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Regulation of protein synthesis and degradation in L8 myotubes

Effects of serum, insulin and insulin-like growth factors

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We have examined the regulation of protein turnover in rat skeletal myotubes from the L8 cell line. We measured protein synthesis by the rates of incorporation of radiolabelled tyrosine into protein in the presence of a flooding dose of non-radioactive tyrosine. We monitored degradation of proteins labelled with radioactive tyrosine by the release of acid-soluble radioactivity into medium containing excess non-radioactive tyrosine. Extracellular tyrosine pools and intracellular tyrosyl-tRNA equilibrate rapidly during measurements of protein synthesis, and very little reutilization of the radiolabelled tyrosine occurs during degradation measurements. Measured rates of protein synthesis and degradation are constant for several hours, and changes in myotube protein content can be accurately predicted by the measured rates of protein synthesis and degradation. Most of the myotube proteins labelled with radioactive tyrosine for 2 days are degraded, with half-lives ($t_{\frac{1}{2}}$) of approx. 50 h. A small proportion (< 2.5%) of the radiolabelled proteins are degraded more rapidly ($t_{\frac{1}{2}}$ < 10 h), and, at most, a small proportion (< 15%) are degraded more slowly ($t_{\frac{1}{2}}$ > 50 h). A variety of agents commonly added to primary muscle cell cultures or to myoblast cell lines (18% Medium 199, 1% chick-embryo extract, antibiotics and antifungal agents) had no effect on rates of protein synthesis or degradation. Horse serum, fetal bovine serum and insulin stimulate protein synthesis and inhibit the degradation of long-lived proteins without affecting the degradation of short-lived proteins. Insulin-like growth factors (IGF)-1 and -2 also stimulate protein synthesis and inhibit protein degradation. The stimulation of protein synthesis and the inhibition of protein degradation are of similar magnitude (a maximum of approx. 2-fold) and display similar sensitivities to a particular anabolic agent. Insulin stimulates protein synthesis and inhibits protein degradation only at supraphysiological doses, whereas IGF-1 and -2 are effective at physiological concentrations. These and other findings suggest that IGFs may be important regulators of skeletal muscle growth during the fetal and early neonatal periods.

INTRODUCTION

Rates of muscle protein turnover can be modified in many situations, such as trauma [1], immobilization [2], compensatory growth [3], contractile activity [4], diabetes [5] and muscle wasting diseases [6]. Muscle protein turnover has been measured *in vivo* [5], in perfused muscle [7], in incubated muscle [8], in cultured myoblasts [9] and in cultured myotubes [10].

Skeletal-muscle cells can be grown *in vitro* as primary cultures [11] or established cell lines [12]. Myoblasts in culture can be induced to fuse into multinucleated myotubes, which accumulate muscle-specific proteins [13]. Differentiation in culture is incomplete, however, in that myotubes express embryonic and/or neonatal isoforms of many contractile proteins [14,15] and enzymes [16,17]. Furthermore, cultured myotubes are similar to embryonic muscle in the distribution and functional properties of acetylcholine receptors [18,19], the lack of functional acetylcholinesterase activity [18], and the expression of low numbers of insulin receptors [20]. Thus cultured muscle cells provide a unique model system for studying fetal/neonatal muscle under a variety of culture conditions where net nitrogen balance can range from positive to negative.

Many growth factors and hormones undoubtedly interact in the regulation of muscle growth in the fetus or newborn. One group of potentially important regulators includes insulin and the insulin-like growth factors (IGFs). Insulin stimulates muscle growth in adults [5], but may only be permissive for normal fetal growth [21]. IGF-1 levels are relatively low in fetal serum compared with adult serum [22], but fetal tissues are very responsive to IGF-1 [23]. Levels of IGF-2 (previously designated multiplication-stimulating activity) are highest in fetal- and neonatal-rat serum, and decline gradually in the first few weeks after birth [22,24].

The relative contributions of changes in protein-synthesis and -degradation rates during tissue growth or atrophy vary for different cell types and developmental

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; HBSS, Hanks' balanced salt solution; HBSS-Tyr, Hanks' balanced salt solution containing 2 mM non-radioactive tyrosine; FBS, fetal bovine serum; HS, horse serum; CEE, chick embryo extract; TCA, trichloroacetic acid; IGF, insulin-like growth factor; hIGF-1, human insulin-like growth factor-1; rIGF-2, rat insulin-like growth factor-2; BSA, bovine serum albumin; RIA, radioimmunoassay.

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stages. Published studies concerning the regulation of protein turnover in primary chick myotubes [10,25–28] and in myoblasts from the L6 rat cell line [9,29–31] suggest that both protein-synthesis and -degradation rates are subject to regulation. The degree to which each process is regulated has been carefully compared in myoblasts [29], but not in myotubes. Establishing the mechanism of protein accumulation in cultured myotubes is important, because the highest muscle growth rates *in vivo* occur during the fetal and neonatal periods [32].

MATERIALS AND METHODS

Materials

L-[*ring*-3,5-^{3}H]Tyrosine (sp. radioactivity 25–50 Ci/mmol) and L-[U-^{14}C]tyrosine (sp. radioactivity 350–495 mCi/mmol) were purchased from ICN (Irvine, CA, U.S.A.). Fetal-bovine serum (FBS) was from K.C. Biologicals (Lenexa, KS, U.S.A.). Heat-inactivated horse serum (HS), Dulbecco's modified Eagle's medium with high glucose (DMEM), Medium 199, Earle's balanced salt solution, Hanks' balanced salt solution (HBSS) with and without Ca^{2+}/Mg^{2+}, chick-embryo extract (CEE), nystatin and penicillin/streptomycin solution were from Grand Island Biological Co. (Grand Island, NY, U.S.A.). Cystosine 1-β-D-arabinofuranoside (cystosine arabinoside), fluorescamine and radioimmunoassay (RIA)-grade bovine serum albumin (BSA; fraction 5) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Soluscint A and Liquiscint were from National Diagnostics (Manville, N.J., U.S.A.). Human IGF-1 (hIGF-1) was purchased from Collaborative Research (Lexington, MA, U.S.A.) or Amgen Biologicals (Thousand Oaks, CA, U.S.A.). Partially purified rat IGF-2 (rIGF-2) was generously given by James R. Florini (Department of Biology, Syracuse University, Syracuse, NY, U.S.A.). The rIGF-2 was purified from conditioned medium of cells of the Buffalo rat liver line and analysed by h.p.l.c. as described in ref. [30]. The preparation contained 25% Peak 3 and 60% Peak 2 as described in ref. [33]. Sterile non-cytotoxic bovine insulin (24.7 units/mg by U.S.P. rabbit assay; 26.5 units/mg by RIA) was from Collaborative Research. Highly purified crystalline pig insulin (26.8 units/mg by U.S.P. rabbit assay) was given by Dr. Ronald Chance, Lilly Research Laboratories (Indianapolis, IN, U.S.A.). Insulin was dissolved and stored at −20 °C. hIGF-1 was stored at −20 °C as supplied, and rIGF-2 was solubilized in 0.25 M-acetic acid, diluted in DMEM, and stored at −20 °C. Insulin and IGFs were diluted to their final concentrations in the presence of RIA-grade BSA on the day of each experiment.

Cell culture

L8 rat skeletal-muscle cells were provided by Dr. J. W. C. Bird (Bureau of Biological Research, Rutgers University, Piscataway, NJ, U.S.A.) and Dr. Fred J. Roisen (Department of Anatomy, University of New Jersey School of Medicine and Dentistry, Piscataway, NJ, U.S.A.). The L8 muscle-cell line was originally established in 1969 by serial passaging of myoblasts isolated from primary rat skeletal-muscle cultures prepared from newborn Wistar rats [12]. Frozen stocks, kept in liquid N_2, were thawed every few weeks and were maintained by repeated subculturing at low density on 100 mm-diameter culture dishes. Cells were used for only 3–7 passages after thawing. Undifferentiated myoblasts were grown at 37 °C in a humidifed atmosphere of CO_2/air (1:9) in DMEM containing 20% (v/v) FBS. DMEM was routinely supplemented with 44 mM-$NaHCO_3$, 110 mg of sodium pyruvate/ml, 100 units of penicillin/ml, 100 μg of streptomycin/ml and 100 units of nystatin/ml. The cells were removed with 0.125% trypsin/1 mM-EDTA in Ca^{2+}/Mg^{2+}-free HBSS and transferred to 12-well or 6-well dishes before experiments. Cells were grown in the presence of 20% FBS until they reached confluency; at this point the medium was replaced by DMEM supplemented with 5% HS. This procedure minimizes differentiation until the cells are confluent and sychronizes fusion [34]. L8 myotubes grown under similar conditions have a fusion index of 60–90% [35]. We further enriched for myotubes by addition of cytosine arabinoside to the cultures for 24–36 h at a final concentration of 10 μM after the cells became confluent. The cytosine arabinoside eliminates most myoblasts remaining after differentiation [36]. Once fusion was complete, the medium was changed to DMEM containing 10% FBS before radiolabelling.

Measurement of protein synthesis

Rates of protein synthesis were determined by monitoring the incorporation of [^{3}H]tyrosine into acid-insoluble material. Cultures were incubated in non-radioactive experimental medium for various periods of time. The culture medium was replaced with experimental medium containing 1.5–5 μCi of [^{3}H]tyrosine/ml and non-radioactive tyrosine up to 2.0 mM. Myotubes were incubated with [^{3}H]tyrosine for 1 h, the radiolabelling medium was removed, and the monolayers were rinsed rapidly five times with ice-cold HBSS containing 2 mM-non-radioactive tyrosine (HBSS-Tyr). Monolayers were fixed for at least 10 min in 10% (w/v) trichloroacetic acid (TCA) on ice [29]. Protein was thoroughly scraped off the dishes in the TCA, chilled for at least 1 h, and spun down for 2 min in a Beckman Microfuge at 9600 $g_{max.}$. Separate experiments showed that this procedure removed most of the cellular protein (94–97% as compared with protein extraction in 0.1 M-NaOH/0.1% sodium deoxycholate). The pellet was washed once with 10% TCA and dissolved in 1.0 ml of 1.0 M-NaOH/1% sodium deoxycholate. A small portion was taken for protein analysis by the Fluram method [37], with BSA as a standard, and a measured volume was neutralized with 10 M-HCl before determination of radioactivity in a Packard 3255 Tri-Carb liquid-scintillation spectrometer. Quenching was determined with an automatic external standard. Rates of incorporation were normalized for protein content in each well. In one experiment (results shown in Fig. 5*a*), myotubes were deprived of serum before incubation with or without HS.

Measurement of protein degradation

Rates of protein degradation were determined by monitoring the release of acid-soluble radioactivity into the culture medium at various intervals after radiolabelling proteins with [^{3}H]tyrosine and/or [^{14}C]phenylalanine. Radiolabelling was begun after cell fusion was complete. Long-lived proteins were radiolabelled with 0.2–1.0 μCi of [^{3}H]tyrosine/ml for 2 days in DMEM supplemented with 10% FBS. Cells were rinsed once in HBSS-Tyr and then placed in DMEM supplemented with 10% FBS and 2 mM-tyrosine for 1–2 h at 37 °C to allow degradation of very-short-lived proteins. The cells were then rinsed twice in HBSS-Tyr and transferred

to non-radioactive experimental medium. Short-lived proteins were radiolabelled with 2.2–5 μCi of [^{3}H]-tyrosine/ml for 1 h in DMEM supplemented with 10% FBS. Cells were rinsed once with HBSS-Tyr and chased for 5–10 min in DMEM supplemented with 10% FBS and 2 mM non-radioactive tyrosine to allow for release of intracellular acid-soluble radioactivity [38]. Cells were rinsed twice in HBSS-Tyr and transferred to experimental medium.

Samples (usually 0.5 ml) of the culture medium were taken at various intervals, and were adjusted to the same serum concentration by addition of whole serum to aid protein precipitation. TCA was added to a final concentration of 10% (w/v). After standing at 4 °C for at least 1 h, samples were spun in a Microfuge for 2 min at 9600 $g_{max.}$, and acid-soluble radioactivity was determined.

At the end of an experiment, the remaining medium was aspirated and the monolayer rinsed once with HBSS-Tyr. Cell-associated radioactivity was determined after dissolving the monolayer in 0.1 M-NaOH/0.1% sodium deoxycholate and neutralizing the samples with 1 M-HCl. Rates of degradation were calculated by determining the amount of radioactivity appearing in the medium at various times after correction for volume changes owing to successive sampling. The total radioactivity released into the medium plus that remaining in the monolayer at the end of the experiment represents the initial radioactivity in the cells. The percentage of protein degraded at any time point equals the acid-soluble radioactivity present in the medium at that time divided by the initial radioactivity. In some experiments acid-insoluble radioactivity in the medium was determined by washing TCA precipitates once in 5.0% TCA and then dissolving the pellet in 0.5 ml of 95–97% formic acid.

For calculations of the fraction of short- and long-lived proteins, regression lines were fitted to the time points occurring after short-lived proteins had been chased away. Disappearance of short-lived proteins was determined by calculating half-lives between individual time points and short segments on a well-by-well basis, and testing for significant differences in half-lives of adjacent regions by Student's t test.

For experiments where tyrosine concentrations less than that normally present in DMEM (0.46 mM) were required, a modified DMEM was prepared by addition of the following components to Earle's balanced salt solution at the concentrations normally present in DMEM: $NaHCO_3$, $MgSO_4$, $CaCl_2$, $Fe(NO_3)_3$, amino acids (except tyrosine), sodium pyruvate and glucose. MEM Vitamin Solution (100×) was used as a stock; in this case the final vitamin concentrations are the same as found in DMEM except for *myo*-inositol, which is 11% higher. Media containing 0 and 2 mM-tyrosine were mixed together to give intermediate concentrations of this amino acid.

Measurement of specific radioactivity of tyrosyl-tRNA

We used the methods described by Martin *et al.* [39] to extract aminoacyl-tRNAs from myotubes. In brief, RNA from a single 100 mm dish of myotubes was extracted with 2×1 ml of phenol acetate buffer (1:1, v/v) to remove protein. Cellular RNA together with 250 μg of carrier yeast tRNA was precipitated twice in ethanol at −20 °C, and the precipitate was redissolved in buffer and chromatographed on a Sephadex G-100 column. The tRNA elution peak was identified by the A_{260}, and the tRNA fractions were pooled and deacylated by adjusting to pH 8.8 with NH_3. The released amino acids were separated from the tRNA by chromatography on a Sephadex G-25 column.

The specific radioactivity of the tyrosine was determined by the dual-radioisotope technique described by Van Venrooij *et al.* [40]. Growing myoblasts were radiolabelled with 2 μCi of [^{3}H]tyrosine/ml in the presence of 2.0 mM non-radioactive tyrosine for 8 days. At this time the specific radioactivity of tyrosine in myotube proteins was > 90% of that of tyrosine in the medium (results not shown). The [^{3}H]tyrosine associated with tRNA was used to calculate the nmol of tyrosine recovered. [^{14}C]Tyrosine was added to the medium containing [^{3}H]tyrosine, and cells were harvested at increasing times. All of the ^{3}H and ^{14}C radioactivity associated with the isolated RNA fraction appeared to be tyrosyl-tRNA, on the basis of the its comigration with tRNAs on Sephadex G-100 and its release with NH_3. The time course of ^{14}C radioactivity accumulating in the RNA fraction was followed as an indication of the rate at which equilibration was reached between the extracellular and tyrosyl-tRNA pools.

Some cells were radiolabelled for 8 days with both [^{3}H]- and [^{14}C]-tyrosine. These cultures were switched to medium containing [^{3}H]tyrosine only, and the loss of ^{14}C from the tRNA was monitored.

Statistical analysis

Data from wells incubated under different conditions were compared by Student's t test.

RESULTS

Equilibration of pools of extracellular and intracellular tyrosine and of tyrosyl-tRNA during measurements of protein synthesis

Radiolabelled tyrosine and phenylalanine were used to measure protein synthesis and degradation in myotube cultures because these amino acids are neither synthesized nor catabolized by skeletal muscle [7,8,25,41]. The incorporation of [^{3}H]tyrosine into acid-precipitable material was linear for at least 3 h (results not shown). In all the following experiments, protein synthesis was measured over a 1 h period after various preincubation times in the absence of label.

Non-radioactive 2 mM-tyrosine was added to the experimental medium for measurements of both protein synthesis and protein breakdown, because this is the maximal amount of tyrosine that can be dissolved in DMEM. During measurements of protein synthesis the specific radioactivity of the intracellular pool appears to have reached a stable value quickly, since the first time point (20 min) in our measurements of the linearity of [^{3}H]tyrosine incorporation is on the linear portion of the curve, and the regression line extrapolates back very close to the origin (results not shown).

We also measured tyrosine incorporation at different tyrosine concentrations under conditions of constant specific radioactivity (Fig. 1). Incorporation was decreased with no added non-radioactive tyrosine, probably because extracellular and intracellular pools had not equilibrated. Incorporation of 0.5 mM-tyrosine was 89% of that at 2.0 mM, so increasing the concentration of

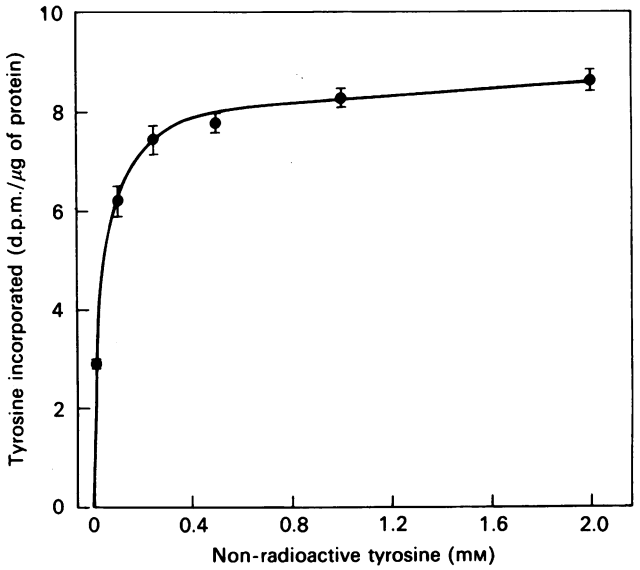

Fig. 1. Effect of tyrosine concentration on [^{3}H]tyrosine incorporation

Myotubes grown on 12-well dishes were rinsed twice with HBSS and placed in DMEM made up to contain 0–2 mM non-radioactive tyrosine at constant tyrosine specific radioactivity (2 μCi/mmol). Monolayers were incubated for 60 min, rinsed five times with ice-cold HBSS-Tyr, and processed for incorporation of acid-soluble radioactivity as described in the Materials and methods section. Values are means $\pm$ S.D. ($n = 6$). In this and all the following Figures, absence of an error bar indicates that 1 S.D. falls within the datum point.

tyrosine in the medium appears to saturate intracellular tyrosine specific radioactivity.

These results do not rule out the possibility that internal amino acid pools exist that cannot be flooded [42]. We examined this question by determining the specific radioactivities of tyrosine in the medium and of tyrosyl-tRNA. [^{14}C]Tyrosine added for various times before isolation of tyrosyl-tRNA shows that intracellular tyrosine pools used for charging tRNAs are 67% and 95% equilibrated with extracellular tyrosine at 5 and 15 min respectively (Table 1). Similar results apply to cells maintained for 48 h in the absence of serum (results not shown). Therefore, any underestimation of protein synthesis will occur only during the first few minutes of radioisotope incorporation, and will amount to < 10% error in a 1 h measurement.

Reutilization of radioisotope in measurements of protein degradation

Flooding of the intracellular pools should minimize reutilization of radiolabelled tyrosine released from degraded proteins and thereby avoid underestimation of breakdown rates. To rule out a direct effect of tyrosine on protein degradation, myotubes were radiolabelled simultaneously with [^{3}H]tyrosine and [^{14}C]phenylalanine, and breakdown rates were measured in the presence of increasing concentrations of non-radioactive tyrosine (Fig. 2). Non-radioactive phenylalanine (2.0 mM) was added to the medium, since Mayorek *et al.* [43] have shown that phenylalanine at 1.2 mM maximally inhibits reutilization of [^{14}C]phenylalanine from prelabelled proteins in skeletal-muscle cultures.

Table 1. Equilibration of extracellular tyrosine and intracellular tyrosyl-tRNA

Specific radioactivities of [^{3}H]- and [^{14}C]-tyrosine were measured as described in the Materials and methods section: (*a*) incorporation of [^{14}C]tyrosine into tRNA during a typical protein-synthesis experiment in the presence of 10% FBS and 2 mM non-radioactive tyrosine; (*b*) loss of radioactivity from tRNA during a typical degradation experiment in the presence of 10% FBS and 2 mM non-radioactive tyrosine. Values given are means $\pm$ S.D. for three to six samples.

(*a*) Protein-synthesis measurements

Time (min)	Sp. radioactivity (d.p.m./nmol of tyrosine)	Equilibration (%) (tRNA/medium)
0	21 $\pm$ 9	3
1	155 $\pm$ 83	23
5	449 $\pm$ 141	67
15	642 $\pm$ 146	95
30	584 $\pm$ 132	87
Medium	673 $\pm$ 61	100

(*b*) Protein-degradation measurements

Time (min)	Sp. radioactivity (d.p.m./nmol of tyrosine)	Equilibration (% loss of radioactivity)
0	702 $\pm$ 133	0
15	478 $\pm$ 128	32
30	100 $\pm$ 33	86
90	83 $\pm$ 18	88
1200	17 $\pm$ 20	98

Raising the tyrosine concentration to 0.5 mM maximally stimulated release of [^{3}H]tyrosine into the culture medium in the presence and absence of serum (Fig. 2*a*). In contrast, tyrosine in the same concentration range had no effect on the release of [^{14}C]phenylalanine into the medium (Fig. 2*b*). These results suggest that reutilization of [^{3}H]tyrosine is maximally suppressed at 0.5 mM-tyrosine.

To determine whether some reutilization persists even in the presence of non-radioactive tyrosine [42], we measured the specific radioactivity of [^{14}C]tyrosine linked to tRNA at different times during degradation measurements in medium containing 2 mM-non-radioactive tyrosine. Table 1 shows that most (> 86%) of the [^{14}C]tyrosine is chased from tRNA within 30 min in degradation medium. The loss of [^{14}C]tyrosine from tRNA is slightly slower in cells maintained without serum, but reutilization is > 85% suppressed within 90 min (results not shown).

Secretion of acid-precipitable radioactivity

Radiolabelled proteins and peptides are released into the medium during degradation measurements, probably as a result of protein secretion, cell death, and release of incompletely hydrolysed proteins from their sites of digestion (L. Isenman & J. F. Dice, unpublished work). Acid-precipitable radioactivity released into the medium represents less than 2% of the total radioactivity incorporated by the myotubes in most experiments, which were terminated within 13 h. In addition, there were no major differences between cells incubated in the presence

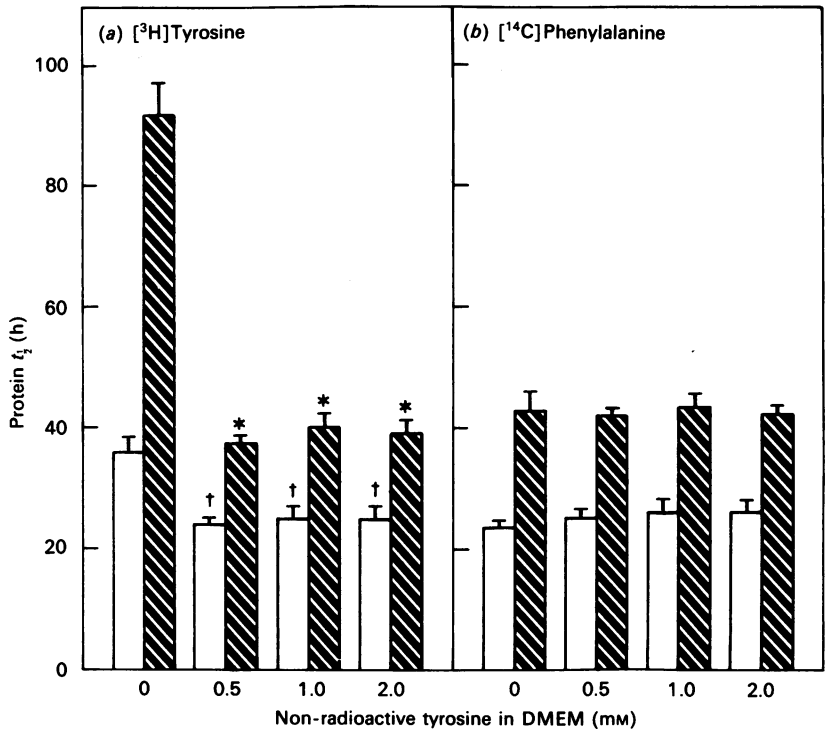

Fig. 2. Effect of tyrosine concentration on apparent half-lives of proteins labelled with [^{3}H]tyrosine (*a*) and [^{14}C]phenylalanine (*b*)

Myotubes grown on 12-well dishes were radiolabelled for 2 days with 0.6 μCi of [^{3}H]tyrosine/ml and 0.2 μCi of [^{14}C]phenylalanine/ml in DMEM supplemented with 10% FBS. Cultures were rinsed twice with HBSS + 2.0 mM-phenylalanine and incubated for 10.75 h in degradation medium. Degradation medium consisted of DMEM made up to contain 2.0 mM non-radioactive phenylalanine and 0–2 mM non-radioactive tyrosine supplemented either with (▨) or without (□) 10% HS. Monolayers were rinsed twice with HBSS containing 2 mM non-radioactive phenylalanine and processed for determination of radioactivity as described in the Materials and methods section. The data are means ± S.D.: [n = 5 (▨) or 4 (□)]. *$P < 0.001$ versus DMEM + 0 Tyr (10% HS added); †$P < 0.001$ versus DMEM + 0 Tyr (no HS added).

or absence of serum (results not shown). It is difficult to correct for this secreted protein in degradation measurements because of the complexity of its origin. Nevertheless, we have recalculated protein half-lives after adding the acid-insoluble radioactivity in the medium to the acid-soluble radioactivity for each degradation time point. We have also recalculated protein half-lives after adding acid-precipitable radioactivity to cellular radioactivity remaining at the end of the experiment. The calculated half-lives were only slightly altered (< 10%), and differences in degradation rates between groups of myotubes were unchanged.

Breakdown of long-lived and short-lived proteins

Long-lived proteins (radiolabelled for 2 days) are degraded with a half-life of approx. 50 h, whereas short-lived proteins (radiolabelled for 1 h) are degraded with heterogeneous half-lives of < 10 h (results not shown). Regression lines were fitted to the curves in the regions where all the rapidly-turning-over protein had been chased away, and were extrapolated back to the ordinate for estimation of label incorporated into the short-lived pool. The 2-day label resulted in a maximum of 2.5% of total radioactivity incorporated into the short-lived pool, whereas the 1 h label resulted in a maximum of 33% of the radioactivity incorporated into that pool.

We extended our monitoring of degradation of long-lived proteins to 128 h for myotubes maintained in the presence of serum (Fig. 3). A single exponential curve was evident throughout the 128 h period, indicating that longer-lived proteins must comprise less than 15% of the protein radiolabelled under our conditions. Similar conclusions have been reached for other cell types [44].

Protein turnover in different growth media

We examined whether supplementing the culture medium with antibiotic and antifungal agents had any effect on protein turnover, because Moss *et al.* [45] reported that streptomycin inhibited the accumulation of myosin and total protein and delayed the appearance of striations in primary chick myotubes. We grew L8 muscle cells for 5 days in the absence or presence of penicillin, streptomycin and nystatin, and then measured synthesis and breakdown of long-lived proteins. These compounds had no effects on protein synthesis or degradation or on myotube morphology, as determined by light-microscopy (results not shown).

We also examined the effects of medium supplemented with 18% Medium 199, 1% CEE and 10% HS, as this was the medium in which the cells were grown before we obtained them. Medium 199 differs from DMEM in that it contains additional compounds such as purines, pyrimidines, pentose sugars, cholesterol, sodium acetate and oleic acid. CEE is essential for the growth of skeletal-muscle cells in primary culture, but is not required for growth of clonal muscle cell lines [11]. The ability of the Medium 199/CEE/HS mixture to stimulate protein synthesis and inhibit protein degradation is due entirely

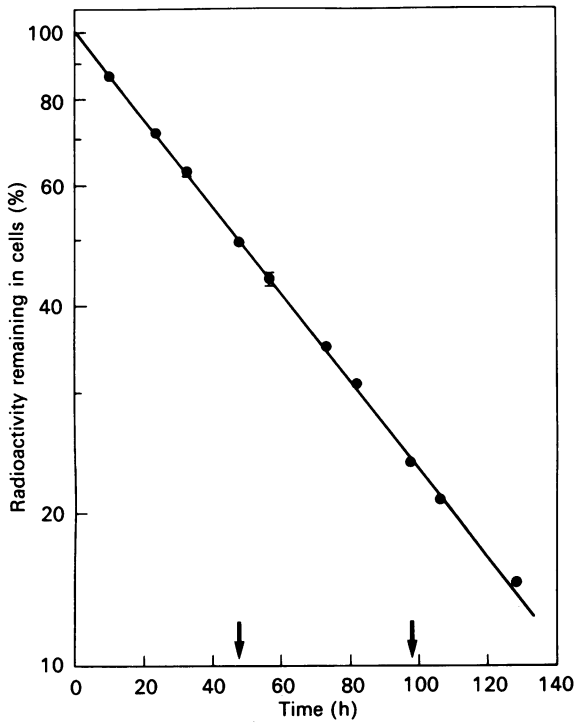

Fig. 3. Breakdown of long-lived proteins studied for 128 h in the presence of serum

Myotubes grown on 6-well dishes were radiolabelled for 2 days with [^{3}H]tyrosine in DMEM containing 10% FBS. Very-short-lived proteins were chased for 2 h in DMEM + 10% FBS + 2 mM-tyrosine before the beginning of degradation measurements. The monolayers were placed in fresh degradation medium at 48 and 97.5 h (arrows) to avoid depletion of medium components. Monolayers were examined by light-microscopy at the end of the experiment and were in good condition. The data are means ± S.D. ($n = 6$ for each time point).

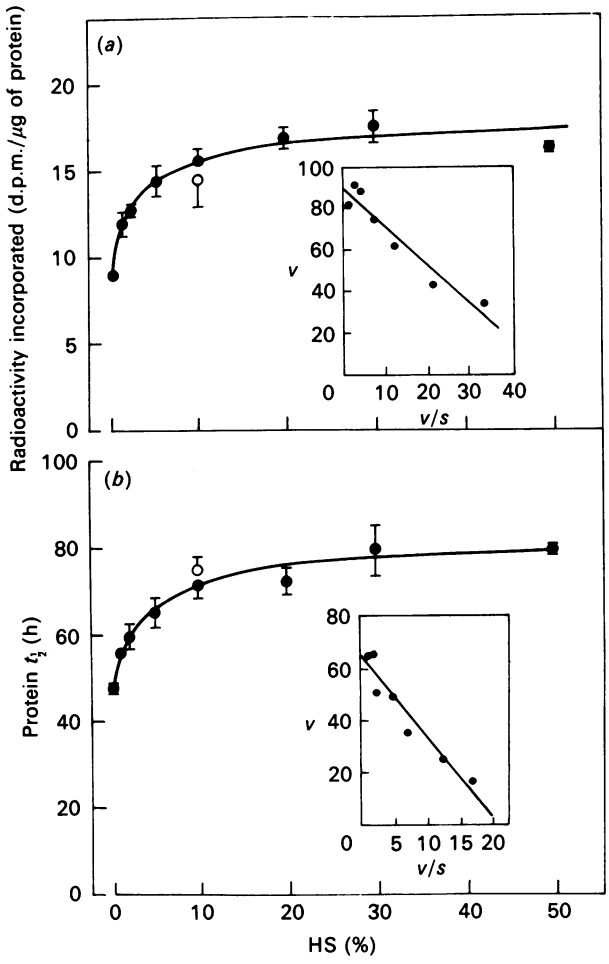

Fig. 4. Dose–response of protein synthesis and breakdown to horse serum

Myotubes were grown on 12-well dishes. The data are means ± S.D. ($n = 4$ for each group). (*a*) Protein synthesis. Myotubes were rinsed once in HBSS and incubated for 14 h in non-radioactive experimental medium consisting of DMEM with or without HS (●); ○, effect of 10% HS in DMEM diluted 1:1 with 0.9% NaCl/0.1% BSA (control for dilution of DMEM by HS at the higher concentrations). Inset, Eadie–Hofstee plot: v = % increase in synthesis rate above basal; s = % HS. (*b*) Protein degradation. Myotubes were incubated in degradation medium for 10.5 h. Inset, Eadie–Hofstee plot: v = % increase in half-life above basal. Other symbols are the same as in (*a*).

Table 2. Degradation of short- and long-lived proteins in the presence of serum

Short-lived proteins and long-lived proteins were radiolabelled for 1 h and 2 days respectively. For measurement of long-lived proteins, myotubes were chased overnight in the presence of serum. Myotubes were then incubated for 90 min in degradation medium containing no serum or 10% serum, and the medium was sampled at 30 min intervals (short-lived proteins) or after 90 min (long-lived proteins). Values represent means ± S.D. ($n = 5$ or 6): *$P < 0.001$ versus DMEM.

Addition to DMEM	$t_{\frac{1}{2}}$ (h)	
	Short-lived proteins	Long-lived proteins
None	6.2 ± 0.4	32 ± 2
FBS	6.3 ± 0.6	48 ± 2*
HS	6.0 ± 0.4	49 ± 1*

to the presence of HS (results not shown). For simplicity, we no longer supplement our medium with Medium 199 or CEE.

Effect of serum on degradation of short-lived proteins

Serum inhibits the breakdown of long-lived proteins in L8 myotubes, but does not alter the degradation of short-lived proteins as defined by our label/chase protocol (Table 2). Furthermore, the inability of anabolic agents to modify breakdown rates of short-lived proteins is not due to the short degradation period required for these measurements. Myotubes were radiolabelled for 2 days, chased overnight in the presence of serum to ensure removal of short-lived proteins, and exposed to serum withdrawal for only 90 min. Degradation of long-lived

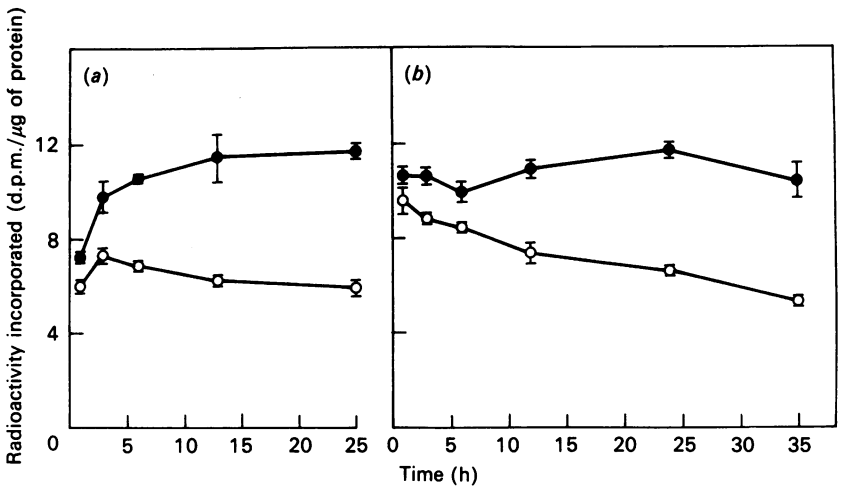

Fig. 5. Time course of protein synthesis in the presence and absence of serum

Myotubes were grown on 12-well dishes. The data are means ± S.D. for $n = 4$–6. (*a*) Serum-deprived cells. Myotubes were last exposed to fresh medium containing 10% FBS 5 days before preincubation. All wells were preincubated for 10.5 h with DMEM in the absence of serum. Monolayers were then incubated for increasing times in non-radioactive medium consisting of DMEM alone (○) or supplemented with 10% HS (●) before synthesis measurements. (*b*) Serum-supplemented cells. In a separate experiment, non-radioactive experimental medium was added to myotubes which had been exposed to fresh growth medium in the previous 2 days. Monolayers were incubated in non-radioactive experimental medium for various periods of time before protein-synthesis measurements. Rates in serum-supplemented medium are all significantly different from the corresponding time points incubated in the absence of serum [$P < 0.001$, except for 1 h time point in (*b*), where $P < 0.005$].

proteins was significantly enhanced in the absence of serum (Table 2).

Horse serum dose–response for stimulation of protein synthesis and inhibition of protein degradation

HS stimulates protein synthesis and inhibits breakdown of long-lived proteins in a dose-dependent fashion (Fig. 4). The data were replotted as the percentage change in [^{3}H]tyrosine incorporation or half-life in Eadie–Hofstee format; results are shown in the insets of Fig. 4. The effects of HS on both synthesis and degradation were half-maximal at 2%. The maximal stimulation of protein synthesis was 90%, and the increase in half-life of long lived proteins was 65%.

Time course of protein synthesis and protein breakdown in the presence and absence of serum

Myotubes were incubated for 5 days without medium changes, followed by overnight incubation in serum-free medium in order to depress protein-synthesis rates. Synthesis rates in serum-free medium remain constant over the following day (Fig. 5*a*). Re-addition of serum stimulates synthesis rates within the first 1 h, and the effect is maximal between 6 and 13 h (Fig. 5*a*). In contrast, when serum-supplemented cells are placed directly in medium with or without HS, synthesis rates in the presence of HS remain constant, whereas rates in serum-free medium decline continuously over a 35 h period (Fig. 5*b*). FBS regulates protein synthesis and degradation in a similar fashion to HS (results not shown).

The time course of regulation of long-lived-protein breakdown is illustrated in Fig. 6(*a*). Protein half-lives in serum-supplemented medium are approx. 50 h. Myotubes deprived of serum initially almost double their rates of protein breakdown, but the increased degradation gradually declines over the next 2 days. Eventually, breakdown rates in serum-deprived cells become comparable with those in serum-supplemented cells (Fig. 6*b*).

Comparison of predicted and measured changes in cellular protein content

We calculated expected changes in myotube protein content in the presence and absence of serum by using the formula:

$$\frac{dP}{dt} = K_s - K_d(P)$$

where P = protein content (μg), t = time (h), K_s = synthesis rate (μg of protein synthesized/h), and K_d = degradation rate (fraction of protein degraded/h).

We used data from experiments where changes in protein content and the time course of changes in protein synthesis and degradation were measured in serum-deprived and serum-supplemented medium. Half-lives in cells grown from the same myoblast precursors averaged 30 h ($K_d = 0.0231$) in the absence of serum and 50 h ($K_d = 0.0139$) in its presence. The contribution of short-lived proteins to the degradation rate was ignored, since they constitute only a minor fraction of the total cell protein. The following information is also needed for these calculations: (1) intracellular tyrosine specific radioactivity is equivalent to extracellular specific radioactivity (Table 1); (2) tyrosine comprises 2.7% of the total amino acid content in rat muscle protein on a molar basis [46]; (3) the average M_r of amino acid residues in muscle protein is 110 (calculated from the data of [46]). We also assumed that changes in synthesis and degradation rates were linear in the interval between samples. The calculated values for protein content agree well with the measured changes in protein content (Fig. 7).

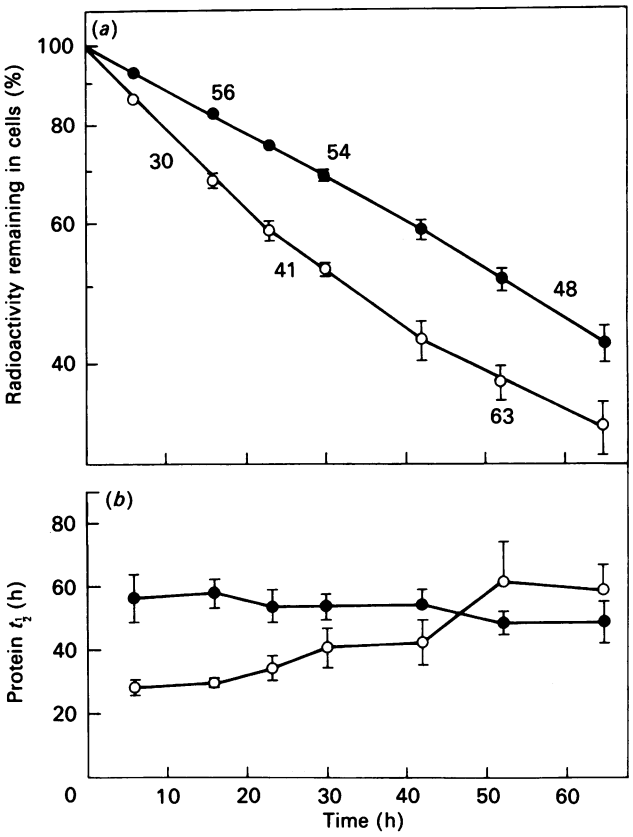

Fig. 6. Time course of long-lived-protein breakdown in the presence and absence of serum

Myotubes grown on 6-well dishes were radiolabelled for 2 days. Very-short-lived proteins were chased for 1.5 h before degradation measurements in medium supplemented with (●) or without (○) 10% HS. The data are means ± S.D. ($n = 6$). (*a*) Time course of protein breakdown. Half-lives calculated by linear regression were significantly different between adjacent segments in serum-deficient medium. These half-lives (h) are displayed next to the appropriate portion of the curve over which they were calculated (0–23 h, 23–42 h, 42–65 h). Values for serum-supplemented medium were calculated over the same segments. (*b*) Half-lives between adjacent time points calculated by linear regression are shown for comparison with Fig. 5.

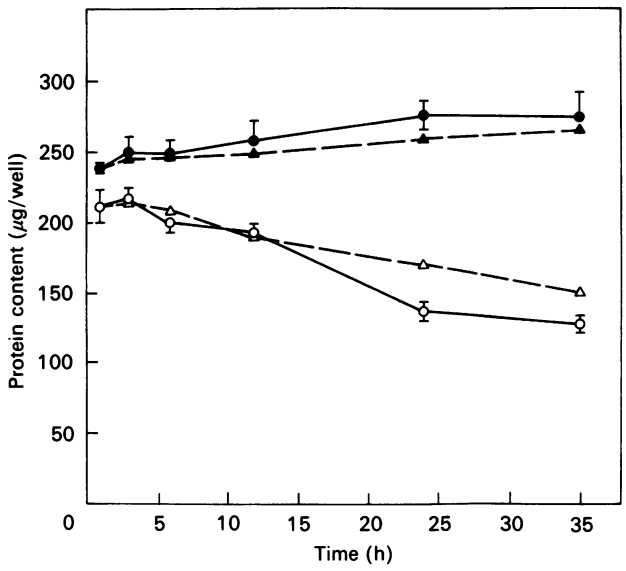

Fig. 7. Comparison of measured changes in protein content with values calculated from rates of protein synthesis and degradation

Protein content was calculated from measured rates of protein synthesis and degradation as described in the text. Experimental medium consisted of DMEM with (●, ▲) or without (○, △) HS: ●, ○, measured values of protein content; ▲, △, predicted values. The data are means ± S.D. ($n = 6$ for each measured value of protein content).

Effects of insulin, IGF-1 and IGF-2 on protein turnover

The response of L8 myotubes to insulin is shown in Fig. 8. Insulin stimulates protein synthesis and inhibits protein breakdown in a dose-dependent fashion. However, half-maximal effects require at least 0.1 μM-insulin, and maximal effects may not have been reached even with 10 μM-insulin. Although the inhibition by 1 nM-insulin in Fig. 8(*b*) was statistically significant ($P < 0.025$), the decrease was only 4%. In two other experiments 1–5 nM-insulin had no effect on protein degradation under conditions where HS, FBS and higher concentrations of insulin all were inhibitory. Highly purified pig insulin (which has the exact amino acid sequence of rat insulin) was indistinguishable from bovine insulin with respect to effects on protein synthesis and breakdown.

The requirement for supraphysiological concentrations of insulin is not likely to be due to rapid inactivation of insulin by the myotubes. Bacitracin, an inhibitor of insulin degradation [47], had no effect on protein breakdown when added to the culture medium at 1 mg/ml, and preincubation of the degradation medium with non-radioactive cells for 9 h did not decrease insulin activity as measured by its ability to inhibit protein degradation in L8 myotubes (results not shown).

hIGF-1 and rIGF-2 are more potent regulators of protein metabolism in L8 muscle cells than is insulin. This is particularly significant in light of the known levels of insulin [48] and IGFs [49] in fetal/neonatal animals. Table 3 shows that 50 ng of hIGF-1/ml (6 nM) was approximately as effective as 1 μM-insulin (Fig. 8) in stimulating protein synthesis and inhibiting protein degradation. rIGF-2 appears to be approximately one-tenth as active as hIGF-1 (Table 3), but the rIGF-2 was not completely purified [33]. Therefore the difference in biological activity is probably less than 10-fold. Table 3 also suggests that hIGF-1 and rIGF-2, when compared with 10% HS, may be somewhat more effective in the inhibition of protein degradation than in the stimulation of protein synthesis.

DISCUSSION

A major advantage of using cultured muscle cells to study the regulation of protein turnover is that experimental methods can be devised for accurate determination of both synthesis and degradation rates. Protein turnover has been characterized in some detail in L6 myoblasts [9,29], but independently derived cell lines and cells at different developmental stages may show differences in their ability to regulate protein synthesis and/or protein degradation ([50]; J. F. Dice, unpublished

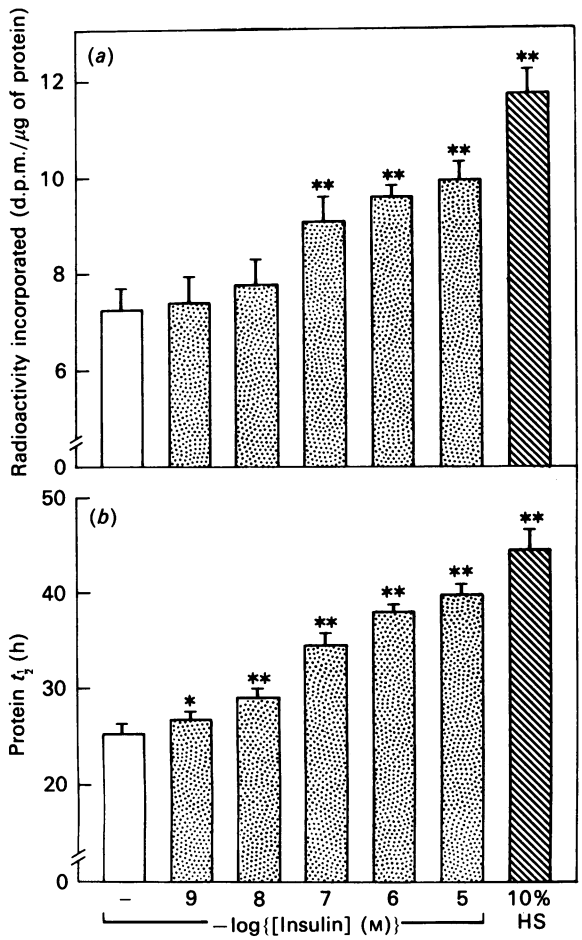

Fig. 8. Dose–response of protein synthesis and breakdown to insulin

Myotubes were grown on 12-well dishes. The data are means ± S.D. for $n = 5$–6 samples: $*P < 0.025$, $**P < 0.001$ versus no insulin. (*a*) Protein synthesis. Myotubes were incubated for 7 h in non-radioactive experimental medium consisting of DMEM + 2 mM non-radioactive tyrosine + 0.5 mg of RIA-grade BSA/ml supplemented with (▨) or without (□) insulin or 10% HS (▩). Protein synthesis was measured as described in the Materials and methods section. Note that the incubation with and without insulin was for 7 h (for experimental convenience), whereas most other experiments used 10–15 h incubations. The stimulation of protein synthesis by insulin may be greater with a longer incubation time (e.g. see Fig. 5), but dose–response relationships should not change. (*b*). Protein degradation. Very-short-lived proteins were chased for 1 h in DMEM + 10% FBS + 2 mM non-radioactive tyrosine, and protein degradation was monitored for 13.25 h.

Table 3. Effect of hIGF-1 and rIGF-2 on protein synthesis and degradation

Values represent means ± S.D. ($n = 6$); $*P < 0.001$ versus DMEM. For protein-synthesis measurements myotubes were incubated for 9 h in non-radioactive experimental medium consisting of DMEM + 2 mM-tyrosine + 0.5 mg of RIA-grade BSA/ml with or without hIGF-1, rIGF-2 or HS. Protein degradation was also monitored for 9 h. Abbreviation: nd, not determined.

Addition to DMEM	Protein synthesis (d.p.m./μg of protein)	Protein degradation ($t_{\frac{1}{2}}$, h)
None	6.2 ± 0.4	26 ± 2
hIGF-1 (50 ng/ml)	9.8 ± 0.8*	38 ± 2*
hIGF-1 (500 ng/ml)	10.6 ± 0.5*	45 ± 3*
rIGF-2 (50 ng/ml)	6.4 ± 0.3	28 ± 1
rIGF-2 (500 ng/ml)	9.2 ± 0.6*	36 ± 3*
rIGF-2 (4000 ng/ml)	n.d.	43 ± 2*
HS (10%)	13.0 ± 0.5*	46 ± 2*

work). Activities of proteolytic enzymes in L8 muscle cells have recently been reported [51], but rates of protein turnover have not.

We added 2 mM-tyrosine to the media during synthesis and degradation measurements in order to flood intracellular amino acid pools. This brings intracellular and extracellular tyrosine pools near equilibrium within 5–15 min for measurements of synthesis and inhibits most reutilization of radiolabelled tyrosine within 30 min for measurements of protein degradation. These results indicate that our measured rates of protein synthesis are unlikely to be in error by more than 10%. Measured rates of degradation for long-lived proteins are completely unaffected by isotope reutilization, since we incubate radiolabelled cells for 1–2 h in non-radioactive medium containing serum before beginning degradation measurements. However, degradation rates of short-lived proteins measured over 1–2 h (Table 2) may be underestimated by as much as 2-fold.

The correspondence between measured values of protein content and those predicted from our measurements of protein-synthesis and -degradation rates (Fig. 7) confirms that our methods are accurate. Recalculating predicted protein content by using K_s or K_d values that varied by more than about 15% from measured values gave clearly less satisfactory curves for predicted protein content.

Long-lived proteins appear to constitute the vast majority of cell protein in L8 myotubes, as in other cell types [52,53]. However, the fraction of radiolabel incorporated into short-lived proteins can be enhanced by applying a short radiolabelling period. The identity of the 2.5% of cellular proteins that are short-lived is not clear. Several individual proteins are known to be rapidly degraded [54], but this short-lived fraction undoubtedly also includes proteins that do not fold properly after synthesis and the maturation of secreted and organelle proteins (e.g. signal peptidase activity) [53].

The apparent single-exponential decay of long-lived proteins over a 6-day period (Fig. 3) was somewhat surprising, since a wide range of half-lives has been reported for individual proteins in cultured myotubes, e.g. 14 h for the acetylcholine receptor [55], 29 h for myosin light chain 1f [56], 75 h for the M-isoenzyme of creatine kinase [57], 142 h for actin [58] and 20–200 h for myosin heavy chain [59,60]. Our rather uniform half-life of 50 h is due in part to our label/chase protocol. The very long-lived proteins will not have been radiolabelled to equilibrium in 2 days. Nevertheless, our results imply that a large fraction of myotube proteins have a half-life near 50 h. Similar results have been reported in fibroblasts, but the half-life is slightly longer [44].

L8 myotubes show stimulated protein synthesis and

inhibited protein breakdown in response to serum, insulin and IGFs. The inhibition of protein breakdown is restricted to long-lived proteins as has been reported for a variety of other mammalian cells [10,38,52,53]. The concentrations of serum, insulin or IGFs required for half-maximal effects on synthesis and degradation are similar, and both synthesis and degradation can be regulated as much as 2-fold. An enhancement of protein synthesis coupled with a decrease in breakdown may account for the dramatic increase in fetal muscle protein content late in gestation [61].

Rates of protein synthesis and degradation and their alterations in response to serum, insulin and IGFs depend on the nutritional status of the cells before measurements. Cells preincubated overnight in serum-free medium and then placed in medium containing serum show a rapid increase in protein-synthesis rates, but maximal effects require 6–13 h (Fig. 5*a*). When cells previously maintained in the presence of serum are then incubated in its absence, a decrease in protein-synthesis rate is apparent within the first 1 h and continues to magnify over the next 35 h (Fig. 5*b*). Degradation experiments are analogous to the experiment in Fig. 5(*b*) in that the cells are radiolabelled in fresh medium containing FBS. Differences in degradation rates between cells subsequently maintained in the presence or absence of serum are maximal within the first few hours, and then gradually decline after 15–24 h (Fig. 6).

A similar limitation of enhanced proteolysis has been extensively characterized in human fibroblasts [44,62]. In these cells only 30 % of the protein can be degraded at an enhanced rate in response to serum withdrawal, and the enhanced degradation declines as these proteins are depleted from the cell [63]. Fibroblast proteins that are degraded more rapidly in response to serum depletion contain specific peptide sequences related to Lys-Phe-Glu-Arg-Gln, and their depletion can be quantified with antibodies raised against the pentapeptide [63]. Our preliminary results show that immunoreactive proteins also exist in L8 myotubes, and these proteins are preferentially degraded in response to serum withdrawal (H.-L. Chiang, E. A. Gulve & J. F. Dice, unpublished work).

L8 myotubes show accelerated protein synthesis and decreased protein breakdown only at supraphysiological concentrations of insulin (Fig. 8), since the plasma concentration of insulin in late-fetal/neonatal rats ranges from 0.3 to 2 nM [48]. Insulin-dependent regulation of protein turnover in cultured myoblasts [9,23,29,31] and myotubes [10,26–28] has been reported by a number of groups. In nearly all cases only supraphysiological doses of insulin were effective. The dose–responses of primary embryonic-chick breast myotubes [28] and L6 myoblasts [31] are similar to those of L8 myotubes in terms of magnitude as well as sensitivity. The only reports of insulin acting at physiological concentrations in cultured muscle cells are those by Airhart *et al.* [27], who reported that primary embryonic-chick leg myotubes increase protein-synthesis rates in response to physiological concentrations of insulin, and by Standaert *et al.* [64], who found in BC3H-1 myocytes that glucose and amino acid transport increased at physiological insulin concentrations. C2 mouse myotubes show inhibited breakdown to a small but significant extent at 1 nM-insulin and are about one order of magnitude more sensitive to insulin than are L8 myotubes (E. A. Gulve & J. F. Dice, unpublished work). Possible explanations for these differences in insulin sensitivity in different muscle cultures include the different stages of development at the time when primary cultures are prepared or cell lines established [65–67], different myoblast populations at a given embryonic age [65,67], or maturational events occurring *in vitro* [68,69]. For example, muscle insulin sensitivity varies in response to contractile activity [70]. In any case, the low sensitivity of protein turnover to insulin in cultured muscle cells is in contrast with that in adult muscle *in vivo* [5] and *in vitro* [71].

hIGF-1 and rIGF-2 appear to be more potent than insulin in regulating protein turnover in L8 myotubes (Table 3). IGF-1 and IGF-2 also regulate protein synthesis and protein degradation in L6 myoblasts [23,31], and IGF-2 has been shown to regulate protein turnover in primary chick myotubes [28]. In all cases examined, cultured muscle cells are more sensitive to IGF-1 than to IGF-2 ([23,31]; the present work). However, fetal serum contains more IGF-2 (1.8 μg/ml; 220 nM) than IGF-1 (0.28 μg/ml; 34 nM) [49], so both growth factors are likely to play a role in growth stimulation.

Finally, we wish to re-emphasize the relevance of these findings for the growth of fetal and neonatal skeletal muscle. The half-life of cellular protein in cultured myotubes is similar to that of fetal muscle, but is much less than that of adult muscle *in vivo* (32), and protein accumulation during fetal growth *in vivo* occurs by both increased protein synthesis and decreased protein degradation [61].

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