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Creatine and the Control of Myosin Synthesis in Differentiating Skeletal Muscle

(cell culture/chick embryo/heavy chain/protein synthesis/DNA synthesis)

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ABSTRACT **These experiments provide evidence that creatine, an end product of contraction unique to muscle, is involved in the control of muscle-protein synthesis. Skeletal muscle cells formed both *in vitro* and *in vivo* synthesize myosin heavy chain faster when supplied creatine *in vitro*. The response is apparent within four hours after addition of creatine to the culture medium, and is dependent on concentration over a range of 10–100 μM creatine. The effect seems to be selective for cell-specific proteins(s), since the rate of total protein synthesis is unaffected.**

Since hypertrophy and atrophy of skeletal muscle result from increased and decreased muscular activity, one might postulate that a product of muscular activity stimulates contractile protein synthesis. Creatine is a likely candidate for such an effector, since it is a unique product of activity. We have investigated the possibility that creatine functions in this way by manipulating the external concentration of creatine available to differentiating skeletal muscle cells *in vitro*. We have found that increased concentrations of extracellular creatine selectively stimulates 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 (1, 2). Mononucleated muscle cells were isolated from breast muscle from 12-day chick embryos and suspended in complete culture medium [87.5% of Eagle's Minimum Essential Medium, Grand Island Biological Co.; 10% of selected horse serum, Grand Island Biological Co.; and 2.5% of 11-day chick-embryo extract]. The cells were counted and plated on collagen-coated dishes (Falcon Plastics) at densities of about 10^6 cells per 60-mm dish and about 4×10^6 cells per 100-mm dish. The medium was changed at 24 hr. In such cultures, the cells first proliferate and then fuse to form myotubes. With the lot of horse serum used for the experiments reported here, fusion begins before the 30th hour and is 70–80% complete by the 48th hour of incubation.

Organ cultures were prepared from 14- and 15-day chick embryos. 10 Pieces of breast muscle, cleaned of connective tissue and weighing about 1 mg each, were placed on a stainless-steel screen at the air–medium interface in Falcon organ culture dishes and incubated for 24 hr. The medium and incubation conditions were as for monolayer culture.

Myosin Heavy-Chain Synthesis. The rate of myosin heavy-chain synthesis was measured at various times after differentiation (cell fusion) in cultures growing in 100-mm dishes. Cultures were incubated for 4 hr with 10 μCi of L-[4,5-^{3}H]-leucine (Schwarz–Mann, 50 Ci/mmol) in leucine-free Eagle's Minimum Essential Medium (minimal medium) (Grand Island Biological Co.) with 3–5% horse serum.

The same qualitative effects of creatine on myosin heavy-chain synthesis occur in medium containing 3, 5, or 10% horse serum. Since there is less dilution of the specific activity of [^{3}H]leucine with lower serum concentrations, 3 or 5% serum concentrations were used during the incubation with [^{3}H]leucine for most experiments. Individual 100-mm culture dishes in over 25 different experiments were assayed by the following procedure: Each plate was washed three times with Hank's balanced salt solution, then the cells were scraped and homogenized in a tightly fitting Dounce homogenizer in 20 mM NaCl–2 mM phosphate buffer (pH 7) at 4° (3)—conditions under which myosin is not soluble. Myosin-containing material was centrifuged at 800 × *g* and solubilized by addition of 1% sodium dodecyl sulfate (NadodecylSO$_4$)–1% dithiothreitol–50 mM phosphate buffer (pH 7) and heating at 60° for 30 min or at 100° for 5 min. This procedure was equivalent to dissociation by sulfonation (4). Myosin heavy-chain was isolated by disc gel electrophoresis; either 3.3% NadodecylSO$_4$–polyacrylamide gels (4) or 4% NadodecylSO$_4$–urea polyacrylamide gels (5) were used with equivalent results. Under these conditions, myosin heavy-chain forms a single band. Frozen gels were sliced into 1 mm-thick discs, and the band corresponding to myosin heavy-chain [identified by comparison with reference gels of rabbit and chick myosin (6)] was extracted by heating at 50° for 1 hr with 0.5 ml of NCS Solubilizer (Amersham–Searle). In each experiment, this measurement was performed in duplicate for each variable studied. The samples were counted in toluene–PPO–POPOP in a scintillation counter.

The rate of myosin heavy-chain synthesis in explants of muscle tissue was measured in the same way. The explants were immersed in medium (3% horse serum in leucine-free minimal medium) containing 10 μCi of [^{3}H]leucine for 3 hr, during which the incorporation of isotope was linear. At the end of the pulse, the explants from each dish were homogenized, and myosin was extracted as described above. Aliquots of the homogenate were taken to measure incorpora-

Abbreviations: minimal medium, Eagle's Minimum Essential Medium.

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tion of isotope into total protein and to measure total DNA content.

Total Protein Synthesis. Total protein synthesis in tissue culture was measured with replicate plates or explants. Labeled protein from monolayer cultures was collected as 5% trichloroacetic acid-insoluble material on glass filters (Whatman, GF/C) and counted in toluene–PPO–POPOP. About 85–95% of the radioactivity measured by this method is resistant to extraction in 5% perchloric acid for 10 min at 70° (7). Cl_3CCOOH-insoluble material from explants was extracted with 5% Cl_3CCOOH for 10 min at 70°, washed twice (each) with cold 5% Cl_3CCOOH and absolute ethanol–ether 3:1, solubilized by heating in 0.1 ml of NCS Solubilizer for 30–60 min at 50°, and counted in toluene–PPO–POPOP.

DNA Synthesis in Tissue Culture. The rate of DNA synthesis was measured by the rate of incorporation of [^{3}H]thymidine (Schwarz–Mann, 20 Ci/mmol) per μg of DNA per dish. After a 1-hr pulse (1 μCi/ml in minimal medium with 5% horse serum), the plates were washed three times with Hank's solution, and Cl_3CCOOH-precipitable material was collected by scraping. DNA was extracted from this material by heating with 5% perchloric acid for 10 min at 70°. An aliquot was counted in Bray's scintillation fluid. The amount of DNA per dish was measured by the methods of Burton (8) or Hinegardner (9).

Histological Procedure. Explants were fixed with Bouin's solution, embedded in paraffin, sectioned, and stained with Delafield's hematoxylin by standard histological techniques.

RESULTS

Fig. 1 shows the relationship between the concentration of extracellular creatine and the rates of myosin heavy-chain and total protein synthesis in 3-day-old monolayer cultures of differentiating muscle cells. There is more than a 100% increase in the rate of myosin heavy-chain synthesis over the range 10–100 μM creatine. In the range 10–25 mM creatine (not shown), the percent increase in the rate of myosin heavy-chain synthesis is 20–50%. Table 1 and Fig. 2 present data from several experiments in which the rates of total protein and myosin heavy-chain synthesis were measured in cultures grown with or without 5 mM of creatine for 1–4 days. There is no increase in the rate of myosin heavy-chain synthesis in creatine-supplied cultures until after the cells have begun to fuse (between 25 to 30 hr of incubation). The percent increase in the rate of myosin heavy-chain synthesis increases from about 40% on day 2, to about 80% on day 3, to more than 130% on day 4. In contrast, there is little or no change in the rate of total protein synthesis in creatine-supplied cultures compared to controls through 3 days of incubation. There may be an increase in total protein synthesis in older cultures, especially by day 4 of incubation, reflecting the fact that myosin synthesis comprises a relatively larger proportion of total protein synthesis by this time. The data presented in Table 1 indicate that the rate of myosin heavy-chain synthesis increases from 2–5% of the rate of total protein synthesized on days 2 and 3 to about 10% on day 4.

The length of time it takes for creatine to effect the rate of myosin heavy-chain synthesis is shown in Table 2. Cultures of muscle cells were permitted to fuse and were then exposed to creatine and [^{3}H]leucine for 4 hr (46–50 hr of incubation). By the fourth hour, the accumulation of labeled

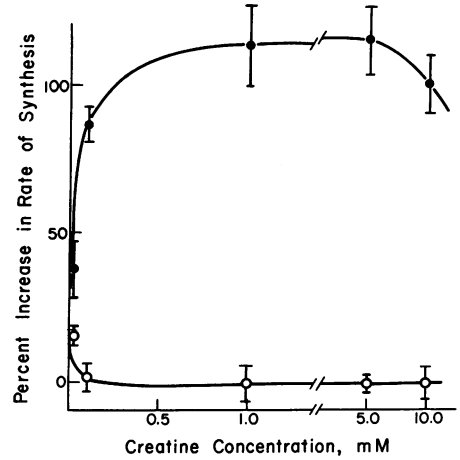

FIG. 1. The relationship between the concentration of extracellular creatine and the rates of myosin heavy-chain (●) and total protein (○) synthesis in 3-day-old monolayer cultures. 10 μM–10 mM creatine was added to cultures immediately after plating. The rates of synthesis were measured between 73 and 77 hr by the incorporation of [^{3}H]leucine (see *Methods*). By this time fusion was complete. Data are expressed as percent increase in rate of creatine-supplied cultures compared to control cultures; *brackets* indicate the percent deviation from the mean of duplicate determinations.

amino acid into myosin heavy-chain in creatine-supplied cultures is different from controls, and is the same as in cultures supplied creatine continuously from the time of plating.

Although creatine appears to stimulate the synthesis of at least this one cell-specific protein, it is possible that it does so indirectly. For example, it is possible that creatine enhances the attachment of myoblasts to the culture dishes, stimulates myoblast proliferation, or increases the number of myoblasts that fuse and begin to synthesize contractile proteins. Each possibility could result in increased myosin synthesis in creatine-supplied cultures relative to control cultures.

These possibilities were tested as follows: Creatine was supplied to cultures either on plating or after cell fusion was complete. It can be seen in Fig. 2 that the increase in the rate of myosin heavy-chain synthesis was the same whether creatine was added before or after fusion had occurred. Furthermore, even though present in muscle cell cultures, creatine had no effect on the rate of myosin synthesis until after cell fusion. These results indicate that creatine affects only cells already synthesizing muscle protein, and does not affect cellular events during myoblast proliferation or during cell fusion.

This interpretation is further supported by results from experiments measuring the plating efficiency, the DNA content, and the time-to-the-beginning of fusion in creatine-supplied cultures and controls. The number of cells attached 6 hr after plating in the presence or absence of creatine (1–5 mM) is the same (±7%). The amount of DNA synthesized by various times of incubation is also the same (±10%) in creatine-supplied cultures and controls. If creatine were to promote earlier fusion, the rate of myosin synthesis in creatine-supplied cultures would increase earlier than in control cultures, and at any particular time would be higher than in control cultures. Fig. 3 shows that creatine does not alter the rate of cell fusion or proliferation during the culture

TABLE 1. *Rates of synthesis of total protein and myosin heavy-chain*

Monolayer culture Days of culture	Hr of pulse	Total protein synthesis (cpm/dish) Control	Total protein synthesis (cpm/dish) +5 mM creatine	Myosin heavy-chain synthesis (cpm/dish) Control	Myosin heavy-chain synthesis (cpm/dish) +5 mM creatine	% Increase in rate of myosin synthesis
2	46–50	—	—	7,160 ± 250	9,800	37
	48–52	178,000 ± 17,000	176,000 ± 16,000	4,400 ± 850	5,700	30
	50–54	332,000	346,000 ± 3,000	5,880	8,540	45
	51–55	—	—	3,220 ± 260	4,310 ± 90	38
3	66–70	176,000 ± 4,000	241,000 ± 32,000	5,000 ± 800	9,130 ± 700	82
	68–72	219,000 ± 2,200	192,000 ± 7,000	4,500 ± 270	8,000 ± 500	78
	70–74	154,000	173,000	1,770	3,000	70
	73–77	250,000 ± 5,000	248,000 ± 6,000	2,100 ± 270	4,520 ± 600	115
4	90–94	112,000 ± 17,000	173,000 ± 12,000	9,050 ± 950	21,200 ± 2,300	134

Organ culture Age of embryo, days	Total protein synthesis (cpm/μg of DNA) Control	Total protein synthesis (cpm/μg of DNA) +5 mM creatine	Myosin heavy-chain synthesis (cpm/μg of DNA) Control	Myosin heavy-chain synthesis (cpm/μg of DNA) +5 mM creatine	% Increase in rate of myosin synthesis
14	8,400 ± 800	9,600 ± 2,100	320	700	120
15	8,800 ± 1,600	9,650 ± 1,200	470 ± 70	800 ± 60	70
	—	8,560 ± 1,000	100 ± 10	225 ± 50	125

Rates of total protein and myosin heavy-chain synthesis were determined by measurement of the incorporation of [^{3}H]leucine into Cl_3CCOOH-precipitable protein and into myosin heavy-chain isolated by disc gel electrophoresis, respectively. Data represents the average of duplicate culture dishes. The creatine concentration in all experiments was 5 mM.

period. In this experiment, advantage was taken of the observations that DNA synthesis ceases in myoblasts as they fuse to form multinucleated cells, and fusion is accompanied by a distinct drop in incorporation of [^{3}H]thymidine (2, 3, 13–15). It is clear from Fig. 3 that fusion begins between the 27th and 29th hour in both creatine-supplied and control cultures, and that the rate of DNA synthesis is the same in creatine-supplied cultures as in controls.

In a few experiments, we have studied the effect of creatine on embryonic skeletal muscle cells that have differentiated *in vivo*. In 15-day-old embryos, about 70% of the myoblasts have fused into myotubes that synthesize contractile proteins (16). Explants of pectoral muscle from 14- and 15-day chick embryos were maintained in organ culture. Viability of muscle cells in these explants was tested by histological examination of sectioned tissue; virtually every cell was viable for more than 24 hr. The rates of myosin heavy-chain and total protein synthesis in these explants were measured. As shown in Table 1, the addition of 5 mM creatine to the culture medium doubled the rate of myosin heavy-chain synthesis in these skeletal muscle explants, just as in muscle cells that differentiated *in vitro*. The rate of total protein synthesis is increased only slightly (10–15%).

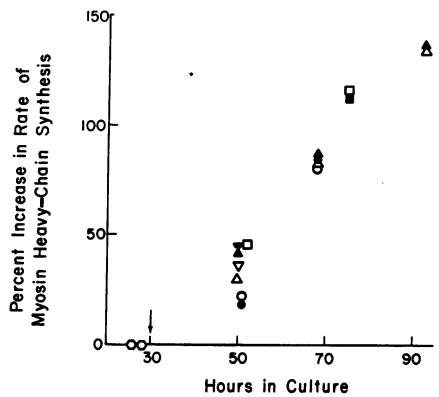

FIG. 2. Percent increase in rate of myosin heavy-chain synthesis in cultures supplied 5 mM creatine before and after fusion. The beginning of fusion is indicated by the *arrow*. This figure presents data from separate experiments, each represented by a different symbol, positioned at a time corresponding to the midpoint of the 4-hr pulse. *Open symbols* are values obtained for cultures in which 5 mM creatine was added at 6 hr of incubation; *filled symbols* are values obtained for cultures in which 5 mM creatine was added between 42 and 46 hr of incubation, i.e., after fusion.

DISCUSSION

These experiments demonstrate that skeletal muscle cells formed either *in vitro* or *in vivo* synthesize myosin heavy-chain faster when supplied creatine *in vitro*. The response is apparent within 4 hr after addition of creatine to the culture medium, and is concentration dependent over the range of 10–100 μM. Creatine-supplied cultures synthesize myosin heavy-chain faster in well-developed myotubes than in newly-

TABLE 2. [^{3}H]*Leucine incorporation into myosin heavy-chain (cpm/dish)*

Hours	Control	Creatine supplied at zero time	Creatine supplied at 26 hr
1	1,810 ± 230	2,750 ± 340	1,850 ± 140
2	2,340 ± 350	3,120 ± 260	2,470 ± 440
3	4,390	5,270 ± 450	4,830 ± 500
4	7,160 ± 250	9,800	10,000 ± 2,250

Cultures were supplied 5 mM creatine at zero time, at 46 hr after plating, or not at all (control). Isotope incorporation into myosin heavy-chain was measured by gel electrophoresis. Data are averages of duplicate culture dishes ± the range.

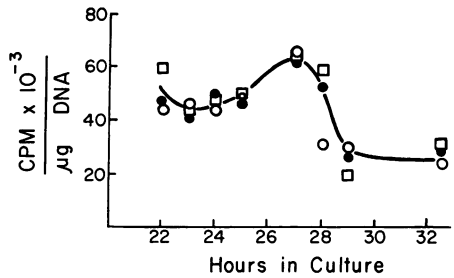

FIG. 3. The rate of [^{3}H]thymidine incorporation per μg of DNA as a function of time in culture for 1 mM (○) and 5 mM (□) creatine-supplied cultures, and control (●). Cultures were supplied creatine within 6 hr after plating.

formed myotubes. This effect is specific, since the rate of total protein synthesis is relatively unaffected by the addition of creatine. Since myosin is a stable protein *in vivo* (10, 11) and *in vitro* (12), it is unlikely that degradative processes alone account for the stimulation in the rate of synthesis of myosin heavy-chain in response to creatine.

The selectivity to specific protein synthesis suggests that creatine has some role other than involvement in a system energizing general protein synthesis or in cell nutrition. The results are consistent with the idea that creatine is a positive-feedback effector in a system relating muscular activity and muscle-protein synthesis. Its specificity in this role, and the effect of deprivation of the cultures of creatine (since medium with horse serum and embryo extract contains creatine) on myosin synthesis remain to be tested. Creatine is not synthesized in muscle, but is transported from its site of synthesis in liver and enters muscle via a membrane transport system (17). Since this transport system regulates the intracellular creatine concentration, it will be important to establish the relationship between the developmental appearance of this transport system, the intracellular creatine concentration, and the stimulation of synthesis of contractile protein.

In these experiments, creatine had its effect after myotubes had formed, i.e., after fusion and the onset of contractile protein synthesis. In these cells, creatine could function at any of several levels in protein synthesis. It is possible that creatine alters the rate of myosin heavy-chain degradation as well as synthesis. It could affect translation or transcription, or be involved in mechanisms for control of myofilament assembly. It remains to be determined if creatine also stimulates the synthesis of other muscle cell-specific proteins, such as actin, myosin light-chains, and creatine kinase.

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