A REEXAMINATION OF THE EFFECTS OF CRÉATINE 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 [3H]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 x 10⁶ 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% CO₂ 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 [3H]leucine into cultures was initiated by transferring cultures into fresh leucine-free medium with 4% HS, plus 20 µCi [3H]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
been detected with assurance. A significant difference. Differences as small as 10% could have was adequate for statistically significant discrimi-

tion between cultures was small and 3H-incorporation into total creatine was insignificant. Variability be-

tween cultures with regard to 3H-incorporation into total protein or HC were insignificant. Variability be-

between controls and creatine-supplied cultures was small and 3H-incorporation into total creatine was insignificance. A significant differ-

ence ($P < 0.001$) 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 x $10^{-6}$/dish) were 3.7 for total protein and 0.48 for myosin. In all three measurements the experimen-
tals 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. O2-
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 [crea-
tine], depth of medium does affect creatine ac-
cumulation. However, in 5-ml cultures the presence or absence of 5 mM creatine produced no significant differences in [3H]leucine incorporation into total protein or into myosin (Fig. 1). In par-
ticular, 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 homog-
enate. 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 [creatinine], 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 dia-
yzed 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 accu-
mulated by the cells between each medium change. A 5,700-fold range in extracellular [crea-
tine] 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 $\mu$M creatine) accumulated $\sim$8
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 vivo (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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