

SPECIFICITY OF CREATINE IN THE CONTROL OF MUSCLE PROTEIN SYNTHESIS

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ABSTRACT

This study provides additional evidence that creatine, an end product of contraction unique to muscle, is involved in the control of muscle protein synthesis. Creatine is shown to stimulate selectively the rate of synthesis of two major contractile proteins, actin and myosin heavy chain, in cultures of differentiating skeletal muscle. Creatine affects only the rate of synthesis and not the rate of degradation. Several creatine analogs are as effective as creatine in stimulating muscle protein synthesis, creatinine and amino acids such as arginine and glycine are not. Creatine stimulates myosin heavy chain synthesis twofold in cultures of embryonic muscle grown in either normal or dialyzed media.

INTRODUCTION

In a previous report (1), we presented evidence that creatine, a product of muscular activity, is involved in the control of muscle protein synthesis. Increasing the amount of creatine available to differentiating skeletal muscle cells formed both *in vitro* (in monolayer culture) and *in vivo* (explants maintained in organ culture) stimulates the rate of myosin heavy chain synthesis twofold. The response is concentration-dependent over the range of 10–100 μM and maximal over the range 100 μM –25 mM creatine. The stimulation is specific since the rates of total protein and DNA synthesis are not affected by creatine. Creatine functions by stimulating muscle protein synthesis in differentiated cells rather than by causing cells to differentiate.

In this communication, we present results fur-

ther defining the role of creatine in the control of muscle protein synthesis. To determine whether creatine stimulates the synthesis of other cell-specific proteins, we have compared the rate of actin synthesis in cultures supplied with creatine to the rate in control cultures. To determine whether creatine affects the rate of myosin degradation as well as its rate of synthesis, we have measured the half-lives of myosin heavy chain and total protein in cultures grown with and without creatine. The ability of other compounds to substitute for creatine in stimulating muscle protein synthesis was studied by measuring the rates of total protein and myosin heavy chain synthesis in cultures supplied with compounds structurally related to creatine. The compounds tested are arginine and glycine (the amino acids from which creatine is synthe-

sized), ornithine, creatinine (the anhydride of creatine formed in muscle), and several creatine analogs synthesized by Kenyon and coworkers (2). We also report the effect of using dialyzed medium on the rates of total protein and myosin heavy chain synthesis.

MATERIALS AND METHODS

Skeletal Muscle Tissue Culture

Details of the preparation of monolayer cultures of embryonic skeletal muscle have been described (3, 4). Mononucleated muscle cells were isolated from breast muscle from 11–13-day chick embryos and suspended in complete culture medium (87.5% of Eagle's minimum essential medium and 10% of selected horse serum [both from Grand Island Biological Co., Grand Island, N. Y.], and 2.5% of 11-day chick embryo extract). The cells were counted and suspended in complete medium in collagen-coated dishes (Falcon Plastics, Div. of B-D Laboratories, Inc., Los Angeles, Calif.), at a density of $4-6 \times 10^6$ cells per 100-mm dish. The medium was changed after 24 h except when noted. In these cultures the cells proliferate and then fuse to form myotubes. With the lots of horse serum used in the experiments reported here, fusion began by the 20th h and was 70–80% complete by the 40th h of incubation.

Myosin Heavy Chain and Actin Synthesis

Rates of myosin heavy chain and actin synthesis were measured as previously described (1). Cultures were incubated for 4 h with 10 or 15 μ Ci of L-[4,5- 3 H]leucine, 50 Ci/mmol (Schwarz Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.), in leucine-free Eagle's minimum essential medium with 3–5% horse serum. After washing each plate with Hanks' balanced salt solution, the cells were scraped and homogenized in a tightly fitting Dounce homogenizer in 20 mM NaCl-2mM phosphate buffer (pH 7) on ice (5). Material containing contractile protein was collected by centrifugation and solubilized by adding 1% sodium dodecyl sulfate (SDS)-1% dithiothreitol-50 mM phosphate buffer (pH 7) and heating at 100°C for 5 min. In some experiments, myosin was further purified by extraction in 20 mM sodium pyrophosphate-1 mM Mg^{++} , pH 9.5 (5) before the dissociation step. Myosin heavy chain and actin were isolated by disk gel electrophoresis using 4% SDS-urea polyacrylamide gels (6). Those regions of the gel containing myosin heavy chain and actin, identified by comparison with reference gels of rabbit and chick myosin (7) and of rabbit actin (8), were extracted by heating 1-mm thick gel slices at 50°C for 1 h in 0.5 ml of NCS solubilizer (Amersham/Searle Corp., Arlington Heights, Ill.), or Protosol (New England Nuclear, Boston, Mass.). The samples were counted in toluene-2,5-diphenyloxazole/1,4-bis[2-(5-phenyloxazolyl)]benzene (PPO/

POPOP) in a scintillation counter. In each experiment this procedure was performed on replicate tissue culture plates in duplicate for each variable studied.

In these experiments, the subunits of tropomyosin and troponin migrate close to actin on the gel. To verify that the data presented for the rate of actin synthesis are not significantly affected by contaminating polypeptides, actin was selectively isolated from the crude actomyosin-containing precipitate before being subjected to electrophoresis. The actomyosin-containing precipitate was washed with water and acetone and then dried. Actin was solubilized from the acetone powder by extraction with 0.5 mM ATP, 0.2 mM $CaCl_2$, and 20 mM Tris chloride, pH 7.5. After centrifugation, the actin-containing supernate was treated with SDS-dithiothreitol-phosphate buffer and subjected to electrophoresis as described above. As reported by Sender (6), this procedure resulted in a single band migrating the same as actin prepared from adult rabbit. The gels were treated as described above. The percent increase in the rate of actin synthesis stimulated by creatine was the same using either procedure.

Total Protein Synthesis

The rate of total protein synthesis was measured in one of two ways. Labeled protein from duplicate plates was collected as 5% trichloroacetic acid-insoluble material on glass filters (Whatman, GF/C) and counted in toluene-PPO/POPOP in a scintillation counter. Alternatively, two 100- μ l aliquots of cell homogenate prepared as described above were solubilized in 0.5 ml of NCS solubilizer or Protosol and counted in toluene-PPO/POPOP in a scintillation counter. This procedure gave results equivalent to those obtained by precipitating the protein in such aliquots with 5% trichloroacetic acid, washing with absolute ethanol-ether 3:1, solubilizing in NCS solubilizer, and counting in toluene-PPO/POPOP.

DNA synthesis

The amount of DNA per dish was measured using the method of Hinegardner (9).

RESULTS

Fig. 1 shows the effect of supplying 5 mM creatine to monolayer cultures of differentiating muscle cells on the rates of synthesis of total protein, myosin heavy chain, and actin as a function of time in culture. Creatine does not affect the rate of total protein synthesis at any time in the 7-day culture period. In contrast, the percent increase in the rate of myosin heavy chain synthesis increases from about 40% at the end of day no. 2 to about 80% at the end of day no. 3, to about 120% in cultures 3.5-days old and older in creatine-supplied cultures. Qualitatively, creatine affects the rate of

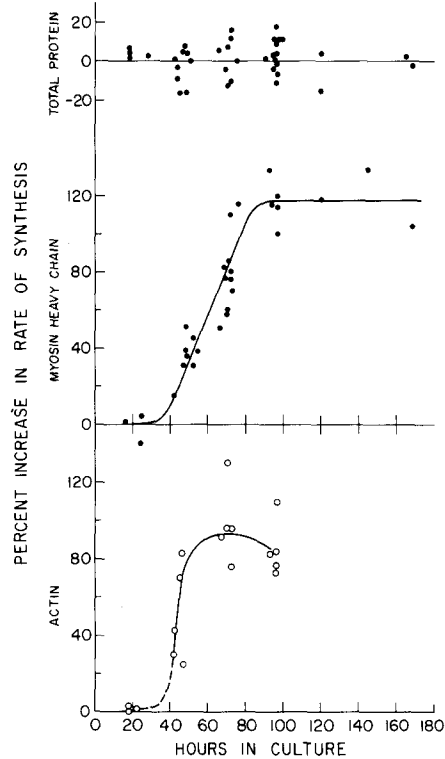


FIGURE 1 The percent increase in the rate of synthesis of total protein, myosin heavy chain, and actin in cultures supplied with 5 mM creatine from zero time compared to control cultures plotted against time in culture. The rates of synthesis were determined as described in Materials and Methods. The data points are positioned at a time corresponding to the midpoint of the 4-h pulse and are accurate to within 10%.

synthesis of actin and myosin heavy chain the same, i.e., no stimulation is observed until cell fusion is essentially complete and there is a twofold increase in the rate of synthesis by day no. 3. These results suggest that skeletal muscle cells in culture synthesize at least two major contractile proteins faster when supplied with creatine, and they do this only after overt cell differentiation has occurred.

Since it is possible that creatine affects the rate of myosin degradation as well as its rate of synthesis, the turnover rates of myosin heavy chain and total protein were measured in cultures grown with and without creatine. Cultures were pulse labeled for 4 h on day no. 2 or 3, and radioactive medium was replaced with complete culture medium, with and without creatine. At various times during the following 30 h the amount of isotope incorporated into myosin heavy chain and total

protein was measured. Half-lives were determined from the time required for loss of half of the original radioactivity incorporated. This experiment measures protein turnover during the period of cell differentiation when the rate of synthesis of myosin heavy chain and total protein increases linearly.

Fig. 2 shows the results of several experiments in which the loss of radioactivity from myosin heavy chain and total protein was measured at various times during the 30-h period following exposure of the cultures to [^3H]leucine. Creatine does not affect the rate of degradation of either myosin heavy chain or total protein. Within experimental error (10–20%), the data follow first order kinetics typically observed for proteins (10). Half-lives for myosin heavy chain and total protein are ~ 15 h and ~ 22 h, respectively.

To determine whether other compounds can

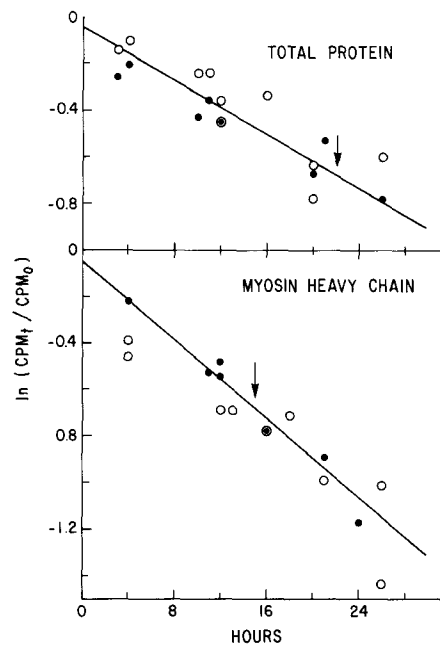


FIGURE 2 The logarithm (\ln) of the ratio of cpm remaining at time (t) to original cpm for total protein (upper portion) and myosin heavy chain (lower portion) plotted against time (hours) in culture. The open circles refer to data for creatine-supplied cultures; filled circles to controls. Cultures were pulse labeled for 4 h with [^3H]leucine on day no. 2 or 3; radioactive medium was replaced with complete culture medium. At various times during the following 30 h, the amount of radioactivity remaining in myosin heavy chain and total protein was determined as described in Materials and Methods.

TABLE I
Effect of Amino Acids and Creatine-Analogs on the Rates of Synthesis of Total Protein and Myosin Heavy Chain

	Structure	Ratio of experimental/control	
		Total protein synthesis	Myosin Heavy chain synthesis
I Creatine (<i>N</i> -methyl- <i>N</i> -amidinoglycine)		(1) 0.8 ± 0.1 (2) 1.3 ± 0.1	(1) 2.2 ± 0.5 (2) 2.2 ± 0.2
II Arginine		(1) 1.0 ± 0.1 (2) 1.0 ± 0.2 (3) 1.0 ± 0.1	(1) 0.9 ± 0.1 (2) 0.8 ± 0.1 (3) 1.4 ± 0.3
III Ornithine		(1) 1.0 ± 0.1 (2) 0.9 ± 0.1	(1) 1.0 ± 0.1 (2) 1.0 ± 0.1
IV Glycine		(1) 1.0 ± 0.1 (2) 1.1 ± 0.1	(1) 1.2 ± 0.1 (2) 0.8 ± 0.1
V Creatinine (1-methyl-2-amino-2-imidazolin-4-one)		(1) 0.8 ± 0.1 (2) 1.0 ± 0.1	(1) 1.0 ± 0.1 (2) 1.1 ± 0.2
VI 1-carboxymethyl-2-iminoimidazolidine		(1) 1.0 ± 0.2 (2) 0.8 ± 0.1 (3) 0.9 ± 0.2 (4) 1.0 ± 0.2 (5) 1.4 ± 0.2	(1) 1.4 ± 0.2 (2) 1.4 ± 0.1 (3) 1.6 ± 0.2 (4) 2.3 ± 0.3 (5) 2.6 ± 0.2
VII <i>N</i> -methylamidino- <i>N</i> -methylglycine		(1) 1.1 ± 0.2 (2) 0.8 ± 0.1 (3) 0.9 ± 0.1 (4) 0.9 ± 0.1	(1) 1.4 ± 0.2 (2) 1.2 ± 0.1 (3) 1.1 ± 0.1 (4) 1.0 ± 0.2
VIII <i>N</i> -ethyl- <i>N</i> -amidinoglycine		(1) 1.0 ± 0.2 (2) 1.2 ± 0.1 (3) 1.1 ± 0.1	(1) 2.8 ± 0.3 (2) 2.3 ± 0.2 (3) 1.8 ± 0.2
IX 1-carboxymethyl-2-iminohexahydropyrimidine		(1) 0.9 ± 0.1 (2) 1.2 ± 0.1 (3) 1.0 ± 0.2	(1) 2.0 ± 0.2 (2) 2.2 ± 0.2 (3) 2.0 ± 0.3

Rates of total protein and myosin heavy chain synthesis were determined by measurement of the incorporation of [³H]leucine into cell homogenates and into myosin heavy chain isolated by disk gel electrophoresis, respectively, in 3- or 4-day old cultures. The data represent the ratio of cpm/dish, determined from the average of duplicate culture dishes, for experimental compared to control cultures. The number of entries for each compound represent different experiments. Cultures were supplied with the test compound from zero time, and the concentrations tested were 1 mM and 5 mM for I and VIII, 5 mM for II-V, and 1 mM for VI, VII, and IX. The cultures were not fed after 24 h. The amount of DNA per dish was within 10% of control for each compound tested.

TABLE II
Effect of Dialyzing Medium on the Rates of Synthesis of Total Protein and Myosin Heavy Chain

	Exp.	Total protein synthesis (cpm/ μ g DNA)	Myosin heavy chain synthesis (cpm/ μ g DNA, $\pm 10\%$)	Percent Increase in rate of myosin synthesis
Control	1	149,000 \pm 13,500	360	—
	2	134,000 \pm 6,300	350	—
+ 5 mM Creatine	1	161,000 \pm 1,500	800	120
	2	151,000 \pm 7,800	610	75
Dialyzed medium	1	138,000 \pm 3,600	500	40
	2	120,000 \pm 6,000	420	20
Dialyzed medium + 5 mM creatine	1	138,000 \pm 1,800	880	145
	2	121,000 \pm 10,600	860	145

Rates of total protein and myosin heavy chain synthesis were determined by measurement of the incorporation of [3 H]leucine into cell homogenates and into myosin heavy chain isolated by disk gel electrophoresis, respectively, in 3- or 4-day old cultures. The cultures were not fed after 24 h. Data represent the average of duplicate culture dishes plus or minus the range.

substitute for creatine in stimulating muscle protein synthesis, the rates of synthesis of myosin heavy chain and total protein in cultures supplied with compounds structurally related to creatine were compared to the corresponding rates in creatine-supplied and control cultures. All experiments were performed on day no. 3 and 4 when the stimulation of myosin synthesis in response to creatine is maximal (see Fig. 1). The concentrations of analog used were 1 and 5 mM; creatine concentrations of 1 and 5 mM give maximal response (1). The results, given in Table I, are expressed as the ratio of rates of synthesis of experimental to control. Arginine and glycine, the amino acids from which creatine is synthesized, ornithine, structurally related to arginine, and creatinine, formed nonenzymatically from creatine and creatine phosphate in muscle (11), do not substitute for creatine in stimulating myosin synthesis.

Several creatine analogs synthesized by Kenyon and coworkers (2) as potential substrates of creatine kinase were tested to determine how well they could substitute for creatine and also to explore the possibility that creatine kinase is involved in the mechanism of creatine action. The latter possibility would be supported if only those analogs with high V_{max} stimulated myosin heavy chain synthesis. The relative V_{max} values for the analogs tested are 90% for VI, 32% for VIII, and nondetectable for VII and IX (2). Of these four analogs, VI, VIII, and IX are as effective as creatine in selectively stimulating myosin heavy chain synthe-

sis, only the acyclic counterpart of VI is not. Since both reactive and nonreactive substrates of creatine kinase stimulate myosin synthesis, creatine kinase may not be involved in the mechanism of creatine action.

Creatine appears to function by stimulating differentiated muscle cells to synthesize more contractile protein and not by initiating synthesis in undifferentiated cells (1). It seems unlikely that the small amount of creatine present in the tissue culture medium (from horse serum and embryo extract) functions in the control of contractile protein synthesis since creatine selectively stimulates myosin synthesis in fetal mouse hearts maintained in serum- and embryo extract-free medium in organ culture (12). Nevertheless, it was of interest to determine the rates of myosin heavy chain and total protein synthesis in cultures supplied with creatine-depleted medium. Complete culture medium was dialyzed against 10 vol of minimum essential medium for 24 h with one change of the dialysate at 4°C. Cultures grown in dialyzed medium appeared the same as control cultures under phase microscopy. The rates of total protein and myosin heavy chain synthesis were determined in cultures grown in normal and dialyzed media with and without creatine. The results from two representative experiments are shown in Table II. The rate of total protein synthesis was not affected by growing the cultures in dialyzed medium or by supplying creatine to either normal or dialyzed media. There is a small increase in the rate of myosin heavy chain synthe-

sis in cultures grown in dialyzed medium compared to control cultures, suggesting that dialysis removes some substance(s) detrimental to myogenesis. The rate of myosin heavy chain synthesis in cultures supplied with creatine in both normal and dialyzed media was stimulated twofold.

DISCUSSION

The experiments reported here further define the role of creatine in the control of muscle protein synthesis. These experiments demonstrate that creatine selectively stimulates the rate of synthesis of the two major contractile proteins, actin and myosin, in cultures of differentiating skeletal muscle. It seems likely that all of the contractile proteins are synthesized faster in response to creatine, but any increase in the rate of synthesis of the relaxing proteins remains to be measured. The data presented in Fig. 1 shows that creatine stimulates the rate of actin and myosin heavy chain synthesis approximately twofold by day no. 3. Although it is possible that between 40 and 70 h the rate of actin accumulation is more rapid than it is for myosin, there is not sufficient data for the rate of actin synthesis to justify such a conclusion.

Increased accumulation of myosin heavy chain in creatine-supplied cultures (observed using polyacrylamide gels stained for protein) is due solely to an increase in rate of synthesis; creatine does not block myosin degradation. In work-induced muscle hypertrophy *in vivo*, there is a decrease in catabolism of sarcoplasmic protein but not of myofibrillar protein (13). In creatine-induced hypertrophy, no change in catabolism of myofibrillar or total protein is observed. The values of the half-lives of myosin heavy chain and total protein (~15 h and ~22 h, respectively) reported here compare favorably with values obtained by Reporter (14) using skeletal muscle tissue culture of embryonic rat leg (18–21 h and 21 h for myosin and total protein, respectively). However, these values are very different from those reported for myosin from adult skeletal muscle growing *in vivo*: 20 or 30 days for rat (15) and rabbit (16). A value of 150 days has also been reported for rabbit myosin (17). Half-lives of the contractile proteins in cardiac muscle *in vivo* are much shorter: 7.6 days for rabbit myosin (18), and 11–12 days for rat myosin heavy and light chains, actin, and tropomyosin (19). The half-life of both myosin and total protein in cultures of beating heart cells was recently reported to be 4–6 days (20).

Several compounds structurally related to creatine were tested for their effectiveness in substituting for creatine in stimulating contractile protein synthesis. Neither arginine and glycine, the amino acids from which creatine is synthesized, nor creatinine, the form in which creatine is released from muscle, can substitute. Several creatine analogs, however, are as effective as creatine in stimulating myosin synthesis. These include both reactive and nonreactive substrates for creatine kinase, suggesting that creatine kinase may not be involved in the mechanism of creatine action. It is possible that analog VII did not substitute for creatine because it cannot enter muscle cells; the stimulation of contractile protein synthesis may be determined by the specificity of a membrane transport system. This seems unlikely since the other analogs tested apparently entered muscle cells and functioned at the site of creatine action, but, in the absence of radioactive analogs, influx was not measured.

The effect of using dialyzed medium with and without creatine on the rate of protein synthesis was measured. Cultures grown in dialyzed medium synthesize myosin 20–40% faster than control cultures, suggesting that dialysis removes substance(s) detrimental to myogenesis. Cultures supplied with 5 mM creatine in either normal or dialyzed media synthesize myosin twice as fast as the corresponding control cultures.

Creatine is not synthesized in muscle but is transported from its site of synthesis in the liver and enters muscle via a membrane transport system (20). In the experiments reported here, it is assumed that creatine influx also occurs in myotubes developing in culture and that intracellular creatine stimulates muscle protein synthesis. The influx of [¹⁴C]creatine into differentiating skeletal muscle in culture has been measured, and the results support this assumption (see footnote 1).

These experiments provide additional evidence that creatine functions in the control of muscle protein synthesis. Experiments to determine the mechanism of this effect remain to be performed.

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