Inhibition of Intracellular Proteolysis in Muscle Cultures by Multiplication-stimulating Activity

COMPARISON OF EFFECTS OF MULTIPLICATION-STIMULATING ACTIVITY AND INSULIN ON PROTEOLYSIS, PROTEIN SYNTHESIS, AMINO ACID UPTAKE, AND SUGAR TRANSPORT

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The effects of the insulin-like growth factor, multiplication-stimulating activity (MSA), on chick myotube cultures were investigated. In serum-free media, MSA at levels reported to be present in fetal serum (5 ng/ml) significantly inhibited overall rates of protein degradation and stimulated protein synthesis and amino acid uptake. Half-maximal effects on protein degradation (−30%), synthesis (+25%), and amino acid uptake (+50%) occurred at approximately 0.05 µg/ml. In contrast, 10−2−10−3-fold higher concentrations (5 µg/ml) were required to stimulate transport of the glucose analog 2-deoxyglucose. The results indicate that MSA is an effective anabolic agent regulating protein metabolism and amino acid uptake, but not sugar transport in these cells. Parallel studies conducted with insulin demonstrated similar size effects on protein metabolism and amino acid uptake in serum-free media. However, unlike MSA, insulin levels (10−8 units/ml) well in excess of its normal physiological range were required to produce significant effects. In addition, the relative sensitivity of sugar transport with respect to protein metabolic effects differed for insulin and MSA. Thus, 2-deoxyglucose transport was approximately 10 times more sensitive to insulin than protein synthesis, proteolysis, or amino acid uptake in contrast to MSA where the reverse was true. However, despite the relatively higher sensitivity of sugar transport to insulin, supraphysiological levels (10−5 units/ml) of this hormone were still required for significant stimulation. These results suggest a generally low insulin sensitivity in cultured chick myotubes relative to adult tissues. In contrast, the effects of MSA are consistent with a possible role of this or similar factors in regulating growth and development of embryonic muscle.

Protein turnover occurs as an integral part of normal cellular metabolism, and cells possess highly regulated mechanisms for both protein synthesis and degradation (1, 2). States of cellular growth or atrophy result from imbalance between overall rates of synthesis and degradation, and it is evident that regulation of rates of protein degradation is potentially as important as synthesis in controlling growth in cells. Studies utilizing tissue culture have shown that removal of serum from the growth medium results in enhanced rates of overall protein degradation as well as decreased rates of protein synthesis (3–5). Various hormones, amino acids, and other nutrients have been tested with the aim of identifying the putative serum factors responsible for regulation of protein metabolism as well as other growth-related processes (2, 6–12). The most widely studied of these factors is insulin which is known to stimulate protein synthesis and inhibit proteolysis at or near physiological hormone levels in several adult tissues including liver (13), cardiac muscle (14), and skeletal muscle (15, 16). However, in tissue culture systems, the physiological significance of such insulin effects has sometimes been questioned since levels of hormones well above normal have been required (8, 10–12).

More recently, the role of insulin-like growth factors and somatomedins has been studied (17, 18). One family of polypeptides termed multiplication-stimulating activity, first described by Dulak and Temin (18, 20), possesses various growth-promoting properties. The major component of MSA1 is now believed to be the rat form of the somatomedin insulin-like growth factor-II (21). Studies have shown that MSA can stimulate fibroblast growth, sulfation incorporation into cartilage, and [3H]thymidine incorporation into DNA (22). Florini and colleagues (23–25) have shown that MSA is a potent and specific stimulator of muscle differentiation in culture. These observations, along with the finding that MSA is selectively elevated during fetal and neonatal life (26, 27) in rats, suggest that MSA is important during early growth and development of muscle. However, it is not known whether the growth-promoting effects of MSA on muscle involve regulation of proteolysis.

The present investigation has evaluated the sensitivity of protein degradation in differentiated chick myotube cultures to MSA and compared this sensitivity with other growth-related and metabolic processes. For comparative purposes, parallel experiments were carried out with insulin. The results indicate that at physiological levels MSA, but not insulin, has an anabolic effect on myotubes resulting from an inhibition of protein degradation and a simultaneous stimulation of protein synthesis and amino acid uptake.

**EXPERIMENTAL PROCEDURES**

Myotube Cultures—Primary cultures of chick embryo skeletal muscle were prepared by a modification of the method of Bullaro and

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1 The abbreviations used are: MSA, multiplication-stimulating activity; AIB, α-aminoisobutyric acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HBSS, Hanks' balanced salt solution.
Brookman (28). Freshly dissected leg muscle from 10–11-day embryos was minced finely, suspended in growth medium (5 ml), and transferred to a sterile capped test tube. The mince was then dissociated using a Vortex mixer (VWR Scientific Inc.) at full speed for 15 s (3×), and the resulting suspension was diluted with growth medium (10 ml). After standing for 10 min at room temperature, the upper two thirds were sucked off and filtered through Nitril filter aid. The suspension was further diluted with growth medium and plated on nongelatinized tissue culture dishes for 20 min at 37°C in an atmosphere of 5% CO2. The cell suspensions were then drawn off, counted on a hemocytometer, and plated on gelatin-coated dishes at 4060 Hanks' balanced salt solution:Waymouth's medium, 10% selected horse serum, 2% chick serum, 1% antibiotic mixture. The HCO3- concentration was adjusted to 25 mM.

**Measurement of Protein Degradation**—Rates of protein degradation were estimated from the release of acid-soluble radioactivity into the culture medium following labeling of endogenous proteins with [35S]methionine. Growth medium was withdrawn on Day 5 and replaced. Isotope (2 μCi/ml) was added, and the medium was filtered through a 0.2 μm filter and returned to the culture. Twenty-four hours later, the labeling medium was renewed, and the cultures were rinsed (5×) with ice-cold Hanks' balanced salt solution containing 2 mM l-methionine. In most experiments, the cultures were then incubated for 4 h in serum-free medium. During this interval, rapidly turning over proteins will be degraded, resulting in preferential labeling of the more stable proteins. Following this incubation, cells were transferred to experimental media (also containing 2 mM l-methionine) as indicated under "Results," and rates of degradation were determined as described below.

Aliquots (100 μl) of medium were taken and mixed with 100% trichloroacetic acid to give a final concentration of 10%. After standing on ice for 1 h, the samples were spun on a microfuge for 3 min. Aliquots of the supernatants were counted on a Packard Tri-Carb liquid scintillation counter. At the end of the experiment, the medium was analyzed for both total and acid-soluble radioactivity and cell-associated counts/min determined after sonication of the washed monolayers with SNU (0.2% sodium dodecyl sulfate, 2% Norocid P-40, 40 M urea). Rates of degradation are calculated by expressing the acid-soluble radioactivity as a percentage of the total radioactivity recovered from each plate (total counts/min in medium plus cell-associated counts/min) at the end of the experiment (30). Rates are expressed as per cent degradation/culture/unit of time.

**Measurement of Protein Synthesis**—Rates of incorporation of [3H]tyrosine were used to estimate rates of synthesis. Cultures (6 days) were incubated in serum-free medium for 4 h and then transferred to experimental media containing the various additions as indicated under "Results." After incubation for 1 h, the labeling media were aspirated and the cells rinsed (5×) with ice-cold HBSS containing 2 mM l-tyrosine. The cells were then extracted with SNU and analyzed for cell-associated radioactivity.

**Measurement of Amino Acid Uptake**—Cultures (6 days) were incubated in serum-free medium for 4 h and then transferred to preincubation medium containing AIB, 45 μM plus other additions as indicated under "Results." After 2.5 h, the cells were transferred to uptake media (preincubation media plus [3H]AIB, 2.0 μCi/ml) for 40 min at 37°C. Uptake was terminated by aspirating media and rinsing (5×) the cells rapidly in ice-cold HBSS containing 1 mM AIB. The cells were air-dried overnight and extracted with Lowry solution "C." Protein content of extracts was determined by using the Lowry method (31), and radioactivity was determined on Packard Tri-Carb liquid scintillation counter. Control experiments (data not shown) indicated that rates of uptake reached a steady state during the experimental interval. Rates of uptake are expressed as counts/min/mg of protein/40 min.

**Measurement of Sugar Transport**—Cultures (6 days) were incubated in serum-free medium for 4 h and then transferred to preincubation medium (HBSS, 25 mM HCO3 plus additions as indicated) for 1 h at 25°C. Uptake was measured in the same medium except that bicarbonate buffer was replaced with HEPES (10 mM, pH 7.3) and labeled ([3H]2-deoxy-d-glucose, 4 μCi/ml, 1 μM) was added. Cultures were incubated in this medium with constant agitation (25 rpm) for 60 s at 37°C. Media were then rapidly aspirated, and the cultures were washed by successively immersing the trays five times in ice-cold HBSS containing 2-deoxyglucose (1 μM). The entire washing period took less than 45 s. The cultures were then air-dried and analyzed as described for amino acid uptake. Control experiments (data not shown) indicated that uptake of 2-deoxyglucose was linear during the experimental interval. Rates of uptake are expressed at counts/min/mg of protein/min.

**Materials**—Regular bovine insulin (100 units/cc) (bovine/porcine) was a product of Eli Lilly Inc. The concentration of insulin in this product (6.9 × 10^{-4} M) was determined by the Lowry method using crystalline insulin (Sigma) as a standard. MSA was a generous gift of Dr. James A. Florini, Department of Biology, Syracuse University, Syracuse, NY. The preparation isolated from Buffalo rat liver cells in culture contained at least three components, all of which exhibited insulin-like activity. All tissue culture plasticware was obtained from Falcon except for six-well trays which were Costar. Horse and chick serum and Waymouth's medium and HBSS were from Gibco; l-[35S]methionine (specific activity 1100–1400 Ci/mmol) was from American Corp.; and L-[35S]cysteine (specific activity 813 mCi/mmol), α-amino[14C]isobutyric acid (specific activity 56 mCi/mmol) and [2-3H]deoxy-d-glucose (specific activity 3.73 mCi/mmol) were obtained from New England Nuclear.

**RESULTS**

**Serum Regulation of Protein Degradation**—As indicated in Fig. 1, over a 13-h period, in the presence of 10% horse serum (basal conditions), intracellular proteins were degraded at a steady rate of about 1.8%/h (t1/2 = 38 h), in excellent agreement with values reported by Libby and Goldberg (32). Similar cultures grown in serum-free medium (step-down conditions) exhibited increased rates of degradation approximately twice that seen in the presence of serum (Fig. 1). Increased degradation appeared to develop without a lag, was maintained for several hours, and then declined towards levels approaching those seen in the presence of serum after about 10 h. All subsequent measurements were carried out on cultures incubated for 4 h in step-down conditions.

**Regulation of Protein Metabolism and Sugar Transport by MSA**—To determine whether insulin-like growth factor activity can account for the inhibitory effect of serum on intracellular proteolysis, the ability of MSA to regulate this process was examined (Fig. 2A). MSA at concentrations as low as 5 ng/ml, well within the physiological range (23, 27), significantly inhibited the enhanced degradation of proteins seen in cultures incubated under step-down conditions (Fig. 2A). Increased levels of inhibition were observed with higher concentrations of MSA during the experimental interval. Rates of uptake are expressed as counts/min/mg of protein/40 min.
Effects of Multiplication-stimulating Activity on Muscle Cultures

muscle grown in NC medium, 15% horse serum, and consisting almost entirely of cross-striated, multinucleated myotubes were used for all experiments. Prior to the measurement of the various parameters, cultures were incubated (4 h) in serum-free NC medium. Similar results were obtained in at least three independent experiments. A, protein degradation. Cultures, labeled (24 h) with [35S]methionine (2.0 μCi/ml), were transferred to experimental media (NC medium plus L-methionine (2 mM) and MSA as indicated). Six h later, protein degradation was determined from the release of acid-soluble radioactivity as described under “Experimental Procedures.” MSA at 5 ng/ml or greater inhibited (p < 0.005) degradation. n = 6 for each point. B, protein synthesis. Cultures were transferred to experimental media containing [14C]tyrosine (2 μCi/ml) for 1 h. Cultures were washed, extracted with SNU, and incorporation determined as described under “Experimental Procedures.” MSA at all concentrations stimulated (p < 0.1 at 50 ng/ml and p < 0.005 for other concentrations) incorporation, n = 5 or 6 for each point. C, amino acid uptake. Cultures were transferred to NC medium plus AIB (45 μM) and MSA as indicated for 2.5 h. Uptake was measured in the same medium containing [14C]AIB (2 μCi/ml) for 40 min at 37°C. Cultures were rinsed, dried, extracted with Lowry C, and analyzed as described under “Experimental Procedures.” MSA at 5 ng/ml or greater stimulated (p < 0.005) uptake. n = 6 for each point. D, sugar transport. Cultures were transferred to HBSS plus MSA as indicated (1 h, 37°C). Transport was measured in the same medium plus [3H]2-deoxy-D-glucose (4 μCi/ml, 1 mM), HEPES (10 mM) pH 7.3 for 60 s at 37°C. Cultures were rinsed, dried, extracted with Lowry C, and analyzed as described under “Experimental Procedures.” MSA at 5 μg or greater stimulated (p < 0.01) transport. n = 6 for each point.
Effects of Multiplication-stimulating Activity on Muscle Cultures

Six-day-old cultures of chick embryo skeletal muscle, grown in NC medium, 15% horse serum, and consisting almost entirely of cross-striated, multinucleated myotubes were used for all experiments. Prior to the measurement of the various parameters, cultures were incubated (4 h) in serum-free NC medium. Similar results were obtained with at least four independent experiments using different insulin stocks. A, protein degradation. Cultures prelabeled (24 h) with [35S]methionine (20 μCi/ml) were transferred to experimental media (NC medium plus 1-methionine (2 mM) and insulin as indicated). Six h later, protein degradation was determined from the release of acid-soluble radioactivity as described under “Experimental Procedures.” Insulin at 10^{-2} units/ml or greater inhibited (p < 0.01) degradation. n = 6 for each point. B, protein synthesis. Cultures were transferred to experimental media containing [3H]tyrosine (2 μCi/ml) for 1 h. Cultures were washed and extracted with SNU, and incorporation was determined as described under “Experimental Procedures.” Insulin at 10^{-2} units/ml or greater stimulated (p < 0.01) incorporation. n = 5 for each point. C, amino acid uptake. Cultures were transferred to NC medium plus AIB (45 μM) and insulin as indicated for 2.5 h. Uptake was measured in the same medium containing [3H]AIB (2.0 μCi/ml) for 40 min at 37 °C. Cultures were rinsed, dried, extracted with Lowry C, and analyzed as described under “Experimental Procedures.” Insulin at 10^{-2} units/ml or greater stimulated (p < 0.01) uptake. n = 5 for each point. D, sugar transport. Cultures were transferred to HBSS plus insulin as indicated (1 h, 37 °C). Transport was measured in the same medium plus [3H]2-deoxy-D-glucose (4 μCi/ml, 1 mM), HEPEPS (10 mM) pH 7.3 for 60 s at 37 °C. Cultures were rinsed, dried, extracted with Lowry C, and analyzed as described under “Experimental Procedures.” Insulin at 10^{-2} units/ml or greater stimulated (p < 0.01) transport. n = 6 for each point.

In marked contrast to the situation with MSA where 2-deoxyglucose transport was found to be about 1 thousand-fold less sensitive relative to protein metabolism (Fig. 2), significant stimulation of transport of this glucose analog was observed with insulin concentrations of 10^{-2} units/ml (3 × 10^{-4} M) or greater (Fig. 3D), indicating a 10-fold increase in sensitivity compared to the other three processes studied. Furthermore, maximum stimulation of sugar transport by insulin was approximately twice that obtained with high levels of MSA. Thus, insulin and MSA differ considerably in their relative potency towards growth-related functions and glucose transport.

It is noteworthy that despite the relatively high sensitivity of glucose transport to insulin versus MSA in the present studies, stimulation of 2-deoxyglucose transport still required supraphysiological insulin levels (Fig. 3). Thus, our results indicate that chick myotubes are relatively insensitive to insulin in general, and this is not restricted to effects on growth. Control experiments were also carried out to determine whether the low sensitivity to insulin was due to loss of insulin as a result of nonspecific binding or degradation. Radioimmunoassay for insulin indicated that initial losses of hormone during preparation of media and transfer to tissue culture plates were less than 5%. In addition, loss of insulin activity during a typical experiment was evaluated. Media containing the lowest effective concentration of insulin (10^{-2} units/ml) were incubated in the presence of myotubes and then tested for a loss of ability to inhibit protein degradation (not shown). Incubation for periods up to 15 h did not significantly alter the inhibitory activity of medium containing insulin (10^{-2} units/ml), suggesting that nonspecific losses were not sufficient to account for the supraphysiological insulin levels required for effects.

**DISCUSSION**

The results of the present study clearly demonstrate that levels of MSA reported to be present in fetal rat serum (27) inhibit overall rates of protein degradation and simultaneously stimulate protein synthesis and amino acid uptake.

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The effects of insulin on protein degradation, protein synthesis, amino acid uptake, and sugar transport in cultured chick skeletal muscle. Six-day-old cultures of chick embryo skeletal muscle grown in NC medium, 15% horse serum, and consisting almost entirely of cross-striated, multinucleated myotubes were used for all experiments. Prior to the measurement of the various
Thus, MSA has a significant anabolic effect on myotube cultures in the absence of serum. Since the MSA preparation used in these studies contained several components, the potency of the active constituents may be even greater than we have estimated. In addition, it is noteworthy that the chick myotubes appear to be at least as sensitive to rat MSA as were rat muscle cell cultures (23–25). These results together with the previous reports of Florini and colleagues (23–25), demonstrating marked mitogenic action of this agent on proliferating myoblasts, suggest that MSA may have an important role in promoting growth in skeletal muscle, particularly during early development when circulating levels of MSA are selectively elevated (26, 27).

An interesting aspect of the present studies is that sugar transport was found to be approximately 1000 times less sensitive to MSA than protein synthesis, proteolysis, or amino acid uptake. These results clearly indicate a difference in the regulatory specificity of MSA versus insulin and are consistent with the suggestion by King et al. (33), using adipocytes and fibroblasts, that the physiological actions of MSA, mediated via its receptor, are restricted to the regulation of growth effects, but do not involve regulation of “metabolic” activities such as sugar transport. The effect on sugar transport seen only with very high concentrations of MSA may be due to weak cross-reactivity (33) with insulin receptors which function in regulation of glucose metabolism. Similarly, regulation of growth effects by high concentrations of insulin can be explained in terms of low affinity interactions with MSA or perhaps other insulin-like growth factor receptors. If this were true for the chick muscle cultures used here, then one would predict that (a) sugar transport should be responsive to physiological levels of insulin, and (b) sugar transport should exhibit greater sensitivity to insulin than the growth-related processes. Although 2-deoxyglucose transport was found to be more sensitive to insulin than were the other processes, the difference was much smaller than that found with MSA. In addition, despite this greater sensitivity, supra-physiological levels of insulin were still required to significantly stimulate 2-deoxyglucose transport. Thus, there is no clear evidence from these results to suggest that insulin selectively regulates glucose metabolism in cultured chick muscle. In this context, it is also worth noting that in a recent study (34) using affinity labeling of receptors, insulin regulation of both growth and metabolic processes was shown to be mediated via the insulin receptor. It, therefore, appears that functional distinction of insulin and insulin-like growth factors on the basis of the cellular processes which they regulate is not definitive and that in some cases both growth and metabolic events may be regulated via a single type of receptor.

Insulin-dependent regulation of intracellular proteolysis has been reported for a variety of cells in culture including hepatocytes (11), fibroblasts (10, 12), and a variety of mammalian cell lines (8). In most (8, 10–12) but not all (35) cases, supra-physiological levels of insulin were required for significant effects. Recently, Ballard and Francis (36) reported values for insulin sensitivity of protein degradation in the L6 myoblast cell line which are very similar to that obtained for chick muscle in the present studies. Furthermore, our studies have demonstrated clearly that low sensitivity to insulin is not specific to regulation of protein degradation, but is also seen in at least three other cellular processes. Thus, a generally low sensitivity to this hormone appears to be characteristic property of chick muscle and possibly other types of cells in culture. The reasons for this low sensitivity are not known, but control experiments indicate that it cannot be explained by loss of insulin through nonspecific binding or degradation. Since the insulin used in these studies was not derived from chicks, it might be thought that low sensitivity may be explained by species differences. However, this possibility seems highly unlikely since comparative effects of different insulin species have shown at most only very small (0–25%) differences in contrast to the 10–100-fold differences reported here. The possibility that the low sensitivity is due to the absence of insulin receptors would appear to be ruled out by the studies of Candia and Przybyski (37) who demonstrated specific high affinity binding of [125I]-insulin to cultures of chick myotubes. Pollet et al. (38) have reported similar findings for the nonfusing mouse muscle line B3CH-1. However, Ballard et al. (8) found that even cells which exhibit high levels of specific insulin binding can also exhibit low sensitivity to the hormone. Thus, the presence of receptors may not be by itself sufficient to confer high or at least physiological sensitivity. It is possible that in those cases where low sensitivity is observed, a fraction of the receptors are physiologically inactive. In this context, it has been shown that myoblasts, which also have insulin receptors, are insensitive to even pharmacological doses of insulin (37). On the other hand, sensitivity to insulin may be of greater importance in the control of protein metabolism and growth in the adult tissues. Increases in insulin receptor number or affinity may be genetically programmed or modulated by exogenous factors. For example, increased physical activity is known to enhance insulin sensitivity of glucose metabolism in muscle, especially in insulin-dependent diabetes (41). It would, therefore, be of interest to determine whether muscle stretch, electrical stimulation, innervation, or medium conditioning alter cultured myotube sensitivity. In this context, a recent study by Airhart et al. (35) claimed that protein synthesis in cultured myotubes was sensitive to physiological levels (0.8 nM) of insulin. However, as noted by these workers, the effects of insulin at low doses were variable, and in some experiments, stimulation of synthesis was not observed. The source of this variability was not identified, but it was suggested that it may have been attributable to factors in the medium, e.g. cysteine or length of exposure to insulin. Therefore, it seems possible that under certain, as yet undefined, conditions myotubes in culture may be induced to develop insulin sensitivity similar to that seen in the adult tissues.

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Effects of Multiplication-stimulating Activity on Muscle Cultures

Inhibition of intracellular proteolysis in muscle cultures by multiplication-stimulating activity. Comparison of effects of multiplication-stimulating activity and insulin on proteolysis, protein synthesis, amino acid uptake, and sugar transport.

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