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January 1996

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Use of Cell Culture to Study Muscle Growth in Beef Cattle

Timothy Woods
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Summary

Muscle cell proliferation and differentiation were observed microscopically and biochemically. The cell DNA content increased for the first four days of culture, then decreased slightly. The muscle creatine kinase activity increased dramatically throughout the study. Protein turnover was measured in myotubes incubated with either dexamethasone or insulin in serum-free media. Protein degradation was increased with increasing dexamethasone levels, but protein synthesis was not affected. Increasing insulin levels increased protein synthesis and decreased protein degradation. The insulin action at high levels was most likely due to its binding to insulin-like growth factor receptors, which is known to increase protein synthesis. This study demonstrates that bovine primary cultures can be used to study muscle growth.

Introduction

Muscle growth is the primary objective of meat animal livestock producers and represents a major source of amino acids and energy within the animal. Endogenous and exogenous factors that impinge on muscle cell development may influence the animal throughout its life cycle. In the adult animal, treatment with hormones, such as anabolic steroids or insulin, can affect muscle metabolism. Attempting to determine a compound's effects on muscle cell development and metabolism can be obscured in animal trials, since other organs and tissues are altering the environment.

Muscle cell culture provides a research tool to determine the direct effects of a specific compound. There are several advantages to cell culture use. First, the cells can be grown as a "pure" culture. Ideally, the cells are of the same type. Secondly, the culture environment can be controlled. The environment includes the atmosphere, temperature, pH, and the available nutrients. Finally, the sample processing can be simple and rapid. Cell culture results permit researchers to look at complex problems in a simplified model; however, these results need to take the complex nature of the animal into account.

There have been many reports using muscle cell culture; however, most reports involve established cell lines from either mouse or rat sources. The definition of a cell line is a cell culture that has been passaged, or transferred to a new culture dish, many times. Many established cell lines have been routinely cultured for years, and the cell characteristics may have changed from the original tissue source with time. Few researchers have used bovine muscle cells in their studies. It is difficult for most researchers to obtain a reliable source of fetal tissue. Nebraska has a number of beef processing facilities available, which would provide a convenient fetal tissue source. The objective of this study is to develop a muscle cell culture system derived from bovine fetal muscle tissue. This cell culture system would permit the study of

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potential economically important compounds and their effects on bovine muscle.

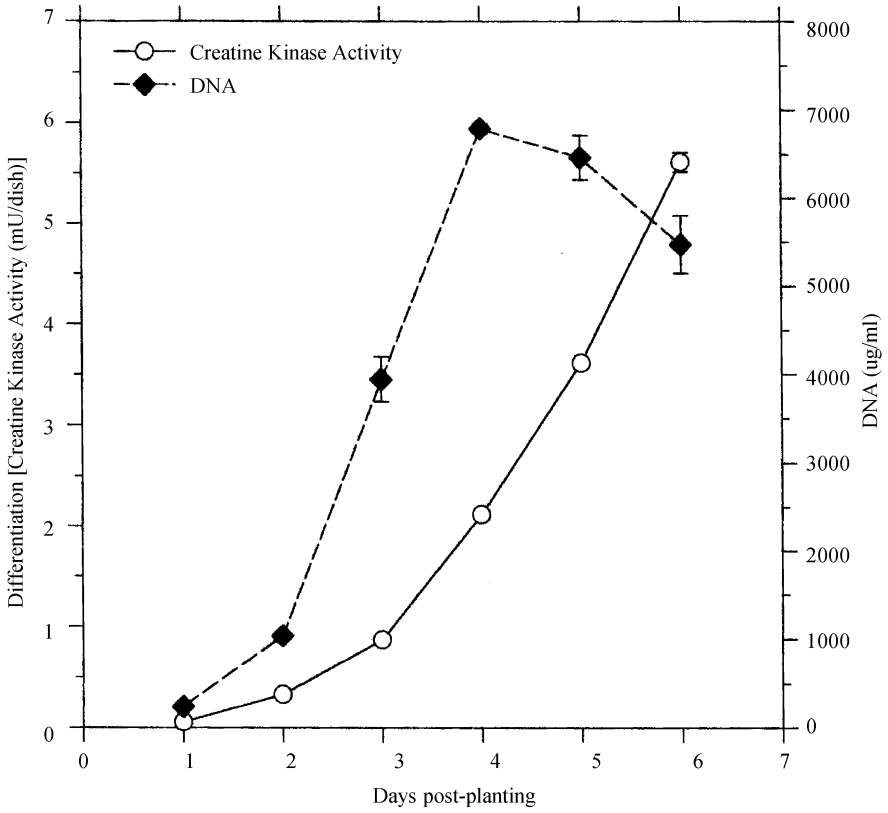
Procedure

Uteri from recently slaughtered cows were obtained from a local slaughter house. These uteri were transported intact to the UNL Meat Research Laboratory. The fetus from each uterus was removed and the crown-rump length measured. The fetal crown-rump length is indicative of the fetus age. For all experiments, 3-4 foeti ranging from 5.5-7.0 inches were processed. The fetus was rinsed with 70 % (v/v) ethanol, and the hindlimbs were dissected. The skin surrounding the hindlimb was peeled from the muscle, and the muscle was dissected from the bone. The muscle was transferred to a clean petri dish and was minced into small pieces. The muscle pieces were placed in a sterile flask containing a phosphate-balanced saline with trypsin and collagenase. Trypsin is a general proteolytic enzyme that removes the undifferentiated cells from the muscle fibers. Collagenase is an enzyme that degrades collagen, a connective tissue component. The muscle and enzyme mixture was incubated at 37°C for one hour. Next, the muscle fragments were separated from the dissociated cells by a low speed centrifugation. The cells in the supernatant were pelleted by centrifugation, and they were resuspended in complete medium (70 % Delbucco's minimal medium (DMEM), 20 % M-199 medium, and 10 % fetal bovine serum (FBS)). The cell number present in the suspension was determined using a Coulter counter, and the cells (approximately 20 x 10^6 cells/flask) were plated onto 75 cm^2 flasks with 15 ml of complete medium. The cells were incubated for one hour, and the medium was replaced with fresh medium. After 48 hours, the media were removed, and the cells released from the plate surface using trypsin. The cell number was determined using a Coulter counter. For experiments, cells were diluted to 2 x 10^5 cells/ml, and 0.5, 1.0, and 2.0 ml of this suspension was added to 24-, 12-, and 6-well plates, respectively. The remaining cells (2 x 10^6 cells/ml) were frozen in 70 % DMEM, 20 % FBS, and 10 % dimethylsulfoxide at -80°C for subsequent experiments.

A growth study was performed with the cells grown on 6-well culture plates. These cells were grown over a 7-day period. Every day, one plate was removed from the incubator, and the media removed from the wells. Each well was washed twice with phosphate-buffered saline, in order to remove any residual media left on the plate. Each plate was frozen at -80°C, until DNA and differentiation (phosphocreatine kinase) assays were performed.

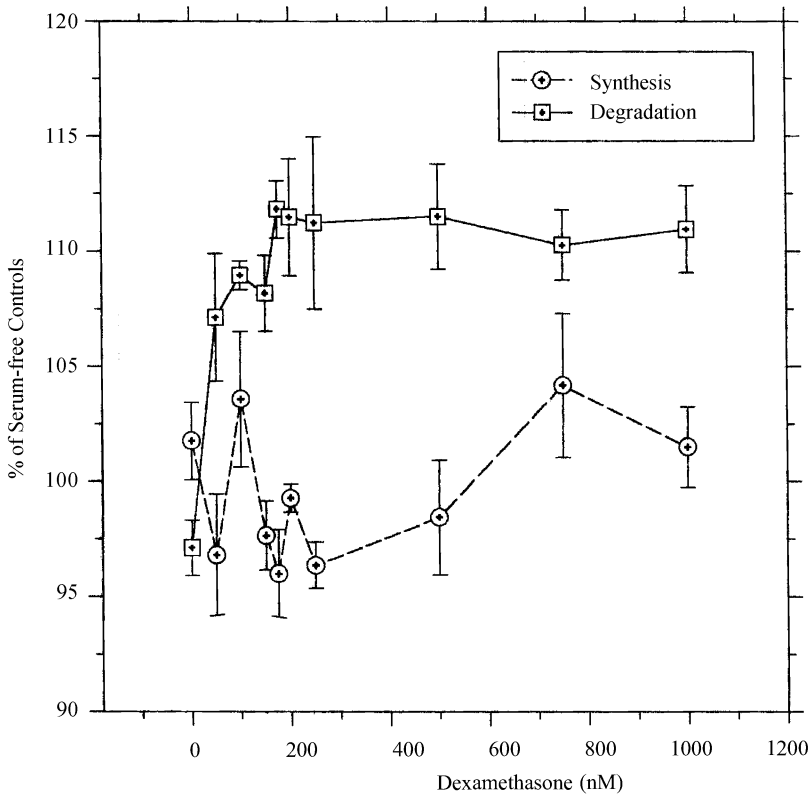
The phosphocreatine kinase is one of several muscle specific marker proteins. Other such marker proteins include myosin, α-actin, desmin, and α-actinin. As muscle cells differentiate by fusing into myotubes, these muscle specific proteins increase in concentration. Therefore, the presence of these proteins indicates the myogenic capacity of the cells. Phosphocreatine kinase activity was determined using a kinetic enzyme assay using a 96-well microplate spectrophotometer. DNA was determined using a fluorometric procedure.

For protein turnover studies, the cells were plated and allowed to grow and fuse into myotubes. The cells were treated with either dexamethasone or insulin in serum-free DMEM. The insulin levels were 0.5, 1.0, 5.0, 10, 50, 75,100, 500, 1000 ng/ml in DMEM. The dexamethasone levels were 50, 100, 150, 175, 200, 250, 500, 750, 1000 nM in DMEM. Protein synthesis was measured by the incorporation of radioactive tyrosine into the myotubes over a four-hour period. Protein degradation was measured by the release of radioactive tyrosine from myotubes over a twenty-hour period. Both protein synthesis and turnover were expressed as a percentage of serum-free controls containing no hormones.



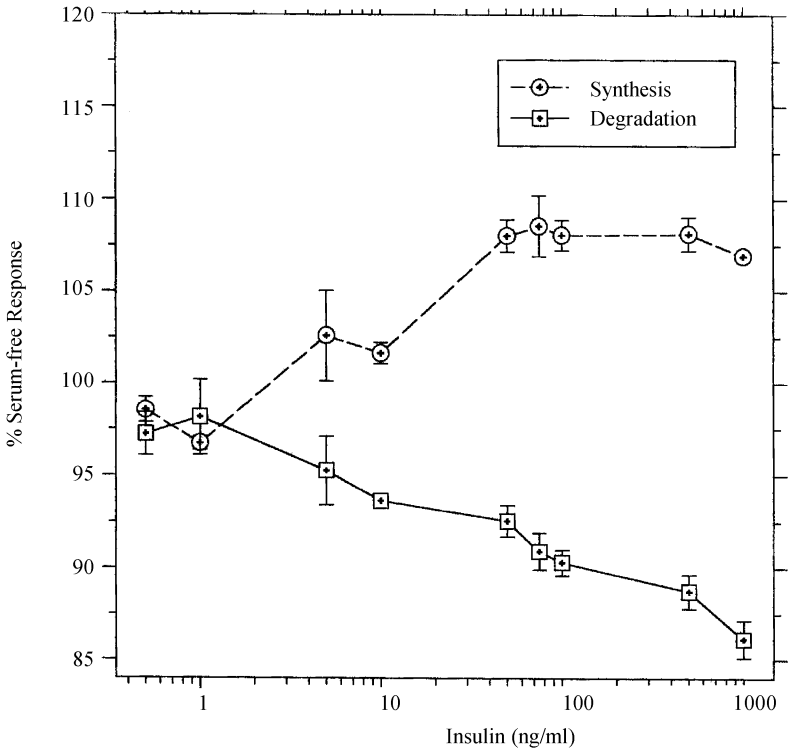
Cells were plated at 1 x 10^5 cells/ml in DMEM/M-199 plus 10% FBS. After 48 h post-plating, the media was changed to DMEM plus 2% Horse serum (HS). Thereeafter, the media was changed every 48 h. Data is expressed as mean ± SEM.

Figure 1. Differentiation and DNA content in bovine primary muscel cell cultures incubated over time.



The test media consisted of DMEM with dexamethasone. The results were compared to serum-free controls (mean ± SEM). Each point represents n = 8.

Figure 2. Protein turnover in bovine primary myotubes incubated with dexamethasone.



The test media consisted of DMEM with insulin. The results were compared to serum-free controls (mean ± SEM). Each point represents n = 8.

Figure 3. Protein turnover in bovine primary myotubes incubated with insulin.

Results

Individual cells had a tapered appearance, when observed under the microscope. The cells multiplied over a 2-3 day period, and the dishes were almost covered with cells by day 3. The cells tended to align parallel to each other at confluence. Fusion of neighboring cells into myotubes could be observed between day 3 and 4 in culture. By day 5, myotubes were the predominant feature of the culture dish.

The DNA content in the dish increased from day 1 to 4, and slightly decreased from day 5 to 6 (Figure 1). The early increase in DNA content is indicative of the cells undergoing rapid proliferation. However, the creatine kinase activity increased throughout the culture (Figure 1), with the increase occurring rapidly after day 3. The increase in creatine kinase and DNA profiles are representative of muscle cells. Creatine kinase is a muscle specific protein, which appears after the cell has begun to undergo differentiation. The decreased DNA content was due to some cell death occurring during the cell fusion into myotubes. Myosin content within the culture increased with differentiation (data not shown). These observations led to the conclusion that muscle cells were isolated from the fetal tissue, and these cells could be viably maintained in culture.

Dexamethasone increased protein degradation but did not influence protein synthesis ($P>.05$) in bovine muscle cell cultures (Figures 2). The increased protein degradation was 12 % of serum-free controls ($P<.05$), when the media contained dexamethasone (Figure 2). This increased degradation occurred between 0 and 200 nM, and the dexamethasone response was maximal at 200 nM.

Insulin altered both protein synthesis and degradation in the bovine muscle cells (Figure 3). The synthesis was increased 8 % compared to serum-free controls ($P<.05$) and was maximal at 75 ng/ml (Figure 3). Protein degradation linearly decreased ($P<.05$)

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in the muscle cells by 10 % (Figure 3). The insulin effects observed at the higher concentrations was likely a pharmacological response of insulin on the muscle cells, rather than a physiological response. This pharmacological response observed represents the insulin binding to the muscle insulin-like growth factor receptors, as well as its insulin receptors. The insulin-like growth factors are potent proteins, which exert a strong growth response and stimulate differentiation in the cells. Protein turnover in muscle cells was shifted towards a net protein accumulation in the muscle cells, when the cells were incubated with insulin.

Future research will proceed with the development of bovine muscle cell clones. These clones are cell lines that have been derived from a single cell. This will provide a useful tool to study the effects of compounds without the interference of other cell types, such as fibroblasts, which may produce localized hormones that may influence the muscle cell culture response. The development of a serum-free culture media will also provide future studies with a controlled nutrient and hormonal environment to grow the cells. With these tools, studies involving the effects of hormones on the development of bovine muscle cells can be readily undertaken.

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