Insulin and Insulin-Like Growth Factor-1
Induce Pronounced Hypertrophy of Skeletal
Myofibers in Tissue Culture

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Running Title: Insulin and IGF-1 Induced Myofiber Hypertrophy

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ABSTRACT

Skeletal myofibers differentiated from primary avian myoblasts in tissue culture can be maintained in positive nitrogen balance in a serum-free medium for at least 6-7 days when embedded in a three dimensional collagen gel matrix. The myofibers are metabolically sensitive to physiological concentrations of insulin (10^{-11} to 10^{-10} M) but these concentrations do not stimulate cell growth. Higher insulin concentrations (5 x 10^{-9} to 10^{-8} M) stimulate both cell hyperplasia and myofiber hypertrophy. Cell growth results from a long term 42% increase in total protein synthesis (P < .001) and a 38% decrease in protein degradation (P < .001). Myofiber diameters increase by 71% to 98% after 6-7 days in insulin-containing medium (P< .001). Insulin-like growth factor-1 but not insulin-like growth factor-2, at 250 ng/ml, is as effective as insulin in stimulating cell hyperplasia and myofiber hypertrophy. This model system provides a new method for studying the long-term anabolic effects of insulin and insulin-like growth factors on myofiber hypertrophy under defined tissue culture conditions.

Index Terms: Insulin; Insulin-like Growth Factors; Skeletal Muscle; Hypertrophy; Hyperplasia; Protein Synthesis; Protein Degradation.
INTRODUCTION

Insulin is a potent anabolic growth factor for skeletal muscle. Although this polypeptide hormone has been extensively studied for many years, the molecular events coupling insulin receptor binding to myofiber growth are still unclear (18). Experiments involving insulin-induced muscle growth in vivo are complicated by the nature of whole animal studies. Organ culture muscle studies are limited to short time periods during which the tissue is in negative nitrogen balance (2). Tissue cultured skeletal muscle cells have been extensively used to study the effects of insulin and insulin-like growth factors on myogenic cell proliferation and differentiation (8,9). Studies of the effects of these growth factors on subsequent myofiber growth in tissue culture have been limited by the inability to maintain differentiated myofibers for extended periods of time in serum free, defined media (1,10,13). We recently reported a new protocol for maintaining differentiated, highly contractile avian myofibers under tension in serum-free medium for up to 14 days (27). This method involves embedding the newly developed myotubes in a three dimensional collagen gel matrix attached to a stainless steel support structure. These cells differentiate into well-striated, neonatal-like myofibers. We now describe a muscle maintenance medium in which insulin and insulin-like growth factor-1 induce rapid and pronounced skeletal myofiber hypertrophy over a period of days. This system provides a new method for studying the long term anabolic effects of insulin-like growth factors on skeletal muscle hypertrophy under defined culture conditions. In addition,
it will allow the in vitro analysis of the complex interactions of growth factors and muscle mechanical activity which synergistically regulate skeletal muscle growth both in vivo (7,15) and in vitro (24,26). Parts of this work have appeared in abstract form (25).
MATERIALS AND METHODS

Cell Cultures

Avian myoblasts are isolated from 11 to 12 days in ovo pectoralis muscle by standard dissection techniques (27). Fertilized chicken eggs are obtained from Beaver River Farm (West Kingston, RI). The cells are plated at a very high density of 5,700 cells/mm² in 1 ml of growth medium and maintained in a humidified 5.0% CO₂ incubator at 37°C. Growth medium is Eagle's basal medium containing 50 U/ml penicillin (Sigma Chemical, St. Louis, MO), 10% horse serum (Hyclone Laboratories, Logan, UT), and 5% chicken embryo extract (85/10/5 medium). Cells are plated and grown in collagen-coated 15 mm diameter tissue culture dishes (Nunc Corp., Roskilde, Denmark). At 48-54 h postplating, the cells are embedded in a collagen gel matrix (27). Briefly, the culture medium is removed and 0.5 ml of ice-cold collagen solution is added. The collagen solution is prepared immediately before use by mixing 85/10/5 medium with rat tail collagen (Type I, Collaborative Research, Bedford, MA) to a final concentration of 200-400 µg/0.5 ml. Sterile sodium hydroxide (0.1N) is added to the chilled solution to maintain a pH of 7.0. Stainless steel wire cloth (mesh no. 50, Newark Wire Cloth, Newark, NJ) is placed inside the perimeter of each well immediately after the collagen gel solution is added to the wells but before polymerization. The screen acts as an "artificial tendon" by providing a surface to which the differentiating cells and collagen gel can attach and be held under tension during long-term growth in defined medium. Culture medium (85/10/5) is added to the top of the hardened collagen gel 24 h after solidifying and changed every 24 h thereafter. After several
days, the collagen gel dehydrates, collapsing on top of the muscle cells as a thin 12-50 \( \mu m \) layer (27) in the same manner as described for other cell types (3). Under these growth conditions, the myogenic cells proliferate and fuse during the first 24 to 72 h in vitro, become striated and highly contractile by 96 to 120 h, and remain so for the duration of the experiments. Day 6 to Day 8 postplating cultures contain 30-40\% of their nuclei in mononucleated cells, the majority of which are fibroblasts. Eighty-five to ninety percent of the noncollagenous protein in the cultures is located in the much larger multinucleated skeletal myofibers based on morphometric analysis of cell volumes (26).

On Day 6-8 postplating, the cultures are rinsed over a 2 h period with Eagle’s basal medium containing 50 U/ml penicillin (1 \( \text{ml/well}, 6 \times 15 \text{ min changes} \)) on a rotary shaker (60 rpm) at 37\(^\circ\)C. Serum-free muscle maintenance medium (mm medium) is added to the cultures (500 \( \mu l/well \)) containing varying concentrations of bovine insulin (Sigma Chemical Co. cat. no. I1882, 24.5 IU/mg protein), insulin-like growth factor-1 (Collaborative Research hIGF-1, Bedford MA), or insulin-like growth factor-2 (MSA, Collaborative Research). MM medium is Eagle’s basal medium containing 1,250 mg/l bovine serum albumin (Sigma Chemical Co. cat. no. A4161), 0.05 mg/l sodium selenite (Sigma Chemical Co. cat. no. S0882), 0.835 mg/l ferrous sulfate (Sigma Chemical Co. cat. no. F8633), 5.0 mg/l linoleic acid (Sigma Chemical Co. cat. no. L1012), and 50 IU/ml penicillin. All reagents are cell culture tested by Sigma. The medium is changed every 24 h for the duration of each experiment.
Biochemical Assays

Glucose utilization by the cells is measured as the disappearance of glucose from the culture medium. One ml fresh mm medium with or without growth factors is added to each culture well and changed at 4-6 h intervals. The cultures are maintained in a 37°C CO₂ incubator on a rotary shaker (60 rpm) during the incubation. The glucose level in the medium is measured with a YSI Glucose/Lactate Analyzer, Model 2000. The reduction in glucose during the 4-6 h incubation is calculated from the difference in medium glucose content relative to medium incubated at 37°C without cells.

Protein synthesis is measured as L-[U-¹⁴C]-phenylalanine (Amersham, Arlington Heights, IL) incorporated into trichloroacetic acid-insoluble material during a 4 h incubation period in the presence of 0.6 mM nonradioactive phenylalanine as previously outlined (26). Incorporation of ¹⁴C-phenylalanine is linear during this time period (26). Results are expressed as DPMs incorporated per ug total noncollagenous protein. This assay method does not give absolute rates of protein synthesis since the specific radioactivity of the charged-tRNA precursor pool is not directly measured. But the method gives accurate relative rates of protein synthesis since the specific radioactivity of charged-tRNA pools in cultured muscle cells are not altered by insulin or insulin-like growth factors (1,10). Aliquots from sonicated samples are also analyzed for total noncollagenous protein, and total DNA content by previously described procedures (26).
Protein degradation rates of long lived proteins are measured by an intermittent perfusion technique. Its validation as an accurate measure of total protein degradation in tissue cultured skeletal muscle cells has been described previously (28). Briefly, cellular proteins are labelled by incubation of the cells in medium containing 0.5 to 1.0 \( \mu \)Ci/ml L-[U-\(^{14}\)C] phenylalanine for 20-24 h at 37°C. The cells are rinsed with Eagle's basal medium containing 25 mM Hepes (pH 7.4) and 1.0 mM nonradioactive phenylalanine. The cultures are shaken for 2 h at 37°C on a rotary shaker (60 rpm) and the rinse medium changed 3 times to remove unincorporated radioactivity. One ml of fresh medium is added and rotary shaking of the culture dishes is continued at 37°C during the entire assay period. The efflux of trichloroacetic acid soluble radioactivity into the medium is followed for 1 to 32 h. Calculation of the percent radioactivity remaining in protein at each time point and protein half-lives are performed as previously described (28). Excess nonradioactive phenylalanine (1.0 mM) in the medium during the degradation assay period prevents significant reutilization of \(^{14}\)C-phenylalanine (28). Comparison of protein degradation rates obtained with this intermittent perfusion technique to one using a nonreutilizable amino acid (2-\(^{3}\)H-phenylalanine) indicates that reutilization of radioactive phenylalanine released from prelabeled proteins is not a complicating factor in the measurement of protein degradation rates in cultured muscle cells (29).

**Morphometry**

To visualize the myofibers, the cells are stained by an enzyme immunocytochemical technique. A monoclonal antibody specific for
avian embryonic fast-myosin heavy chain (EB165) is used (4) together with an avidin-biotinylated secondary antibody reaction coupled to horseradish peroxidase (Vectastain ABC, Vector Laboratories, Burlingame, CA). The stained cells are embedded in Spurr embedding medium (Polysciences, King of Prussia, PA) and observed as whole mounts or thin sections with a light microscope (x10 to x100 oil immersion objectives, total magnification x125 to x1250). Microscopic fields are observed using a Zeiss microscope equipped with a drawing tube attachment focused onto a Numonics 2210 digitizing tablet that is connected to a Compaq Deskpro 286 computer. The stained cells and tablet mouse can be seen while looking through the microscope eyepieces. Morphological measurements are made using morphometry software (SIGMA SCAN, Jandel Scientific, Sausalito, CA) and all data are computer corrected for magnification. Mean myofiber diameters are measured on randomly selected whole mount and thin section fields. In whole mounts, the length and area of each myofiber in the field are measured and the mean diameter calculated. In thin sections, the diameter of each myotube in the field is measured 3-4 times at different points around the cell's circumference, and the minimal value used as the cell's diameter. As outlined previously, both methods give similar results (26).

Statistical analyses of the data are performed by t-tests for paired and unpaired values (PC Statistician Software, Human Systems Dynamics, Northridge, CA).
RESULTS

Primary avian muscle cells cultured for 7-14 days embedded in a three dimensional collagen gel matrix are highly contractile and morphologically well differentiated with several characteristics of neonatal myofibers, including a well developed external lamina layer and well organized myofibrils (27). Sixty to eighty percent of these myofibers stain positive for the neonatal isoform of myosin heavy chain. The collagen embedded myofibers are structurally stronger than non-collagen embedded myofibers, based on their ability to withstand repetitive mechanical stimulation for several days without rupturing (26). The myofibers can be maintained for several weeks under tension in unsupplemented medium (27). Myofiber cell densities remain constant under these culture conditions.

Addition of physiological concentrations of insulin (10^{-14} to 10^{-12} M) to the Day 7-Day 8 cultures in mm medium stimulates glucose utilization by 42-54% during the first 4 h after its addition (Figure 1, Insert). Glucose utilization returns to control levels after this time period in 10^{-11} to 10^{-12} M (data not shown). Increasing the insulin concentration to 10^{-11} M or greater produces a longer term increase in glucose utilization which can be measured over a 24 h period. Insulin concentration of approximately 10^{-10} M produces a secondary increase in glucose utilization and this increase is also maintained for at least 24 h (Figure 1, Main Graph).

Supraphysiological insulin concentrations (10^{-9} to 10^{-6} M) also stimulate noncollagenous protein and DNA accumulation in the
cultures after two to three days of incubation (Figure 2), indicating an anabolic response of the cells to high insulin concentrations. Physiological concentrations of insulin are ineffective in stimulation of either protein or DNA accumulation in the cultures (Figure 2). Similar results were obtained in two additional experiments (data not shown). The cells' anabolic response to high insulin concentrations (based on total noncollagenous protein accumulation) occurs in muscle cultures 7 to 10 days postplating, and the response does not increase in older cultures (Table 1). Insulin stimulation of cell proliferation is significantly greater 7 to 10 days postplating than 13 to 16 days postplating, when control culture DNA content is almost twice that of Day 7 to 10 cultures (Table 1).

Cultures treated with high insulin concentrations for 5 days have larger diameter myotubes than myotubes in unsupplemented medium (Figure 3). It is evident from Figure 3C that cells incubated for 5 days in unsupplemented mm medium are structurally well maintained. Many of the myotubes increase to 20 to 40 μm in diameter after 5 to 7 days in the presence of insulin. Mean myofiber diameter increases 98% (P < .001) with 6-7 days of incubation in 10⁻⁸ M insulin (Day 7 to Day 14 postplating) and 78% (P < .001) for Day 14 to Day 19 postplating cultures (Figure 4). Myofibers diameters are 20-38% (P < .01) greater in insulin containing mm medium than in cultures supplemented with 10% serum and 5% embryo extract during this time period (Figure 4). Insulin at high levels thus induces rapid and pronounced skeletal myofiber hypertrophy in defined mm medium.
From 1 to 6 days of incubation of Day 7 to 8 postplating muscle cultures in mm medium without insulin there is a small, nonsignificant 9.5% decrease in protein synthesis rates and a significant, 21% (P < .001) increase in protein degradation rates compared to cultures incubated in complete 85/10/5 medium (data not shown). Insulin at 10⁻⁶ to 10⁻⁵ M stimulates protein synthesis rates by 22% to 41% (P < .001) after either 3 days (Figure 5A) or 6 days of incubation (Figure 5B). Insulin at 10⁻⁶ M concentration reduces total protein degradation rates by 36-39% (P < .001) after either 1 day (Figure 6A) or 6 to 7 days of incubation (Figure 6B), based on calculation of mean protein half lives from the slopes of the degradation rates shown in Figure 6. During the first day of incubation in mm medium with insulin, protein degradation is reduced to a level similar to that seen in 85/10/5 medium (Figure 6A). Insulin is less effective than 85/10/5 in reducing protein degradation over 6 days of incubation (Figure 6B). Insulin thus stimulates cell growth by a long term stimulation of protein synthesis rates and inhibition of protein degradation rates.

Insulin-like growth factor-1 (IGF-1) and insulin-like growth factor-2 (multiplication stimulating activity, IGF-2) also stimulate noncollagenous protein and DNA accumulation in skeletal muscle cultures after 3 days of incubation in mm medium (Figure 7). IGF-1 is a more potent stimulator of cell growth than IGF-2, significantly stimulating noncollagenous protein accumulation at a 10-fold lower concentration than IGF-2 and to a 2.2 greater extent at the highest concentration tested - 5 x 10⁻⁷ g/ml (Figure 7A). IGF-1 significantly stimulates DNA accumulation at a 50 x
lower concentration than IGF-2 and to a 1.4 x greater extent than IGF-2 at 5 x 10^-7 g/ml (Figure 7B).

Microscopic analysis of Day 7 to 12 postplating cultures indicates that significant myofiber hypertrophy occurs after 5 days in the presence of 5 x 10^-7 g/ml IGF-1 but not IGF-2 (Figure 8). Morphometric measurement of the mean myofiber diameter confirms this conclusion (Figure 9). IGF-1 stimulates mean myofiber diameter by 79% (P < .001) after 5 days of incubation and is as effective in stimulating myofiber hypertrophy as high concentrations of insulin (60%, P < .001).

IGF-1-induced muscle growth results from long term stimulation of protein synthesis rates (Figure 10) and inhibition of protein degradation rates (Figure 11). IGF-2 at high concentrations also stimulates protein synthesis rates (Figure 10) and inhibits protein degradation rates (Figure 11) but is much less potent than IGF-1. IGF-1 at 10^-7 g/ml stimulates protein synthesis by 62% (P < .01) and IGF-2 is 2.6 x less effective (24% increase, P < .03). IGF-1 or IGF-2 at 2.5 x 10^-7 g/ml reduces total protein degradation rates by 28% (P < .001) and 9% (P < .02), respectively, after 3 days of incubation (Figure 11) based on calculation of mean protein half lives from the slopes of the degradation curves in Figure 11. IGF-1 is thus 3.2 x more effective than IGF-2 in inhibiting protein degradation. IGF-1 is less effective than 85/10/5 in inhibiting protein degradation during longer term incubations, as is the case with insulin inhibition of protein degradation (Figure 6B).
DISCUSSION

The major finding in this study is that insulin and IGF-1 can induce rapid and pronounced skeletal myofiber hypertrophy under the defined conditions of tissue culture. This ability to induce hypertrophy in vitro results from: 1) the development of a new culture system whereby highly contractile, differentiated myofibers can be maintained under tension in unsupplemented medium for extended periods of time; and 2) the development of a serum-free muscle maintenance medium which contains all the necessary components for growth factor-induced hypertrophy. Prior to the developments described in this paper, the analysis of insulin and insulin-like growth factor-induced myofiber hypertrophy was confined to in vivo studies (14,20). Extensive studies of the anabolic effects of these growth factors on protein turnover have been performed with organ-cultured skeletal muscle, but even under the best of conditions (2), these tissues are in negative nitrogen balance and hypertrophy does not occur. Tissue cultured skeletal muscle cells have been used to study the short term protein turnover effects of these growth factors (8,9,10) but the culture conditions could not sustain long term cell growth leading to myofiber hypertrophy. These short term studies found that insulin and IGF-1 stimulate protein synthesis (1,10,17) and inhibit protein degradation (10,13,17). Similar results occurred in the present study, but these alterations in protein turnover are maintained for 5-7 days and result in pronounced myofiber hypertrophy. The new model system described in this paper makes it possible to study all of the multi-faceted steps necessary for myofiber hypertrophy
under defined tissue culture conditions.

The hypertrophy response to either insulin or IGF-1 in tissue culture requires high doses of the growth factors. IGF-2, even at high concentrations, does not stimulate hypertrophy after 5 days of incubation, even though protein synthesis increases and protein degradation decreases, as reported previously (10,13). Longer exposure times in IGF-2 may be necessary to stimulate hypertrophy. Similar anabolic effects of insulin on tissue cultured skeletal muscle at supraphysiological levels have been reported by others (8,11). Numerous possibilities exist for why high concentrations of insulin are required for growth in vitro. The cells are metabolically sensitive to physiological insulin concentrations (Figure 1) during the first hours of exposure, but become insensitive to these levels after 3-4 h of incubation (see RESULTS). A similar diminished response of cultured myotubes to insulin stimulated protein synthesis with time has been reported (1) and may result from the development of insulin resistance in the cultured cells (6). High insulin concentrations may overcome this resistance. Insulin and IGF-1 stimulate the synthesis and secretion of insulin-like growth factor binding proteins in cultured muscle cells (16) and these proteins may bind and partially inactivate the anabolic effects of the growth factors (12). High levels of insulin and IGF-1 may be necessary to overcome this partial inactivation. Other mechanisms may also exist for inactivation and/or degradation of the growth factors during the long time periods of the experiments performed in the present study. A third possible reason for the high doses of growth factors
needed to stimulate myofiber hypertrophy is the recent finding that growth factors interact synergistically in stimulating cell growth. Thus, anabolic steroids potentiate the response of cultured cells, including skeletal muscle, to IGF-1 (5,22). By combining several different defined growth factors in mm medium, myofiber hypertrophy might occur at lower doses of insulin and insulin-like growth factors. A fourth possibility is that since insulin and insulin-like growth factors cross react in cultured skeletal muscle with each other’s membrane receptors with varying affinities (21), high doses of insulin and IGF-1 may be required to induce myofiber hypertrophy because the growth responses are transduced via receptors of low affinity. Finally, muscle use can dramatically alter the sensitivity of skeletal muscle to insulin in vivo (7), and mechanical stimulation of cultured muscle cells may increase their growth response to more physiological levels of growth factors (23).

The cultures utilized for the present study contain a mixed population of postmitotic, multinucleated myofibers and proliferating, mononucleated cells made up of myoblasts and fibroblasts. Insulin and IGF-1 stimulate both myofiber hypertrophy and cell proliferation in the mixed cultures, as would be expected from their known effects on skeletal muscle growth in vivo (7,15) and in vitro (8,9). The proportion of growth factor induced changes in protein synthesis and degradation rates in these different cell populations in the present study are not known; serum alters both processes to a similar extent in myofibers and fibroblasts (28,29). Insulin and IGF-1 stimulation of hyperplasia
may be important in this model system for myofiber growth. Neonatal to adult myofiber hypertrophy in vivo requires the continued proliferation and fusion of myogenic cells to already formed myofibers (19). In vitro the growth factors may stimulate myofiber hypertrophy by activating myoblasts to proliferate and fuse into existing myofibers, or they may act directly on the postmitotic muscle fibers to stimulate their growth. Experiments are in progress to distinguish between these possibilities.

In summary, cultured skeletal myotubes can be induced by insulin and insulin-like growth factor-1 to undergo rapid and pronounced hypertrophy under defined culture conditions. This new model system may assist in studying the second messengers involved in the hypertrophy response to growth factors and to mechanical stimulation in vitro.
FOOTNOTES

"Vandenburgh et al. In Preparation.

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REFERENCES


TABLE 1
Insulin's Growth Stimulating Activity at Different Culture Ages

<table>
<thead>
<tr>
<th>Culture Age (Days)</th>
<th>Insulin (10⁻⁴ M)</th>
<th>Total Noncollagenous Protein (µg/well) ± SE</th>
<th>%</th>
<th>Total DNA (µg/well) ± SE</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-10</td>
<td>-</td>
<td>259.3 ± 8.6</td>
<td>8.6 ± 0.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>339.8 ± 9.1'</td>
<td>31%</td>
<td>11.3 ± 0.5''</td>
<td>31%</td>
</tr>
<tr>
<td>10-13</td>
<td>-</td>
<td>313.0 ± 5.7</td>
<td>12.5 ± 0.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>397.0 ± 5.3'</td>
<td>27%</td>
<td>12.9 ± 0.6</td>
<td>3%</td>
</tr>
<tr>
<td>13-16</td>
<td>-</td>
<td>353.3 ± 2.7</td>
<td>15.0 ± 0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>433.8 ± 6.2'</td>
<td>23%</td>
<td>17.8 ± 0.5''</td>
<td>19%</td>
</tr>
</tbody>
</table>

Cultures are grown for 7-13 days in complete medium and extensively rinsed as outlined in MATERIALS AND METHODS before incubation for 3 days in mm medium ± insulin. Cultures are fed fresh medium every 24 h. Total noncollagenous protein and DNA are measured as outlined in MATERIALS AND METHODS. Each value is the mean ± SE of 4 samples and statistical analyses are by t-tests for unpaired values. *P < .001  **P < .005
FIGURE LEGENDS

Figure 1. Neonatal myofibers are metabolically sensitive to insulin. Myofibers grown for 8 days in complete medium (10% serum, 5% embryo extract), are rinsed extensively and incubated in a serum free muscle maintenance medium (mm medium) with varying concentrations of insulin for 4 h (insert) or 24 h. Glucose utilization is measured after 4 h (insert) or at 6 h intervals over a 24 h period (Main Graph) as outlined in MATERIALS AND METHODS. Each point is the mean ± SE of 4 (insert) or 16 (main graph) samples. For the 24 h incubation period, glucose utilization rates do not change significantly from the first 6 h interval to the final 6 h interval (data not shown). The data is therefore combined for the 4 time intervals. Statistical analyses are by t-tests for unpaired values.

Figure 2. High concentrations of insulin stimulate cell growth. Muscle cultures are grown as outlined in the legend to Figure 1 and switched to varying insulin concentrations in mm medium on Day 7 postplating. Medium is changed every 24 h. After three days of incubation total noncollagenous protein (A) and total DNA (B) content are measured as outlined in MATERIALS AND METHODS. Each point is the mean ± SE of 4 samples and statistical analyses are by t-tests for either unpaired values (A), or paired values for samples from $10^{-6}$ to $10^{-4}$ M insulin (B).

Figure 3. High insulin concentrations stimulate myofiber growth. Myofibers are grown for five days (Day 7 to Day 12 postplating) in
mm medium containing ± 10⁻⁷ M insulin. Medium is changed every 24 h. The cultures are fixed and immunocytochemically stained with a monoclonal antibody (EB165) against fast embryonic avian myosin heavy chain. Day 12 cultures in the absence (A,C) or presence (B,D) of 10⁻⁷ M insulin. At the higher magnification (C,D), the myofibers are seen to be well striated. Striations are more difficult to resolve in the cells grown in 10⁻⁵ M insulin because of their greater thickness. Bar equals 25 μm in A,B and 15 μm in C,D.

Figure 4. Insulin induces myofiber hypertrophy. Mean minimal myofiber cross sectional diameters are measured in thin sections as outlined in MATERIALS AND METHODS on cultures treated for 5 to 7 days in mm medium with or without 10⁻⁴ M insulin starting at Day 7 (left) or Day 14 (right) postplating. Each bar represents 28-30 measurements ± SE in randomly chosen thin sections made by three independent observers and statistical analyses of the combined data are by t-tests for unpaired samples of no insulin versus 10⁻⁴ M insulin.

Figure 5. Insulin stimulates long-term protein synthesis rates. Day 7 to 8 muscle cultures are grown as outlined in the legend to Figure 1 and switched to mm medium containing varying insulin concentrations for either 3 days (A) or 6 days (B). The medium is changed every 24 h. Protein synthesis rates are measured during a 4 h incubation at the end of these times as outlined in MATERIALS AND METHODS. Each point is the mean ± SE of 4 samples and statistical analyses are by t-tests for unpaired values. Error bars
Figure 6. Insulin inhibits long-term protein degradation rates. Day 7 to 8 muscle cultures are grown as outlined in the legend to Figure 1 and switched to mm medium containing no supplements, 10⁻⁶ M insulin, or 85/10/5 medium for either 1 day (A) or 6 days (B). The medium is replaced every 24 h. Cell proteins are labelled with "C-phenylalanine for 20 to 24 h as outlined in MATERIALS AND METHODS starting on either Day 7 to 8 (A) or Day 13 (B). The labelled cells are rinsed for 2 h and the efflux of trichloroacetic acid soluble radioactivity is measured in the medium over a 32 h period. Each point is the mean ± SE of 4 values and statistical analyses are by t-tests for unpaired value. Error bars are smaller than symbol size where not shown.

Figure 7. IGF-1 and IGF-2 stimulate muscle cell growth. Muscle cultures are grown as outlined in the legend to Figure 1 and switched to varying IGF-1 or IGF-2 concentrations in mm medium for three days starting 7 to 10 days postplating before measuring total noncollagenous protein (A) and total DNA (B) content as outlined in MATERIALS AND METHODS. Medium is changed every 24 h. Each point is the mean ± SE of 4 samples and statistical analyses are by tests for unpaired values.

Figure 8. IGF-1 but not IGF-2 stimulates neonatal myofiber growth. This experiment is the same as outlined in the legend to Figure 3. Control myofibers grown in mm medium without supplements are shown
in Figure 3A. Myofibers grown for 5 days in $2.5 \times 10^{-7} \text{ g/ml}$ of either IGF-1 (A) or IGF-2 (B) are shown in this Figure. Medium is changed every 24 h. Bar equal 25 $\mu$m.

Figure 9. IGF-1 but not IGF-2 induces neonatal myofiber hypertrophy. This experiment is similar to that outlined in the legend to Figure 4 with $2.5 \times 10^{-7} \text{ g/ml}$ IGF-1 or IGF-2 added in mm medium at Day 7 postplating and the cultures fixed and stained on Day 12 postplating. Each bar represents 18-27 measurements $\pm$ SE on whole mounts in randomly chosen sections as outlined in MATERIALS AND METHODS by two independent observers and statistical analyses are by t-tests for unpaired samples of the combined data.

Figure 10. IGF-1 and IGF-2 stimulate long-term protein synthesis rates. Day 7 to 8 myofiber cultures are grown as outlined in the legend to Figure 1 and switched to mm medium contains varying concentrations of either IGF-1 or IGF-2 for 3 days. The medium is changed every 24 h. Protein synthesis rates are measured during a 4 h incubation at the end of the 3 days as outlined in MATERIALS AND METHODS. Each point is the mean $\pm$ SE of 4 samples and statistical analyses are by t-tests for unpaired values (IGF-1) or paired values (IGF-2, data combined from $1.0 \times 10^{-7}$ to $2.5 \times 10^{-7} \text{ g/ml}$). Error bars are smaller than symbol size where not shown.

Figure 11. IGF-1 and IGF-2 inhibit long-term protein degradation rates. Day 7 to 8 muscle cultures are grown as outlined in the legend to Figure 1 and switched to medium containing $2.5 \times 10^{-7} \text{ g/ml}$
IGF-1 or IGF-2 for 5 days. The medium is changed every 24 h. Cell proteins are labelled with ¹⁴C-phenylalanine for 20 to 24 h on Day 10 to 11 and protein degradation measured from Days 11 to 12 as outlined in MATERIALS AND METHODS. Each point is the mean ± SE of 4 values and statistical analyses are by t-tests for unpaired samples. Error bars are smaller than symbol size where not shown.
FIGURE 2

A

TOTAL PROTEIN
(μg/well × 10⁻¹)

*P < .001

mm medium - no insulin

B

TOTAL DNA
(μg/well)

**P < .03

mm medium - no insulin

INSULIN (M)

DM11PRO.GRA
DM11DNA.GRA
DM11PRO.PAC
DM11PRO.PLT
FIGURE 4

MYOFIBER DIAMETER (µm)

DAY 7 - DAY 14  DAY 14 - DAY 19

- 85/10/5
- no insulin
- 10^{-6} M insulin

** P<.004
* P<.001

DM7-DM10.GRA
DM7-DM10.PAG
DM7-DM10.PLT
FIGURE 5

A

PROTEIN SYNTHESIS (TCA ppt dpm/μg protein)

mm medium - no insulin

* P<.003
** P<.001

B

* P<.02
** P<.001

mm medium - no insulin

DM11D3A.GRA
DM11D6A.GRA
DM11A.PAG
DM11A.PLT
FIGURE 6

A

% RADIOACTIVITY REMAINING IN PROTEIN

*P<.001

○ ○ 85/10/5
● ● mm medium - no insulin
△ △ mm medium + 10^{-6}M insulin

B

TIME (hours)

*P<.001

DM12A.GRA
DM12B.GRA
DM12ABC.PAG
DM12ABC.PLT
FIGURE 7

A

TOTAL PROTEIN
(µg/well x 10^-1)

IGF-1

IGF-2

mm medium only

B

TOTAL DNA
(µg/well)

mm medium only

*P<.05

**P<.005

***P<.001

CONCENTRATION (g/ml)

DM16PRO.GRA
DM16DNA.GRA
DM16PDR.PAG
DM16PDR.PLT
FIGURE 9

MYOFIBER DIAMETER (μm)

- DAY 7 CONTROL
- DAY 12 CONTROL
- DAY 12 INSULIN
- DAY 12 IGF-1
- DAY 12 IGF-2

* P < .001
FIGURE 10

PROTEIN SYNTHESIS
(TCA ppt dpm/μg protein)

IGF-1

IGF-2

*P<.01

**P<.03

CONCENTRATION (g/ml)

10^{-9} 10^{-8} 10^{-7}

DM16F1B.GRA
DM16F1.B.PAG
DM16F1B.PLT