A REEXAMINATION OF THE EFFECTS OF CREATINE ON MUSCLE PROTEIN SYNTHESIS IN TISSUE CULTURE

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ABSTRACT

Experiments designed to test the hypothesis that intracellular creatine level regulates the synthesis of muscle specific proteins have failed to demonstrate any creatine regulatory effect. Manipulation of the extracellular creatine in culture medium over a 5,700-fold range (1.3–7.4 mM) was successful in altering intracellular total creatine by only a factor of 20 (1.4–42 mg creatine/mg protein), an indication that muscle cells are able to regulate intracellular creatine levels over a wide range of external creatine concentrations. Alterations of cell creatine had no effect on either total protein synthesis or synthesis of myosin heavy chain. Methods were perfected to measure total creatine, and incorporation of [^{3}H]leucine into total protein and purified myosin heavy chain from the same culture dish to avoid the possibility of variation between dishes. The creatine analog 1-carboxymethyl-2-iminohexahydropyrimidine (CMIP) previously reported to stimulate myosin synthesis in culture was found to depress creatine accumulation by cells and depressed total protein synthesis and synthesis of myosin heavy chain. This inhibitory action of CMIP is consistent with the reported competitive inhibition of creatine kinase and presumed interference with energy metabolism.

KEY WORDS myosin · creatine analog · muscle differentiation · oxygen tension

The hypothesis that creatine regulates muscle protein synthesis led to investigations concluding that creatine stimulated the rate of myosin and actin synthesis in chick embryo skeletal muscle culture (3, 4). Reexamination now with more reliable levels of isotope incorporation, and with proof that creatine penetrates myoblasts, failed to confirm earlier results.

MATERIALS AND METHODS

Skeletal Muscle Tissue Cultures

Cultures of 11- to 12-d chick embryo pectoral myoblasts were grown on collagen-coated 60-mm plastic petri dishes (10). Cells were plated at ~1.25 × 10^6 cells/dish in 3, 5, or 8 ml "complete medium" (88% Eagle's minimal essential medium, [MEM]; 10% horse serum, [HS]; 2% chick embryo extract, [EE]). Medium was changed at 24 h, and every 48 h thereafter. Experimental cultures received creatine at 24 h and at each medium change. HS lots selected for promoting differentiation, and a 95% air + 5% CO_2 atmosphere, were used. Myoblasts fused into myotubes within 60 h, and many myotubes were striated by 5 d.

Protein Synthesis Measurement

Rates of myosin heavy chain (HC) and total protein synthesis were measured (3). Incorporation of [^{3}H]leucine into cultures was initiated by transferring cultures into fresh leucine-free medium with 4% HS, plus 20 μCi [^{3}H]leucine (Amersham Searle Corp., Arlington Heights, Ill., 59 Ci/mmol) and with variable carrier leucine to maintain [leucine] during rapid synthesis (total [leucine], 0.068–400 μM; 4 μM was usual). Cultures were thrice rinsed with Hanks' solution after 2–4 h incorporation, scraped, and

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homogenized in Teflon tissue grinders or sonicated either in 20 mM NaCl, 2 mM Pi, pH 6.8, or in myosin extraction solution (300 mM KCl, 100 mM Pi, 5 mM $MgCl_2$, 5 mM ATP, pH 6.8). Total protein (6), ^{3}H-incorporation into total TCA-precipitable protein, and total cellular creatine were measured in duplicate aliquots of crude homogenate. Most of the homogenate was processed for sodium dodecyl sulfate (SDS) gel electrophoresis (11) of HC. Extraction solution was used for myosin purification from individual cultures. ^{3}H-incorporation into total protein was counted on crude homogenate precipitated (18% TCA) on 0.45-μm Millipore filters (Millipore Corp., Bedford, Mass.). Incorporation of [^{3}H]leucine into HC was measured in appropriate slices from an SDS gel electrophoretogram of crude homogenate, purified myosin, or pellet from 20 mM NaCl + 2 mM Pi solution (pellet was resuspended in 1% SDS, 1% DTT, pH 8.8, boiled for 5 min, and electrophoresed on 5–7.5% polyacrylamide gels in the presence of SDS [11]).

For partial myosin purification (see reference 12) dishes were scraped and homogenized in extraction solution, and insoluble proteins and cell debris were removed by centrifugation. Myosin was precipitated at low ionic strength, resuspended at high [salt] and subjected to SDS gel electrophoresis.

Creatine Measurements

Small amounts of creatine in cells and culture medium were detected fluorimetrically (1). The *o*-carboxyphenylglyoxal of this assay reacts with creatine and other guanidines and *N,N*-disubstituted guanidines. The produce is maximally excitable at 390 nm, and emits maximally at 495–500 nm. The major interfering substance is arginine, which in this work was 80–90% removed together with proteins by $Ba(OH)_2$-$ZnSO_4$ precipitation. The sensitivity was ~50 pmol of creatine. Duplicate aliquots, 50–100 μl, from crude homogenate, medium, or its components (MEM, HS, EE) were taken for creatine assay. Sample volume was adjusted to 1 ml with water; 0.5 ml 0.15 M $ZnSO_4$ and 0.5 ml 0.15 M $Ba(OH)_2$ were added sequentially, and the suspension was centrifuged at 1,500 *g*. Then 0.5 ml of 1% ninhydrin (95% ethanol) was added, followed by 0.5 ml of 10% KOH (95% ethanol), with rapid mixing to start the alkaline ninhydrin reaction. Creatine fluorescence was measured after 10 min and was linear with amount between 50 and 300,000 pmol. No attempt to distinguish between creatine and its phosphate was made; the method hydrolyzes the latter, so that all values are expressed as total creatine.

RESULTS

Protein Synthesis

Under the conditions of Ingwall et al. (3, 4)—1.4 mm medium depth, ± 5 mM creatine, 4-h incorporation of 59 Ci/mmol [^{3}H]leucine, no carrier leucine, 4% HS, no EE, in 60-mm dishes—differences between control and creatine-supplied cultures with regard to ^{3}H-incorporation into total protein or HC were insignificant. Variability between cultures was small and ^{3}H-incorporation was adequate for statistically significant discrimination. Differences as small as 10% could have been detected with assurance. A significant difference ($P < 0.001$) in total cell creatine (8 μg/dish for control; 30 μg/dish for experimental) was achieved. In control 5-d cultures the total protein was 1.1 mg/dish; the rates of synthesis (in cpm × 10^{-6}/dish) were 3.7 for total protein and 0.48 for myosin. In all three measurements the experimentals were within 6% of controls, and error bars (SEM) were overlapping.

To improve growth and incorporation 5 ml instead of 3 ml of medium were often used. O_2-diffusion in 5-ml cultures is adequate as judged by cell morphology, growth, and lack of lipid accumulation (7, 8, 13, 14). At very high [creatine], depth of medium does affect creatine accumulation. However, in 5-ml cultures the presence or absence of 5 mM creatine produced no significant differences in [^{3}H]leucine incorporation into total protein or into myosin (Fig. 1). In particular, no effects of creatine on myosin synthesis were observed at 3–5 d, the time of maximal effect reported previously (3, 4).

In some cultures, myosin was partially purified (12), and its incorporation of radio-leucine was compared with the incorporation into the HC band of the electrophoretogram of crude homogenate. The HC band of the myosin coincided exactly with the HC band of purified myosin (Fig. 1). Fig. 1 also shows that in 3-d cultures addition of 5 mM creatine had no effect on total protein or myosin synthesis and that results were similar for purified myosin or myosin separated in SDS gels of crude homogenate.

Creatine Accumulation

We wish to study accumulation from a range (1.3 μM–7.4 mM) of extracellular [creatine], but complete medium already contains ~40 μM creatine. Creatine was removed from sterile HS and EE by dialysis against sterile Hanks' solution. In this process growth-promoting substances were evidently also removed, since cultures grown in dialyzed medium plus creatine did not grow as well as undialyzed controls. Commercial creatinase proved too impure to use for creatine removal.

Cells accumulated creatine from the medium at all extracellular concentrations, e.g., at 1.3 μM, >60% of the creatine in the medium was accumulated by the cells between each medium change. A 5,700-fold range in extracellular [creatine] produced only a 20-fold difference in cellular [creatine].

Because of its possible effect on the creatine-

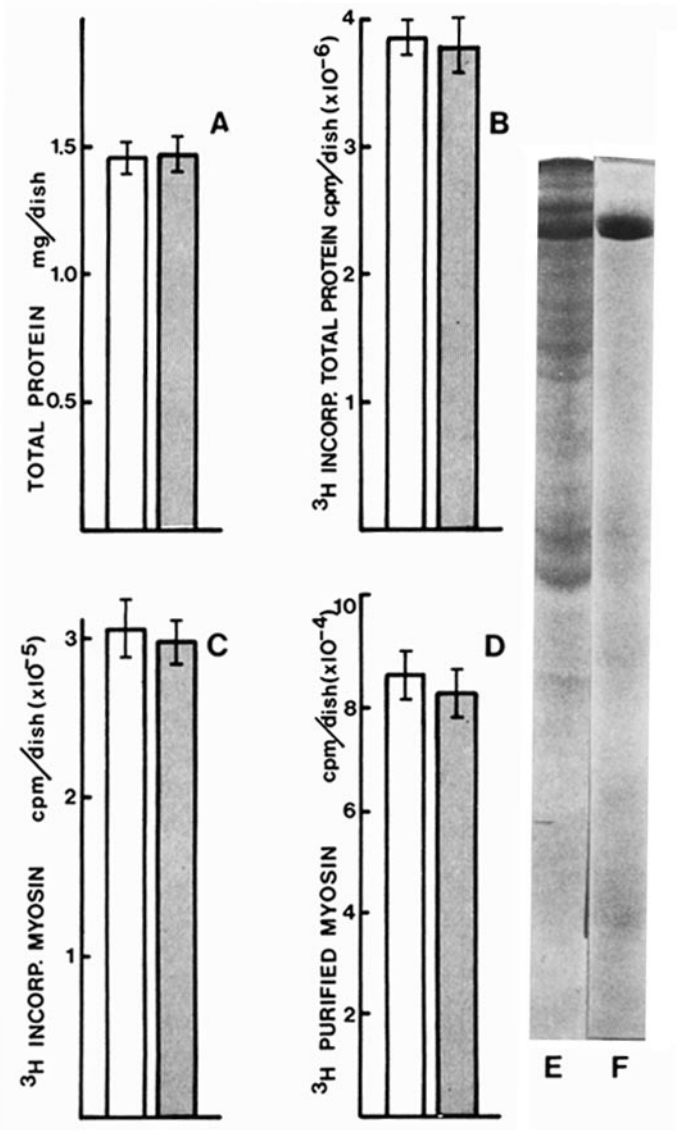

FIGURE 1 Lack of effect of 5 mM creatine on growth, total protein synthesis, and synthesis of myosin in 3-d-old cultures. 5 ml medium/dish; 5 mM creatine supplied from 24 h. Unshaded bars: control cultures. Shaded bars: plus 5 mM creatine cultures. (*A*) Total protein/dish. (*B*) [^{3}H]leucine incorporation into TCA-precipitable protein. (*C*) [^{3}H]leucine incorporation into myosin HC separated by SDS gel electrophoresis. (*D*) [^{3}H]leucine incorporation into myosin HC purified by the method of Tonomura et al. (12) and separated by SDS acrylamide gel electrophoresis. (*E*) Photograph of gel electrophoretogram of crude homogenate used for detection of myosin synthesis in *C*. (*F*) Photograph of gel electrophoretogram of myosin heavy chain purified and used in *D*. Both *E* and *F* are of proteins from the same culture dish.

growth relation, we investigated cellular creatine concentration as a function of depth of culture medium. Increase in depth has two effects: O_2-flux decreases and the available nutrients increase. With medium changes every 48 h, a depth of 2.3 mm (5 ml/dish) optimized growth and differentiation. Increasing the depth to 3.7 mm (8 ml/dish) depressed differentiation and promoted lipid accumulation. Cells grown for 5 d in 3 or 5 ml of control medium (40 μM creatine) accumulated ~8

μg creatine/mg protein while accumulation in 8 ml medium was depressed 30% (5.6 μg/ml; $P < 0.001$). Total protein of the cultures at 3, 5, and 8 ml of medium was 1.3, 2.2, and 1.9 mg/dish, respectively. The nutrient-limited growth at 3 ml was significantly different from 5- or 8-ml cultures ($P \leq 0.01$). Increasing the medium creatine to 5 mM significantly increased the intracellular creatine (500% increase at 3 ml, 360% at 5 ml, and 240% at 8 ml, $P < 0.001$) but did not alter the total protein/dish or incorporation of [^{3}H]leucine into total protein or myosin relative to controls.

Effect of CMIP on Myosin Synthesis

The effect of the creatine analog 1-carboxymethyl 1-2-iminohexahydropyrimidine (CMIP) on myosin and actin synthesis was previously studied (4, 9). It was reasoned that if CMIP stimulated synthesis, it would probably not be by involvement in nonspecific energy metabolism (always a possibility for creatine itself), as it is a poor substrate for creatine kinase and is not phosphorylated (5). It was reported that CMIP doubled specific muscle protein (myosin and actin) synthesis without stimulating total protein synthesis. We have reinvestigated this issue on four culture series. As usual, 5 mM extracellular [creatine] had no effect on the incorporation (cpm $\times 10^{-6}$/dish) of radio-leucine into either total protein or purified myosin (2.7 and 0.080, respectively); on the other hand, CMIP significantly reduced both measures (2.1 and 0.49, respectively, $P < 0.01$).

Control cultures accumulated creatine from the medium as in previous experiments but the presence of 1 mM CMIP markedly depressed the uptake to 36% of control levels ($P < 0.01$).

CONCLUSION

Completely contrary to previous reports (3, 4, 9) by other investigators and M. F. Morales, it was found in this work that creatine has no effect on myosin synthesis (incorporation of radio-leucine into myosin), or on total protein synthesis. Furthermore, CMIP was found to inhibit rather than to stimulate myosin synthesis. These conclusions apply to circumstances identical to the previous ones, and also to circumstances that elicited much more growth and myosin synthesis than in the earlier work. The rate of myosin synthesis in this work (12–13% of total protein synthesis) is higher than that reported in developing chick leg muscle *in ovo* (3.6–4%) (2) and apparently reflects selected conditions for differentiation. Myosin synthesis rate in previous work (3, 4) was a considerably lower fraction (2%) than the in vivo rate. In this work, as contrasted with the previous work, it was separately shown that manipulation of extracellular [creatine] does change intracellular [creatine], so that absence of an effect on synthesis cannot be ascribed to failure of creatine penetration.

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