Effect of creatine on contents of myosin heavy chain and myosin-heavy-chain mRNA in steady-state chicken muscle-cell cultures

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Embryonic-chick muscle cells reach a steady state with respect to protein metabolism after approx. 1 week in cell culture. To determine if this steady state could be altered by the administration of agents that have been reported to stimulate myosin heavy-chain synthesis, 7-day muscle-cell cultures were treated with 0–1 mM-creatine. Incorporation of [³H]leucine into myosin heavy chain was stimulated by 30–40% at the optimum creatine concentration (0.2 mM), but this stimulation was blocked when actinomycin D (10 µg/ml) was also present. However, the quantity of myosin-heavy-chain mRNA as measured by hybridization *in vitro* was only 15% higher in creatine-treated cultures, and was therefore not entirely responsible for the observed effect. It is important to note that creatine only exerted its action on myosin-heavy-chain synthesis rate in steady-state cultures; creatine was ineffective in altering this rate in rapidly differentiating 3-day muscle cultures. Finally, muscle-cell cultures that had been grown for the entire 7-day culture period in the presence of 0.2 mM-creatine were assayed for quantity of myosin heavy chain. Control and creatine-treated cultures contained 12.7 ± 1.5 and 20.5 ± 1.8 µg/dish respectively. In conclusion, creatine apparently enhances the quantity of myosin heavy chain in steady-state embryonic muscle-cell cultures, but it probably does not mediate regulation of myosin content in adult skeletal muscle.

The mechanism by which the frequency or intensity of contractile activity in skeletal-muscle cells can indirectly regulate myofibrillar protein content is not known. However, several previous investigations have implicated a possible role for creatine in this process. Creatine has been reported to stimulate the incorporation of radioactive amino acids into myofibrillar proteins in both embryonic muscle-cell cultures and organ cultures (Ingwall *et al.*, 1972; Ingwall & Wildenthal, 1976), although subsequent re-evaluation of these data was unable to demonstrate stimulation (Fry & Morales, 1980). The observations that electrical stimulation (Brevet *et al.*, 1976) and Ca²⁺-ionophore treatment (Kameyama & Etlinger, 1979; Young *et al.*, 1981) stimulate myosin synthesis can also be rationalized as indirect actions of creatine. Finally, the ionophore A23187 apparently stimulates myofibrillar protein synthesis by promoting transcription of

new RNA (Kameyama & Etlinger, 1979; Young *et al.*, 1981).

The present experiments examined the ability of creatine to perturb the steady state that exists in 1-week-old muscle-cell cultures. The specific goals were (1) to measure the ability of creatine to stimulate the incorporation of [³H]leucine into myosin heavy chains in the presence and absence of actinomycin D, (2) to measure the effect of creatine on the concentration of myosin-heavy-chain mRNA, and (3) to measure the ability of creatine to affect the quantity of myosin-heavy-chain protein in steady-state muscle-cell cultures. A preliminary report of this work has appeared (Young & Denome, 1982).

Experimental

Materials

Creatine and actinomycin D were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). [³H]Leucine, [5-³H]dCTP and a kit for nick translation of cloned probes were purchased from

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Amersham (Arlington Heights, IL, U.S.A.). The Ca^{2+} ionophore A23187 was a gift from Dr. Robert Hamill of Eli Lilly Co. (Indianapolis, IN, U.S.A.). The liquid-scintillation cocktail Aquasol was from New England Nuclear (Boston, MA, U.S.A.). Fertilized White Leghorn chicken eggs for preparation of cell cultures were obtained from Feather Crest Hatchery (Arkadelphia, AL, U.S.A.). All chemicals for electrophoresis were from Bio-Rad (Richmond, CA, U.S.A.); other chemicals and biochemical reagents were from either Fisher Chemical Co. (Atlanta, GA, U.S.A.) or Sigma Chemical Co. The quail myosin-heavy-chain complementary-DNA clone, cC128, was obtained from Dr. Charles Emerson (University of Virginia, Charlottesville, VA, U.S.A.).

Experimental design

For all experiments in which the rate of incorporation of [^3H]leucine into protein was measured, 7-day cultures were employed. Cells were re-fed with complete cell-culture medium (consisting of 85% Eagle's Minimum Essential Medium, 10% horse serum, 5% chick-embryo extract, 50 units of penicillin/ml, 50 μg of streptomycin/ml and 2.5 μg of fungizone/ml) containing 10 μCi of [^3H]leucine/ml, and pulse labelled for 4 h. Selected concentrations of creatine, actinomycin D or A23187 were also present during the pulse-labelling period either singly or in the appropriate combinations as described in individual experiments in the text. When the concentration of myosin-heavy-chain mRNA was measured, cells were re-fed with fresh culture medium in the presence or absence of creatine 4 h before polyribosome isolation.

Cell cultures

Muscle-cell cultures were prepared from the leg muscle of 12-day chick embryos as described by Young *et al.* (1975).

Incorporation of radioactive amino acids into protein

At the end of the pulse-labelling period, the dishes were rinsed once with cold iso-osmotic saline (0.9% NaCl) solution and twice with cold 0.25M-NaCl/0.02M-Tris/HCl, pH 7.4. Cells were scraped with a plastic spatula into 0.5 ml of the above buffer. Cells were then homogenized with 20 strokes of a 7 ml Dounce-type glass homogenizer, and the homogenate was centrifuged at 12000g for 10 min. The NaCl concentration in the supernatant was decreased 10-fold by the addition of cold water, and the myofibrillar protein fraction was allowed to aggregate overnight at 2°C. Myofibrillar proteins were collected by centrifugation at 3000g for 0.5 h, and electrophoresis was performed as described by Young *et al.* (1979). After

staining the gels with Coomassie Blue, myosin-heavy-chain bands were sliced out and dissolved in 30% (v/v) H_2O_2 by heating at 50°C for 3 h.

The radioactivity incorporated into total cellular protein was determined by precipitation of samples of the initial cell homogenate with 5% (w/v) trichloroacetic acid (Riebow & Young, 1980). Radioactivity was then measured by liquid-scintillation spectrometry.

Measurement of myosin-heavy-chain content of muscle cultures

The procedure for measuring myosin-heavy-chain content of creatine-treated cell cultures was identical with that described above for myosin-heavy-chain synthesis rates up to and including the step where the precipitated myofibrillar protein was dissolved for electrophoresis. The quantity of myosin heavy chain was measured on polyacrylamide gels that had been cross-linked with NN' -diallyltartardiamide as described by Young *et al.* (1980).

The total quantity of protein in each culture was determined from small samples of the original cell homogenate by the method of Ehresman *et al.* (1973). Total myotube nuclei and total nuclei per culture dish were counted by using Giemsa-stained cultures (Young *et al.*, 1979).

Extraction of RNA from muscle-cell cultures

Cells were scraped from 10 cm-diam. dishes, lysed with 0.5% Triton X-100 and fractionated on linear 10–40% sucrose gradients in 0.25M-NaCl/0.01M-MgCl₂/0.02M-Tris/HCl, pH 7.2, as detailed by Young *et al.* (1975). Polyribosome distribution was monitored at 254 nm to collect non-polyribosomal and polyribosomal fractions. Both types of particles were collected by centrifugation at 105000g for 16 h and were dissolved in 15 mM-Tris/HCl, pH 7.4. A quantity of proteinase K equal to one-tenth the quantity of ribosome material was added, followed by incubation at 37°C for 2 h. RNA was precipitated twice at –20°C in 67% (v/v) propan-2-ol/0.2M-sodium acetate, pH 6.0, and finally dissolved in distilled water. Myosin-heavy-chain mRNA was quantified by hybridization against the ^3H -labelled quail myosin-heavy-chain complementary-DNA clone cC128. The plasmid DNA was radioactively labelled by nick translation in the presence of [^3H]dCTP. Hybrid formation was analysed as detailed by Dym *et al.* (1979), except that radioactivity (c.p.m.) instead of percentage hybrid formation was plotted versus R_0t values.

Results

The ability of creatine to affect the metabolism of myosin heavy chain is dependent in part on the

developmental stage in cell culture. Thus it is crucial to understand the relationship between myosin-heavy-chain synthesis rate, myosin-heavy-chain accumulation and the onset of myoblast fusion under the specific conditions used in this investigation (Fig. 1). Special emphasis is placed on the fact that muscle cultures approach a steady state with respect to myosin-heavy-chain synthesis rate and myosin-chain content after approx 1 week.

To test indirectly whether creatine exerted its effect on muscle cells by transcriptional mechanisms, 7-day muscle cultures were pulse-labelled for 4 h with [^3H]leucine in the presence of 0–1 mM-creatine and in the presence or absence of actinomycin D (10 $\mu\text{g}/\text{ml}$). In the absence of actinomycin D, creatine increased [^3H]leucine incorporation into myosin heavy chain by approx. 50% (Fig. 2). In the presence of actinomycin D, the ratio of myosin-heavy-chain synthesis to total protein synthesis did not change significantly (Fig. 2). Although actinomycin D is toxic to cells after prolonged exposure, the brief pulse-labelling period used for these experiments did not alter either the morphology or the number of cells as determined by light microscopy.

The rate of myosin-heavy-chain synthesis increases sharply from approx. 5000 molecules/min per nucleus on day 3 in culture to approx. 22000

molecules/min per nucleus on day 7 (Fig. 1). To examine the effect of creatine at different stages of differentiation, muscle cells at 3 and 7 days in culture were treated with 0.2 mM-creatine during

Fig. 2. Effect of creatine on the fraction of total radioactivity in myosin heavy chain (MHC) in the presence (○) and absence (●) of actinomycin D (10 $\mu\text{g}/\text{ml}$)

Each point represents the mean $\pm$ S.E.M. for four experiments, with quadruplicate determinations in each experiment.

Fig. 1. Relationship of muscle-cell fusion, myosin-heavy-chain (MHC) synthesis rate and myosin-heavy-chain concentration in embryonic-chick muscle-cell cultures

Each point represents the mean $\pm$ S.E.M. for four experiments in which triplicate cultures were examined. Cells were grown in 15 cm-diameter dishes for these experiments.

pulse-labelling in the presence of [^3H]leucine. Incorporation into myosin heavy chain was increased by 28.4% by creatine in 7-day cultures, but only by 4.8% in 3-day cultures (Table 1). Total protein-synthesis rate and protein quantity were not affected by the creatine treatment at either age (Table 1).

The ionophore A23187 enhances the incorporation of [^3H]leucine into myosin heavy chain in a pattern virtually identical with that shown in Fig. 2 for creatine (Young *et al.*, 1981). Experiments were therefore conducted to determine if the two agents act by a common mechanism. If so, the combined effects of the two agents should be the same as the effect of each of them separately. In this particular set of experiments (five experiments, quadruplicate samples in each), 0.2mM-creatine by itself stimulated myosin-heavy-chain synthesis by 45%, 0.2 μM -A23187 stimulated the synthesis by 38% and creatine and A23187 together stimulated the synthesis by 41% (results not shown). These results are consistent with the conclusion that they act by a common mechanism.

Because use of actinomycin D relies on the assumption that no cellular processes other than transcription are affected, an independent test of this possibility was conducted. RNA was isolated from the polyribosomal and non-polyribosomal compartments of muscle cells after treatment with creatine for 4h under conditions identical with those for the pulse-labelling experiments shown in Table 1 and Fig. 2. Whereas the total quantity of RNA in the two fractions of muscle cells was not

altered by creatine, the quantity of myosin-heavy-chain mRNA in the creatine-treated cells was 13.5% higher in the polyribosomal compartment and 15.7% higher in the non-polyribosomal compartment (Table 2). Even though creatine consistently caused an increase in the quantity of myosin-heavy-chain mRNA, it should be emphasized that the variability of hybridization experiments is fairly large, and the data are not different at the $P=0.05$ level. Table 2 also shows that the distribution of myosin-heavy-chain mRNA between the polyribosomal and non-polyribosomal compartments was similar in the control and creatine-treated cells. Thus the increase in the quantity of myosin-chain mRNA caused by creatine is much less than the increase in the rate of incorporation of [^3H]leucine into myosin heavy chain usually observed in pulse-labelling experiments (cf. Fig. 2 and Table 1). It may be possible that creatine also alters intracellular amino acid pools in such a way as to increase fortuitously the apparent synthesis rate of myosin chain in pulse-labelling experiments.

Experiments described thus far involved only a brief exposure of cells to creatine (4h), after which either radioactivity (c.p.m.) or mRNA content was assessed. Additionally, these experiments contain inherent unverifiable assumptions (e.g. no effect on amino acid pools, specificity of actinomycin D etc.). Because of these uncertainties, the most direct and assumption-free alternative for testing the effect of creatine on myosin-heavy-chain metabolism was to measure the quantity of myosin

Table 1. *Effect of 0.2mM-creatine on myosin-heavy-chain synthesis rate, total protein-synthesis rate and total protein content of 3-day and 7-day muscle-cell cultures*

Protein content and protein-synthesis rates were determined as described in the Experimental section. Each value represents the mean $\pm$ S.E.M. for five experiments in which all measurements were performed in quadruplicate. * $P<0.05$.

Culture ...	3 Day		7 Day	
	Control	+ Creatine	Control	+ Creatine
$10^{-3} \times$ Radioactivity in myosin heavy chain (c.p.m.)	3.31 ± 0.45	3.47 ± 0.52	28.5 ± 2.3	$36.6 \pm 2.4^*$
$10^{-5} \times$ Radioactivity in total protein (c.p.m.)	5.73 ± 0.18	5.75 ± 0.12	9.22 ± 0.53	9.45 ± 0.54
Total protein/culture (mg)	3.40 ± 0.15	3.36 ± 0.14	6.11 ± 0.17	6.18 ± 0.16

Table 2. *Effect of 0.2mM-creatine on the quantity of total RNA and on the quantity of myosin-heavy-chain mRNA in the polyribosomal and non-polyribosomal compartments of 7-day muscle-cell cultures*

Cells were grown for 7 days in 10cm culture dishes. Total RNA in the two compartments was quantified by its A_{260} . Myosin-heavy-chain mRNA in the two compartments was quantified by hybridization against a quail myosin-heavy-chain complementary-DNA clone as described in the Experimental section. Each value represents the mean $\pm$ S.E.M. for eight experiments, in which all measurements were made in duplicate. The numbers listed indicate the quantity of RNA in one 10cm culture dish.

	Control	+ Creatine	Stimulation (%)
Polyribosomal RNA (μg)	741 ± 99	763 ± 112	3.0
Non-polyribosomal RNA (μg)	430 ± 56	453 ± 81	5.3
Polyribosomal myosin mRNA (ng)	96.9 ± 8.8	110 ± 15.1	13.5
Non-polyribosomal myosin mRNA (ng)	38.3 ± 3.3	44.3 ± 4.9	15.7

Table 3. *Effect of 0.2mM-creatine on myosin-heavy-chain content, total protein content, number of myotube nuclei and percentage fusion in 7-day muscle-cell cultures*

Muscle cultures in 6cm dishes were exposed to 0.2mM-creatine for the entire 7 days of culture; culture medium was replenished daily. Each value represents the mean $\pm$ s.e.m. for eight experiments, each of which contained six replicate cultures.

	Control	+ Creatine
Myosin heavy chain (μ g)	12.71 $\pm$ 1.48	20.49 $\pm$ 1.79
Total protein (mg)	1.19 $\pm$ 0.07	1.23 $\pm$ 0.07
10 ⁻⁶ $\times$ No. of myotube nuclei	3.18 $\pm$ 0.17	2.96 $\pm$ 0.16
Fusion (%)	66.9 $\pm$ 0.6	66.8 $\pm$ 0.9

heavy chain that accumulated in muscle cultures after exposure to optimum concentrations of creatine for several days. Accordingly, muscle cultures were grown for 7 days in culture medium that had been supplemented with 0.2mM-creatine, and the quantity of myosin heavy chain was then measured and compared with that in control cultures. These data show clearly that the quantity of myosin heavy chain was higher by approx. 60% in the creatine-treated cells (Table 3). Despite the prolonged treatment with creatine, the total quantity of protein per culture, the number of nuclei found in multinucleated myotubes and the percentage fusion were virtually identical in the two treatments. These data leave little doubt that, under the specific set of conditions employed for this study, myosin-heavy-chain accumulation is enhanced by the presence of creatine.

Discussion

Conflicting results concerning the ability of creatine to stimulate myosin synthesis have been reported. This lack of reproducibility may be attributed to several factors. Stage of differentiation may be partially responsible, since incorporation of [³H]leucine into myosin heavy chain is accelerated by creatine in 7-day cultures, but not in rapidly differentiating 3-day muscle cultures. A second factor may be variation in batches of either horse serum or embryo extract. Extremes in the effects of different lots of serum on myosin-heavy-chain metabolism have been previously reported (Young & Dombroske, 1981); however, the serum used in the present study was screened to ensure that it supported myosin-heavy-chain synthesis to at least 20000 molecules/min per nucleus.

Even when constant lots of horse serum and embryo extract are employed in controlled experiments such as those reported in Table 3, considerable variation in the quantity of myosin heavy chain was still observed. For example, in the control experiments reported in Table 3, the results ranged from a minimum of 7 μ g/culture to a maximum of 18 μ g/culture. Interestingly, a plot of

Fig. 3. *Relationship between the percentage increase in myosin-heavy-chain (MHC) content in creatine-treated cultures and the quantity of myosin heavy chain in simultaneously analysed controls*

The data used for this plot are from the eight individual experiments that were averaged in Table 3.

percentage stimulation of myosin heavy-chain concentration in creatine-treated cultures versus myosin-heavy-chain content of simultaneously analysed controls revealed an inverse relationship ($r^2 = 0.6$) that extrapolated to zero percentage stimulation of myosin-heavy-chain content at approx. 21 μ g of myosin heavy chain per culture (Fig. 3). These data suggest that 0.2mM-creatine may not be capable of enhancing myofibrillar protein accumulation beyond a minimal amount.

The central issue in this investigation is whether or not creatine is the molecular signal linking altered contractile activity with changes in transcriptional rate of the myofibrillar-protein gene family in adult skeletal muscle. Muscle cannot synthesize creatine and must depend on the liver for it, and the apparent stimulation by creatine may merely reflect the addition of creatine back to the minimum concentration required for embryonic muscle to function properly. Involvement of creatine could provide a logical explanation for coupling between contractile activity and gene expression, because prolonged contractile activity

would diminish the supply of phosphocreatine and simultaneously cause a transient increase in the concentration of free creatine. Free creatine could then interact with the nucleus to alter transcription of myofibrillar protein mRNA. However, this seems unlikely, since myosin-heavy-chain mRNA contents are not significantly higher in creatine-treated cells.

Other considerations also argue against creatine as the molecular signal directly linking contractile activity with gene expression in adult skeletal muscle. First, even though 0.2 mM creatine would cause an increase in myosin-heavy-chain content of embryonic muscle-cell cultures (Table 3, Fig. 3), the maximum quantity of myosin heavy chain accumulated per unit of DNA never exceeds the quantity found in embryonic muscle tissue, and this quantity is approximately one-tenth of that of adult muscle tissue (cf. Orcutt & Young, 1982). Secondly, the intracellular concentration of creatine is relatively unresponsive to drastic changes in extracellular creatine concentration (Fry & Morales, 1980). Thirdly, because of the speed and equilibrium of the creatine kinase reaction, the concentration of free creatine could probably be elevated for only a brief, transient, period of time. Enhanced transcription of some of the myofibrillar genes (especially myosin heavy chain) would seem to necessitate signals of a more stable nature than that provided by creatine. Because chicken myosin-heavy-chain genes are at least 30 000 bases in size (Barringer *et al.*, 1982), and because the maximum transcription rate of RNA polymerase is approx. 50 nucleotides/s, at least 10 min would be required for synthesis of an entire primary transcript of myosin heavy-chain mRNA. It seems unlikely that free creatine concentration would be elevated for such a prolonged period of time. Fourthly, even though creatine tended to increase the quantity of myosin heavy-chain mRNA, this increase was not as large as the increase in synthesis rate typically observed and was not significant at $P = 0.05$. Finally, it seems clear from Fig. 1 that degradation rates of myosin heavy chain are modulated at approx. day 7, since there is a change in its accumulation rate at the same time that the synthesis rate has approached its maximal value. The prolonged effects of creatine on myosin-heavy-chain accumulation could therefore have resulted from its indirect effects on breakdown rate of either myosin heavy chain or myosin-heavy-chain mRNA.

In summary, the most logical unifying explanation for the present data and published data is as

follows. A minimal concentration of creatine is required in embryonic muscle cells for normal cell function. As creatine is added up to this minimal value, an apparent stimulation of myosin-heavy-chain synthesis occurs; addition of creatine above this minimum has no effect. The link between contractile activity and myofibrillar-gene expression in adult skeletal muscle probably requires a less transient signalling mechanism than could be provided by creatine. This as yet undefined signalling mechanism might be Ca^{2+} -activated (and therefore directly related to changes in contractile activity) and might result in the formation of more stable products that could then interact with the nucleus to enhance myofibrillar-gene transcription.

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