

Effect of Cortisol on Calpains in the C2C12 and 3T3-L1 Cells

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Abstract The present study was carried out to understand the effect of cortisol on calpain system in the C2C12 and 3T3-L1 adipocyte cells under co-culture system. Cells were co-cultured by using transwell inserts with a 0.4 μm porous membrane to separate C2C12 and 3T3-L1 preadipocyte cells. Each cell type was grown independently on the transwell plates. Following cell differentiation, inserts containing 3T3-L1 cells were transferred to C2C12 plates. Ten microgram per milliliter of cortisol was added to the medium. Following treatment for 3 days, the cells in the lower well were harvested for analysis. Calpains such as μ -calpain, m-calpain, and calpastatin were selected for the analysis. RT-PCR results indicated the significant increase in the mRNA expression of μ -calpain, m-calpain, and calpastatin. In addition, the confocal microscopical investigation indicated the cortisol treatment increases calpain expression in the C2C12 and 3T3-L1 cells. Taking all these together, cortisol treatment with co-culture system shows most reliable status of calpains expression in the cells, which is quite distinct from one-dimensional monocultured cells.

Keywords Calpains · RT-PCR · C2C12 · Protein

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Introduction

Calpains are a class of proteins which belongs to the Ca^{2+} dependent, non-lysosomal cysteine proteases [1]. There are three major types of calpains expressed in skeletal muscle, namely μ -calpain, m-calpain, and their specific inhibitor, calpastatin. μ -calpain and m-calpain are ubiquitously expressed in muscle, which require micro and millimolar concentration of Ca^{2+} for their activation, respectively [2]. Even though, this proteolytic system is distributed throughout the animals, their exact physiological and pathological functional role has not yet been studied well. Calpains play a vital role in the cellular functions such as muscle cell differentiation, cell death, and protein degradation of myofibrils and neurofilaments [3–5]. Calpains modulate the enzyme activity, including key signaling molecules and induce specific cytoskeletal rearrangements [6–9].

There are several endogenous regulators of calpain such as calcium, phospholipids, calpain smaller subunit, and calpastatin [10–12]. Calpains are also activated in the conditions of muscle wasting [13]. The changes in calpain activity over time corresponds to morphological changes in muscle [14]. Calpains disrupt myofibrillar proteins such as troponin-T, troponin-I, desmin, filamin, C-protein, titin, nebulin, vimentin, and vinculin [15–18]. Goll et al. [19] have proposed that the calpains play a significant role in myofibrillar protein degradation, especially in the disassembly of the myofibrillar proteins during early stages of turnover. Inhibition of calpain activity in a L8 myoblast cell line, leads to reduced protein degradation [20]. In vivo, the treatment of rats with a calpain inhibitor prevents sepsis-induced increase in muscle protein degradation [21].

Regulation of calpain activity has not yet been understood well at the cellular level. Studies on the calpain regulation are very limited. Calpains expression could be modulated by hormones such as insulin and corticosterone and insulin growth factor 1 during myogenesis of embryonic myoblasts in culture [22]. Patel and Lane [23] have suggested that calpain is required for the differentiation of 3T3-L1 preadipocytes into adipocytes. Calpain is expressed in preadipocytes and its level decreases during differentiation. Calpain degrades the cyclin-dependent kinase inhibitor, p27 during the mitotic phase of 3T3-L1 preadipocyte differentiation (23).

Cortisol is a steroid hormone secreted from the zona fasciculata of the adrenal cortex. It is released in response to stress and a low level of blood glucocorticoids. Ertbjerg et al. [24] reported that the stress hormone (epinephrine) administered pigs showed an increased quantity of extractable μ -calpain from longissimus dorsi muscle. It was suggested that low energy stores leads to increased calpain-induced proteolysis in pig muscle. Sensky et al. [25] reported that no observable effect was found on calpain activity during slaughter after 1 week administration of stress hormone in pigs. Administration of 2 $\mu\text{g/ml}$ stress hormone for 18 h increased μ -calpain, m-calpain, and calpastatin activities in a C2C12 cell homogenate [26]. The biochemical effect of cortisol on calpain proteolytic system in muscle and fat cells is limited. The objective of the present study was to investigate the effect of cortisol on the calpain proteolytic system in the co-cultured C2C12 and 3T3-L1 cells.

Materials and Methods

Materials

All chemicals and laboratory wares were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) and Falcon Labware (Becton-Dickinson, Franklin Lakes, NJ, USA), respectively.

Cell Culture

C2C12 and 3T3-L1 preadipocyte cells were incubated at a density of 8,000 cells/cm² and grown in Dulbecco's modified Eagle's medium (DMEM) containing 10 % fetal bovine serum and antibiotics at 37 °C in 5 % CO₂. Confluent 3T3-L1 preadipocytes were induced to differentiate with a standard differentiation medium consisting of DMEM medium supplemented with 10 % FBS, 250 nM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, 5 µg/ml insulin, and antibiotics. Preadipocytes were maintained in this differentiation medium for 3 days. Cultures were re-fed every 2 days to allow 90 % cells to reach fully differentiation before co-culturing. C2C12 cells were grown to 90 % confluence and changed into differentiation medium and fed with fresh differentiation medium every day.

Co-culture of C2C12 and 3T3-L1 Preadipocytes

C2C12 and 3T3-L1 preadipocytes cells were co-cultured using transwell inserts with a 0.4 µm porous membrane to separate the cells. Each cell type was grown independently on the transwell plates. Following cell differentiation, inserts containing adipocytes were transferred to myotube plates and inserts containing myotubes were transferred to adipocyte plates [27].

Experimental Groups

The experimental groups were designated as follows: control, cortisol with monoculture, and cortisol with co-culture.

Treatment of Cells

Cortisol was freshly diluted in the medium before treatment. The cultures were then incubated with medium containing with 10 µg/ml cortisol for 3 days at 37 °C in 5 % CO₂ prior to harvesting.

Cell Viability

Cell viability was measured by 2 % trypan blue staining. Neubauer chamber was used to count the viable cells in each group such as control, cortisol with monoculture, and cortisol with co-culture.

Reverse Transcriptase Polymerase Chain Reaction Analysis

Total RNA was isolated from the control and treated cells with Trizol reagent according to the manufacturer's protocol. The first-strand cDNA was synthesized from 1 µg of the total RNA using the M-MLV reverse transcriptase with the anchored oligo d(T)_{12–18} primer. Reverse transcription polymerase chain reaction (RT-PCR) was performed using a cDNA equivalent of 10 ng of total RNA from each sample with primers specific for rat µ-calpain (GenBank NM_001110504.1), m-calpain (GenBank NM_009794.3), and calpastatin (GenBank NM_009817.3) and a housekeeping gene GAPDH. The reaction was carried out in 10 µl using SYBR Green Master Mix (Invitrogen) according to the manufacturers' instructions. Relative ratios were calculated based on the 2^{-ΔΔCT} method [28]. PCR was monitored using the MiniOpticon Real-Time PCR System (Bio-Rad).

Immunocytochemistry

Cells grown on glass cover slips in six-well plates were cultured in DMEM. At the indicated time, the cells were fixed for 10 min in 3 % paraformaldehyde in phosphate buffered saline (PBS) and then washed twice in PBS. Cells were permeabilized in 0.1 % Triton X-100 in PBS for 10 min and then washed twice in PBS. Blocking was performed in 3 % bovine serum albumin (BSA) in PBS for 30 min, and the cells were then incubated with monoclonal μ -calpain and m-calpain antibodies (Santa Cruz Biotechnology, Inc) for 12 h at 4 °C in PBS-1 % BSA. After three washes in PBS, the cells were incubated with a secondary fluorescein isothiocyanate (FITC)-conjugated antibody for 1 h at room temperature and then washed three times in PBS. The cover slips were mounted on fluorescent mounting medium and visualized with fluorescence microscope.

Statistical Analysis

All the values are expressed as means \pm SEM. Statistical analysis was performed using SPSS version 16.0 (Statistical Package). Student's *t*-test was performed to determine the differences between control and treatments. $P < 0.05$ was considered to be significant.

Results and Discussion

Results

Effects of Cortisol on C2C12 and 3T3-L1 Cell Viability

The mean percentage of viable C2C12 cells was 98, 97 and 95 % in the control, cortisol with monoculture, and cortisol with co-culture, respectively. The mean percentage of viable 3T3-L1 cells was 96, 95 and 96 % in the control, cortisol with monoculture, and cortisol with co-culture, respectively.

Total Protein

Total protein content reduced 32.4 and 19.8 % in the cortisol with monoculture and co-culture groups of C2C12 cells, respectively. Total protein content reduced 28.7 and 22.8 % in the cortisol with monoculture and co-culture groups of 3T3-L1 cells, respectively (Fig. 1).

mRNA Expression

The μ -calpain, m-calpain, and calpastatin mRNA expressions were calculated and quantitated in the C2C12 and 3T3-L1 cells. The μ -calpain mRNA expression significantly increased 70 and 41.4 % in the cortisol with monoculture and co-culture group of C2C12 samples, respectively, whereas it was increased significantly 45.5 and 22.7 % in the cortisol with monoculture and co-culture group of 3T3-L1 cells, respectively (Fig. 2). Expression of m-calpain was significantly increased 94.4 and 60.6 % in the cortisol with monoculture and co-culture group of C2C12 cells, respectively, whereas m-calpain mRNA expression was significantly increased 95.2 and 61.9 % in the cortisol with monoculture and co-culture group of 3T3-L1 cells, respectively (Fig. 3). Calpastatin mRNA expression was significantly increased 33.33 and 41.7 % in the

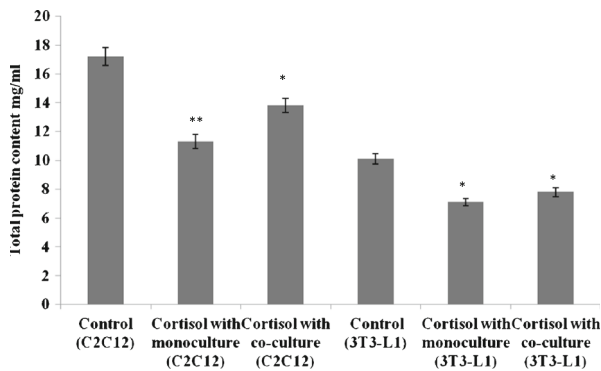


Fig. 1 Total protein content in the C2C12 and 3T3-L1 cell homogenate. C2C12 and 3T3-L1 preadipocytes cells were co-cultured using transwell inserts with a 0.4 μm porous membrane to separate the cells. Total protein content in the C2C12 and 3T3-L1 cells were estimated by the standard method

cortisol with monoculture and co-culture group of C2C12 cells, respectively, whereas it was increased 28.6 and 14.3 % in the cortisol with monoculture and co-culture group of 3T3-L1 cells, respectively (Fig. 4).

Immunofluorescence Staining and Laser-Scanning Confocal Microscopy

To examine changes in the calpains expression in the C2C12 and 3T3-L1 cells, we used immunofluorescent staining with calpains. Mono and co-cultured C2C12 and 3T3-L1 cells showed increased μ -calpain and m-calpain expression. Both calpain isoforms were detected by immunofluorescence combined with confocal microscopy. Distribution of μ and m-calpains in C2C12 cells were presented in Fig. 5. Distribution of μ and m-calpains in 3T3-L1 adipocyte cells were presented in Fig. 6.

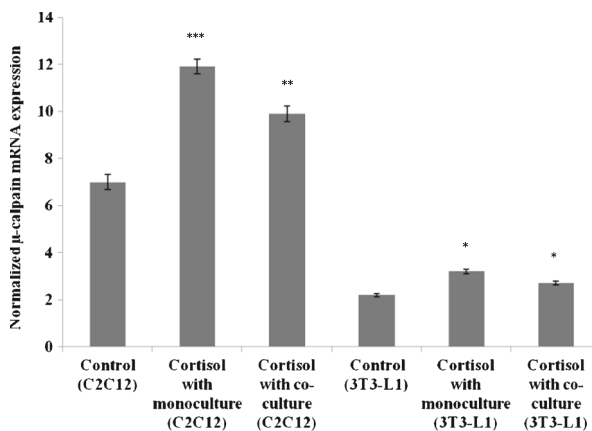


Fig. 2 RT-PCR of μ -calpain in the C2C12 and 3T3-L1 samples. The cultures were incubated with medium containing with 10 $\mu\text{g}/\text{ml}$ cortisol for 3 days. Total RNA was isolated from the control and treated cells with Trizol reagent. The first-strand cDNA was synthesized from 1 μg of the total RNA using the M-MLV reverse transcriptase with the anchored oligo d(T)_{12–18} primer. PCR was monitored using the Mini Opticon Real-Time PCR System

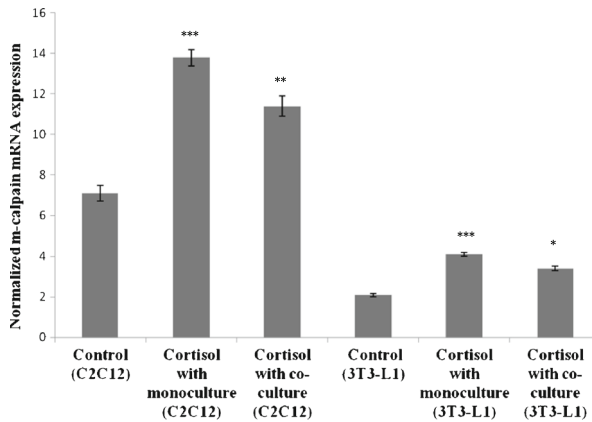


Fig. 3 RT-PCR of m-calpain in the C2C12 and 3T3-L1 samples. The cultures were incubated with medium containing with 10 $\mu\text{g}/\text{ml}$ cortisol for 3 days. Total RNA was isolated from the control and treated cells with Trizol reagent. The first-strand cDNA was synthesized from 1 μg of the total RNA using the M-MLV reverse transcriptase with the anchored oligo d(T)_{12–18} primer. PCR was monitored using the Mini Opticon Real-Time PCR System

Discussion

The calpain system has been suggested to play a vital role in the degradation of myofibrillar proteins by making specific cleavages that produce thick and thin filaments from myofibril surface [19, 29]. In our present co-culture study, cortisol administration reduced total protein content 34.3 and 19.8 % in the cortisol with monoculture and co-culture group of C2C12 cells, whereas it was 28.7 and 22.8 % reduction in the 3T3-L1 cells, respectively (Fig. 1) and agrees with result of Erbjerg et al. [26]. A possible relationship exists between the increased level of calpains and the decreased level of total protein in the cells.

The total amount of muscle mass depends on the rates of both protein synthesis and protein degradation in the animals [30]. A high level of calpastatin may in turn result in decreased

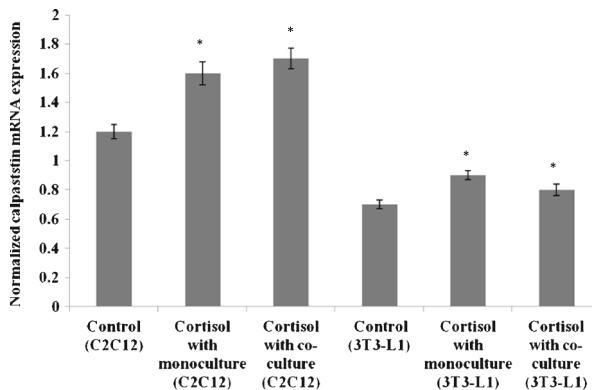


Fig. 4 RT-PCR of calpastatin in the C2C12 and 3T3-L1 samples. The cultures were incubated with medium containing with 10 $\mu\text{g}/\text{ml}$ cortisol for 3 days. Total RNA was isolated from the control and treated cells with Trizol reagent. The first-strand cDNA was synthesized from 1 μg of the total RNA using the M-MLV reverse transcriptase with the anchored oligo d(T)_{12–18} primer. PCR was monitored using the Mini Opticon Real-Time PCR System

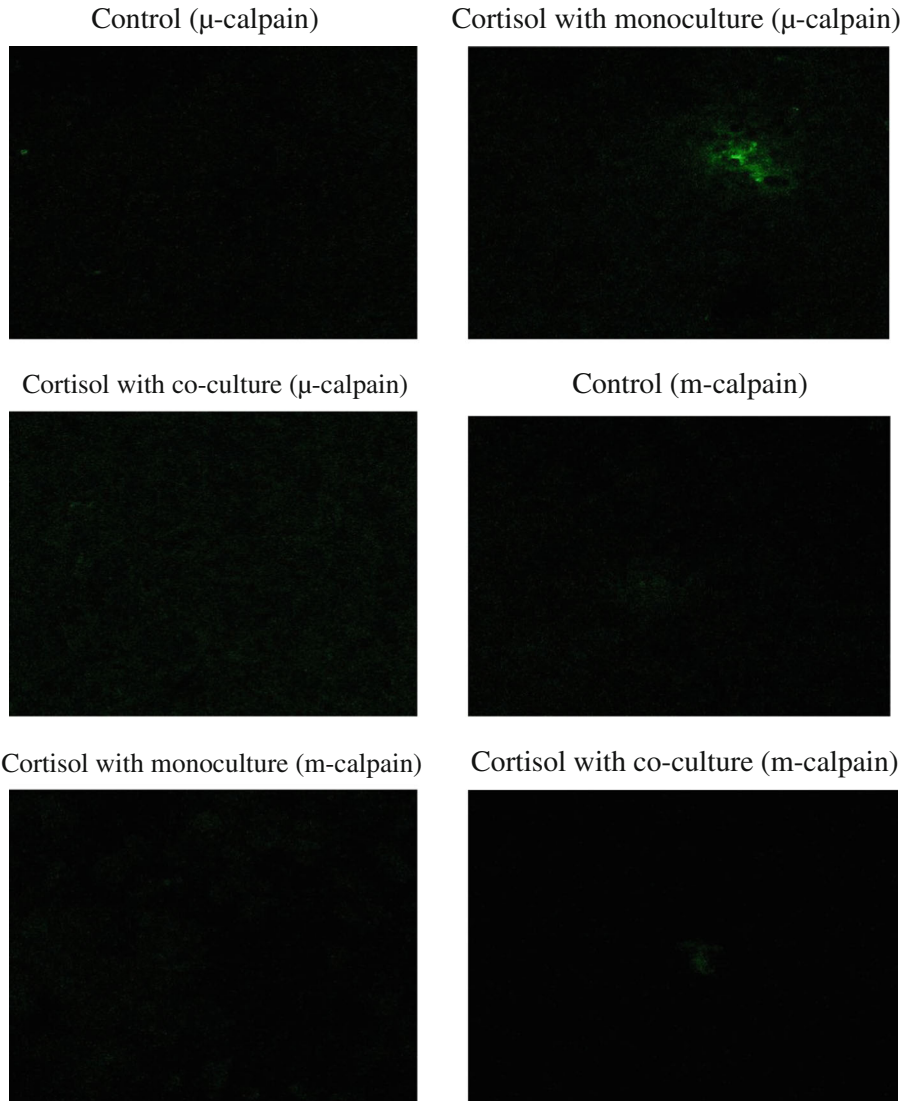


Fig. 5 Immunofluorescence in C2C12. Cells grown on glass cover slips in six-well plates were cultured in DMEM. At the indicated time, the cells were fixed for 10 min in 3 % paraformaldehyde in PBS and then washed twice in PBS. Blocking was performed for 30 min and the cells were then incubated with monoclonal μ -calpain and m-calpain antibody for 12 h at 4 °C in PBS-1 % BSA. Cells were incubated with FITC-conjugated antibody. The cover slips were mounted on fluorescent mounting medium and visualized on fluorescence microscope

proteolysis by calpains after slaughter, and hence more tough meat. Synthetic β -adrenergic agonists improve muscle growth. A diet supplemented with β -adrenergic agonists affect the calpain system in sheep and cattle [31, 32]. Chronic administration of β -adrenergic agonists resulted in an increased level of calpastatin and in a decreased μ -calpain to calpastatin ratio, and this is in contrast to the observed increase in μ -calpain activity in porcine muscle following stress hormone injection 15 h before slaughter [24].

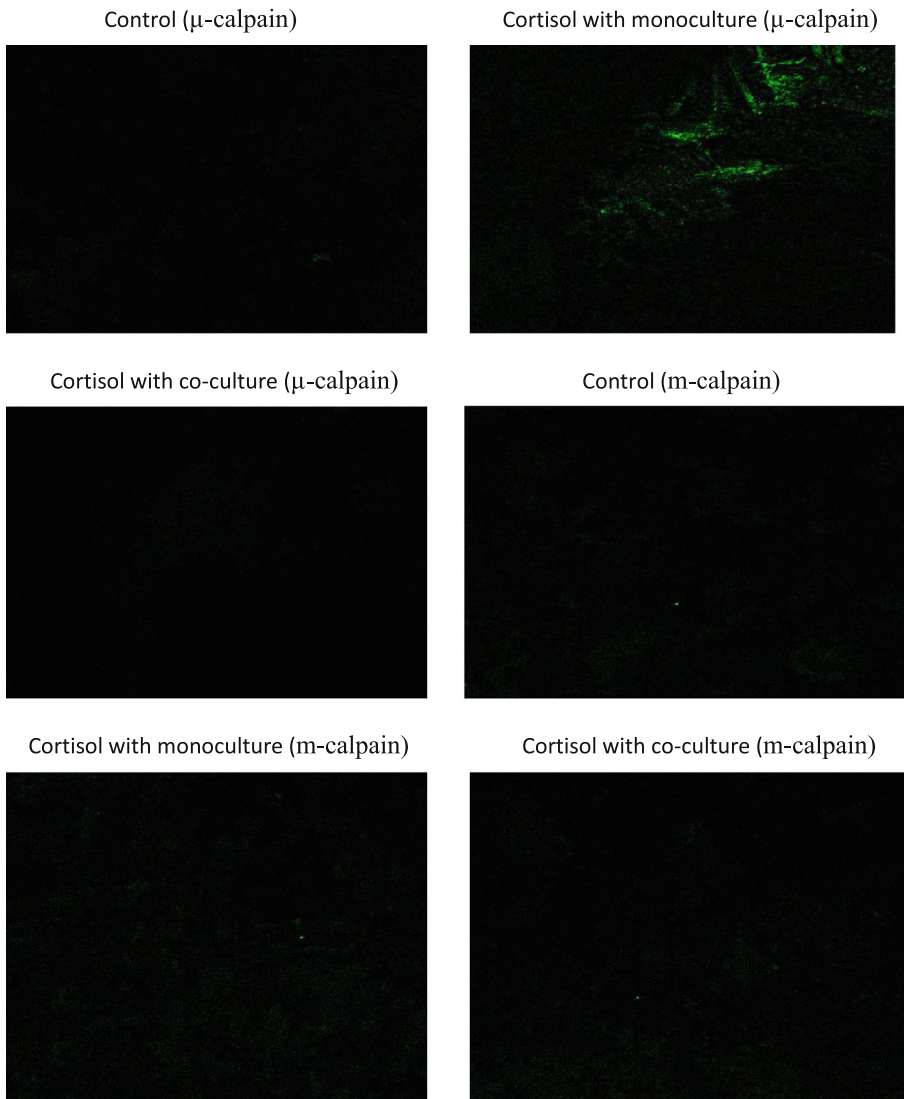


Fig. 6 Immunofluorescence in 3T3-L1 adipocytes. Cells grown on glass cover slips in six-well plates were cultured in DMEM. The cells were fixed for 10 min in 3 % paraformaldehyde in PBS and then washed twice in PBS. Blocking was performed for 30 min, and the cells were then incubated with monoclonal μ -calpain and m-calpain antibodies for 12 h at 4 °C in PBS-1 % BSA. Cells were incubated with FITC-conjugated antibody. The cover slips were mounted on fluorescent mounting medium and visualized on fluorescence microscope

The differences in the results could be due to many factors such as acute versus chronic effects, differences in receptor subtype binding and receptor desensitization, which influence the response of adrenergic agonists [33]. Stress hormone, epinephrine is known to stimulate β -adrenergic receptors and β -adrenergic receptor stimulation increased calpain activity in heart muscle cells during hypoxia [34].

Calpains expression increased in the muscle wasting [35, 36]. Calpain expression increased in the rat and rabbit skeletal muscle during fasting [13]. Muscle protein degradation in

starvation is in order to provide amino acids for gluconeogenesis for energy requirement. However, the cellular mechanism of skeletal muscle atrophy during fasting is not clearly understood. Cortisol is secreted in response to a low blood glucose level and involve in the hormonal regulation of protein metabolism. Elevated the circulating stress hormone over a 7-day period increased the calpastatin activity in cardiac and skeletal muscles [37]. Administration of stress hormone in pigs increased skeletal muscle μ -calpain activity [26]. It was proposed that that low energy stores in vivo lead to increased calpain-induced proteolysis in pig muscle. The present study used a co-culture of C2C12 and 3T3-L1 cells that does not have connection with blood circulation or a nervous system. Therefore, this is the first study that shows a direct effect of cortisol on the calpain system in muscle and fat cells.

Conclusion

Our experimental data demonstrate that the cortisol treatment increases the μ - and m-calpain expressions in C2C12 and 3T3-L1 cells. From the overall result, it is concluded that that cortisol may be involved in the regulation of protein metabolism through activation of the calpain system in C2C12. Increased expression of calpain in 3T3-L1 cells suggests the complete cessation of differentiation and the beginning of lipolysis for energy requirements.

References

1. Koohmaraie, M., & Geesink, G. H. (2006). *Meat Science*, 74, 34–43.
2. Goll, D. E., Thompson, V. F., Li, H. Q., Wei, W., & Cong, J. Y. (2003). *Physics Review* 83, 731–801.
3. Blomgren, K., Kawashima, S., Saido, T. C., Karlsson, J. O., Elmered, A., & Hagberg, H. (1995). *Brain Research*, 684, 143–149.
4. Squier, M. K. T., & Cohen, J. J. (1996). *Cell Death and Differentiation*, 3, 275–283.
5. Temm-Grove, C. J., Wert, D., Thompson, V. F., Allen, R. E., & Goll, D. E. (1999). *Experimental Cell Research*, 247, 293–303.
6. Dourdin, N., Bhatt, A. K., Dutt, P., Greer, P. A., Arthur, J. S., Elce, J. S., et al. (2001). *Journal of Biological Chemistry*, 276, 48382–48388.
7. Fox, J. E. (1999). *Thrombosis and Haemostasis*, 82, 385–391.
8. Glading, A., Lauffenburger, D. A., & Wells, A. (2002). *Trends in Cell Biology*, 12, 46–54.
9. Glading, A., Uberall, F., Keyse, S. M., Lauffenburger, D. A., & Wells, A. (2001). *Journal of Biological Chemistry*, 276, 23341–23348.
10. Melloni, E., Salamino, F., & Sparatore, B. (1992). *Biochimie*, 74, 217–223.
11. Saido, T. C., Nagao, S., Shiramine, M., Tsukaguchi, M., Yoshizawa, T., Sorimachi, H., et al. (1994). *FEBS Letters*, 346, 263–267.
12. Michetti, M., Salamino, F., Tedesco, I., Averna, M., Minafra, R., Melloni, E., et al. (1996). *FEBS Letters*, 392, 11–15.
13. Ilian, M. A., & Forsberg, N. E. (1992). *Biochemistry Journal*, 287, 163–171.
14. Dahlmann, B., Kuehn, L., Reinaue, H., Kay, J., & Stauber, W. T. (1989). *Contributions to Nephrology*, 73, 127–136.
15. Nelson, W. J., & Traub, P. (1983). *Molecular and Cellular Biology*, 3, 1146–1156.
16. Goll, D. E., Dayton, W. R., Singh, I., & Robson, R. M. (1991). *Journal of Biological Chemistry*, 266, 8501–8510.
17. Croall, D. E., & Demartino, D. N. (1991). *Physiological Reviews*, 71, 813–847.
18. Di Lisa, F., De Tullio, R., Salamino, F., Barbato, R., Melloni, E., Siliprandi, N., et al. (1995). *Biochemistry Journal*, 308(5), 7–61.
19. Goll, D. E., Kleese, W. C., & Szpacenko, A. (1989). In D. R. Campion, G. J. Hausman, & R. J. Martin (Eds.), *Animal growth regulation* (pp. 141–182). New York: Plenum Publishing Corporation.
20. Huang, J., & Forsberg, N. E. (1998). *Proceedings of the National Academy of Sciences of the United States of America*, 95, 12100–12105.

21. Fareed, M. U., Evenson, A. R., Wei, W., Menconi, M., Poylin, V., Petkova, V., et al. (2006). *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*, 290, R1589–R1597.
22. Cottin, P., Brustis, J. J., Poussard, S., Elamrani, N., Broncard, S., & Ducasting, A. (1994). *Biochimica et Biophysica Acta*, 1223, 170–178.
23. Patel, Y. M., & Lane, M. D. (1999). *Proceedings of the National Academy of Sciences of the United States of America*, 96, 1279–1284.
24. Ertbjerg, P., Henckel, P., Karlsson, A., Larsen, L. M., & Møller, A. J. (1999). *Journal of Animal Science*, 77, 2428–2436.
25. Sensky, P. L., Parr, T., Bardsley, R. G., & Buttery, P. J. (1996). *Journal of Animal Science*, 74, 380–387.
26. Ertbjerg, P., Moira, A. L., & Peter, P. P. (2000). *Biochimie*, 82, 197–201.
27. Sun, X., & Zemel, M. B. (2008). *Journal of Nutritional Biochemistry*, 19, 392–399.
28. Pfaffl, M. W. (2001). *Nucleic Acids Research*, 29, e45.
29. Goll, D. E., Thompson, V. F., Taylor, R. G., & Christiansen, J. A. (1992). *Biochimie*, 74, 225–237.
30. Goll, D. E., Otsuka, Y., Nagainis, P. A., Shannon, J. D., Sathe, S. K., & Muguruma, M. (1983). *Journal of Food Biochemistry*, 7, 137–177.
31. Higgins, J. A., Lasslett, Y. V., Bardsley, R. G., & Buttery, P. J. (1988). *British Journal of Nutrition*, 60, 645–652.
32. Koochmaraie, M., Shackelford, S. D., Muggli-Cockett, N. E., & Stone, R. T. (1991). *Journal of Animal Science*, 69, 4823–4835.
33. Mersmann, H. J. (1989). In D. R. Campion, G. J. Hausman, & R. J. Martin (Eds.), *Animal growth regulation* (pp. 337–357). New York: Plenum Publishing Corporation.
34. Iizuka, K., Kawaguchi, H., & Yasuda, H. (1991). *Japanese Circulation Journal*, 55, 1086–1093.
35. Dayton, W. R., Schollmeyer, J. V., Chan, A. C., & Allen, C. E. (1979). *Biochimica et Biophysica Acta*, 584, 216–230.
36. Arakawa, N., Takashima, M., Kurata, T., & Fujimaki, M. (2000). *Agricultural and Biological Chemistry*, 47, 1517–1522.
37. Parr, T., Sensky, P. L., Arnold, M. K., Bardsley, R. G., & Buttery, P. J. (2000). *Archives of Biochemistry and Biophysics*, 374, 299–305.