MECHANICAL STIMULATION OF C2C12 CELLS INCREASES M-CALPAIN EXPRESSION, FOCAL ADHESION PLAQUE PROTEIN DEGRADATION AND CELL DIFFERENTIATION

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Background

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). Magnetic field stimulation has been shown to affect transcription of specific gene sequences, protein synthesis, the immune system and increase in Ca^{2+} influx (Tatsumi *et al*, 2002).

The past 10 years has seen a dramatic increase in the understanding of how proteolytic enzymes such as calpains can affect the growth of muscle (Goll et al, 1992). In vivo studies have shown that m-calpain is necessary for myoblast fusion leading to the formation of muscle fibres (Barnoy et al., 1998; Joffroy et al., 2000), and that inhibition of this enzyme restricts myotube formation (Temm-Grove et al., 1999). Whether there is a link between stretch- or load induced signaling and m-calpain expression and activation is not known.

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 expression and localization of m-calpain, an enzyme required for myotube formation in vitro.

Materials and methods

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 or fibronectin 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. Where indicated, cells were incubated for 1 h at 37°C in PBS containing a 1:100 dilution of anti-integrin blocking antibody prior to the attachment of the microspheres.

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 immunohistochemical analysis, cells were fixed with 4% paraformaldehyde, permeabilised with saponin and stained the indicated primary antibodies, followed by Alexa 488 secondary antibodies (Molecular Probes, Eugene Oregon). 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 activity of the enzyme creatine phosphokinase (CPK) increases as myoblasts fuse into myotubes. The level of CPK was therefore assayed as a measure of cell differentiation and myotube formation in the different culture conditions, using the CPK assay kit (Sigma, St. Louis, MO), on cell suspensions whose total protein concentration was determined using a BCA kit (Pierce, Rockford, IL), on homogenised cell suspensions.

Results and discussion

Cells mechanically stimulated with laminin coated microspheres show increased CPK activity. The activity of CPK increases as myoblasts differentiate into myotubes. When cells were stimulated with laminin coated microspheres, the CPK activity seen in the culture rose approximately 40% (Figure 1). Similar increases were not seen in any of the control cultures, whether they were exposed to the magnetic field in the absence of spheres or had the microsperes present without being exposed to the magnetic field. No differences in CPK activity were seen when cells were stimulated with fibronectin coated beads. or in cell populations that had been stimulated with fibronectin coated microspheres. These results indicate that mechanical stimulation of myoblasts through laminin but not fibronectin receptors can significantly stimulate myoblast differentiation. Additionally, the response was not due to the simple binding of spheres to ECM receptors or to the exposure of cells to a magnetic field.

Expression of m-calpain but not μ-calpain is up regulated in cells exposed to mechanical stimulation. M-calpain is thought to be an essential enzyme in the differentiation and fusion of myoblasts. Cells mechanically stimulated with laminin coated microspheres express a higher amount of m-calpain, while the expression of the related enzyme μ-calpain is unaffected by this stimulation (Figure 2). Similarly, cells mechanically stimulated with fibronectin coated spheres do not show an increase in m-calpain expression (data not shown).

Mechanical stimulation through laminin receptors causes a re-localization of m-calpain to focal adhesion, followed by a disappearance of the enzyme from these regions. In control cells, m-calpain is found throughout the cytoplasm and in small quantities at the cell surface (Figure 3a). After 30 min. of mechanical stimulation, the amount of enzyme is increased in these focal adhesions, as it is in the whole cell (Figure 3b). 3 hours later, the amount of m-calpain in the cells remains high, but less is found in the focal adhesion sites (Figure 3c). The decrease in staining may be due to autolysis of the enzyme after activation.

Mechanical stimulation via laminin receptors leads to a breakdown of paxillin in focal adhesion complexes. m-calpain is known to associate with focal adhesion complexes at the cell membrane of muscle cells and focal adhesion proteins are known substrates for this enzyme. After mechanical stimulation of C2C12 cells via the laminin receptor, staining for paxillin is not longer found in punctuate regions on the cell surface (Figure 4). Similar results have been seen when cells are stained for talin, and β1-integrin, also known substrates for m-calpain (data not shown). Previous work in our laboratory has shown that blocking m-calpain activity with specific enzyme inhibitors blocks the breakdown of focal adhesion proteins on the cell surface.

Conclusions

Muscle cells are exposed to both a chemically and mechanically active environment during differentiation. During the developmental process, myoblasts come in contact with different extracellular matrix proteins, through which mechanical signals can be generated. We have shown that mechanical signals transmitted through the C2C12 cells interaction with laminin cause an increase in cellular differentiation. At the same

time, this signaling results in not only an increase in the expression of the proteolytic enzyme m-calpain, but a resultant breakdown of focal adhesion proteins on the cell surface. This breakdown may well be due to the activation and subsequent autolysis of the enzyme in this region.

References

Barnoy S, Glaser T, and N.S. Kosower.1998. The calpain-calpastatin system and protein degradation in fusing myoblasts. Biochim Biophys Acta. 1402(1):52-60.

Carsen, J.A, and S.E. Always 1996. Stretch overload-induced satellite cell activation in slow tonic muscle from adult and aged Japanese quail**.** Amer. J. Physiol. Cell. Physiol. 270: 578-584.

Glogauer M and J. Ferrier 1998. A new method for application of force to cells via ferric oxide beads. Eur. J. Physiol. 435: 320-327.

Goll DE, Thompson VF, Taylor RG, and J.A. Christiansen. 1992. Role of the calpain system in muscle growth. Biochimie. 74(3):225-37.

Joffroy S, Dourdin N, Delage JP, Cottin P, Koenig J, and J.J. Brustis. 2000. M-calpain levels increase during fusion of myoblasts in the mutant muscular dysgenesis (mdg) mouse**.** Int J Dev Biol. 44(4):421-8.

Tatsumi, R., A. Hattori, Y. Ikeuchi, J.E. Anderson and R.E. Allen. 2002. Release of hepatocyte growth factor from mechanically stretched skeletal muscle satellite cells and role of pH and nitric oxide. Mol. Biol. Cell. 13:2909-2918.

Temm-Grove CJ, Wert D, Thompson VF, Allen RE, and D.E. Goll. 1999. Microinjection of calpastatin inhibits fusion in myoblasts. Exp Cell Res. 247(1):293-303.

Winchester, P.K., M.E. Davis, S.E. Always, and W.J. Gonyea 1991. Satellite cell activation in the stretch-enlarged anterior latissimus dorsi muscle of the adult quail. Am. J. Physiol Cell Physiol. 260: 206-212.

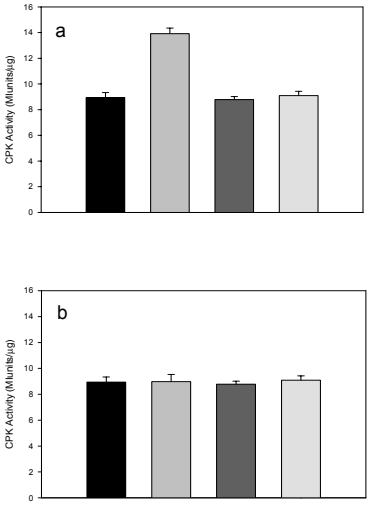

Figure 1. The effect of mechanical stimulation on CPK activity in C2C12 cells. C2C12 cell cultures stimulated with laminin coated microspheres showed a higher CPK activity than controls (a). CPK activity was unaffected by either the presence of, or stimulation by fibronectin coated microspheres (b).

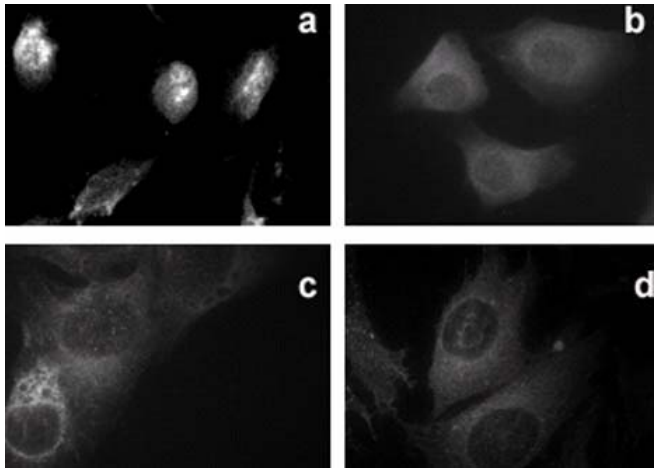

Figure 2. The effect of mechanical stimulation via laminin receptors on m-calpain expression in C2C12 cells. Mechanically stimulated (a,c) and un-stimulated (b,d) myoblasts stained for either m-calpain (a,b) and m-calpain (c,d). Mechanical stimulation increases m-calpain but not m-calpain expression.

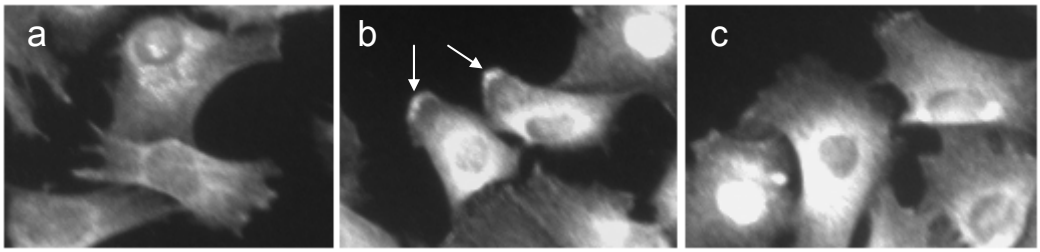

Figure 3. Localization of m-calpain after mechanical stimulation with laminin coated spheres. A. Prior to stimulation. B. 30 min of stimulation. C. 4 h of stimulation. Arrows - focal adhesion complexes. Expression of m-calpain increases shortly after mechanical stimulation begins, and the enzyme relocates to the cell surface. After a longer stimulation time, the staining at the cell surface disappears.

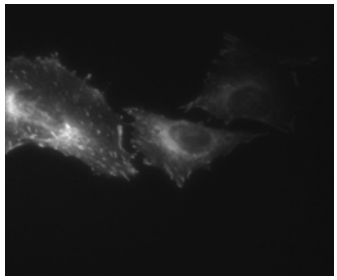

Figure 4. Breakdown of focal adhesions after mechanical stimulation of myoblasts. Stimulated (Bottom) and un-stimulated (Top) myoblasts were stained for the focal adhesion plaque protein paxillin. Paxillin staining can be seen in punctate focal adhesions in un-stimulated cells but not in stimulated cells.