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Amino Acid Pools in Cultured Muscle Cells

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Compartmentalization of cellular amino acid pools occurs in cultures of cardiac and skeletal muscle cells, but the factors involved in this are not clear. We have further defined this problem by analyzing the intracellular free leucine and the transfer-RNA-(tRNA)-bound leucine pool in cultures of skeletal and cardiac muscle incubated with ^{3}H-leucine in the presence and absence of serum and amino acids. Withdrawal of nitrogen substrates caused substantial changes in leucine pool relationships—in particular, a change in the degree to which intracellular free leucine and tRNA-leucine were derived from the culture medium. In separate experiments, the validity of our tRNA measurements was confirmed by measurements of the specific activity of newly synthesized ferritin after iron induction. We discuss the implications of these findings with regard to factors involved in the control of amino acid flux through the cell, as well as with regard to design of experiments using isotopic amino acids to measure rates of amino acid utilization.

Key words: cultured muscle, protein synthesis, amino acid pools, ferritin synthesis

It has become apparent in recent years that compartmentalization of cellular amino acid pools occurs in a number of eukaryotic tissues, including muscle [1,2]. More recently available methods which measure the specific radioactivity of transfer RNA (tRNA) bound amino acids [3–5] have been helpful in defining the problem, as have other methods which identify additional functional pools [eg, 6]. In some cases, equilibration between these pools can be effected when the extracellular amino acid concentration is elevated sufficiently [4,5,7,8]. This, however, may not always be the case [eg, 9].

Amino acid compartmentalization is also known to occur in a variety of cultured cells [6,10–14], but the mechanisms by which this occurs and the factors involved in controlling the flux between compartments remain unclear. One controlling factor may be nutritional. Skeletal muscle cells cultured under nutritionally deprived conditions reutilize the amino acid, leucine, for protein synthesis [6], whereas this appears not to be the case for well-fortified conditions in which the cells actually are growing [11]. We have now evaluated the problem further by studying the effects of nitrogen deprivation on primary cultures of embryonic chick cardiac and skeletal muscle. We also have measured the absolute synthesis rate of an inducible protein, ferritin [15,16],

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in the established L-6 muscle line [17] to evaluate our tRNA-amino-acid-based methods. We discuss these results in the context of the literature and in terms of building an understanding of the significance and meaning of compartmentalization.

METHODS

Cell Culture

Primary cultures of chick embryo skeletal leg and heart muscles were prepared as described [6,11] and cultured as monolayers in 60-mm Falcon dishes in L-15 medium supplemented with fetal bovine serum. Glutamine was excluded from the medium to suppress fibroblast growth [11,18] and no antibiotics were employed at any stage of cell isolation or culture. The skeletal muscle cells fused to form myotubes on day 3 and were used for experiments on day 5. Cardiac cultures beat continuously after day 1 in culture and were used at day 7 or day 8.

L-6 cells were provided by Dr. James Florini (Syracuse University) and cultured as described [15,17]. Briefly, cell stocks were maintained in T-75 or T-150 Falcon flasks in medium containing 70% Dulbecco's Modified Eagle's Medium, 20% M-199, 10% horse serum, and 0.1% gentamycin. For experiments, cells were plated in 60-mm Falcon dishes at 1.5×10^5 cells/dish and continued in the same medium for 2 or 3 d prior to measurements of protein synthesis. In experiments where ferritin synthesis was measured, iron was added as ferric nitrilotriacetate for periods of 2–29 hr. Individual batches of L-6 cells were distributed randomly for use in studies of amino acid pools or of ferritin synthesis.

Protein Synthesis

The medium of primary cultures was changed to medium with or without amino acids (at the concentration found in L-15) and 10% fetal bovine serum, except in each case leucine was added at a concentration of 0.08 mM. Radioactive leucine was added 2 hr later (approximately 200 μCi/ml for medium containing all amino acids and serum and 100 μCi/ml for medium lacking these additions), and the cells were then incubated for 2 hr with the isotope. Isotopic leucine was added to L-6 cultures without a medium change and incubation continued for 2 hr. Leucine was chosen as precursor for reasons of comparison with prior results and because we have no evidence that the concentration of this amino acid affects synthesis [3,6,10,11].

Methods for monitoring ^{3}H-leucine distribution into protein and cellular pools in tissue culture have been described previously in detail [3,11]. Briefly, washed monolayers were lysed in 2 ml of 0.05 M cacodylate buffer, pH 6.8, containing 1% sodium dodecylsulfate (SDS). Small portions of the cell lysate were added to 10% trichloroacetic acid, and the remainder of the lysate was processed by phenol extraction, ethanol precipitation, and mild alkali hydrolysis to isolate the amino acids bound to tRNA. Incorporation of ^{3}H-leucine into protein was monitored in acid-precipitated material collected on glass fiber filters. Additional acid-insoluble samples were hydrolyzed at 110°C for 24 hr. The specific radioactivity of leucine in the extracellular medium, acid-soluble cellular pool, acylated to tRNA and in hydrolyzed protein was determined by allowing the amino acid sample to react with ^{14}C-diaminonaphthalene-5-sulfonylchloride (dansyl chloride) of known specific activity and separating the leucine derivatives by thin-layer chromatography [3]. Absolute and fractional rates of

protein synthesis were calculated as described [3,6,10]. The protein content of 25-μl samples of the cell lysate was determined by the method of Lowry et al [19] using a bovine serum albumin standard prepared in lysate buffer.

Ferritin Synthesis

Ferritin synthesis was measured with the immunoradiometric assay (IMRA) as previously described [15,16]. Briefly, monospecific antibody against rat liver ferritin raised in rabbits and immobilized on Immunobeads (Bio-Rad) was first incubated with antigen (standards or unknowns; conditions of antibody excess) in microfuge tubes, followed by washing and incubation with radioiodinated antibody, and washing and counting of the final complex. The absolute amount of ferritin synthesized per culture after incubation with iron was calculated as the increment in ferritin content in the time period during which iron was present. For determination of the specific activity of newly synthesized ferritin, cultures were incubated with ^{3}H-leucine (150 μCi/ml) for a 2-hr period after addition of iron. The isotopic content of ferritin synthesized in the 2 hr interval was measured from the change in ferritin content over the interval (IMRA) and the total ^{3}H-radioactivity in immunoreactive ferritin [16]. The specific activity of leucine in ferritin was then calculated assuming a molecular weight for the protein of 490,000 and 631 leucine residues per molecule [20]. We used these values as reported for liver ferritin, since skeletal muscle ferritin is predominantly "slow" ferritin [21], which appears indistinguishable from liver ferritin [6].

Materials

Fertile eggs were obtained from Oliver Merrill and Sons, Londonderry; L-15 and M-199 medium from GIBCO and amino acid-free L-15 from M.A. Associates; reagents for SDS polyacrylamide gel electrophoresis from Bio-Rad Laboratories; reagents for liquid scintillation spectrometry from Amersham and New England Nuclear; L-(4,5-^{3}H)-leucine, 50 Ci/mmol and L-(U-^{14}C)-leucine, 342 mCi/mmol, from Amersham Corp.; and N-methyl-^{14}C-dansyl chloride from Amersham Corp., 48mCi/mmol or from RPI, Elk Grove Village, IL, 110 mCi/mmol. The RPI product is supplied in hexane which was evaporated on receipt and resuspended in acetone. Stock solutions of ^{14}C-dansyl chloride were prepared by diluting the radioisotopes with unlabeled dansyl chloride (100 mg/ml in acetone, Pierce Biochem).

RESULTS

Effects of Nitrogen Deprivation in Primary Cultures of Muscle Cells

Table I shows the effects of nitrogen deprivation on leucine pools and protein synthesis in primary cultures of embryonic chick skeletal and cardiac muscle. Leucine concentration and specific activity did not change over the course of the experiment (data not shown). The tRNA/extracellular (tRNA/EC) leucine specific activity ratio was 0.74 for skeletal cells incubated in complete medium, indicating that most of tRNA-bound leucine comes directly from EC free leucine and only a small amount from an intracellular (IC) source. The intracellular/extracellular (IC/EC) leucine specific activity ratio was only 0.17 under the same conditions. There was a dramatic and significant fall in the tRNA/EC ratio under conditions of nitrogen deprivation, whereas the IC/EC ratio did not change. Cultures of cardiac cells responded quite

TABLE I. Effects of Nitrogen Deprivation on Protein Synthesis in Cultured Muscle Cells*

	Skeletal cells		Cardiac cells	
Medium	Complete	Minus serum and amino acids	Complete	Minus serum and amino acids
Specific activities (dpm/pmol)				
tRNA leucine	2,231 ± 87	1,504 ± 136	1,596 ± 172	1,583 ± 152
IC leucine	504 ± 86	417 ± 42	546 ± 71	2,118 ± 246
(ratios)				
tRNA/EC	0.74 ± 0.04	0.52 ± 0.04**	0.54 ± 0.06	0.55 ± 0.07
IC/EC	0.17 ± 0.04	0.14 ± 0.02	0.18 ± 0.05	0.72 ± 0.11**
Protein content (μg/culture)	441 ± 66	456 ± 40	187 ± 36	196 ± 39
Protein synthesis				
dpm/μg prot·hr ($\times 10^{-3}$)	24.2 ± 2.1	15.3 ± 1.1	14.6 ± 1.4	13.7 ± 1.2
pmol/μg prot·hr	11.2 ± 1.2	10.4 ± 1.1	9.3 ± 1.0	8.8 ± 0.8
%/hr	2.8 ± 0.4	3.1 ± 0.4	2.4 ± 0.3	2.2 ± 0.3

*Differentiated cultures of cardiac or skeletal muscle cells were incubated for 2 hr in the presence of complete L-15 medium or in L-15 medium lacking serum and amino acids ($-N_2$) as described in Methods; ^{3}H-leucine (100 μCi/ml for $-N_2$, 200 μCi/ml for complete) was added for the final 2 hr of culture. Data are from three separate experiments and expressed as mean ± SD and normalized to an extracellular leucine specific activity of 3,000 dpm/pmol. Skeletal data are means of six to eight determinations; cardiac data, seven to nine determinations. IC, intracellular; EC, extracellular.
**Significantly different from complete medium value of same cell type, $P < .01$ by Student t test.

differently to nitrogen deprivation: the tRNA/EC leucine specific activity ratio did not change (0.55 vs 0.54) while the IC/EC ratio increased substantially (0.18 in complete medium; 0.72 in nitrogen deprived medium).

Despite these changes in leucine pool relationships, there was no effect of nitrogen deprivation on protein synthesis for either cell type whether expressed in terms of fractional or absolute rates, using tRNA-leucine specific activity as precursor in the rate calculations. The calculated effects of nitrogen deprivation on protein synthesis using EC or IC free leucine specific activities as precursor, of course, gave widely varying numbers (Table II).

Control experiments indicated that the effects of 4-hr nitrogen deprivation (the total duration of this condition) were reversible. For example, 48 hr after addition of serum and amino acids to nitrogen-starved cells, morphology, protein/dish and tRNA-leucine-based rates of protein synthesis were identical to control cells (data not shown). As well, dual-isotope experiments [eg, 22] indicated that in the final 2 hr under nitrogen-deprived conditions (during which synthesis measurements were made) there was no selective effect on the synthesis of sarcoplasmic vs myofibrillar proteins or on the synthesis of the individual myofibrillar protein's myosin heavy chains, myosin light chains, actin, and tropomyosin (data not shown).

Ferritin Synthesis by L-6 Myoblasts

The ability to measure the synthesis of a newly induced protein offered an additional, independent method for determining the specific activity of the precursor

TABLE II. Protein Synthesis by Primary Cultures of Muscle Cells: Effects of Nitrogen Deprivation Calculated Using Different Precursor Pools

	Percent effect	
Precursor	Skeletal cells	Cardiac cells
Extracellular	−35% ($P < .01$)	−6.1% ($P > .10$)
Intracellular	−23% ($P < .05$)	−140% ($P < .01$)
tRNA	+3.8% ($P > .10$)	−4.9% ($P > .10$)

TABLE III. Ferritin Synthesis by Cultured L-6 Myoblasts*

Specific radioactivity (dpm/pmol)		
Medium leucine specific activity	Leucine in newly synthesized ferritin	(b/a)[a]
342 ± 21 (n = 3)	260 ± 48** (n = 3)	0.76 ± 0.12 (n = 3)

*Newly synthesized ferritin was measured by determining immunologically the amount of ferritin present in cultures of L-6 cells before and 2 hr after the addition of 200 μM ferric nitrilotriacetate and 100 μCi/ml ^{3}H-leucine. Ferritin-associated radioactivity was determined similarly 2 hr after iron and isotope administration, and used to calculate the ferritin and thus leucine specific activity. Data expressed as mean ± SD (n).

[a]Values were paired by dish for specific activity of leucine in the medium and in ferritin.

**P < 0.01.

pool, at least for that particular protein. The tRNA/EC leucine specific activity ratio was 0.77±0.06 (mean ± SD, n = 8; leucine concentration approximately 0.4 mM) for these cells, indicating again that tRNA-bound leucine was largely derived from EC free leucine. The fractional synthesis rate was 13.0 ± 2.0% per hr and the absolute rate 26.6 ± 2.0 pmol/μg protein per hr. These rates were considerably higher than for the primary cell cultures (Table I) but are comparable to those reported by Ballard [23].

We next examined iron-induced ferritin synthesis in the L-6 cells [15,16]. The results of such an experiment are shown in Table III. The measured specific activity of leucine incorporated into newly synthesized ferritin was 75.7% of free leucine. This is in excellent agreement with the tRNA data cited above.

DISCUSSION

Sufficient information is available to underscore the complexity of amino acid pools in cultured muscle cells. Results from a variety of experiments indicate that compartmentalization of at least one amino acid, leucine, does occur. This appears to be a functional compartmentalization in at least one case [6]. It appears, as well, that

nutritional factors can alter pool relationships—for example, the relationship of the tRNA-bound leucine pool to the intracellular leucine pool and to the medium. We have reported conditions of nutritional deprivation of primary cultures of skeletal muscle (cells cultured in phosphate-buffered saline) under which the major portion of leucine bound to tRNA is derived from obligatory endogenous sources [6]. At the same time, similar cultures incubated in complete growth medium are quite different in that the majority of tRNA-bound leucine comes directly from extracellular leucine [11] and only a small proportion from intracellular free leucine. Indeed, tRNA-bound leucine can come entirely from the culture medium if the medium leucine concentration is raised (compare data for cells in complete medium, Table II at 0.08 mM leucine with data from Airhart et al [11] at 0.4 mM leucine). Ballard [23] has come to the same conclusions from experiments on cells of the same line, in which case a different means was used to calculate synthesis rates. Much of our argument rests on the validity of our tRNA measurements. The measurements of ferritin synthesis are consistent with the tRNA-based results for the same culture conditions.

The different responses of skeletal and cardiac cultures to nitrogen deprivation are striking, and only partially explainable. It would appear that skeletal muscle cells adapt by increasing the contribution to protein synthesis of leucine derived from endogenous breakdown. Cardiac cells, on the other hand, increase the proportion of leucine for protein synthesis coming from the medium. The adaptive advantage of this latter response may be related to the function of cardiac tissue. It would be most interesting in this regard to compare the effects of nitrogen deprivation on the rate of protein degradation in these two cell types. It would also be important to confirm the validity of tRNA-leucine based rates of synthesis by other, independent means, as by analyses of polyribosomes.

Changes which occur in the source of free leucine found in the intracellular pool are even more difficult to explain. We have, in fact, preferred not to use that pool as a valid precursor for protein synthesis or for leucine oxidation, given the inconsistent results it can provide (Table II) [6,10,11,13]. The changes, whatever their explanation, appear to be due to free amino acids, since lack of serum does not affect the source of amino acids used to charge tRNA [11]. This is an interesting possibility, especially given the role of extracellular amino acids in the regulation of endocytic pathways and breakdown of exogenous proteins in cultured cells [24].

Our results illustrate the caution which must be taken in extrapolating findings regarding pool relationships from one experimental system to another when the purpose is to design experiments for measuring rates of amino acid utilization. Thus, it appears, for example, that measurement of the tRNA precursor may be necessary in experiments designed to measure rates of protein synthesis. It would also appear that use of the method in which medium amino acid concentration is raised until a constant rate of synthesis is calculable using medium amino acid specific activity as precursor does not necessarily lead to accurate estimates of synthesis rates [23,25,26]. This method applied in studies of macrophage [10,12], fibroblast [13], and primary skeletal muscle cultures, the last in nutritionally deprived conditions [6], would lead to erroneous rate calculations, at least in comparison to tRNA-amino-acid-based rates calculated under the same conditions [6,10,12,13].

In summary, we describe new results with studies of primary cultures of skeletal and cardiac muscle cells and L-6 myoblasts which illlustrate the complexity of free amino acid pools in cultured muscle cells. It would appear that one factor regulating

flux between these pools is the availability of exogenous amino acids. The existence of these pools complicates design of experiments examining amino acid utilization by these cells, yet provides further illustration of the degree to which cellular functions are highly compartmentalized.

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