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THE EFFECT OF MECHANICAL STIMULI ON MUSCLE DEVELOPMENT IN VITRO

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Introduction

The process of muscle growth is a central issue in the business of producing animals for meat. At the most fundamental level, the process of muscle development and growth is a complex sequence of events whereby muscle cells respond to a number of stimuli in order to form organised muscle tissue. Increase in muscle mass is greatly influenced by the rate of skeletal muscle protein synthesis, a process that can be altered by mechanical forces. Stretch- or load-induced signaling is now beginning to be understood as a factor which affects the mass and phenotype of muscles as well as the expression of a number of proteins within muscle cells (Carsen *et al.,* 1996; Winchester *et al.,* 1991). Use of magnetic field to produce mechanical forces to stimulate cell populations has been well documented (Glogauer *et al.,* 1998; Hagen *et al.,* 1996). Magnetic field stimulation has been shown to affect transcription of specific gene sequences, protein synthesis, the immune system and increase in Ca^{2+} influx (Michele *et al.,* 2001). Since the number of muscle fibers is fixed at birth and muscle mass is determined in part by fiber number, the mechanisms by which these numbers are determined are of interest to those involved in the study of muscle development.

Objectives

The purpose of this study was to investigate the role of mechanical signals in m-calpain induced muscle cell fusion. The specific goal of this work was to determine whether a mechanically stimulated cell population showed differences in the activity of m-calpain, an enzyme required for myotube formation in vitro.

Methodology

C2C12 cells from ATCC (American Type Culture Collection, Manassus, VA), were maintained under a 5% CO_2 stream at 37°C, plated at 7,500 cells/cm^2 on tissue culture surfaces and grown to 75% confluence in DMEM containing 10% foetal calf serum (FCS).

For stimulation experiments, cells were incubated with laminin coated 1 μm Encapsulated Super-Paramagnetic Microspheres (EMI- 100/40) for 30 minutes to allow

for bead attachment and then rinsed to remove unattached beads. Stimulated cultures were placed on a heating plate maintained at 37 °C under the electromagnet for a period of 6 hrs. Control cultures were kept in incubator at 37 °C during that period.

A magnetic field of 0.5 mT was generated by an electromagnet (Power Generator 0-30 Volts, 0.1-100 Hz; Elcanic A/S, Denmark). The magnet produced alternating MF at frequency of 1 Hz. Magnet was placed 10 mm over the monolayer of cells during the stimulation period. Cells lacking beads but placed under the magnetic field were used as an additional control.

For the determination of myoblast formation and size, cells were fixed with 4% paraformaldehyde, permeablised with saponin and stained with Fluorescein isothiocyanate (FITC) (Sigma, St. Louis, MO), and 4',6-Diamidino-2-phenylindole (DAPI) (Sigma, St. Louis MO). Images were obtained using a Leica DmIRB inverted microscope (Leica, DK) couples to a Coolsnap digital camera (Roper Scientific, DE). Images were obtained and analysed using the Image Pro Plus system (Image House, DK). The results shown are an average of 5 different experiments performed on different days.

To determine calpain activity in the cell cultures, cells were plated at 75% confluence on microtiter plate and incubated for 40 min in the presence of 25 μM CMAC, *t*-BOC-Leu-Met, a membrane-permeable calpain specific fluorogenic substrate. The control cells were placed in an incubator at 37 °C, while the stimulated cells were incubated for 20 min with laminin coated microbeads assay, and then placed in a magnetic field for 5 h. The calpain activity was measured as a function of the rate of change in intracellular fluorescence using a fluorometer with 355-nm exitation and 460-nm emission filters.

For immunochemistry experiments, cells were cultured and stimulated as described above. The cells were removed from each dish by scraping, boiled in sample buffer for 5 minutes, and then centrifuged. Protein determination, using the BCA protein determination system (Pierce Scientific, Rockford, IL), was performed on each supernatant and the protein concentrations of each sample were adjusted to the same value. An aliquot containing 10 μg protein was added to each well of an 8-16% gradient gel and run for 90 min at 126 V. The proteins were transferred to a nitrocellulose filter and blocked for 1 h in 3% milk in Tris-buffered saline (TBS). The blots were incubated in a 1:500 dilution of the specified primary antibody in the 3% milk buffer overnight at 4^{o}C, rinsed thoroughly with TBS and incubated in a 1:10,000 dilution of alkaline phosphatase conjugated secondary antibody in TBS for 1 h at room temperature. After thorough rinsing in TBS, the protein bands were visualized using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) and quantified using NIH Image

Results & Discussion

Mechanical stimulation increases myotube frequency but decreases the size of myotubes formed. C2C12 cells are able to fuse and form myotubes *in vitro* after switching the cells from media containing foetal calf serum to medium containing horse serum. The size and frequency of myotube formation can be visualized and quantified by staining cell membranes and nuclei. When cell cultures are mechanically stimulated through their laminin receptors, the myotubes formed contain significantly ($P<0.001$) fewer nuclei than cultures of un-stimulated cells containing the coated beads (Fig 1a). Mechanical stimulation through laminin receptors significantly increases the number of

myotubes formed in culture ($P<0.001$) (Figure 1b). Few if any myotubes can be seen in cultures stimulated with fibronectin coated beads. Therefore, mechanical stimulation via laminin but not fibronectin receptors has a significant effect on myotube formation *in vitro*.

Expression of m-calpain but not μ-calpain is up regulated in cells exposed to mechanical stimulation Protein extracts from control C2C12 cells and stimulated C2C12 cells were resolved in 8% polyacrylamide gel, transferred onto membrane and detected using antibodies against m- and m-calpain (Fig 2). Mechanically stimulated cells show a 20-fold increase in m-calpain expression as compared to non-stimulated controls. Expression of μ-calpain is unaffected by mechanical stimulation. Similar controls were performed on cells stimulated with fibronectin coated microspheres without an increase in m-calpain expression.

Mechanical stimulation increases calpain activity in C2C12 cells. The level of fluorescence of a calpain specific substrate, CMAC, *t*-BOC-Leu-Met, was used to determine the level of calpain activity in the cells (Fig 3). The substrate becomes fluorescent only after specific cleavage by calpains. At time 0, before any mechanical stimulation was applied to the cultures, the level of calpain activity was not significantly different in the two cell populations. During the time course of the experiment, the control cells did not show any increase in calpain activity. The cells which had been stimulated with the laminin coated microspheres showed a sharp increase in calpain activity after 2.5h, after which time no further increases were observed.

Conclusions

We have shown that mechanical signals transmitted through the C2C12 cells interaction with laminin cause an increase in cellular differentiation. This signaling results in an increase in the number of myotubes formed in the cultures, with each individual myotube containing fewer nuclei. Mechanical stimulation increases not only the expression of m-calpain but also the overall activity of calpain in the cells.

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Tables and Figures

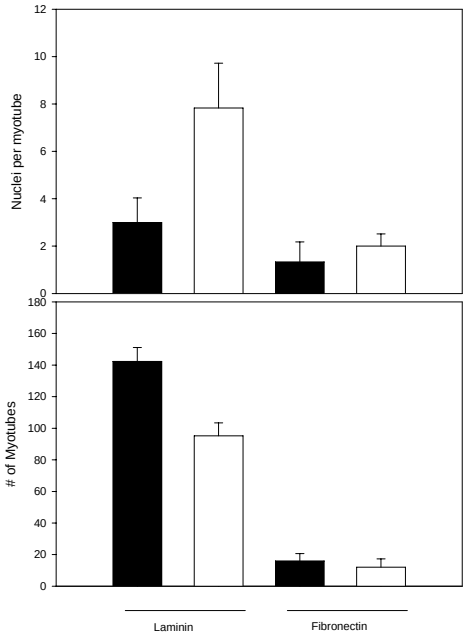
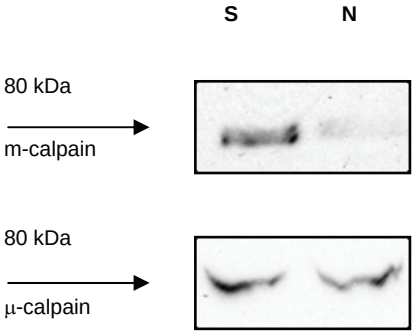


Figure 1. Frequency and size of myotubes formed by C2C12 cell populations in the presence and absence of mechanical stimulation. The number of nuclei per myotube (a) and the total number of myotubes (b) in cultures of cells stimulated with microspheres (black) as compared to un-stimulated controls. Stimulation with laminin coated microspheres causes an increase in the number of myotubes formed, but the resultant myotubes are significantly smaller. Few if any myotubes are seen when cultures are stimulated with fibronectin coated microspheres. Error bars - ± SE.



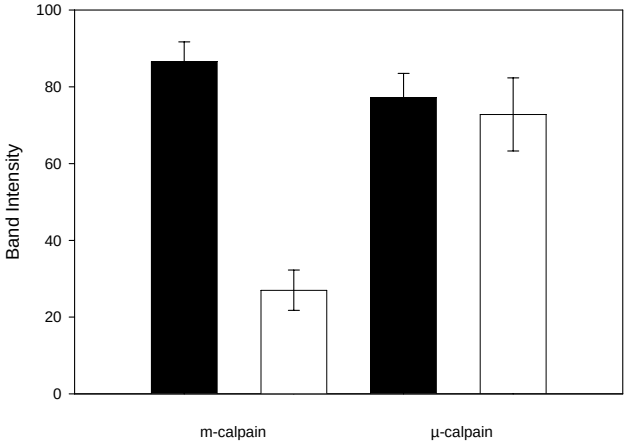


Figure 2. Protein extracts from control C2C12 cells (N) and stimulated C2C12 cells (S) were resolved in 8% polyacrylamide gel, transferred onto membrane and detected using antibodies against m- and μ-calpain. Mechanical stimulation increases m-calpain expression threefold but does not have an affect on μ-calpain expression. (Black-stimulated; White, control).

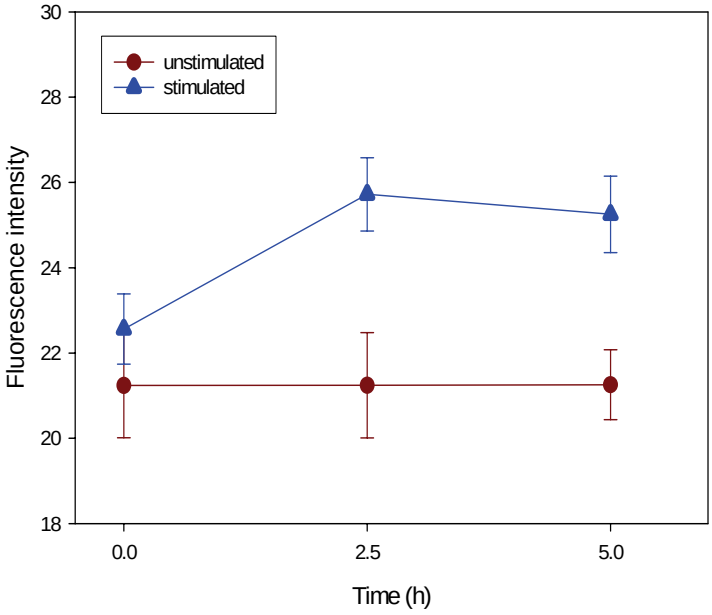


Figure 3. Quantitation of calpain activity using a fluorescent substrate probe. The control cells did not show an increase in calpain activity over the course of the experiment, while mechanically stimulated cells showed a marked increase in calpain activity by 2.5 h after which time no further increases were observed.