Effects of stress hormone cortisol on the mRNA expression of myogenin, MyoD, Myf5, PAX3 and PAX7

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Abstract The present investigation was carried out to evaluate the effect of stress hormone cortisol on the myogenic markers in the C2C12 cells co-cultured with 3T3-L1 preadipocytes. Co-culturing was achieved by transwell inserts with a 0.4 µm porous membrane. C2C12 and 3T3-L1 cells were grown independently on the transwell plates. After differentiation, inserts containing 3T3-L1 cells were transferred to C2C12 plates for co-culturing. 10 µg/µl of cortisol was added to the medium. After 72 h of treatment, C2C12 cells

which were in the lower well were harvested for analysis. RT-PCR analysis of myogenic markers such as of myogenin, MyoD, Myf5, PAX3 and PAX7 showed a significant reduction in the mRNA expression of these myogenic markers. In addition, cortisol increased calpain activity, which led to accelerated protein degradation, which in turn reduced the myogenic rate. In conclusion, cortisol treatment reduced mRNA expression of myogenic markers in the co-cultured C2C12 cells, which is quite distinct from one dimensional mono-cultured C2C12 cells.

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Abbreviations

MyoD	Myogenic determination
Myf5	Myogenic factor 5
PAX3 and PAX7	Paired box gene 3 and Paired box gene 7
FBS	Fetal bovine serum

Introduction

Growth, development and differentiation of fat and muscle cells are regulated by several endocrine, paracrine and autocrine factors. The interaction between muscle and fat cells, plays a vital role in myogenesis and adipogenesis. Fat and muscle interaction was important in fat, lean deposition and the

energy utilization in growth and development (Dodson et al. 1997). Skeletal muscles of the vertebrate body are derived from the dorsal portion of somites (Buckingham 1992; Wachtler and Christ 1992). Dermomyotome gives rise to two cell types such as myoblasts and fibroblasts. Myoblasts are the precursors of skeletal muscle fibres and express myogenic factors such as MyoD (Weintraub et al. 1991; Olson 1992). Fibroblasts, express markers common to mesodermal cells, such as collagens and vimentin. Normal fibroblasts fuse with dysgenic mdg (Restoration of dysgenic murine) myoblasts to restore the expression of the membrane calcium channel in the mutant (Chaudary et al. 1989; Courbin et al. 1989). It was suggested that fibroblast nuclei would be exposed to muscle transcription factors due to the infrequent random fusion (Blau et al. 1985). It is believed that the primary fibroblasts will undergo muscle differentiation during co-cultured with myogenic cells.

Cortisol is a steroid hormone secreted from the zona fasciculata of the adrenal cortex. It is released in response to stress and to a low level of blood glucocorticoids. Its primary function is to increase blood sugar through the gluconeogenic pathway. Protein degradation may be accelerated through the proteolytic calpain system. Increased calpain activity leads to increased rate of protein degradation which in turn leads to a reduced rate of myogenesis. Ertbjerg et al. (1999) reported that the administration of cortisol in pigs showed an increased quantity of μ -calpain from the longissimus dorsi muscle. Stress hormone cortisol increases protein degradation and decreases myogenesis. The present experiment was designed to evaluate the effect of stress hormone cortisol on myogenic markers of C2C12 cells, co-cultured with 3T3-L1 cells. In vivo, cells have close interactions with adjacent cells, which are three dimensional. Therefore, three dimensional co-culture study is the best option to understand in vivo cellular functions and interactions.

Materials and methods

Materials

All chemicals and laboratory wares were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

Cell culture

C2C12 and 3T3-L1 preadipocytes (ATCC® CL-173™, Manassas, VA 20108 USA) 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 supplemented with 10 % FBS, 250 nM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, 5 μ g/ml insulin and antibiotics. Preadipocytes were maintained in this differentiation medium for 3 days. Cultures were re-fed every 2–3 days to allow 90 % of the cells to reach complete differentiation. C2C12 cells were grown to 90 % confluence and switched to differentiation medium and fed with fresh differentiation medium every day.

Co-culture of C2C12 and 3T3-L1 cells

Cells were co-cultured by using transwell inserts with a 0.4 μ m porous membrane to separate C2C12 muscle cells and 3T3-L1 preadipocytes. Each cell type was grown independently on the transwell plates. Following cell differentiation, inserts containing adipocytes were transferred to myotube plates (Sun and Zemel 2008).

Treatment of cells

Cortisol was freshly diluted in the medium before treatment. The cultures were then incubated with medium containing ten μ g/ml cortisol for 72 h at 37 °C in 5 % CO₂ prior to harvesting.

Experimental groups

The experimental group designated as follows such as control (without cortisol), monocultured C2C12 cells with cortisol and co-cultured C2C12 cells with cortisol.

Cell viability

Cell viability was measured by 2 % trypan blue staining. The number of viable cells was estimated in each sample such as control, monocultured C2C12 with cortisol and co-cultured C2C12 with cortisol by counting in a Neubauer chamber.

RNA isolation and RT-PCR

Control, monocultured C2C12 with cortisol and co-cultured C2C12 with cortisol cells were lysed in Trizol reagent and total RNA was extracted from all samples according to the manufacturer's protocol. The first-strand cDNA was synthesized from 1 µg of total RNA using the M-MLV reverse transcriptase with the anchored oligo d(T)_{12–18} primer. RT-PCR was performed using a cDNA equivalent of 10 ng of total RNA from each sample with primers specific for myogenin (forward primer: 5'-GGTGCCAGCGAATGC-3', reverse primer: 5'-TGATGCTGTCCACGATGGA-3'), MyoD (forward primer: 5'-CCGCTGAGCAAAGTAAATGA-3', reverse primer: 5'-GCAACCGCTGGTTGGATT-3'), Myf5 (forward primer: 5'-TTCTACGACGGCTCCTGCATA-3', reverse primer: 5'-CCACTCGCGGCACAAACT-3') PAX3 (forward primer: 5'-AGT GAG ATT ACG CGC TAG-3', reverse primer: 5'-CCA GCG GAA GAC CAG AAA C-3'), PAX7 (forward primer: 5'-CACAGCTTCTCCAGCTACTCTG-3', reverse primer: 5'-GAAGCTCAGGGTTCAGTTAGG-3') and the housekeeping gene GAPDH (forward primer: 5'-CACCCTCAAGATTGT-CAGC-3', reverse primer: 5'-TAAGTCCCTCCACGATGC-3'). The reaction was carried out in 10 µl using SYBR Green Master Mix (Invitrogen, Carlsbad, CA, USA) according to the manufacturers' instructions. Relative ratios were calculated based on the $2^{-\Delta\Delta CT}$ method (Pfaffl 2001). PCR was monitored using the Mini Opticon Real Time PCR System (Bio-Rad, Hercules, CA, USA).

Statistical analysis

All values are expressed as mean $\pm$ 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

Cell viability

C2C12 cell viability was determined by 2 % trypan blue exclusion method and counting in a Neubauer

chamber in each group. C2C12 cell viability was determined after treatment. The mean percentage of viable C2C12 cells was 98, 96 and 97 % in control, monocultured C2C12 cells with cortisol and co-cultured C2C12 cells with cortisol, respectively.

mRNA expression

RT-PCR used for mRNA quantitation of myogenesis markers such as myogenin, MyoD, Myf5, PAX3, PAX7. Myogenin mRNA expression was significantly reduced by 24.5 and 20.8 % in the monocultured C2C12 cells with cortisol and co-cultured C2C12 cells with cortisol, respectively (**p* < 0.05 and ***p* < 0.01, Fig. 1). MyoD mRNA expression was significantly reduced by 44 and 40 % in the monocultured C2C12 cells with cortisol and co-cultured C2C12 cells with cortisol, respectively (***p* < 0.01, Fig. 2). Myf5 mRNA expression was significantly reduced by 23.3 and 30 % in the monocultured C2C12 cells with cortisol and co-cultured C2C12 cells with cortisol, respectively (**p* < 0.05, Fig. 3). PAX3 mRNA was significantly reduced by 30 and 30 % in the monocultured C2C12 cells with cortisol and co-cultured C2C12 cells with cortisol, respectively (**p* < 0.05, Fig. 4). PAX7 mRNA expression was significantly reduced by 28.6 and 35.7 % in the monocultured C2C12 cells with cortisol and co-cultured C2C12 cells with cortisol, respectively (***p* < 0.01, Fig. 5). There

Fig. 1 RT-PCR of myogenin in C2C12. The experimental cultures were treated with ten µg/ml cortisol. 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. **p* < 0.05 and ***p* < 0.01

Fig. 2 RT-PCR of MyoD in C2C12. The experimental cultures were treated with ten $\mu\text{g/ml}$ cortisol. 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. ** $p < 0.01$

Fig. 3 RT-PCR of Myf5 in C2C12. The experimental cultures were treated with ten $\mu\text{g/ml}$ cortisol. 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. * $p < 0.05$

was a significant difference between mono and co-culture results. In co-culture, less expression of myogenin (3.7 %) and MyoD (4 %) were found in C2C12 cells compared to the monoculture, whereas Myf5 (6.7 %) and PAX7 (7.1) expression were higher than in monocultured C2C12 cells. PAX3 mRNA expression was similar in both mono and co-cultured C2C12 cells treated with cortisol.

Fig. 4 RT-PCR of PAX3 in C2C12. The experimental cultures were treated with ten $\mu\text{g/ml}$ cortisol. 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. * $p < 0.05$

Calpain activity

We also measured μ -calpain and m-calpain activity in the each group. Calpain activity significantly increased 28 % in the monocultured C2C12 cells with cortisol and 36 % in the co-cultured C2C12 cells with cortisol.

Discussion

The total amount of muscle mass depends on the rate of protein synthesis and protein degradation in the animals (Goll et al. 1983). Reduction in protein degradation is the best way to improve the growth of farm animals. Synthetic β -adrenergic agonists improve muscle growth. A diet supplemented with β -adrenergic agonists affect the calpain system in sheep and cattle (Higgins et al. 1988; Koohmaraie et al. 1991; Koohmaraie and Shackelford 1991; Parr et al. 1992). Chronic administration of β -adrenergic agonists resulted in an increased level of calpastatin and in a decreased μ -calpain to calpastatin ratio which is in contrast to the observed increase in μ -calpain activity in porcine muscle following stress hormone injection 15 h before slaughter (Erthbjerg et al. 1999). Stress hormone is known to stimulate β -adrenergic receptors, and β -adrenergic receptor stimulation increased calpain activity in heart muscle cells during hypoxia (Iizuka et al. 1991).

Fig. 5 RT-PCR of PAX7 in C2C12. The experimental cultures were treated with ten $\mu\text{g}/\text{ml}$ cortisol. 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. ** $p < 0.01$

Muscle protein degradation was accelerated during starvation 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 is involved in the hormonal regulation of protein metabolism. Elevating the circulating epinephrine over a 7-day period increased the calpastatin activity in cardiac and skeletal muscle (Parr et al. 2000). Calpain induced proteolysis leads to reduced rate of muscle formation. The present study, using a co-culture of C2C12 and 3T3-L1 cells is the best model to mimic in vivo muscle formation. Therefore, this is the first study that shows a direct effect of cortisol on the myogenesis in a co-culture system.

Conclusion

From the experimental data, it is clear that cortisol treatment reduces the expression of myogenin, MyoD, Myf5, PAX3 and PAX7 and increases μ - and m-calpain activity in C2C12 cells. From the overall result, it is concluded that cortisol may be involved in the regulation of protein metabolism through activation of the calpain system.

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