Changes in protein metabolism of ovine primary muscle cultures on treatment with growth hormone, insulin, insulin-like growth factor I or epidermal growth factor

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ABSTRACT

Methods for the primary culture of muscle cells from fetal sheep were developed which gave high yields of cells. Myoblasts were grown in vitro, and allowed to fuse to form contractile multinucleate myotubes; these could be maintained in a good condition for at least 2 weeks. Protein turnover in these differentiated cultures was examined for sensitivity to each of four potentially anabolic peptide hormones and growth factors: insulin, insulin-like growth factor I (somatomedin C), epidermal growth factor and growth hormone. Insulin was found to have no effect except at high concentrations (1 μmol/l), compatible with its role as a somatomedin analogue. Insulin-like growth factor I was active at lower levels (1 nmol/l) but the cultures were not as responsive to it as were primary rat muscle cultures or differentiated L6 cells, which were tested in similar experiments. The maximum stimulation of protein synthesis observed with the ruminant system was only 16%. Epidermal growth factor was highly anabolic for primary cultures from sheep muscle, and the cells were very sensitive to it, half-maximal stimulation of protein synthesis being seen with concentrations as low as 20 pmol/l. No effects of bovine growth hormone were seen in the ovine system. However, an inhibition of protein breakdown was found with high concentrations (0.1 μmol/l) in the L6 rat myoblast cell line. It was found that the culture conditions used could affect the observed responses of protein synthesis and degradation, despite withdrawal of serum from the incubation media 22 h before testing.


INTRODUCTION

The hormonal control of skeletal muscle growth in ruminants is poorly understood, despite its obvious importance to the livestock industry. So far, attempts to relate circulating hormone concentrations to rates of muscle growth have yielded ambiguous results (for review see Etherton & Kensinger, 1984). Many current ideas are based on data from non-ruminants, and it may be that as a result of adaptation to a specialized form of nutrition, ruminants have evolved mechanisms that are quantitatively or even qualitatively different from those in other animals.

Skeletal muscle fibres are syncytiata; they are formed in the fetus through the fusion of precursor myoblasts. The number of fibres in the muscles of most mammalian species is fixed by the time of birth. Post-natal muscle growth occurs by a combination of two processes: (1) the recruitment of more nuclei from satellite cells, especially at the ends of muscle fibres, allowing longitudinal growth, and (2) the deposition of more muscle protein within individual syncytia and an increase in girth (for reviews see Burleigh, 1974; Campion, 1984).

Although rates of protein synthesis within muscles can be measured directly in vivo, rates of protein degradation normally have to be calculated indirectly (for review see Millward, Bates, Brown et al. 1985). Intact skeletal muscle fibres are difficult to isolate for studies in vitro, since they are often many centimetres in length and need to be held under tension to function normally (Goldberg, Tischler, De Martino & Griffin, 1980; Baracos & Goldberg, 1985). Also they cannot be maintained long enough for measurements of protein degradation to be carried out directly (e.g. Harris, Maltin, Palmer et al. 1985; Wijayasinghe,
Milligan & Thompson, 1984). Studies of the other aspect of growth, satellite cell proliferation and recruitment, require lengthy electron microscopic investigations when carried out in vivo. Thus there are many reasons why use of a tissue culture system with ruminant muscle would be of advantage. Gospodarowicz, Weseman, Moran & Lindstrom (1976) cultured fetal bovine myoblasts and showed that fibroblast growth factor is a potent mitogen for them, whereas growth hormone, insulin, testosterone and epidermal growth factor had little effect. Daubas, Caput, Buckingham & Gros (1981) also used bovine myoblasts in their studies on the regulation of the synthesis of muscle-specific proteins during differentiation. However, neither group of workers has examined the effects of hormones and growth factors on protein turnover in cultured muscle.

In this paper we describe a method for the culture of fetal sheep myoblasts which gives good cell yields and allows the subsequent maintenance in vitro of fused, differentiated muscle cultures over a period of up to 2 weeks. Using these cells of ruminant origin, we have measured the effects of some potentially anabolic peptides and growth factors on protein synthesis and protein degradation. We have compared these with responses in the L6 myogenic rat cell line, which has previously been used as a model for muscle growth (Kotts, Dayton, Cornelius & Allen, 1982; Ballard & Francis, 1983).

MATERIALS AND METHODS

Media and buffers

The phosphate-buffered saline (PBS-D) used in the enzymatic dissociation of fetal sheep muscle and in the subculture of cells contained NaCl (144 mmol/l), KCl (5·4 mmol/l), glucose (25 mmol/l), sucrose (14 mmol/l), Na₂HPO₄ (5 mmol/l), penicillin (50 units/ml), streptomycin (50 µg/ml) and phenol red (1µg/ml) (Yasin, Van Beers, Nurse et al. 1977). These were dissolved in double glass-distilled water adjusted to pH 7·5 at 22°C and filtered through a 0·22 µm membrane filter before use. Minimal PBS (PBS-M) used in the procedures for labelling and harvesting cell cultures was obtained in tablet form (Flow Laboratories, Irvine, Ayrshire) and autoclaved before use. It contained NaCl (137 mmol/l), KCl (2·7 mmol/l) and Na₂HPO₄ (3·2 mmol/l).

Dulbecco's modification of Eagle's medium (DMEM) was obtained as a sterile solution lacking glutamine (Flow Laboratories). This was added to produce a final concentration of 4 mmol/l from a sterile stock solution before use. Fetal calf serum (FCS; batch 29084116), donor horse serum (DHS) and chick embryo extract (CEE) were all obtained from Flow Laboratories. The CEE was supplied diluted 50% in PBS solution. Before use it was inactivated by heating at 65°C for 10 min and then precipitated proteins were removed by centrifugation at 20000 g for 30 min. Penicillin (100 i.u./ml) and streptomycin (100 µg/ml) were routinely added to culture media. They were obtained as a combined sterile stock either from Flow Laboratories or from Gibco Ltd (Uxbridge, Middx). Tissue-culture grade glutamine powder and crystalline grade bovine serum albumin (BSA) were from Sigma Chemical Co. Ltd (Poole, Dorset).

Sterile Petri dishes were Corning tissue-culture grade, except where the 'Primaria' surface was used. The latter were obtained from Falcon Products, Becton Dickinson UK Ltd (Cowley, Oxfordshire).

Peptide hormones and growth factors

Bovine pancreatic insulin (Sigma; 26·2 i.u./mg) was dissolved in 0·9% (w/v) NaCl containing HCl (10 mmol/l) as a concentrated stock. Insulin-like growth factor I (IGF-I; somatomedin C) was a recombinant analogue of human IGF-I with threonine substituted for methionine at position 59, but of similar biological potency to the natural molecule (Amgen Biologicals Inc. obtained through Amersham International plc, Bucks). It was dissolved in 0·9% (w/v) NaCl containing HCl (10 mmol/l) and 0·1% (w/v) BSA. Epidermal growth factor (EGF), purified from the mouse by the method of Savage & Cohen (1972), was a gift from Dr K. Brown (AFRC Institute of Animal Physiology, Babraham, Cambs) and was stored dissolved in cell growth medium containing 0·1% BSA carrier. Bovine growth hormone (GH; Miles Laboratories Inc., U.S.A.) was dissolved in PBS-M containing 0·1% BSA. All peptides were sterilized by filtration through a 0·22 µm pore filter and stored as frozen aliquots at -20°C.

Primary culture of fetal sheep muscle

Suffolk cross Scotch half-breed ewes were mated with a Suffolk ram and slaughtered at 8 weeks of pregnancy. The fetuses were removed under aseptic conditions and the hind limb muscles carefully dissected out. The tissue was minced with scissors in a minimal quantity of PBS-D before transfer to a neutral protease solution. This contained Dispase (Boehringer Corporation (London) Ltd, Lewes, East Sussex) at 4 mg/ml in PBS-D. Dissociation of the tissue was
complete after incubation at 37°C for 1·5 h with trituration at half-hourly intervals. The suspension was filtered through sterile nylon mesh (20 µm pore size, Henry Simon Ltd, Special Products Division, Stockport, Cheshire) to remove coarse debris, and diluted 1:1 with PBS-D. Cells were sedimented at 250 g for 10 min. Yields at this stage were 1–2 × 10⁸ cells per g tissue. They were washed with PBS to remove excess enzyme before seeding at approximately 10⁵ cells/cm² on Primaria plastic Petri dishes in 12·5% (v/v) FCS in DMEM. The medium was changed after 24 h to remove the majority of unattached cells. After 48 h monolayers were harvested using a solution in PBS-D containing 0·05% trypsin (1 in 250 grade; Difco Laboratories, East Molesey, Surrey) and 0·02% (w/v) EDTA. Aliquots were frozen in liquid nitrogen until required.

Cultures were selected for myogenic cells before seeding in the experimental dishes. Thawed aliquots of cells were incubated overnight in 8% FCS in DMEM to allow recovery. They were then harvested using 4 mg Dispase/ml in DMEM, and subjected to differential plating. After incubation in untreated plastic tissue culture dishes for 40 min at 37°C, attached cells (mainly fibroblasts) were discarded and the remainder (about 20%) seeded in 24-well Primaria tissue culture trays at about 10³ cells/cm² in 8% FCS growth medium. After 5 days this medium was replaced by 1% FCS-maintained medium. This caused a slowing of cell proliferation and differentiation into an array of small myotubes. Experiments were carried out approximately 2 weeks after initial seeding of the trays.

Cells were also grown similarly in 10% FCS, with 5% (v/v) DHS as a maintenance medium. Here confluence was attained more rapidly and experiments were carried out 9–10 days after initial seeding.

Rat primary muscle culture

New born Wistar rats were stunned and killed by cervical dislocation, and total hind limb muscles removed aseptically. Digestion of tissues to release myogenic cells was carried out substantially as described for primary sheep muscle culture. Cells were grown in a medium containing 10% FCS and 2% CEE in DMEM. Myogenic cells were selected by differential plating and seeded at 10⁴/cm² in 24 well ‘Primaria’ dishes. Maintenance medium contained 2% FCS and 1% CEE. Cells were tested for hormonal responses 9 days after initial seeding.

L6 cell culture

Our stock was obtained from the American Type Culture collection of cell lines as a frozen stock of Yaffé’s third passage material (Yaffé, 1968). This was propagated for two passages in 10% FCS before a number of clonal isolates were made. These were expanded and a rapidly growing isolate which fused readily in our hands was selected for further study. Twenty-four well ‘Primaria’ culture trays were seeded with these cells at 10⁶/cm² in 10% FCS. As the cultures neared confluence, the medium was changed to 5% DHS for maintenance. Experiments were carried out 7–10 days after seeding.

Radioisotope techniques

Methods were modified from those of Ballard (1982).

Measurements of protein degradation

Cell monolayers were labelled for 2 days with 0·37 mBq L-[3,5³H]tyrosine/ml (1·6 TBq/mmol; Amersham International plc) in maintenance medium. Each well was then washed with PBS-M and cells were incubated for 22 h in DMEM containing 0·1% BSA as the only addition. At the start of the experimental period wells were washed twice more with PBS-M before the test media were added. These were DMEM containing 0·1% BSA plus peptide hormones. Each well contained 0·2 ml. The concentration of tyrosine in DMEM was 0·4 mmol/l which should have been sufficient to prevent significant reincorporation of released tyrosine counts (see Ballard & Francis, 1983).

After 24 h the medium was harvested from each well and monolayers were washed carefully with PBS-M and then with two successive aliquots of 5% (w/v) trichloroacetic acid (TCA). Washings from each well were pooled with the medium and 10% TCA added to bring the final concentration to 5%. Tubes were left to stand at 0°C and acid precipitates centrifuged down. The supernatants were sampled in duplicate, precipitates redissolved in NaOH (0·5 mol/l) and also sampled. Trays containing the acid-precipitated cell protein were frozen until analysed. Monolayers were dissolved in NaOH (0·5 mol/l) at 37°C for 2 h and duplicate samples taken for radioactive counting. Each sample was counted in ‘Optiphase’ scintillation fluid (Fisons) with the addition of a small quantity of formic acid (0·1 ml/5 ml) to aid emulsification of alkaline samples.

Measurements of protein synthesis

Techniques were similar to those described above except that cells were not prelabelled and L-[3,5³H]tyrosine (0·37 mBq/ml) was included in the test media. Within an experiment, care was taken to ensure that the specific activity of the tyrosine in each incubation was identical. The experimental labelling period was 6 h during which incorporation was linear with time. After this all media and washings were discarded. After solubilization of monolayers in NaOH...
(0·5 mol/l), two 0·2 ml samples were assayed for protein content by the method of Lowry, Rosebrough, Farr & Randall (1951).

Statistical analysis of results
Experiments were set up using a split-plot design. Except where indicated, each material was tested using three separate trays of cells and in each tray triplicate wells were used, i.e. nine wells per treatment point. Results were processed to give per cent acid soluble counts for each well in the 24-h degradation period or monolayer specific activities in synthesis experiments. In either case analysis of variance was carried out, allowing tray-to-tray differences as a source of variation. Variance ratios were used to test for the overall significance of treatment-specific differences. Treatments were compared with the control in each experiment using Student's t-test with the pooled standard error of the difference (S.E.D.).

RESULTS
Cell culture techniques
Our method for the growth of sheep muscle cells in culture was devised as a result of an extensive series of experiments. In these, the specific activity of the marker enzyme creatine phosphokinase (CPK) in fused cultures was used as an index of the success of techniques in promoting the growth and differentiation of myogenic rather than fibroblastic cells.

Differential plating was found to be the most satisfactory method for enrichment. Myogenic cells attach more slowly than fibroblasts to tissue culture plastic and so can be selected by preplating of the cell suspension (Richler & Yaffé, 1970). Repetition of this step was not found to give worthwhile results. It is difficult to obtain an exact measure of the proportion of myogenic cells in a primary muscle culture. CPK activity can give an estimate but published results cannot easily be used as standards; for example, some strains of L6, a muscle cell line, have values as low as 0·2 i.u./mg protein (Ewton, Erwin, Pegg & Florini, 1984) while the best primary cultures from human muscle have values of greater than 1·0 i.u./mg protein (Yasin et al. 1977; Zuurveld, Oosterhof, Veerkamp & Van Moerkerk, 1985). Our specific activities were usually in the region 0·4–0·6 i.u./mg at 30 °C. An alternative approach is to seed cells at clonal density (5–15/cm²) and score the colonies so generated for the ability to form myotubes (Hill, Crace, Fowler et al. 1984). However the plating efficiency of cells under these conditions is reduced and it is not known whether myoblasts and fibroblasts are differentially affected. Cells selected by differential plating were over 70% myogenic as judged by this method.

The differentiation of sheep muscle cells in culture was followed (Fig. 1). Cells were seeded at 1·5 x 10³/cm² in a number of 35 mm dishes, each containing 1·5 ml growth medium (10% FCS in DMEM). On the second day the medium was renewed, and on the fifth it was changed to a maintenance medium (2% FCS). At intervals, dishes were harvested using trypsin and EDTA in PBS-D and the recovered cell numbers counted in a haemocytometer. During the initial logarithmic phase the cell doubling time was about 15 h. When the cells neared confluence they began to fuse, and numbers became difficult to count, owing to loss of the fragile multinucleate myotubes during harvesting. Cells were scraped off other dishes into PBS-M, disrupted by sonication, and protein and CPK activity in the extract determined. Cell protein continued to increase slowly after cell fusion. Significant levels of CPK first appeared at the start of cell fusion and increased over the following 2 days to over 100 times that of the prefusion cultures.

Differentiated cultures contained numerous myotubes which developed internal tension and were highly refractile when observed by phase-contrast microscopy. Spontaneous contractions were also occasionally observed. Cell cultures grown as described remained in a good condition for up to 2 weeks after the initiation of fusion.
### Table 1. Effects of peptide hormones and growth factors on protein turnover in cultured ovine muscle cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Grown in 8% FCS; maintained in 1% FCS</th>
<th>Grown in 10% FCS; maintained in 5% DHS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Synthesis† [3H]tyrosine incorporation (d.p.m./μg protein)</td>
<td>Breakdown† (% d.p.m. acid soluble)</td>
</tr>
<tr>
<td>Control</td>
<td>1020</td>
<td>19-65</td>
</tr>
<tr>
<td>Insulin 1 µmol/l</td>
<td>1082</td>
<td>18-95</td>
</tr>
<tr>
<td>IGF-I 0-13 nmol/l</td>
<td>1024</td>
<td>20-02</td>
</tr>
<tr>
<td>13 nmol/l</td>
<td>1182**</td>
<td>18-23**</td>
</tr>
<tr>
<td>EGF 1-7 nmol/l</td>
<td>1268***</td>
<td>18-06**</td>
</tr>
<tr>
<td>34 nmol/l</td>
<td>1303***</td>
<td>17-64***</td>
</tr>
<tr>
<td>GH 1 nmol/l</td>
<td>1042</td>
<td>19-73</td>
</tr>
<tr>
<td>100 nmol/l</td>
<td>1044</td>
<td>20-56</td>
</tr>
<tr>
<td>Pooled S.E.D. (16 d.f.)</td>
<td>± 55-0</td>
<td>± 0-485</td>
</tr>
</tbody>
</table>

*P<0.05; **P<0.01; ***P<0.001 compared with controls. For details see Materials and Methods.

IGF-I, insulin-like growth factor I; EGF, epidermal growth factor; FCS, fetal calf serum; DHS, donor horse serum.

†Protein synthesis measurements were made over 6 h and protein breakdown over 24 h. In all experiments, overall treatment-specific differences were significant (P<0.001) using analysis of variance.

### Protein synthesis and degradation in cultured sheep muscle cells

Fused primary cultures of sheep muscle cells were incubated for 22 h in a medium containing only BSA in DMEM in order to reduce any serum effects on the cells. They were then challenged with one of four peptide hormones and growth factors. Insulin was used at 1 µmol/l, a concentration which had been found by other workers to have anabolic effects on muscle cells (Florini, Nicholson & Dulak, 1977; Ballard, 1982; Askanas, Gallez-Hawkins & King-Engel, 1984). The other three materials were each tested at two concentrations (see Table 1).

Protein synthesis was measured as the incorporation of [3H]tyrosine into acid-insoluble material over a 6-h period, and breakdown as the release of counts over a 24-h period from cells prelabelled with [3H]tyrosine.

Co-ordinate–opposite responses were seen when cells were used which had been grown in 8% FCS and maintained in 1% FCS, i.e. increases in protein synthesis were accompanied by decreases in degradation, leading to an overall anabolic effect (Table 1). The non-significant level of response to insulin was surprising. The lower concentration of IGF-I also produced no effects but the cells responded to the higher IGF concentration, and strongly to both levels of EGF.

In contrast, some non-co-ordinate effects (i.e. changes only in synthesis or in degradation) were obtained when cells from the same preparation were grown and maintained in the richer medium normally used for L6 culture (10% FCS followed by 5% DHS, Table 1). Here insulin (1 µmol/l) stimulated protein synthesis more strongly, but responses to EGF and IGF-I were reduced. Epidermal growth factor decreased protein breakdown but insulin and IGF-I had no effects. Growth hormone treatment produced no significant effects on protein metabolism in either experiment.

The results regarding EGF were further examined in an experiment in which sheep cells maintained in 1% FCS were used. The dose–response behaviour of protein synthesis with EGF was examined. It can be seen from Fig. 2a that the cells were not only responsive to the growth factor but also highly sensitive, half-maximal effects being achieved at a concentration of around 20 pmol/l. The maximal response was 25%, in good agreement with previous results using these growth conditions.

Sheep cells maintained in the low serum medium (1% FCS) were similarly tested for their dose–response behaviour to IGF-I and to insulin. It was shown that protein synthesis was only stimulated by the supraphysiological concentrations of insulin which serve as a somatomedin analogue (Ewton & Florini, 1980; De Vroede, Romanus, Standaert et al. 1984) (Fig. 2c). The cells were much more sensitive to IGF-I (Fig. 2b); the half-maximal response occurring at around 0-6 nmol/l (4 mg/ml). The maximal response was given at 10 nmol/l, but once again was only 15-5% over control rates of synthesis.

The possibility was investigated that these poor responses could be due to the presence of interfering insulin-like growth factors in the BSA carrier used, as described by Etherton & Kensinger (1984). Any such

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contaminants might both increase basal rates of protein synthesis and down-regulate receptors on the cells. Control experiments were carried out in which cells were preincubated in the presence of 0.1% BSA or in growth medium alone. They were then treated with growth medium alone, growth medium with a 0.1% BSA supplement, or growth medium, BSA and IGF-I (13 nmol/l). Although a response to IGF-I was always obtained, no effects of BSA were noted in several experiments under a variety of conditions with both L6 cells and sheep cells. The crystalline grade of BSA used should also be deficient in carrier proteins which could possibly have interfered with the assay (Zapf, Schoenle & Froesch, 1985).

**Protein turnover in primary rat muscle culture and L6 cells**

Protein synthesis in primary rat cells responded similarly to that in sheep primary culture with the test peptides, except that greater responses were found with insulin and IGF-I at 13 nmol/l (Table 2). However, when the L6 cell line was examined (Table 2) major differences were seen. Epidermal growth factor produced no response even at the higher concentration. The largest effects were with insulin (1 μmol/l) and IGF-I (0.13 nmol/l) both of which produced a co-ordinate inhibition of protein breakdown and stimulation of synthesis. The lower concentration of IGF-I (13 nmol/l) produced a statistically significant stimulation of protein synthesis but had no effect on degradation, suggesting that the sensitivity of the two processes to IGF-I might be different. Contrary to the results in sheep cells a significant direct effect of bovine GH was seen, breakdown of protein was reduced by 9.5% with the higher (0.1 μmol/l) concentration. In another similar experiment a reduction of 5.4% \( (P < 0.02) \) was seen and in a test on a rat primary culture consisting mainly of fibroblasts a reduction of 7.5% \( (P < 0.02) \). From this evidence alone it is not clear whether GH affects only protein breakdown *in vitro* or whether prolonged incubation (more than 6 h) would reveal effects on protein synthesis as well.

In a separate experiment wells in a single tray of L6 cells were treated with varying concentrations of IGF-I and protein synthesis was measured (six wells per treatment). The results are shown in Table 2. It was found that 13 nmol/l achieved a maximum response and that the dose giving half-maximal stimulation was below 1.3 nmol/l (10 ng/ml).

**DISCUSSION**

Fetal muscle was used in preference to that from adult animals as a much higher number of viable cells could be released by enzymic digestion and a much greater proportion of these were myogenic. Results reported by other workers suggested that the behaviour of fetal myoblasts in culture is closely similar to that of satellite cells from adult animals. Their patterns of protein synthesis and differentiation are indistinguishable (Whalen, Bugaisky, Butler-Browne & Pinset, 1984). However, some differences have been reported, e.g. the mitogenic responses to insulin of rat cells (Crace, Hill & Milner, 1985). Any cultured muscle cell differs from normal adult tissue in that it is an aneural preparation but in many systems myosin isoforms characteristic of neonatal and adult animals are synthesized (e.g. Buckingham, Minty, Robert *et al.* 1982; Hastings & Emerson, 1982; Bandman, 1984; Whalen *et al.* 1984). In our system a good level of differentiation was implied both by the development of internal tension within myotubes and by the 100-fold increase in the marker enzyme CPK observed during maturation.


**Table 2. Effects of peptide hormones and growth factors on protein turnover in cultured muscle cells of rat origin**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Synthesis† ([3H] tyrosine incorporation (d.p.m./µg protein))</th>
<th>Breakdown† (% d.p.m. acid soluble)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Newborn rat muscle</td>
<td>L6 cells</td>
</tr>
<tr>
<td>Control</td>
<td>3019</td>
<td>1560</td>
</tr>
<tr>
<td>Insulin 1 µmol/l</td>
<td>3559*</td>
<td>2032***</td>
</tr>
<tr>
<td>IGF-I 0-13 nmol/l</td>
<td>2964</td>
<td>1719***</td>
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<tr>
<td>1-3 nmol/l</td>
<td>3851**</td>
<td>2030***</td>
</tr>
<tr>
<td>13 nmol/l</td>
<td></td>
<td>1669**</td>
</tr>
<tr>
<td>130 nmol/l</td>
<td></td>
<td>1803***</td>
</tr>
<tr>
<td>EGF 1-7 nmol/l</td>
<td>3985***</td>
<td>27-77</td>
</tr>
<tr>
<td>34 nmol/l</td>
<td>3836**</td>
<td>27-64</td>
</tr>
<tr>
<td>GH 1 nmol/l</td>
<td>2855</td>
<td>27-21</td>
</tr>
<tr>
<td>100 nmol/l</td>
<td>3127</td>
<td>25-35***</td>
</tr>
<tr>
<td>Pooled S.E.D.</td>
<td>± 234-4</td>
<td>± 43-7</td>
</tr>
<tr>
<td>d.f.</td>
<td>16</td>
<td>16</td>
</tr>
</tbody>
</table>

*P < 0.05, **P < 0.01, ***P < 0.001 compared with controls.
IGF-I, insulin-like growth factor; EGF, epidermal growth factor.
†Protein synthesis measurements were made over 6 h and protein breakdown over 24 h. In all experiments overall treatment-specific differences were significant (P < 0.001) using analysis of variance.

Our cultures contained a majority of myogenic cells, but also a significant proportion of non-fusing 'fibroblasts'. These are hard to remove entirely, and could be regarded as an advantage in studies of muscle growth, since their presence allows interaction between different cell types. Non-myogenic cells represent a substantial proportion (25-40%) of the nuclei in mature muscle (Dadoune, 1980; Campion, 1983).

Rates of protein breakdown seen in the experiments presented here are much higher than those which might be expected *in vivo*, but they agree well with those seen by other workers with L6 cells using shorter or no preincubation (Ballard & Francis, 1983; Smith, 1985). Larger treatment-specific differences might have been observed if shorter preincubation and experimental times had been used in the present study; however these might have only reflected differences in the rapidly turning over proteins (see Neville, Neville & Harrold, 1983; Bieńkowski, 1985). We, however, wished to study the more stable majority population which includes myofibrillar and other structural proteins (Dadoune, 1980).

One result which may have wider implications for tissue culture systems was the finding that manipulation of culture conditions can influence the subsequent sensitivity of cells to peptide hormones and growth factors. Co-ordinate–opposite effects on protein synthesis and breakdown were observed in ovine muscle cells with IGF-I if 1% FCS had previously been used as a maintenance medium. However, when 5% DHS was used instead, responsiveness of protein degradation to this peptide was abolished, resulting in an overall non-co-ordinate effect. This occurred despite withdrawing serum 22 h before the start of the experiment. An explanation may possibly be found in differing rates of metabolism in the cultures and the two regimes; however down-regulation and/or uncoupling of some growth factor receptors in the richer medium may also play a part.

Co-ordinate–opposite effects are the pattern most usually observed when the influence of peptide growth factors on protein turnover in cultured cells is examined, though non-co-ordinate responses have been reported in transformed cell lines and in senescent fibroblasts (Ballard & Read, 1985). Here senescence caused a relative insensitivity of protein synthesis to insulin, IGF-II and EGF, whilst protein breakdown was controlled normally.

In *vivo*, co-ordinate–opposite responses in protein turnover are seen only rarely and often under abnormal conditions, as when insulin is administered to diabetic rats (Pain, Alberte & Garlick, 1983; Garlick, Preedy & Reeds, 1985). Anabolic growth responses are usually the result of co-ordinate–parallel effects (Goldberg et al. 1980; Millward et al. 1985). When female rats were treated with the androgenic agent trenbolone (Vernon & Buttery, 1978) and when trenbolone or zeranol (an oestrogenic agent) were administered to sheep (Sinnett-Smith, Dumelow & Buttery, 1983) increased growth resulted in all cases. However, rates of protein synthesis in skeletal muscle were decreased, implying that protein breakdown must have been reduced even more. Recent work in this laboratory (J. H. R. D. Correia, J. B. Soar & P. J. ...
Buttery, unpublished observations) has shown that similar effects occurred when growing lambs were treated with the β-agonist clenbuterol. Repartitioning between fat and lean tissue was observed, with increased growth of skeletal muscle, but rates of protein synthesis were increased in only one of the three muscles studied. More work is clearly needed to show how responses observed in vivo relate to those in tissue culture with purified growth factors. It is likely that a complex series of interactions involving both the factors and their receptors is involved.

While insulin produces clear differences in muscle protein turnover in diabetic rats, and promotes protein deposition in L6 cells at near-physiological concentrations (Ballard & Read, 1985), its role in normal protein metabolism is much less well documented. Lipogenesis in ruminant adipocytes is less insulin-sensitive than found in other species, and there is little general correlation between plasma insulin levels and growth (for review see Prior & Smith, 1982; Etherton & Kensinger, 1984). Sharpe, Buttery & Haynes (1986) noticed a negative correlation between the plasma free glucocorticoid:insulin ratio and growth rate with lambs on a variety of treatments. The correlation was stronger than that between plasma-free glucocorticoids alone and growth. We found that, in sheep muscle culture, insulin by itself was inactive except at the supraphysiological concentrations at which it might be expected to bind to IGF-I receptors. Previous workers (Gospodarowicz et al. 1976) also showed that it was a poor mitogen for bovine myoblasts. Taken together, these findings suggest that the role of insulin in the ruminant may be a permissive one, rather than the hormone being responsible directly for anabolism.

A causative relationship between circulating IGF-I concentrations and growth rate in lambs has not been established but circumstantial evidence for it exists (Wangness, Olsen & Martin, 1980; Spencer, Garssen & Hart, 1983). Our cultured sheep cells were less responsive to IGF-I than primary rat muscle cells or the L6 rat myoblast cell line, but even so the maximal 16% increase in protein synthesis combined with a 7% drop in breakdown could cause massive anabolic effects in vivo. The sensitivity of the cells to IGF-I was within a plausible physiological range, half-maximal effects on protein synthesis occurring at around 1 nmol/l. Concentrations active in vivo are hard to define since almost all circulating insulin-like growth factors are bound to carrier proteins; the affinity of a partially purified carrier for IGF-I has been measured as 1–2 nmol/l (Zapf et al. 1985).

Little is known of the metabolic roles in vivo of EGF, though it seems likely to have importance at least in the development of the young animal (Das, 1982), and in the functioning of the digestive tract (Brown & Blay, 1985). In primary sheep muscle cultures, concentrations as low as 20 pmol/l were shown to give a half-maximal stimulation of protein synthesis. It is not known whether one cell sub-population within the culture such as fibroblasts responded or all did. The fact that primary rat muscle cultures, which also contained fibroblasts did respond to EGF, whereas the purely myogenic L6 line did not, argues for this being a fibroblast response. Gospodarowicz et al. (1976) found that EGF was not mitogenic for fetal bovine myoblasts; however, protein deposition and cell division were related but not always linked processes. A mutant myoblast line has EGF responsiveness (Lim & Hauschka, 1984) indicating that it is possible for myogenic cells to express this phenotype.

Human primary muscle cultures show increases in the activity of CPK, a muscle-specific enzyme, in response to this factor (Askanas et al. 1984). If only fibroblastic cells in our cultures responded to EGF, then their protein turnover must have been drastically altered, for example protein synthesis must have been about 190% of that in untreated cells. Further work using more highly purified myoblasts is needed to resolve the question.

Our experiments showed no direct effects of bovine GH on protein turnover in sheep muscle cells. Similarly Gospodarowicz et al. (1976) showed that ovine GH was not a mitogen for bovine myoblasts. These results are not unexpected, as most physiological responses in vivo are thought to be mediated by somatomedins (Daughaday, Hall, Raben et al. 1972).

L6 cells did respond to bovine GH at high concentration (0·1 µmol/l); protein degradation was decreased, with no effect on synthesis. Previous workers studying the interaction of GH with L6 cells have found that at 0·1–1·0 µmol/l it stimulated mitosis (Florini et al. 1977) and amino-isobutyric acid uptake (Ewton & Florini, 1980). These results collectively agree well with the findings of Albertsson-Wikland, Eden & Isaksson (1980) who showed a direct effect of bovine GH on amino acid and sugar transport during incubation in vitro of rat diaphragms. It is of interest that the direct activity of bovine GH in rat systems has been clearly demonstrated, whereas the rat and human hormones were reported to have no effects on L6 cells (Ewton & Florini, 1980) and we were also unable to find a direct effect of bovine GH on ovine muscle cells. As suggested by Ewton & Florini (1980), the high concentrations needed for a response implicated a breakdown product of the hormone as the active factor. A number of biologically active fragments of bovine GH have been identified (e.g. Liberti, 1981).

Taken as a whole, our studies in vitro showed clear-cut differences from cells used previously to study hormonal effects on growth. Differentiated
primary cultures of sheep muscle cells were found to be insensitive to insulin, and only responsive to a limited extent to IGF-I in contrast to the L6 rat muscle cell line. They also showed no direct effects with GH, whereas it inhibited protein degradation in L6 cells when used at high concentration. None of these findings are contradicted by the findings of previous workers using whole animal or organ systems; we suggest that the observed differences could be evidence of species-specific dissimilarities in the hormonal responsiveness of muscle between ruminants and rodents.

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REFERENCES


