim 23^\circ\text{C}$ and $\sim 55\%$, respectively.

Voluntary activity. During weeks 6 and 7, each rat was assigned randomly to a cage (35 $\times$ 24 $\times$ 20 cm) equipped with a running wheel (10 cm wide and 37 cm diameter), thereby permitting the rat to run at will. The cages were located at the same level in the racks. Total 24-h voluntary activity was determined once for each rat. The total number of wheel revolutions was registered on a counter at 6 A.M. and 6 P.M. The recording of daily activity level was performed randomly for the three groups of rats.

The behavioral pattern of each rat in the cage dur-

ing a 3-h period with the lights on was determined by video recording. The percent of the period spent either walking, standing using only the hind limbs, or resting with the animal in a recumbent position on the cage floor was determined.

Endurance capacity. During week 8, endurance swimming capacity with a relatively low or a high work load was determined for each rat. Swimming was performed in a cylindrical tank with a diameter and depth of ~ 55 cm. The water temperature was adjusted to $\sim 30^\circ\text{C}$. The fur was shampooed to avoid the buoyancy effects of air bubbles. For the relatively low and high work loads, a weight equivalent to ~ 2.5 or 5% of body weight, respectively, was attached with a rubber band around the waist of each rat. Exhaustion was defined as the point when the rat was submerged and could not swim to the water surface within 10 s. There was a 3-d rest interval between the two tests, and the order of the tests was random.

Electromyographic activity. After 8 weeks of dietary manipulation and 3 d of recovery from the final swimming test, the rats were anesthetized with sodium pentobarbital (5 mg/100 g body weight, I.P.). Under aseptic conditions, bipolar EMG recording electrodes were implanted into the left soleus (a predominantly slow plantarflexor) and the left extensor digitorum longus (EDL, a predominantly fast dorsiflexor) muscles, as described by Roy *et al.* [24]. Briefly, two enamel-insulated wires were inserted into the belly of each muscle in parallel with the muscle fibers (~ 2 mm apart) via a 23-gauge needle. The needle was withdrawn carefully, and ~ 0.5 mm of insulation was stripped from each wire. The section of the wire with the insulation removed was implanted into the midbelly of the muscle. Each wire was secured with a suture at its entry and exit from the muscle, so that the stripped portion of the wire in the muscle was fixed. A wire for a common ground (insulation stripped for about 2 mm) was implanted in the back region. One ground wire and four recording wires were then passed subcutaneously and exited through a small skin incision at the dorsal region of the neck. The skin was sutured. A small ball of solder was placed at the end of each wire, and the ends of the wires were kept outside the skin. A lamp shade-like collar, which did not interfere with food or water consumption, was placed around the neck of the rat to protect the externalized wires until the EMG recordings were performed 2–3 d later. A topical antiseptic (nitrofurazone, Furacin) was applied to the incision area, and tetracycline (50 mg/100 ml) was added to the drinking water on the day before surgery and for the following 2 d to prevent infection.

The EMG recordings were performed 2–3 d after a complete recovery from surgery. The exposed portions of wires at the base of the neck were connected to recorders by using flexible insulated cables. The EMG signals were amplified ($\times 1,000$, band width of 5 Hz to 3 kHz, AB-621G, Nihon Kohden, Tokyo, Japan) and recorded on an FM cassette tape recorder (XR-5000, TEAC, Tokyo, Japan) at 4.8 cm/s and on a polygraph paper recorder. The recordings were performed once for a 10-h period during the light phase for each rat.

The amplified raw EMG signals stored in the cassette tape recorder were processed by a PowerLab/16sp (ML795, AD Instruments Inc., Australia) analog-to-digital (A/D) converter, digitized at 2 kHz per channel, and stored on disk (Power Mac G3 computer, Apple, Cupertino, CA, USA). The integrated areas of the EMG bursts (IEMG) were determined by using a computer software package (Chart v3.6.1/s, AD Instruments Inc., Australia). The total mean integrated EMG activity per hour (IEMG/h) was calculated as IEMG times the number of bursts [25]. Computer software was used to detect and display the start and end of each EMG burst based on a given threshold level above the baseline noise for each channel [26] and averaged over 10 repetitions of each behavior.

Phosphorus compound levels. Two days after the EMG recordings, the high-energy phosphate levels in the resting right calf muscles were estimated by using ^{31}P -nuclear magnetic resonance (^{31}P -NMR) spectroscopy (BEM 170/200, Otsuka Electronics, Osaka, Japan), as described elsewhere [11, 16]. In a previous study [16], the soleus and the lateral gastrocnemius muscles from rats treated similarly were freeze-clamped with a pair of aluminum tongs cooled in liquid nitrogen, and the high-energy phosphate levels were measured biochemically [19, 27–29]. These results were compared with the data obtained by using ^{31}P -NMR in the present study, since the data from the *in vivo* ^{31}P -NMR analyses may represent values for the gastrocnemius and not necessarily for the soleus, which is located in the deep (close to the bone) region of calf. The responses of the phosphorus compounds to changes in creatine content were similar for both analyses [16]; thus, the non-invasive ^{31}P -NMR procedures were used in the present study. Furthermore, the responses of the high-energy phosphate levels following the depletion and/or loading of creatine were identical for the plantarflexors and dorsiflexors. Therefore, only the data obtained from the plantarflexors are reported in the present study.

The rats were overdosed with 15–20 mg sodium pentobarbital per 100 g body weight, and the soleus

and EDL muscles were removed rapidly from the right (non-implanted) limb, cleaned of excess fat and connective tissue, and wet weighed. The muscles were stretched gently to approximate the *in vivo* optimal length, pinned on a cork at the proximal and distal tendons, and rapidly frozen in isopentane cooled in liquid nitrogen. Subsequently, the mid-portion (~ 8 mm) of the muscle belly was cut, mounted on a cork with the fibers oriented perpendicular to the cork by using optimal cutting temperature (OCT) compound (Miles, Elkhart, IN, USA), and frozen in liquid nitrogen for cross-sectional analyses. All samples were stored at -80°C until analyzed.

Immunohistochemical and histochemical analyses. Serial transverse cross sections ($10\text{ }\mu\text{m}$ thick) were cut in a cryostat at -20°C for immunohistochemical analyses. The MHC profile of individual fibers was determined by using monoclonal antibodies specific to rat slow or fast MHC isoforms, i.e., primary antibodies NCL-MHCs and NCL-MHCf (Novocastra Laboratories Ltd., UK), respectively, as described previously [30]. Briefly, the avidin-biotin immunohistochemical procedure was used for the localization of primary antibody binding according to the instructions for kits PK-6102 and AK-5010 (Vector Laboratories, Burlingame, CA, USA). Phosphate-buffered saline was used as a buffer for all immunoglobulin G-class primary antibodies, and tris (hydroxymethyl) aminomethane-buffered saline as the buffer for all immunoglobulin M-class primary antibodies. The stained images were incorporated into an image processing system (Color Image Processor, SPICCA-II, Nihon Avionics, Tokyo, Japan). Approximately 120 fibers from the deep (near the bone) region of each muscle were analyzed. They were classified as slow, fast, or hybrid (reacting for both slow and fast MHC), according to the staining profile [30].

Quantitative histochemical analyses of single fiber succinate dehydrogenase (SDH), α -glycerophosphate dehydrogenase (GPD), and myosin ATPase activities were determined as described previously [31, 32]. The images of the same fibers analyzed for MHC expression were digitized as gray level pictures on a computer-enhanced image processing system (Nihon Avionics, Tokyo, Japan) and stored on magnetic optical disks. An array of picture elements was quantified to 256 gray levels that then were converted automatically to an optical density (OD). Furthermore, the cross-sectional area (CSA) of each fiber was determined. Enzyme activities were expressed as specific ($\Delta\text{OD}/\text{min}$ by dividing OD by the time of the reaction for each staining procedure) or integrated ($\Delta\text{OD}/\text{min} \times \text{fiber CSA}$) activities.

Table 1. Body weight and ratios of high-energy phosphate levels in the rat calf muscles estimated by ^{31}P -nuclear magnetic resonance spectroscopy.

	Control	Creatine-fed	β -GPA-fed
Body weight (g)	330 $\pm$ 8	360 $\pm$ 9	305 $\pm$ 8 ^{*,†}
(% change)	(+817%)	(+900%)	(+747%)
Pi/PCr	0.15 $\pm$ 0.01	0.06 $\pm$ 0.01 [*]	0.53 $\pm$ 0.09 ^{*,†}
PCr/(PCr+Pi)	0.87 $\pm$ 0.01	0.95 $\pm$ 0.01 [*]	0.67 $\pm$ 0.04 ^{*,†}

Values are mean $\pm$ SEM. $n=5$ /group. Pi, inorganic phosphate; PCr, phosphocreatine. The ratios were calculated from the areas in the ^{31}P -nuclear magnetic resonance spectroscopy. The increase in body weight relative to the initial mean body weight (36 g) is shown in parentheses. ^{*,†}Significantly different from the control or creatine-fed group, respectively, at $p<0.05$.

Statistical analyses. All data are presented as mean $\pm$ SEM. Overall statistically significant differences were determined by using a one-way analysis of variance, and differences among groups were determined by using Scheffe's post hoc tests. The differences were considered significant at the 0.05 level of confidence.

RESULTS

Body weight and high-energy phosphate levels

The mean body weight of the control rats was increased ~ 9 -fold (from ~ 36 to 330 g) during the 9-week experimental period (Table 1). The gain in body weight was inhibited ($\sim 8\%$) by β -GPA feeding ($p<0.05$). In contrast, creatine feeding resulted in a 9% increase ($p>0.05$) in body weight gain compared to control rats.

The contents of PCr and ATP in the β -GPA-fed rats were decreased by ~ 82 and 49%, respectively, compared to control (Fig. 1, $p<0.05$). These results generally agree with previous observations [4]. The PCr and ATP were elevated by creatine feeding ($p<0.05$). The inorganic phosphate (Pi)/PCr ratio was higher in the β -GPA-fed group than in the control group (Table 1, $p<0.05$), whereas Pi concentration was similar in the two groups (Fig. 1). In contrast, the Pi/PCr ratio was lower in the creatine-fed group than in the control group (Table 1, $p<0.05$) because of a decrease in Pi content and an increase in PCr content (Fig. 1, $p<0.05$). The PCr/(PCr+Pi) ratio was lower in the β -GPA and higher in the creatine-fed groups compared to control (Table 1, $p<0.05$).

Voluntary activity

The rats in the control and creatine-fed groups tended ($p>0.05$) to be more active during the dark period in comparison to the light period of the daily cycle (Fig. 2). For the β -GPA-fed rats, the activity

Fig. 1. Mean content of high-energy phosphates in resting calf muscles of anesthetized rats relative to those of control rats (100%, dashed line). The values (mean $\pm$ SEM) were calculated by using the areas of phosphocreatine (A), adenosine triphosphate (ATP, B), and inorganic phosphate (C) in the ^{31}P -nuclear magnetic resonance spectroscopy. The areas of β - and γ -ATP were combined for the ATP value. β -GPA, β -guanidinopropionic acid. $n=5$ /group. ^{*,†}Significantly different from the control or creatine-fed group, respectively, at $p<0.05$.

level was significantly lower during the light period than in the dark period ($p < 0.05$). The voluntary activity level during the light period for the β -GPA-fed group also was lower than for the other two groups ($p < 0.05$), and the activity level during the dark period

Fig. 2. Daily voluntary activity levels (number of wheel revolutions) for control, creatine-fed, and β -GPA-fed rats. Values are mean $\pm$ SEM. $n=5$ /group. *† Significantly different from the control, creatine-fed group, or the activity level in light time, respectively, at $p < 0.05$.

tended ($p > 0.05$) to be lowest in the β -GPA-fed rats.

Clear differences in the activity patterns during the lights-on period (see MATERIALS AND METHODS for description) were observed among groups (Fig. 3). The β -GPA-fed rats spent $\sim 94\%$ of their time at rest in a recumbent position. In contrast, the mean percentage of time at rest for the control and creatine-fed groups was ~ 78 and 73% , respectively, and these values were less than that for the β -GPA-fed rats ($p < 0.05$). The control and creatine-fed rats spent $\sim 16\%$ and 24% of the time standing, whereas this value was only $\sim 4\%$ for the β -GPA-fed rats ($p < 0.05$). The percent of time spent walking was quite small and similar for all groups, i.e., 6, 3, and 2% for the control, creatine-fed, and β -GPA-fed rats, respectively.

Electromyographic activity

The soleus was more active than the EDL during resting, standing, and walking in all groups (Figs. 4–6, $p < 0.05$). The EMG activity levels in both the soleus and the EDL were greater during standing and walking than during resting (Fig. 5, $p < 0.05$). The magni-

Fig. 3. Sedentary cage activity patterns for control, creatine-fed, and β -GPA-fed rats during the light time. The data are expressed as a percent of time spent resting in a recumbent position on the floor, bipedal standing, and walking. *† Significantly different from the control or creatine-fed group, respectively, at $p < 0.05$.

Fig. 4. Patterns of electromyogram (EMG) activity in the soleus and extensor digitorum longus (EDL) muscles of a representative rat from each group during resting, bipedal standing, and quadrupedal walking. No significant differences among the groups were observed in the frequency of the EMG spikes during walking. All records are normalized to the same amplitude. Note that the time intervals are different for each activity.

Fig. 5. Integrated levels of electromyogram (EMG) in the soleus and extensor digitorum longus (EDL) muscles of each group during ~5 min of resting (A), standing (B), or walking (C). Values are mean $\pm$ SEM. $n=5$ /group. $\dagger$, $\S$ Significantly different from the soleus or from the standing and walking period in the same group at $p<0.05$.

Fig. 6. The overall mean integrated electromyogram (EMG) in the soleus and extensor digitorum longus (EDL) muscles during a 10-h period. Values are mean $\pm$ SEM. $n=5$ /group. $*$, $\dagger$ Significantly different from the control, creatine-fed group, or soleus of the same group, respectively, at $p<0.05$.

tude of the soleus EMG during resting, standing, and walking tended ($p>0.05$) to be lower in the β-GPA-fed group than in the others. The mean total integrated soleus EMG activity determined during a ~10-h recording period was less in the β-GPA-fed group than in the control and creatine-fed groups (Fig. 6, $p<0.05$). There were no effects of feeding either creatine or β-GPA on the EMG levels of the EDL (Figs. 4–6).

Endurance capacity

Swimming time to exhaustion at low intensity (2.5% of body weight attached) was longer in β-GPA-fed rats than in control rats (Fig. 7A, $p<0.05$). In contrast, at a higher intensity (5% of body weight attached) it was shorter in β-GPA-fed rats than in control or creatine-fed rats (Fig. 7B, $p<0.05$). Creatine feeding had no significant effect on either swimming test.

Muscle and fiber size

Both the absolute and relative (expressed as percent

Fig. 7. Swimming time to exhaustion with a weight equivalent to either 2.5% (A, low work intensity) or 5% (B, high work intensity) of body weight (BW) attached. Values are mean $\pm$ SEM. $n=5$ /group. $*$, $\dagger$ Significantly different from the control or creatine-fed group, respectively, at $p<0.05$.

Table 2. Characteristics of the soleus muscle and fibers.

	Control	Creatine-fed	β -GPA-fed
Muscle wet weight (mg) (%BW $\times 10^{-3}$)	149 $\pm$ 6 (43.4 $\pm$ 0.8)	162 $\pm$ 4 (44.7 $\pm$ 1.0)	114 $\pm$ 3 ^{*,†} (36.4 $\pm$ 1.5 ^{*,†})
Fiber type (%)			
Slow	81 $\pm$ 4	81 $\pm$ 1	97 $\pm$ 2 ^{*,†}
Hybrid	4 $\pm$ 1	8 $\pm$ 1	3 $\pm$ 1 [†]
Fast	15 $\pm$ 3	11 $\pm$ 4	—
CSA (μm^2)			
Slow	2,862 $\pm$ 209	2,959 $\pm$ 177	1,865 $\pm$ 177 ^{*,†}
Hybrid	1,496 $\pm$ 113	2,123 $\pm$ 161 [*]	1,524 $\pm$ 97 [†]
Fast	1,479 $\pm$ 109	2,155 $\pm$ 193 [*]	—
mATPase ($\Delta\text{OD}/\text{min} \times 10^{-3}$)			
Slow	22.6 $\pm$ 2.4	21.0 $\pm$ 2.2	20.8 $\pm$ 0.9
Hybrid	25.7 $\pm$ 4.0	21.4 $\pm$ 2.0	19.7 $\pm$ 1.3
Fast	31.7 $\pm$ 3.1	28.4 $\pm$ 3.1	—
I·mATPase ($\Delta\text{OD}/\text{min} \times \mu\text{m}^2$)			
Slow	66.3 $\pm$ 11.3	63.9 $\pm$ 10.1	35.8 $\pm$ 4.0
Hybrid	40.2 $\pm$ 3.6	46.2 $\pm$ 2.0	32.2 $\pm$ 1.2 [†]
Fast	48.6 $\pm$ 10.1	63.5 $\pm$ 11.7	—
SDH ($\Delta\text{OD}/\text{min} \times 10^{-3}$)			
Slow	37.0 $\pm$ 5.2	35.1 $\pm$ 6.0	55.9 $\pm$ 6.0
Hybrid	40.6 $\pm$ 13.3	37.9 $\pm$ 4.8	60.3 $\pm$ 6.4
Fast	53.5 $\pm$ 10.9	51.2 $\pm$ 8.4	—
I·SDH ($\Delta\text{OD}/\text{min} \times \mu\text{m}^2$)			
Slow	105 $\pm$ 14	103 $\pm$ 25	106 $\pm$ 18
Hybrid	61 $\pm$ 20	80 $\pm$ 19	92 $\pm$ 10
Fast	85 $\pm$ 22	110 $\pm$ 31	—

Values are mean $\pm$ SEM. Number of muscles, 5/group; mean number of muscle fibers analyzed, 120/muscle. BW, body weight; CSA, cross-sectional area; OD, optical density; mATPase, myosin adenosine triphosphatase; SDH, succinate dehydrogenase; I·mATPase, integrated mATPase; I·SDH, integrated SDH. ^{*,†}Significantly different from the control or creatine-fed group, respectively, at $p < 0.05$.

body weight) weights of the soleus and EDL were smaller for the β -GPA-fed rats than in the control or creatine-fed rats (Tables 2 and 3, $p < 0.05$). In general, the changes in fiber size were consistent with the muscle weight adaptations. The mean CSA of the slow fibers in the soleus was smaller in the β -GPA-fed group than in the others ($p < 0.05$). Furthermore, the MHC hybrid fibers in the soleus were smaller in the β -GPA-fed group than in the creatine-fed group ($p < 0.05$). In contrast, the mean CSA of the hybrid and fast fibers in the soleus were larger in the creatine-fed rats than in the control rats ($p < 0.05$). All fiber types in the EDL tended to be smaller in the β -GPA rats than in the control rats, but the differences were insignificant ($p > 0.05$). The overall mean fiber CSAs (all phenotypes combined) for both the soleus and the EDL were smaller in the β -GPA group (1,855 and 1,219 μm^2 , respectively) than in the control (2,600 and 1,495 μm^2 , respectively) and creatine-fed (2,804 and 1,496 μm^2 , respectively) groups ($p < 0.05$). There were no differences in weight or fiber size of the EDL between the control and creatine-fed groups.

Muscle fiber type distributions and myosin ATPase activities

A chronic feeding of β -GPA resulted in a higher proportion of slow fibers and a lower proportion of fast fibers in both muscles (Tables 2 and 3, $p < 0.05$). In fact, the soleus of β -GPA-fed rats had 97% slow fibers and no fast fibers (compared to 15 and 11% fast fibers in the control and creatine-fed groups). The percentage of fast fibers in the EDL was lower in the β -GPA-fed group than in the others ($p < 0.05$). The fiber type distributions for both the soleus and the EDL were similar in control and creatine-fed rats.

The mean specific and integrated activities of myosin ATPase of all types of fibers in both muscles tended ($p > 0.05$) to be reduced by β -GPA feeding. In contrast, these activities in the creatine-fed rats were either similar to or slightly higher than in the control rats ($p > 0.05$). The only significant differences were a lower integrated myosin ATPase activity in the hybrid fibers of the soleus in the β -GPA-fed group than in the creatine-fed group (Table 2, $p < 0.05$) and a lower specific activity of myosin ATPase for slow fibers of

Table 3. Characteristics of the extensor digitorum longus muscle and fibers.

	Control	Creatine-fed	β -GPA-fed
Wet weight (mg) (%BW $\times 10^{-3}$)	164 $\pm$ 2 (48.6 $\pm$ 1.6)	174 $\pm$ 5 (48.3 $\pm$ 1.6)	127 $\pm$ 5 ^{*,†} (40.9 $\pm$ 1.1 ^{*,†})
Fiber type (%)			
Slow	3 $\pm$ 4	3 $\pm$ 1	8 $\pm$ 1 ^{*,†}
Hybrid	53 $\pm$ 2	52 $\pm$ 2	56 $\pm$ 2
Fast	44 $\pm$ 2	45 $\pm$ 2	36 $\pm$ 1 ^{*,†}
CSA (μm^2)			
Slow	897 $\pm$ 116	844 $\pm$ 137	633 $\pm$ 21
Hybrid	1,815 $\pm$ 148	1,773 $\pm$ 232	1,509 $\pm$ 201
Fast	1,150 $\pm$ 63	1,219 $\pm$ 222	897 $\pm$ 42
mATPase ($\Delta\text{OD}/\text{min}\times 10^{-3}$)			
Slow	21.2 $\pm$ 1.1	23.9 $\pm$ 1.1	18.1 $\pm$ 0.5 [†]
Hybrid	32.6 $\pm$ 1.1	30.2 $\pm$ 1.8	29.9 $\pm$ 0.9
Fast	28.4 $\pm$ 3.1	30.0 $\pm$ 1.6	25.9 $\pm$ 1.1
I·mATPase ($\Delta\text{OD}/\text{min}\times \mu\text{m}^2$)			
Slow	19.0 $\pm$ 0.7	18.9 $\pm$ 3.9	11.5 $\pm$ 1.8
Hybrid	59.0 $\pm$ 6.0	45.0 $\pm$ 11.3	53.0 $\pm$ 4.9
Fast	31.6 $\pm$ 1.1	36.6 $\pm$ 9.6	23.2 $\pm$ 2.1
SDH ($\Delta\text{OD}/\text{min}\times 10^{-3}$)			
Slow	31.3 $\pm$ 4.9	32.0 $\pm$ 5.6	46.8 $\pm$ 5.6 ^{*,†}
Hybrid	22.2 $\pm$ 4.9	30.6 $\pm$ 12.0	34.5 $\pm$ 7.7
Fast	35.2 $\pm$ 4.2	34.9 $\pm$ 7.0	56.7 $\pm$ 7.7 ^{*,†}
I·SDH ($\Delta\text{OD}/\text{min}\times \mu\text{m}^2$)			
Slow	28.1 $\pm$ 3.0	27.2 $\pm$ 10.6	29.6 $\pm$ 9.8
Hybrid	39.7 $\pm$ 10.6	54.2 $\pm$ 10.6	52.1 $\pm$ 12.1
Fast	40.3 $\pm$ 3.8	42.4 $\pm$ 12.1	50.8 $\pm$ 9.1
GPD ($\Delta\text{OD}/\text{min}\times 10^{-3}$)			
Slow	5.28 $\pm$ 0.68	11.38 $\pm$ 0.68	5.18 $\pm$ 0.38 [†]
Hybrid	8.97 $\pm$ 0.98	11.38 $\pm$ 0.68	6.18 $\pm$ 0.38 [†]
Fast	7.76 $\pm$ 0.68	11.91 $\pm$ 0.38 [*]	7.24 $\pm$ 0.38 [†]
I·GPD ($\Delta\text{OD}/\text{min}\times \mu\text{m}^2$)			
Slow	4.74 $\pm$ 0.38	4.70 $\pm$ 0.50	2.34 $\pm$ 0.50 ^{*,†}
Hybrid	16.28 $\pm$ 1.88	17.17 $\pm$ 1.88	10.96 $\pm$ 1.51 [†]
Fast	8.91 $\pm$ 0.75	14.52 $\pm$ 2.14 [*]	6.49 $\pm$ 0.75 [†]

Values are mean $\pm$ SEM. Number of muscles, 5/group; mean number of muscle fibers analyzed, 120/muscle. BW, body weight; CSA, cross-sectional area; OD, optical density; mATPase, myosin adenosine triphosphatase; SDH, succinate dehydrogenase; GPD, α -glycerophosphate dehydrogenase; I·mATPase, integrated mATPase; I·SDH, integrated SDH; I·GPD, integrated GPD. ^{*,†} Significantly different from the control or creatine-fed group, respectively, at $p<0.05$.

the EDL in the β -GPA-fed group than in the creatine-fed group (Table 3).

Metabolic enzyme activities

The specific activity of SDH in single muscle fibers of both muscles tended to be increased by β -GPA feeding ($p>0.05$), although only the activities of the slow and fast fibers in the EDL were significantly higher than in the control and creatine-fed rats (Tables 2 and 3). Creatine feeding had no effect on the mean SDH activities in any fiber type of either muscle. Furthermore, the integrated SDH activities were unaffected in any fiber type of either muscle by creatine or β -GPA feeding.

The activity levels of GPD in the soleus were near

the threshold for detection. Therefore, no data are presented for the soleus. The specific and integrated GPD activities of the fast fibers of the EDL were increased by creatine feeding (Table 3, $p<0.05$). In contrast, the specific and integrated activities of each fiber type were lower in the β -GPA-fed group than in the creatine-fed group ($p<0.05$). Moreover, the integrated GPD activity of the slow fibers was lower in the β -GPA-fed group than in the control group ($p<0.05$).

DISCUSSION

Body weight and high-energy phosphate levels are reduced in β -GPA fed rats. The high-energy phosphate concentrations in the skeletal

muscles of β -GPA-fed rats were lower than in the control rats, as reported previously [6, 10–11, 14–18]. In contrast, high-energy phosphate levels were elevated in the creatine-fed group. The lower mean body weight of rats fed β -GPA in the current study is consistent with our previous results, which showed that the slower rate of body weight gain is associated with a reduction in fat accumulation [10].

β -GPA reduces neuromuscular activity levels, while elevating the oxidative potential of the muscle. It is well known that the rats are generally active during dark time. Blewett and Elder [33] reported that the EMG activity in soleus and plantaris of rats was $\sim 40\%$ greater during dark time than during light time. The same patterns of voluntary activity were also observed in the current study. The total daily voluntary activity of β -GPA-fed rats, especially during the light phase of the daily cycle, was significantly lower than of the control and creatine-fed rats. These observations are in contrast to the observations of Moerland *et al.* [5], who reported that the level of daily voluntary running activity of β -GPA-fed mice was not different from control. The reasons for these contrasting results are unknown, but could be due to differences in the methods used to assess the activity levels and/or to a species difference in the response.

A detailed analysis of the activity data in the present study shows that the EMG levels of the soleus, a predominantly slow ankle plantarflexor, were lower in rats with depleted high-energy phosphates than in the control or creatine-fed rats. Compared to the other groups, the β -GPA-fed rats spent a higher percentage of time at rest, i.e., when EMG activity levels were relatively low. In contrast, no differences in the EMG levels of the EDL, a predominantly fast ankle dorsiflexor, were observed among groups. This lack of response might be expected, since the EDL is rarely activated even during normal spontaneous movements and is almost silent during resting periods. Although EMG activity in the EDL was observed during walking, the amount of time spent walking was minimal across groups, ranging from only ~ 2 to 6% of the recording time. The present data demonstrate rather clearly that the spontaneous activity of β -GPA rats is below normal levels. The significance of this is twofold. First, it suggests that the nervous system is affected by this diet. Second, it demonstrates that even though the metabolic demands are reduced, the muscle adapts as if it is responding to a greater prolonged metabolic demand. Thus activity could not be a factor in inducing the phenotypic changes observed with the GPA diet.

A shift of muscle fiber phenotype toward a slow-

twitch type was noted in both the soleus and the EDL of rats fed β -GPA. A fiber type transformation with identical patterns was also confirmed by an analysis of MHC expression using gel electrophoresis (unpublished observation). The specific SDH activities for each fiber type in both the soleus and the EDL muscles tended to be elevated by β -GPA feeding. This tendency for an increase in the mitochondrial enzyme activity of single muscle fibers, combined with an increase in the percentage of slow fibers, is consistent with the enzymatic adaptations seen in whole homogenates of both predominantly fast and predominantly slow muscles [11, 14, 16, 18, 21]. Although the activities of most of the glycolytic enzymes are reduced in these muscles [18, 21], resting glucose uptake [34] and GLUT-4 glucose transporter expression [18] are increased. Hexokinase activity also is elevated in β -GPA-fed rats [21]. All of these observations are consistent with an elevated oxidative capacity [35]. Such adaptations in muscle generally are associated with endurance type (long duration, low intensity) training [12]. However, as noted previously it is clear that these muscle adaptations induced by β -GPA feeding were not related to increased muscle activity, since the EMG levels were significantly depressed in these animals.

An increase in mitochondrial mass, often associated with intramitochondrial inclusions observed in myopathies has been observed following β -GPA feeding [36]. Moerland *et al.* [20] reported that the total oxidative capacity of the EDL in β -GPA-fed mice was decreased because of a smaller muscle mass, even though the specific activities (per gram of muscle weight) of mitochondrial enzymes such as cytochrome oxidase and citrate synthase were higher than in controls. The administration of metabolic poisons such as 3'-azido-3'-deoxythymidine decreases the total activity of cytochrome oxidase in muscle [37], implying that β -GPA also may be a metabolic poison. However, the improved endurance swimming capacity observed in the current study and the increased fatigue resistance of the EDL muscle of β -GPA-fed rats, reported in a previous study [8], clearly indicate that the muscle oxidative capacity is not severely compromised. Furthermore, the total activity of SDH per CSA of muscle fiber, i.e., the ISDH, tended to be elevated, although the fiber size was decreased by β -GPA feeding, suggesting an increase in the specific activity of SDH.

Endurance capacity. The greater endurance capacity at a relatively low work intensity observed for the creatine-depleted rats in the present and previous investigations [10, 11] suggests that the associated

elevation in oxidative potential was functionally beneficial. Furthermore, these results demonstrate that there are no negative functional consequences of low resting levels of high-energy phosphates for an endurance type task. On the other hand, the ability to sustain adequate levels of high-energy phosphates in the β -GPA-treated rats appears to be inadequate when a relatively high work intensity is required (Fig. 8). This type of task relies more on fast fibers having higher ATPase activities and more rapid rates of ATP hydrolysis than slow fibers have. Furthermore, many of these fast fibers have a relatively low oxidative capacity. Glycogen content in hind limb muscles of β -GPA-treated rats were even elevated [11]. Thus, the poor work performance at a higher intensity, relative to that in control rats, may not be related to lower anaerobic capacity in response to submersion during swimming or to caloric restriction as a result of pair-feeding (20 g/day).

The beneficial effects of creatine loading on the high-intensity interval running of rats have been reported elsewhere [38]. In a review published by the American College of Sports Medicine, the consensus was that creatine supplementation may enhance exercise performance during repeated short periods of extremely powerful activity, but it does not increase aerobic exercise performance [39]. In another review, it was suggested that creatine supplementation may even be detrimental for endurance type activity, while its effects on high-intensity activity are equivocal [40]. Further, the specific examples of contrasting results obtained from studies examining the effects of creatine supplementation and resistance training in human have been published. Although Willoughby and Rosene [41] reported that long-term creatine supplementation increased muscle strength and size, possibly as a result of increased MHC synthesis, significant augmentation of the mechanical properties or hypertrophy was not observed by Stevenson and Dudley [42]. No beneficial effects of creatine loading were observed in our studies ([8, 9, 11] and present results). In the present study, a single bout of swimming at two intensities was administered to the rats in each group. There was no *a priori* reason to expect an increase in the time to fatigue in the creatine-fed rats. The results showing no difference from control values were consistent for all rats (note the small SEM in Fig. 7).

Although the longer one-half relaxation times in both the soleus [9] and the EDL [8] muscles induced by feeding β -GPA were normalized when the dietary treatment was changed from β -GPA to creatine feeding, no effects of creatine feeding alone were observed in these studies. The endurance running capac-

ity of creatine-loaded rats on a motor-driven treadmill also was identical to that of a control diet group [11]. These observations are generally consistent with the present results, i.e., that the activities of SDH and myosin ATPase were not influenced by creatine loading.

Perspective. The conclusion of the major findings and suggestions obtained from the present study are indicated in Figs. 8 and 9. The present data indicate that the profile of adaptations in skeletal muscles normally associated with chronically elevated levels of neuromuscular activity can be induced while reducing the levels of high-energy phosphates via β -GPA treatment and decreasing the activity levels of the animal (Fig. 8). The type of metabolically-mediated induction of muscle fiber phenotypic adaptations observed normally would be expected to be associated with chronic increases in neuromuscular activity. The observation that β -GPA-fed rats are more susceptible to fatigue at a relatively high work intensity, but more fatigue resistant at a relatively low work intensity than control rats (Fig. 9), indicate the physiological significance of phenotypic changes on *in vivo* performance. We propose that the reduced *in vivo* performance at a relatively high work intensity most likely reflects the recruitment of the higher threshold motor units containing muscle fibers with relatively high myosin ATPase activity levels and relatively low oxidative capacities. The implication of these observations is that the high-energy phosphate concentrations of skeletal muscles represent an important component of the cas-

Fig. 8. Flow diagram suggesting that a shift toward the slow phenotype of muscle fibers can occur in the presence of an increase or decrease in neuromuscular activity. In models of both enhanced (e.g., endurance type training or prolonged chronic electrical stimulation) and decreased neuromuscular activities (by a β -GPA diet), the high-energy phosphates were driven downward. Thus we suggest that the low levels of high-energy phosphates in muscle fibers may be a fundamental biochemical trigger that can induce phenotypic adaptations characteristic of a "slow" muscle type. These adaptations result in an enhanced performance at low work intensities and a depressed performance at high work intensities (also see Fig. 9). HEP, high-energy phosphate; MHC, myosin heavy chain.

Fig. 9. Relationship between the recruitment level (at a low and high work intensity) and time to exhaustion for control and β -GPA-fed rats. *a* and *b*: time to exhaustion for control and β -GPA-fed rats, respectively. The time to exhaustion for the β -GPA-fed rats is enhanced at a low work intensity and depressed at a high work intensity. These comparative graphs reflect the metabolic adaptations observed in the skeletal muscles. Although it is not illustrated in the graph, the muscle atrophy observed in β -GPA-fed rats should require a higher percentage of the motor pools to be recruited to perform any given work task, e.g., at both low and high intensity workloads. This could require a rightward shift of the recruitment curve of the β -GPA-fed rats, partially counteracting the metabolic effect illustrated.

cade of events that control muscle fiber phenotype, whether the high-energy phosphate levels are modulated by diet or by neuromuscular activity levels.

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REFERENCES

- Adams GR and Baldwin KM: Age dependence of rat myosin heavy chain transitions induced by creatine depletion in rat skeletal muscle. *J Appl Physiol* 78: 368–371, 1995
- Adams GR, Bodell PW, and Baldwin KM: Running performance and cardiovascular capacity are not impaired in creatine-depleted rats. *J Appl Physiol* 79: 1002–1007, 1995
- Adams GR, Haddad F, and Baldwin KM: Interaction of chronic creatine depletion and muscle unloading: effects on postural and locomotor muscles. *J Appl Physiol* 77: 1198–1205, 1994
- Mekhfi H, Hoerter J, Lauer C, Wisnewsky C, Schwartz K, and Ventura-Clapier R: Myocardial adaptation to creatine deficiency in rats fed with β -guanidinopropionic acid, a creatine analogue. *Am J Physiol* 258 (Heart Circul Physiol 27): H1151–H1158, 1990
- Moerland TS, Wolf NG, and Kushmerick MJ: Administration of a creatine analogue induces isomyosin transitions in muscle. *Am J Physiol* 257 (Cell Physiol 26): C810–C816, 1989
- Ren J-M, Ohira Y, Holloszy JO, Hamalainen N, Traub I, and Pette D: Effects of β -guanidinopropionic acid-feeding on the patterns of myosin isoforms in rat fast-twitch muscle. *Pflügers Arch* 430: 389–393, 1995
- Mainwood GW, Alward M, and Eiselt B: Contractile characteristics of creatine-depleted rat diaphragm. *Can J Physiol Pharmacol* 60: 120–127, 1982
- Wakatsuki T, Ohira Y, Nakamura K, Asakura T, Ohno H, and Yamamoto M: Changes of contractile properties of extensor digitorum longus in response to creatine-analogue administration and/or hindlimb suspension in rats. *Jpn J Physiol* 45: 979–989, 1995
- Wakatsuki T, Ohira Y, Yasui W, Nakamura K, Asakura T, Ohno H, and Yamamoto M: Responses of contractile properties in rat soleus to high-energy phosphates and/or unloading. *Jpn J Physiol* 44: 193–204, 1994
- Ohira Y, Wakatsuki T, Inoue N, Nakamura K, Asakura T, Ikeda K, Tomiyoshi T, and Nakajoh M: Non-exercise-related stimulation of mitochondrial protein synthesis in creatine-depleted rats. In: *Integration of Medical and Sports Sciences*, ed. Sato Y, Poortmans J, Hashimoto I, and Oshida Y, Karger, Basel, pp 318–323, 1992
- Tanaka T, Ohira Y, Danda M, Hatta H, and Nishi I: Improved fatigue resistance not associated with maximum oxygen consumption in creatine-depleted rats. *J Appl Physiol* 82: 1911–1917, 1997
- Holloszy JO: Biochemical adaptations in muscle. Effects of exercise on mitochondrial oxygen uptake and respiratory enzyme activity in skeletal muscle. *J Biol Chem* 242: 2278–2282, 1967
- Pette D, Muller W, Leisner E, and Vrbova G: Time dependent effects on contractile properties, fibre population, myosin light chains and enzymes of energy metabolism in intermittently and continuously stimulated fast twitch muscles of the rabbit. *Pflügers Arch* 364: 103–112, 1976
- Fitch CD, Jellinek M, Fitts KM, Baldwin KM, and Holloszy JO: Phosphorylated β -guanidinopropionate as a substitute for phosphocreatine in rat muscle. *Am J Physiol* 228: 1123–1125, 1975
- Fitch CD, Jellinek M, and Mueller EJ: Experimental depletion of creatine and phosphocreatine from skeletal muscle. *J Biol Chem* 249: 1060–1063, 1974
- Ohira Y, Saito K, Wakatsuki T, Yasui W, Suetsugu T, Nakamura K, Tanaka H, and Asakura T: Responses of β -adrenoceptor in rat soleus to phosphorus compound levels and/or unloading. *Am J Physiol* 266 (Cell Physiol 35): C1257–C1262, 1994
- Ren J-M and Holloszy JO: Adaptation of rat skeletal muscle to creatine depletion: AMP deaminase and AMP deamination. *J Appl Physiol* 73: 2713–2716, 1992

18. Ren J-M, Semenkovich CF, and Holloszy JO: Adaptation of muscle to creatine depletion: effect on GLUT-4 glucose transporter expression. *Am J Physiol* 264 (Cell Physiol 33): C146–C150, 1993
19. Ohira M and Ohira Y: Effects of exposure to cold on metabolic characteristics in gastrocnemius muscle of frog (*Rana pipiens*). *J Physiol (Lond)* 395: 589–595, 1988
20. Moerland TS and Kushmerick MJ: Contractile economy and aerobic recovery metabolism in skeletal muscle adapted to creatine depletion. *Am J Physiol* 267 (Cell Physiol 36): C127–C137, 1994
21. Shoubridge EA, Challiss RAJ, Hayes DJ, and Radda GK: Biochemical adaptation in the skeletal muscle of rats depleted of creatine with the substrate analogue β -guanidinopropionic acid. *Biochem J* 232: 125–131, 1985
22. Annesley TM and Walker JB: Energy metabolism of skeletal muscle containing cyclocreatine phosphate. Delay in onset of rigor mortis and decreased glycogenolysis in response to ischemia or epinephrine. *J Biol Chem* 255: 3924–3930, 1980
23. Griffiths GR and Walker JB: Accumulation of analog of phosphocreatine in muscle of chicks fed 1-carboxymethyl-2-iminoimidazolidine (cyclocreatine). *J Biol Chem* 251: 2049–2054, 1976
24. Roy RR, Hutchison DL, Pierotti DJ, Hodgson JA, and Edgerton VR: EMG patterns of rat ankle extensors and flexors during treadmill locomotion and swimming. *J Appl Physiol* 70: 2522–2529, 1991
25. Hodgson JA, Wichayanuparp S, Recktenwald MR, Roy RR, McCall G, Day KM, Washburn D, Fanton JW, Kozlovskaya I, and Edgerton VR: Circadian force and EMG activity in hindlimb muscles of Rhesus monkeys. *J Neurophysiol* 86: 1430–1444, 2001
26. Recktenwald MR, Hodgson JA, Roy RR, Riazanski S, McCall GE, Kozlovskaya I, Washburn DA, Fanton JW, and Edgerton VR: Effects of spaceflight on Rhesus quadrupedal locomotion after return to 1 G. *J Neurophysiol* 81: 2451–2463, 1999
27. Guynn RD, Veloso D, and Veech RL: Enzymatic determination of inorganic phosphate in the presence of creatine phosphate. *Analyt Biochem* 45: 277–285, 1972
28. Lamprecht W, Stein P, Heinz F, and Weisser H: Creatine phosphate. *In: Methods of Enzymatic Analysis*, 2nd ed, ed. Bergmeyer HU, Academic Press, New York, pp 1777–1781, 1974
29. Lamprecht W and Trautschold I: ATP determination with hexokinase and glucose-6-phosphate dehydrogenase. *In: Methods of Enzymatic Analysis*, 2nd ed, ed. Bergmeyer HU, Academic Press, New York, pp 2101–2110, 1974
30. Roy RR, Talmadge RJ, Fox K, Lee M, Ishihara A, and Edgerton VR: Modulation of MHC isoforms in functionally overloaded and exercise rat plantaris fibers. *J Appl Physiol* 83: 280–290, 1997
31. Jiang B, Ohira Y, Roy RR, Nguyen Q, Ilyina-Kakueva EI, Oganov V, and Edgerton VR: Adaptation of fibers in fast-twitch muscles of rats to spaceflight and hindlimb suspension. *J Appl Physiol* 73 (Suppl): 58S–65S, 1992
32. Ohira Y, Jiang B, Roy RR, Oganov V, Ilyina-Kakueva E, Marini JF, and Edgerton VR: Rat soleus muscle fiber responses to 14 days of spaceflight and hindlimb suspension. *J Appl Physiol* 73 (Suppl): 51S–57S, 1992
33. Blewett C and Elder GCB: Quantitative EMG analysis in soleus and plantaris during hindlimb suspension and recovery. *J Appl Physiol* 74: 2057–2066, 1993
34. Ohira Y, Ishine S, Tabata I, Kurata H, Wakatsuki T, Sugawara S, Yasui W, Tanaka H, and Kuroda Y: Non-insulin and non-exercise related increase of glucose utilization in rats and mice. *Jpn J Physiol* 44: 391–402, 1994
35. Peter JB, Barnard RJ, Edgerton VR, Gillespie CA, and Stempel KE: Metabolic profiles of three fiber types of skeletal muscle in guinea pigs and rabbits. *Biochemistry* 11: 2627–2633, 1972
36. Ohira Y, Kanzaki M, and Chen C-S: Intramitochondrial inclusions caused by depletion of creatine in rat skeletal muscles. *Jpn J Physiol* 38: 159–166, 1988
37. McCurdy DT III and Kennedy JM: AZT decreases rat myocardial cytochrome oxidase activity and increases β -myosin heavy chain content. *J Mol Cell Cardiol* 30: 1979–1989, 1998
38. Brannon TA, Adams GR, Conniff CL, and Baldwin KM: Effects of creatine loading and training on running performance and biochemical properties of rat skeletal muscle. *Med Sci Sports Exerc* 29: 489–495, 1997
39. Terjung RL, Clarkson P, Eichner ER, Greenhaff PL, Hespel PJ, Israel RG, Kraemer WJ, Meyer RA, Spriet LL, Tarnopolsky MA, Wagenmakers AJ, and Williams MH: American College of Sports Medicine roundtable. The physiological and health effects of oral creatine supplementation. *Med Sci Sports Exerc* 32: 706–717, 2000
40. Demant TW and Rhodes EC: Effects of creatine supplementation on exercise performance. *Sports Med* 28: 49–60, 1999
41. Willoughby DS and Rosene J: Effects of oral creatine and resistance training on myosin heavy chain expression. *Med Sci Sports Exerc* 33: 1674–1681, 2001
42. Stevenson SW and Dudley GA: Dietary creatine supplementation and muscular adaptation to resistive overload. *Med Sci Sports Exerc* 33: 1304–1310, 2001