CALPAIN ACTIVITY IN A CULTURED MUSCLE CELL LINE IS INCREASED BY ADRENALINE

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Background

Knowledge concerning regulation of the cellular level of calpain is very limited. Adrenaline is secreted in response to a low blood glucose level and can participate in the hormonal regulation of protein metabolism. Adrenaline-injected pigs showed an increased amount of extractable μ-calpain from longissimus dorsi muscle (Ertbjerg *et al.*, 1999) and it was proposed that low energy stores in vivo lead to increased calpain-induced proteolysis in pig muscle. Sensky et al. (1996) infused pigs intravenously with adrenaline for 1 week, but in contrast to the more acute effect of adrenaline in the above study, there was no effect on the m- and μ-calpain activity of the muscle at time of slaughter. Raising the circulating adrenaline level over a 7-day period increased the extractable calpastatin activity in cardiac and skeletal muscle (Parr *et al.*, 2000). The adrenaline-induced effect on the calpain system in pigs may have been a result of side-effects from the treatment (e.g. from changes in blood insulin concentrations or from altered nerve responses). The effect of adrenaline on the calpain system in muscle is therefore not well described.

Objectives

The purpose of the present study was to investigate the effect of adrenaline on the calpain system of muscle cells more directly by using a cultured mouse muscle cell line (C2C12).

Methods

Cell culture and calpain extraction

C2C12 cells were maintained at 37°C under a continuous 5% CO_2 stream. The cells were grown to confluence in Dulbecco's modified Eagles medium (DMEM) containing 10% fetal bovine serum. After the cultures reached confluence, the medium was then replaced with DMEM containing 2% horse serum (differentiation medium) and incubated for a further 5 days to stimulate myotube formation. The cultures were then incubated in differentiation medium with or without two μg/ml adrenaline for 18 hours prior to harvesting. For each treatment, ten flasks (80 cm^2) of confluent cells were used. Three repeats of the experiment were performed. Cells were mechanically detached from flasks by cell scraping, and were pooled and then homogenised at 4 °C in a total volume of 32 ml of extraction buffer (50 mM Tris-HCl, 5 mM EDTA, 10 mM monothioglycerol, 150 nM pepstatin A, pH 7.5). Aliquots were withdrawn for protein (100 μl) and calpastatin (400 μl) determination. The cellular homogenate was centrifuged for 20 min at 6,000 x *g*. The supernatant was made up to 500 mM with NaCl (from a 5 M solution) and centrifuged 20 min at 15,000 x g. To determine calpain activity the enzymes were partly purified by chromatography on Phenyl-Spepharose and Mono Q columns essential as described (Ertbjerg *et. al*, 1999).

Calpain activity measurements

Calpain activity was determined using fluorescein isothiocyanate-labeled casein (casein-FITC) as substrate. Calpain activity in fractions eluted from the Mono Q column was assayed by mixing 100 μl samples with 50 μl incubation medium (300 mM Tris, 30 mM mono-thioglycerol, 0.4 mg/ml casein-FITC, 15 mM $CaCl_2$, pH 7.5). After 60 min incubation at 25 °C, the reaction was stopped by addition of 150 μl 10% trichloroacetic acid. Tubes were allowed to stand 15 min at room temperature before centrifugation at 20,000 x *g* for 3 min. A 250 μl aliquot of the supernatant fraction was added 2.5 ml of 500 mM Na_2HPO_4, pH 8.5 with mixing to ensure the entire sample was at the proper pH. Fluorescence was measured using an Aminco-Bowman spectrophotofluorometer with an excitation wavelength of 488 nm and an emission wavelength of 520 nm. A 75 nM standard solution of FITC at pH 8.5 was used to set the output to 5.0 V. Results were corrected for background by using 30 mM EDTA instead of $CaCl_2$ in the incubation medium. One fluorescence unit (FU) of calpain activity was defined as the amount of activity in the assay resulting in the release of casein-FITC peptides per min with fluorescence corresponding to 1 pmol of FITC.

Calpastatin and protein determination

The 400 μl sample from the homogenate extract removed for calpastatin determination was heated at 100 °C for 5 min and centrifuged at 20,000 x *g* for 4 min. The calpastatin activity in the supernatant was determined using various dilutions in the casein-FITC assay containing 5 milliFU of m-calpain. The m-calpain used in the assay was extracted and fractionated from porcine longissimus dorsi muscle as described above after homogenisation of 5 g muscle in six volumes of extraction buffer. Incubation time was 60 min at 25°C. From observation of the decrease in fluorescence, an inhibition curve was generated. The linear part of this curve was used to calculate calpastatin activity. One fluorescence unit of calpastatin activity was defined as the amount that inhibited one FU of porcine muscle m-calpain activity. Protein in cell homogenate and in casein-FITC stock solution was determined by the Bradford procedure.

Results and discussions

Similar chromatograms were obtained for each treatment in the three repeats (results not shown). The peak height of the absorption curve obtained at 280 nm approximately doubled in the fractions with m-calpain as a result of the adrenaline treatment. Adrenaline treatment resulted in a pronounced increase in the measurable amount of μ- and m-calpain activity in the column eluate. Comparing the control cells with the adrenaline-treated cells, there was a significant 3-fold increase of μ-calpain activity, expressed in either

fluorescence units per culture flask ($P<0.05$, Fig. 1) or fluorescence units per mg protein in the cell homogenate ($P<0.05$, results not shown). A similar result was obtained with m-calpain activity approximately increasing 3-fold in the adrenaline-treated cells. The increase in m-calpain activity expressed as either FU per culture flask or FU per mg protein was significant ($P<0.05$). Following adrenaline treatment the calpastatin activity per culture flask increased by 16% ($P<0.01$), and by 36% when expressed per mg of protein ($P<0.001$, Fig. 2). The relative increase of calpains after adrenaline treatment were therefore considerably higher than that of their inhibitor (ca. 300 vs 36%), which may have resulted in increased *in situ* proteolytic activity. The calpain system has been suggested to initiate turnover of myofibrillar proteins by making specific cleavages that release thick and thin filaments from the surface of the myofibril (Goll *et. al,* 1989, 1992). In the present study, adrenaline treatment resulted in a 16% decrease ($P<0.05$) in the total protein content in the C2C12 cell homogenate. It is therefore possible that a relationship exists between the increased level of calpains and the decreased level of total protein in the cell homogenate.

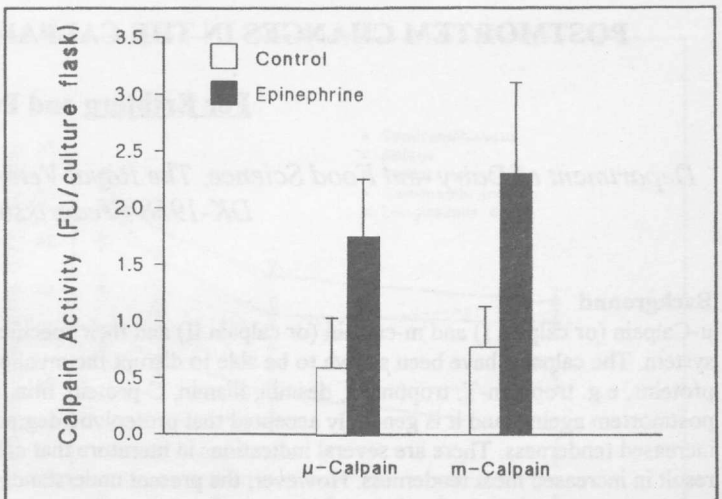

Figure 1. Quantification of the two Ca^{2+}-dependent proteolytic activities in C2C12 cells. Mean ± standard deviation for three separate experiments are shown.

In mammals the total amount of muscle mass depends on the rates of both protein synthesis and protein degradation. A strategy to promote growth of farm animals is to reduce protein degradation. Synthetic β-adrenergic agonists are known to be able to increase muscle growth. When supplemented in the diet, these compounds have been shown to affect the calpain system of sheep and cattle. Administration of β-adrenergic agonists for weeks has, in several studies, been shown to result in an increased level of calpastatin and in a decreased μ-calpain to calpastatin ratio. This is in contrast to the observed increase in μ-calpain activity in porcine muscle following adrenaline injection 15 h before slaughter (Ertbjerg *et al.*, 1999) and the increase in μ- and m-calpain activity in C2C12 cells following adrenaline addition 18 h before harvesting (Fig. 1). The contrasting results could be caused by factors such as acute vs chronic effects, differences in receptor subtype binding and receptor desensitization (down regulation), which are known to influence the response of adrenergic agonist.

Increased levels of calpains have been reported in conditions of muscle wasting. Breakdown of muscle proteins in starvation is known to provide amino acids for gluconeogenesis and energy metabolism, but the cellular mechanisms of skeletal muscle atrophy during fasting is not well understood. Adrenaline is secreted in response to a low blood glucose level and can participate in the hormonal regulation of protein metabolism. Injection of adrenaline in pigs increased skeletal muscle μ-calpain activity (Ertbjerg *et al.*, 1999), and it was proposed that low energy stores in vivo lead to increased calpain-induced proteolysis in pig muscle. However, the adrenaline-induced effect on the calpain system in pigs may have been a result of side-effects from the treatment (e.g. from changes in blood insulin concentrations or from altered nerve responses). The present experiment, using a C2C12 cell culture that does not have connection to blood circulation or a nervous system, is therefore the first study that shows a direct effect of adrenaline on the calpain system in muscle cells.

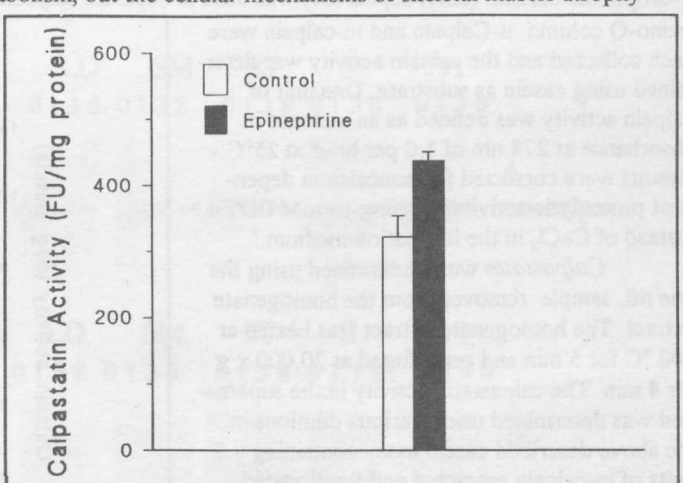

Figure 2. Quantification of calpastatin activity in C2C12 cells. Mean ± standard deviation for three separate experiments are shown.

Further information on the study can be obtained from Ertbjerg *et al.*, 2000.

Conclusions

The data demonstrate that adrenaline treatment increases the level of μ- and m-calpain in C2C12 cells. Overall, the results could suggest that adrenaline may be involved in the regulation of protein metabolism through activation of the calpain system, which in turn has been suggested to initiate turnover of myofibrillar proteins by making specific cleavages that release thick and thin filaments from the surface of the myofibril.

Literature

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