rapid communication

Age dependence of myosin heavy chain transitions induced by creatine depletion in rat skeletal muscle

GREGORY R. ADAMS AND KENNETH M. BALDWIN
Department of Physiology and Biophysics, University of California, Irvine, California 92717

Adams, Gregory R., and Kenneth M. Baldwin. Age dependence of myosin heavy chain transitions induced by creatine depletion in rat skeletal muscle. *J. Appl. Physiol.* 78(1): 368–371, 1995.—This study was designed to test the hypothesis that myosin heavy chain (MHC) plasticity resulting from creatine depletion is an age-dependent process. At weaning (age 28 days), rat pups were placed on either standard rat chow (normal diet juvenile group) or the same chow supplemented with 1% wt/wt of the creatine analogue β-guanidinopropionic acid [creatine depletion juvenile (CDJ) group]. Two groups of adult rats (age ~8 wk) were placed on the same diet regimens [normal diet adult and creatine depletion adult (CDA) groups]. After 40 days (CDJ and normal diet juvenile groups) and 60 days (CDA and normal diet adult groups), animals were killed and several skeletal muscles were removed for analysis of creatine content or MHC distribution. In the CDJ group, creatine depletion (78%) was accompanied by significant shifts toward expression of slower MHC isoforms in two slow and three fast skeletal muscles. In contrast, creatine depletion in adult animals did not result in similar shifts toward slow MIIC isoform expression in either muscle type. The results of this study indicate that there is a differential effect of creatine depletion on MHC transitions that appears to be age dependent. These results strongly suggest that investigators contemplating experimental designs involving the use of the creatine analogue β-guanidinopropionic acid should consider the age of animals to be used.

β-guanidinopropionic acid; creatine analogue

CREATINE DEPLETION via the feeding of creatine analogues has been employed in numerous studies on striated muscle plasticity. This intervention is thought to provide a "passive" (i.e., loading state unchanged) model that can be used to study muscle function, energetics, metabolism, fiber type changes, and, more recently, contractile protein expression.

In general, the information that emerges suggests that chronic creatine depletion results in the alteration of various functional, structural, and metabolic parameters reminiscent of those seen with endurance type training (1). These parameters include decreased shortening of maximal velocity (10), increased endurance (7, 10), increased citrate synthase, and decreased glycolytic enzyme activities (10, 13). Changes in muscle fiber type or myosin heavy chain (MHC) distribution indicative of increased type I MHC expression have also been reported (6, 9, 13). Such changes would also support a shift from a "fast" to a "slower" phenotype.

In a recent study, Adams et al. (1) employed creatine depletion in an attempt to counter the decreased expression of type I MHC normally observed in rat hindlimbs when the animals are tail suspended to unload these muscles. On the basis of the literature, we were surprised to find that several muscles from fully ambulatory animals fed a diet containing the creatine analogue β-guanidinopropionic acid (β-GPA) failed to demonstrate altered MHC expression. This lack of response occurred despite significant depletion (65%) of the creatine in a sample muscle. On examination of the methods employed in various studies that reported major fiber type or MHC shifts, we found one consistent difference: the age of the animals. Studies reporting significant effects of creatine depletion on the fiber type profiles of muscles have uniformly employed very young (e.g., weaning) animals (6, 9, 13). The animals used by Adams et al. were young adults (initial body wt >220 g, age 9 wk). This led us to speculate that the imposition of creatine depletion at a time when animals were rapidly growing might have a greater effect on myosin isoform expression, which is absent or less pronounced in older animals.

The present study was undertaken in an attempt to elucidate a possible age/developmental stage dependence of creatine depletion with regard to MHC isoform expression. Taken together with previously published findings of Adams and co-workers (1), the results indicate that the muscles of juvenile rats respond differently to this intervention compared with animals exposed to creatine depletion as adults. These findings are of importance for the interpretation and comparison of various studies found in the literature as well as the design of future studies employing this model.

METHODS

β-GPA was synthesized from β-alanine and cyanamide (Sigma Chemical, St. Louis, MO) (12). Twelve Sprague-Dawley rat pups were obtained from a single litter. At weaning (age 28 days, initial wt 62 ± 2 g), these animals were removed from the mother and separated into two groups. Seven rats were placed on a pelleted diet consisting of standard rat chow plus 1% wt/wt β-GPA [creatine depletion juvenile (CDJ) group]. The remaining five animals were placed on a standard rat chow diet [normal diet juvenile (NDJ) group]. Sixteen female Sprague-Dawley rats were obtained as adults (initial wt 207 ± 3 g, age 8 wk) from the same closed breeding colony and randomly assigned to a normal diet adult or a

creatine depletion adult group. All animals were housed in standard vivarium cages and were allowed food and water ad libitium.

At the end of 40 days, the four males plus three females from the CDJ group and three males plus two females from the NDJ group were anesthetized with pentobarbital sodium. The vastus lateralis (VL) muscle from one leg was then dissected free and quick frozen for the determination of creatine content. The soleus (SOL), plantaris (PLN), medial gastrocnemius (MG), vastus intermedius (VI), tibialis anterior (TA), and extensor digitorium longus (EDL) muscles were excised from the opposite leg, trimmed of fat and connective tissue, weighed, placed in precooled glycerol, and stored at −20°C for subsequent myosin determination. Animals were then killed by an overdose of pentobarbital sodium.

At the end of 68 days, the animals in the normal diet adult and creatine depletion adult groups were anesthetized with pentobarbital sodium and the SOL and PLN muscles were harvested as above.

Myofibril extraction. Muscle samples were homogenized in ~20 vol of a solution containing (in mM) 250 sucrose, 100 KCl, and 5 EDTA. The homogenate was centrifuged and resuspended successively in three solutions: *1*) 250 mM sucrose, 100 mM KCl, and 5 mM EDTA; *2*) 0.5% Triton X and 175 mM KCl; and *3*) 150 mM KCl. The final pellet was resuspended in 1 ml of 150 mM KCl. The protein concentration of this solution was determined with the biuret method (3). An aliquot of myofibril suspension was added to a solution containing 50% vol/vol glycerol, 100 mM $Na_4P_2O_7$, and 5 mM EDTA at a concentration of 1 mg/ml and stored at −20°C.

Myosin analysis. The myofibril fraction was analyzed to determine relative content of type I, IIa, IIx, and IIb MHCs in the muscles harvested. Five-microliter samples of stored myofibril solution were added to 35 μl of denaturing buffer [62.5 mM tris(hydroxymethyl)aminomethane, 20% glycerol, 1% β-mercaptoethanol, 2.3% sodium dodecyl sulfate (SDS), and 0.05% bromophenol blue] and heated at 100°C for 2 min. After this treatment, a 16-μl aliquot (2 μg) was withdrawn for analysis by SDS-polyacrylamide electrophoresis (PAGE).

Samples were analyzed via SDS-PAGE as previously described (1). Separating gels contained 30% vol/vol glycerol, 8% wt/vol acrylamide (2% cross-link), 1% wt/vol glycine, and 0.4% wt/vol SDS. Stacking gels contained 40% vol/vol glycerol and 4% wt/vol total acrylamide (2.7% total acrylamide). Gels were run at 275 V for ~22 h. Gels were stained for 1 h with Brilliant Blue G 250 dye (Sigma Chemical) and then destained with 25% methanol and 5% acetic acid. MHC bands were scanned with a laser densitometer (Molecular Dynamics, Sunnyvale, CA). The peaks of interest were identified in the digitized densitometric data sets, and the area of each peak was determined by integration.

Creatine assay. Frozen VL muscles were pulverized in liquid N_2 and extracted in perchloric acid (4). Creatine was assayed by using standard enzymatic techniques (4).

Statistical analyses. All values are reported as means ± SE. Treatment effects were determined by Student's *t*-test using the Instat software package (Graphpad Software, San Diego, CA). The 0.05 level of confidence was accepted for statistical significance. Analyses of all percent data were performed on arcsine-transformed values to correct for nonnormal distribution. Percent data presented represent the nontransformed values.

RESULTS

As is commonly reported for studies using β-GPA, the body weights of the animals placed on the analogue diet tended to be lower than those of the animals on the normal diet (Table 1). The large variability in juvenile rat body weights resulted from the inclusion of both male and female rats from the original litter. The diet-induced differences in body weight were similar in both males (−15%) and females (−16%) (results not shown). The body weights of adult rats depleted of creatine averaged ~11% less than those of the normal diet control rats (Table 2). Except for absolute muscle weights and total body weight, no differences were found between males and females in any parameters measured. The data from males and females were therefore combined for all subsequent analysis.

TABLE 1. *Effect of creatine depletion on body weight, muscle-to-body weight ratio, and creatine content in juvenile rats*

	NDJ	CDJ
Body weight, g	260±25	213±19
Muscle/body wt, mg/g		
VI	0.30±0.02	0.35±0.04
SOL	0.39±0.01	0.37±0.01
PLN	1.16±0.02	0.92±0.03*
MG	2.39±0.12	1.98±0.09*
TA	1.89±0.05	1.65±0.05*
EDL	0.41±0.03	0.33±0.01*
LV	2.50±0.07	2.67±0.04
Creatine content, μmol/g		
VL	9.96+0.60	1.90±0.12*

Values are means ± SE. NDJ, normal diet juvenile (n = 5); CDJ, creatine-depleted juvenile (n = 7); VI, vastus intermedius; SOL, soleus; PLN, plantaris; MG, medial gastrocnemius; TA, tibialis anterior; EDL, extensor digitorum longus; LV, left ventricle; VL, vastus lateralis. * Significantly different compared with NDJ, $P < 0.05$.

The muscle-to-body weight ratios of the four muscles composed of myofibers expressing predominantly fast MHC isoforms (PLN, MG, TA, and EDL) were significantly lower for the CDJ group (Table 1). The predominantly slow MHC-expressing muscles, i.e., VI and SOL, as well as the left ventricle of the creatine-depleted group had muscle-to-body weight ratios similar to the NDJ group. A similar trend, involving lower normalized fast muscle weight, was seen in the adult rats (Table 2).

The diet containing 1% wt/wt β-GPA resulted in significant depletion of creatine in VL muscles that was similar in both juvenile (−81%) and adult (−78%) rats (Tables 1 and 2).

In the predominantly slow VI and SOL muscles of juvenile rats, creatine depletion resulted in significant

TABLE 2. *Effect of creatine depletion on body weight, muscle-to-body weight ratio, and creatine content in adult rats*

	NDA	CDA
Body weight, g	280±5	250±4
Muscle/body wt, mg/g		
SOL	0.56±0.02	0.54±0.01
PLN	1.21±0.01	1.01±0.01*
Creatine content, μmol/g		
VL	7.70±0.59	1.70±0.13*

Values are means ± SE. NDA, Normal diet adult (n = 8); CDA, creatine-depleted adult (n = 8). * Significantly different compared with NDA, $P < 0.05$.

TABLE 3. *Effect of creatine depletion on MHC distribution in juvenile rats*

	Type I	Type IIa	Type IIx	Type IIb
VI				
NDJ	70.8±4.0	18.1±1.1	8.2±1.8	2.8±1.7
CDJ	80.9±2.8	11.6±2.2*	5.4±0.9	2.0±1.0
SOL				
NDJ	96.9±1.0	3.1±1.0		
CDJ	100*	0*		
PLN				
NDJ	2.9±0.2	10.4±0.4	37.9±1.9	48.9±1.9
CDJ	6.9±0.6*	12.7±0.3*	41.4±0.9	39.1±0.8*
MG				
NDJ	5.4±0.7	5.9±0.2	21.6±1.1	67.0±0.7
CDJ	11.1±0.5*	6.2±0.3	24.4±1.1	58.2±1.2*
TA				
NDJ	1.1±0.3	7.4±0.5	17.5±0.4	74.0±0.6
CDJ	2.3±0.2*	9.4±0.7*	22.2±0.3*	66.1±0.9*
EDL				
NDJ	1.3±0.1	12.3±0.5	23.6±1.5	62.8±2.0
CDJ	2.3±0.2*	15.4±0.5*	34.4±0.5*	48.0±0.7*

Values are means ± SE; n = 5 and 7 rats for NDJ and CDJ, respectively. Myosin heavy chain (MHC) isoforms are expressed as percentage of total MHC pool. * Significantly different compared with NDJ, $P < 0.05$.

declines in the proportion of type IIa MHC. In SOL, the decline in type IIa was reflected in a shift to expression of 100% type I MHC as detected by SDS-PAGE and densitometry (Table 3). In the four fast muscles, creatine depletion resulted in declines in the proportion of type IIb MHC ranging from 11 to 24%. In these muscles, the decline in percentage of type IIb was offset by varying increases in the putatively slower isoforms (types IIx, IIa, and I). In all four fast muscles (PLN, MG, TA, and EDL), creatine depletion in juvenile rats resulted in small increases in type I MHC.

In contrast to the results seen in the juvenile rats, no shift toward slower MHC isoforms was seen in either the representative slow SOL or fast PLN muscles of adult rats depleted of creatine (Table 4).

DISCUSSION

The primary purpose for the present study was to examine the age dependence of creatine depletion-induced muscle phenotype plasticity. Several previously published studies reported significant effects of creatine depletion on the fiber type or myosin distribution of rodent hindlimb muscles (6, 9, 13, 15). For example,

TABLE 4. *Effect of creatine depletion on MHC distribution in adult rats*

	I	IIa	IIx	IIb
SOL				
NDA	92.5±1.2	7.1±1.0	0.5±0.001	
CDA	91.0±1.4	8.8±1.3	0.4±0.001	
PLN				
NDA	6.7±0.7	10.9±0.5	53.8±2.1	28.7±2.4
CDA	6.5±0.6	11.3±0.8	45.7±2.2*	36.4±3.0

Values are means ± SE; n = 8 rats for both NDA and CDA. MHC isoforms are expressed as percentage of total MHC pool. * Significantly different compared with NDA, $P < 0.05$.

Matoba et al. (6) found a shift from 96 to 100% type I fibers in SOL and from 74% type IIb to 96% type IIa in EDL muscles of rats fed a 1% β-GPA diet. If we assume that there is a general correspondence between myosin adenosinetriphosphatase histochemistry and the type of MHC expressed (2), the results obtained with juvenile rats in the current study are in qualitative accord with the findings of these previously published reports (Table 3). However, creatine depletion does not appear to be a powerful stimulus for the upregulation of slower (e.g., types I and IIa) MHC isoforms in adult rats (Table 4).

This differential effect should be taken into account when one designs or interprets studies using β-GPA to manipulate skeletal muscle phenotype. An example of this caveat is found when the results of Matoba et al. (6) and Adams et al. (1) are compared. In both studies, creatine depletion was employed in an attempt to prevent the shift toward faster MHC or fiber type expression, which is known to result from hindlimb unloading during tail suspension in rats (14). Matoba et al. (6) used juvenile rats and found that creatine depletion not only prevented a decrease in slow fiber types but actually increased slower fiber type proportions in suspended animals. In a study of similar design but that used adult rats, Adams et al. (1) found that creatine depletion did not alter the loss of type I MHC and had only minor effects, retarding the increase in IIb MHC in some muscles.

In summary, this brief communication is intended to provide a cautionary note to investigators contemplating experimental designs involving the use of the creatine analogue β-GPA. Our results clearly indicate a differential effect of creatine depletion on MHC transitions that appears to be age dependent.

This research was supported by National Acronautics and Space Administration (NASA) Grant NAG-2555 and National Institute of Arthritis and Musculoskeletal and Skin Diseases Grant AR-30346 (K. M. Baldwin) and the NASA Space Biology Research Associate Program (G. R. Adams).

Address reprint requests to K. M. Baldwin.

Received 10 August 1994; accepted in final form 22 September 1994.

REFERENCES

1. **Adams, G. R., F. Haddad, and K. M. Baldwin.** Interaction of chronic creatine depletion and muscle unloading: effects on postural and locomotor muscles. *J. Appl. Physiol.* 77: 1198–1205, 1994.
2. **Adams, G. R., B. M. Hather, K. M. Baldwin, and G. A. Dudley.** Skeletal muscle myosin heavy chain composition and resistance training. *J. Appl. Physiol.* 74: 911–915, 1993.
3. **Gornall, A. G., C. J. Bardawell, and M. M. David.** Determination of serum proteins by means of the biuret method. *J. Biol. Chem.* 177: 751–756, 1949.
4. **Lowery, O.** *A Flexible System of Enzymatic Analysis.* New York: Academic, 1972, p. 158–160.
5. **Mainwood, G. W., and J. T. Zepetnek.** Post-tetanic responses in creatine depleted rat EDL muscle. *Muscle Nerve* 8: 774–782, 1985.
6. **Matoba, H., T. Wakatuki, and Y. Ohira.** β-Guanidinopropionic acid suppresses suspension-induced changes in myosin expression in rat skeletal muscle (Abstract). *Med. Sci. Sport Exercise* 25: S157, 1993.
7. **Meyer, R. A., T. A. Brown, B. L. Krilowicz, and M. J. Kushmerick.** Phosphagen and intracellular pH changes during con-

traction of creatine-depleted rat muscle. *Am. J. Physiol.* 250 (*Cell Physiol.* 19): C264–C274, 1986.

8. **Meyer, R. A., J. M. Foley, G. R. Adams, and S. J. Harkema.** Energy cost of contraction is unchanged in rat skeletal muscle chronically depleted of ATP and creatine (Abstract). *Physiologist* 35: 198, 1992.
9. **Moerland, T. S., N. G. Wolf, and M. J. Kushmerick.** Administration of creatine analogue induces isomyosin transitions in muscle. *Am. J. Physiol.* 257 (*Cell Physiol.* 26): C810–C816, 1989.
10. **Petrofsky, J. S., and C. D. Fitch.** Contractile characteristics of skeletal muscles depleted of phosphocreatine. *Pfluegers Arch.* 384: 123–129, 1980.
11. **Ren, J. M., C. F. Semenkovich, and J. O. Holloszy.** Adaptation of muscle to creatine depletion: effect on GLUT-4 glucose transporter expression. *Am. J. Physiol.* 264 (*Cell Physiol.* 33): C146–C150, 1993.
12. **Rowley, G. L., A. L. Greenleaf, and G. L. Kenyon.** On the specificity of creatine kinase: new glycocyamines and glycocyamine analogues related to creatine. *J. Am. Chem. Soc.* 93: 5542–5551, 1971.
13. **Shoubridge, E. A., R. A. J. Challiss, D. J. Hayes, and G. K. Radda.** Biochemical adaptation in skeletal muscle of rats depleted of creatine with the substrate analogue β-guanidinopropionic acid. *Biochem. J.* 232: 125–131, 1985.
14. **Thomason, D. B., R. E. Herrick, D. Surdyka, and K. M. Baldwin.** Time course of soleus muscle myosin expression during hindlimb suspension and recovery. *J. Appl. Physiol.* 63: 130–137, 1987.
15. **Wiseman, R., and M. J. Kushmerick.** Myosin isoform changes in skeletal muscle of mice treated with synthetic analogues of creatine (Abstract). *FASEB J.* 8: A10, 1994.