Cortisol Effect on Heat Shock Proteins in the C2C12 and 3T3-L1 Cells

Sambandam Ravikumar · Pandurangan Muthuraman

Abstract The present study was carried out to understand the effect of cortisol on heat shock protein system (Hsps) in the C2C12 and 3T3-L1 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 cells. Each cell type was grown independently on the Transwell plates. After cell differentiation, inserts containing 3T3-L1 cells were transferred to C2C12 plates and inserts containing C2C12 cells transferred to 3T3-L1 plates. Ten micrograms per microliter of cortisol was added to the medium. Following 72 h of treatment, the cells in the lower wells were harvested for analysis. Heat shock proteins (Hsps) such as Hsp27, Hsp70, and Hsp90 were selected for the analysis. The qRT-PCR results showed the significant increase in the mRNA expression of as Hsp27, Hsp70, and Hsp90. In addition, confocal microscopical investigation showed the cortisol treatment increases Hsps expressions in the mono and co-cultured C2C12 and 3T3-L1 cells. From the results, we concluded that the cortisol increases Hsps expression in the co-cultured C2C12 and 3T3-L1 cells, which is differed from one-dimensional mono-cultured C2C12 and 3T3-L1 cells.

Keywords Co-culture · Hsps · qRT-PCR · C2C12 · 3T3-L1 cells

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Introduction

To evaluate the biological system, animals would be a commonly preferred model; whereas laboratory animals may be secondary. However, several differences occur between laboratory animals and common animals such as environmental factors and etc. Recently, mono-culture system has been used in biological and clinical investigation. Recently, they have started to use the co-cultured system in the biological experiments. Living tissues have highly ordered, complex, architecture and interactions between adjacent cells which differs from the in vitro mono-cultured cells, which are absent (Dodson et al. 1997). The three-dimensional co-culture systems would be a more reliable and preferred model in the biological experiments to evaluate the complex interactions between adjacent cell types.

Heat shock proteins (Hsps) are molecular chaperones which play a vital role in repairing damaged proteins and protein folding. Hsps increases when cells are exposed to high temperature which causes oxidative damage (Feder and Hofmann 1999; Kregel 2002). There are 10 different forms of Hsps present in organisms (Muthuviveganandavel et al. 2008). Stress hormones such as catecholamines and cortisol influence the Hsps expression. Catecholamine increases Hsps expressions in mammals, fish, and invertebrates (Udelsman et al. 1993; Murphy et al. 1996; Paroo and Noble 1999; Lacoste et al. 2001; Currie et al. 2008). The main aim of the present investigation were (a) to evaluate the effect of cortisol on Hsps expressions in C2C12 and 3T3-L1 cells and (b) to compare the variation between the mono and 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) and Falcon Lab ware (Becton-Dickinson, Franklin Lakes, NJ), respectively.

Cell culture. C2C12 and 3T3-L1 cells were incubated at a density of 7,000 cells/cm^2 and grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C in 5% CO_2. 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 1% penicillin/streptomycin. 3T3-L1 cells were maintained in this differentiation medium for 3 days. Cultures were re-fed every 2–3 d to allow 90% of the cells to reach complete differentiation before co-culturing. C2C12 cells were grown to 90% confluence and changed into differentiation medium and fed with fresh differentiation medium every d (Muthuraman and Ravikumar 2013).

Co-culture of C2C12 and 3T3-L1 cells. C2C12 and 3T3-L1 cells were co-cultured by using transwell inserts with a 0.4-μm porous membrane to separate. Each cell type was grown independently on the transwell plates. Following cell differentiation, inserts containing 3T3-L1 were transferred to C2C12 cell plates, and inserts containing C2C12 were transferred to 3T3-L1 plates (Sun and Zemel 2008).

Experimental groups. The experimental group was designated as follows group I: control C2C12 and 3T3-L1 cells (mono-culture), group II: mono-culture C2C12 and 3T3-L1 cells with cortisol, and group III: co-cultured C2C12 and 3T3-L1 cells with cortisol.

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 72 h at 37°C in 5% CO_2 prior to harvesting.

Cell viability. Cell viability was measured by 2% trypan blue staining. The number of viable cells was estimated by a counting in a Neubauer chamber in each sample such as groups I, II, and III.

qRT-PCR. Groups I, II, and III cells were lysed in Trizol reagent and total RNA was extracted from all the samples according to the manufacturer's protocol. The first-strand complementary DNA (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. qRT-PCR was performed using a cDNA equivalent of 10 ng of total RNA from each sample with primers specific for Hsp27 (forward: 5′-ACCATT CCCGTCACCTTCC-3′, reverse: 5′-TCTTTACTTGTTTC CGGCTGTT-3′), Hsp70 (forward: 5′-CGTGATGACCGC CCTGAT-3′, reverse: 5′-CGGCTGGTTGTCCGAGTA-3′), and Hsp90 (forward: 5′-TTGGCTATCCCATCACTC-3′, reverse: 5′-TTCTATCTCGGGCTTGTC-3′), and a housekeeping gene GAPDH (forward: 5′-CACCCTCAAGATTGTC AGC-3′, reverse: 5′-TAAGTCCCTCCACGATGC-3′). The reaction was carried out in 10 μl using SYBR Green Master Mix (Invitrogen, Carlsbad, CA) according to the manufacturers' instructions. Relative ratios were calculated based on the $2^{-\triangle\triangle}{}_{CT}$ method (Pfaffl 2001). PCR was monitored using the MiniOpticon Real Time PCR System (Bio-Rad, Alfred Nobel Drive Hercules, CA).

Immunocytochemistry. Following growth and differentiation of C2C12 and 3T3-L1 cells on glass coverslips in 6-well plates. Mono and co-cultured C2C12 and 3T3-L1 cells are treated with cortisol for 72 h. After the treatment, the cells were fixed with 3% formaldehyde in PBS solution for 10 min and then washed in PBS twice. Cells were permeabilized in 0.1% Triton X-100 in PBS for 10 min and then washed in PBS twice. Then, cells were blocked with 3% BSA in PBS for 30 min. After blocking the cells were incubated with primary Hsp27, Hsp70, and Hsp90 antibodies (mouse, monoclonal, 1:500, Santa Cruz Biotechnology, Inc., Texas, USA) for 12 h at 4°C in 1% bovine serum albumin (BSA) in PBS. Following several 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 coverslips were mounted on fluorescent-mounting medium and visualized with fluorescence microscope.

Statistical analysis. All the values are expressed as means± 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.
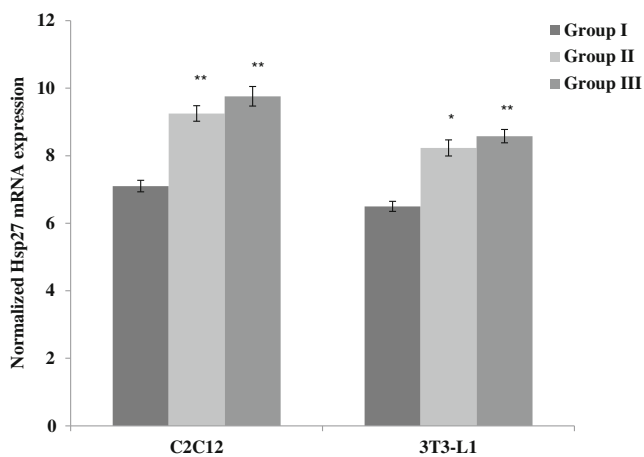
Results and Discussion

Cell viability C2C12 and 3T3-L1 cell viability was determined by 2% trypan blue exclusion method and counting in a Neubauer chamber in each group. C2C12 and 3T3-L1 cell viability was determined after treatment. The mean percentage of viable C2C12 cells 99%, 96%, and 97% in groups I, II, and III sample, respectively. The mean percentage of viable 3T3-L1 cells 98%, 97%, and 97% in groups I, II, and III sample, respectively.

Effect of cortisol on Hsps mRNA expression levels. Hsp27, Hsp70, and Hsp90 mRNA expressions were determined and quantitated in the C2C12 and 3T3-L1 cells. Hsp27 mRNA expression significantly increased 30% and 37.5% in groups II and III C2C12 cells, respectively. Hsp27 mRNA expression increased significantly 26.7% and 32% in the groups II and III 3T3-L1 cells, respectively (Fig. 1). Hsp70 mRNA expression significantly increased 21% and 47.4% in the groups II and III C2C12 cells, respectively. Hsp70 mRNA expression significantly increased 25% and 37% in the groups II and III 3T3-L1 cells, respectively (Fig. 2). Hsp90 mRNA expression significantly increased 23.5% and 29.2% in the groups II and III C2C12 cells, respectively. Hsp90 mRNA expression significantly increased 19.3% and 25.6% in the groups II and III 3T3-L1 cells, respectively (Fig. 3).
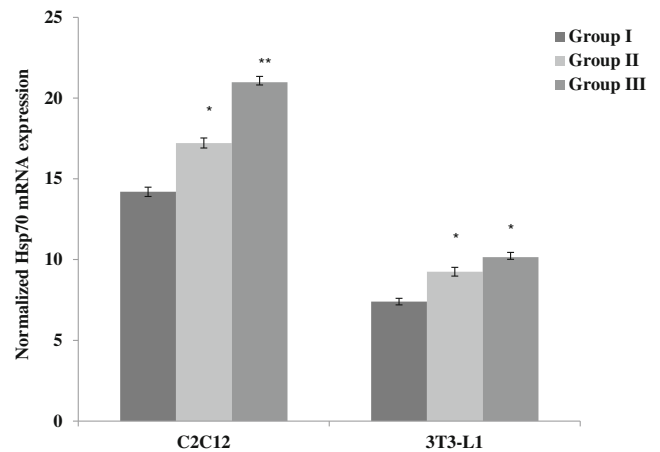
Immunocytochemical analysis. Confocal analysis of C2C12 and 3T3-L1 cells immunostained with Hsps antibodies showed that Hsp27, Hsp70, and Hsp90 distributed throughout the cell. Interestingly, the Hsp27 protein was present abundantly in the mono and co-cultured C2C12 and 3T3-L1 cells (Fig. 4). Hsp70 protein was present abundantly in the mono and co-cultured C2C12 and 3T3-L1 cells (Fig. 5). Hsp90 protein was found in the mono and co-cultured C2C12 and 3T3-L1 cells (Fig. 6).

Variations in the result between mono and co-cultured C2C12 and 3T3-L1 cells. Hsps expression mRNA in groups II and III

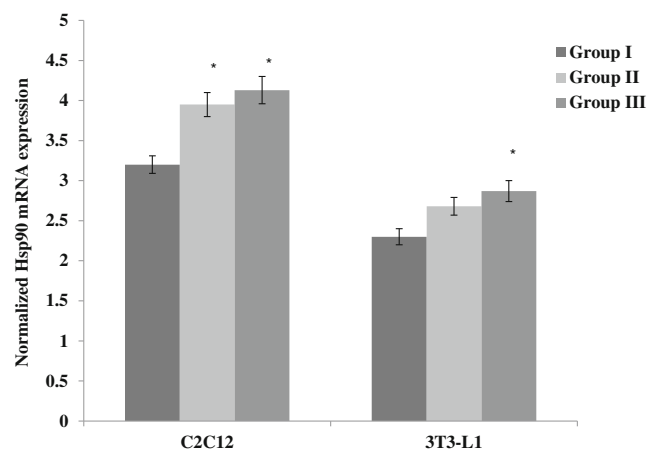

$*p < 0.05$ and $**p < 0.01$

Figure 2 qRT-PCR of Hsp70 in the C2C12 and 3T3-L1 cells. The cultures were incubated with medium containing 10 μg/ml cortisol for 3 d. 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. The significance of differences between the control and treated group was assessed using Student's t test ($*P < 0.05$, $N=3$). $*P < 0.05$ and $**P < 0.01$. *Group I* control C2C12 and 3T3-L1 cells (mono-culture). *Group II* mono-cultured C2C12 and 3T3-L1 cells with cortisol. *Group III* co-cultured C2C12 and 3T3-L1 cells with cortisol.
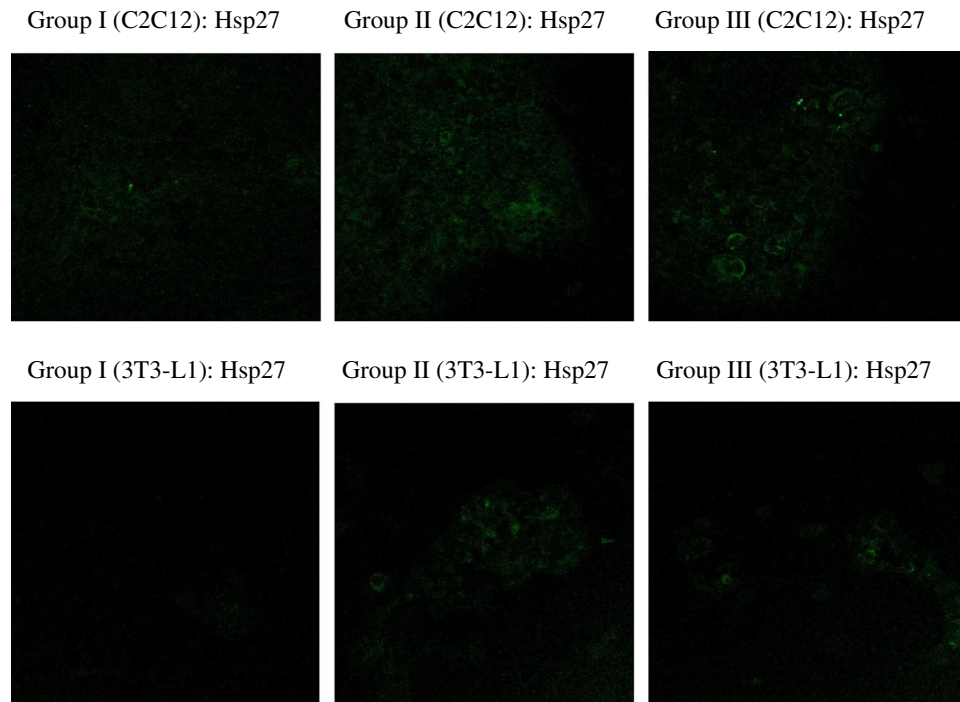
significantly varied from their respective controls. However, there are considerable variations in result between co-culture and mono-culture experiments. There are 7.6%,

$*p < 0.05$ and $**p < 0.01$

Figure 1 qRT-PCR of Hsp27 in the C2C12 and 3T3-L1 cells. The cultures were incubated with medium containing 10 μg/ml cortisol for 3 d. 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. The significance of differences between the control and treated group was assessed using Student's t test ($*P < 0.05$, $N=3$). $*P < 0.05$ and $**P < 0.01$. *Group I* control C2C12 and 3T3-L1 cells (mono-culture). *Group II* mono-cultured C2C12 and 3T3-L1 cells with cortisol. *Group III* co-cultured C2C12 and 3T3-L1 cells with cortisol.

$*p < 0.05$ and $**p < 0.01$

Figure 3 qRT-PCR of Hsp90 in the C2C12 and 3T3-L1 cells. The cultures were incubated with medium containing 10 μg/ml cortisol for 3 d. 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. The significance of differences between the control and treated group was assessed using Student's t test ($*P < 0.05$, $N=3$). $*P < 0.05$ and $**P < 0.01$. *Group I* control C2C12 and 3T3-L1 cells (mono-culture). *Group II* mono-cultured C2C12 and 3T3-L1 cells with cortisol. *Group III* co-cultured C2C12 and 3T3-L1 cells with cortisol.

Figure 4 Immunofluorescence in C2C12 and 3T3-L1 cells. Cells grown on glass coverslips in 6-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 Hsp27 antibody for 12 h at 4°C in PBS-1% BSA. Cells were incubated with FITC-conjugated antibody. The coverslips were mounted on fluorescent mounting medium and visualized on fluorescence microscope. *Green* Hsp27. *Group I* control C2C12 and 3T3-L1 cells (mono-culture). *Group II* mono-cultured C2C12 and 3T3-L1 cells with cortisol. *Group III* co-cultured C2C12 and 3T3-L1 cells with cortisol.
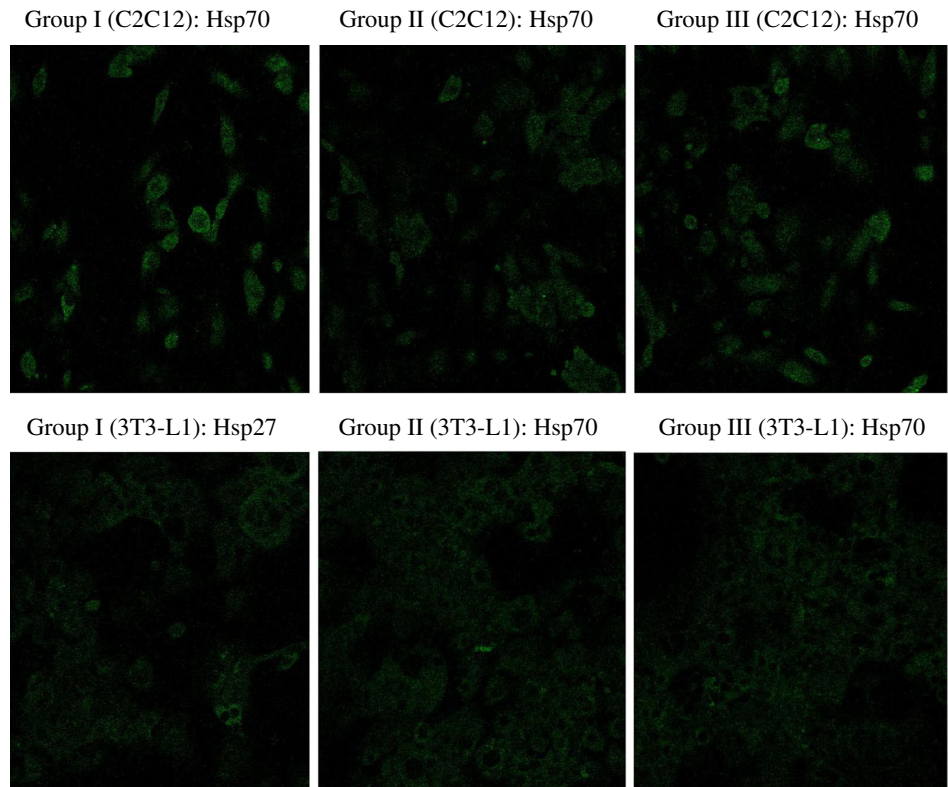

Group I (C2C12): Hsp27 | Group II (C2C12): Hsp27 | Group III (C2C12): Hsp27

Group I (3T3-L1): Hsp27 | Group II (3T3-L1): Hsp27 | Group III (3T3-L1): Hsp27

Green: Hsp27

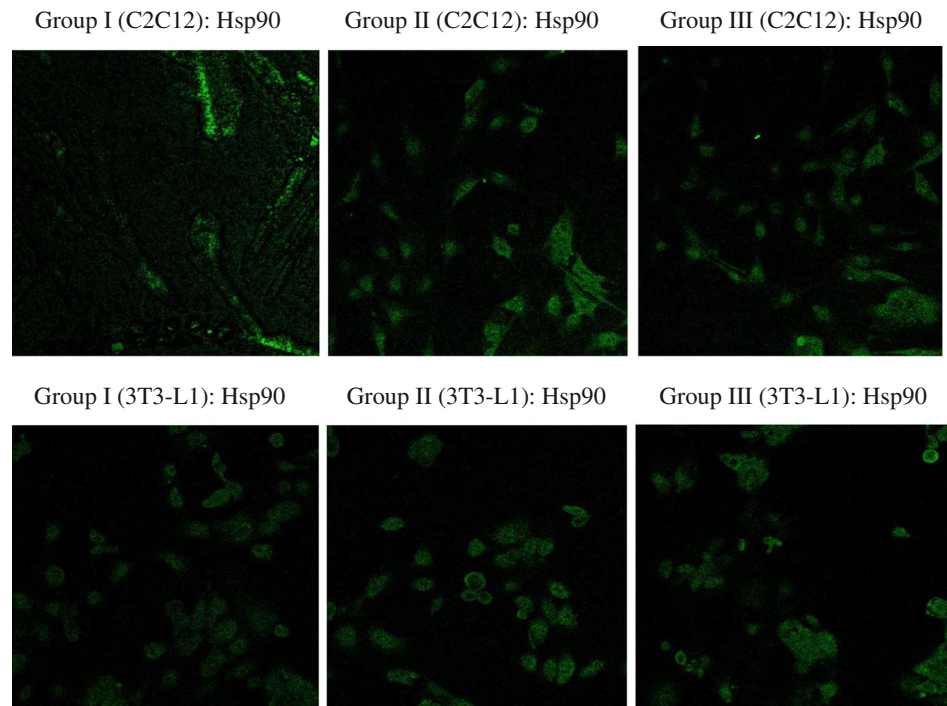16.4%, and 5.7% differences observed between mono- and co-cultured C2C12 Hsp27, Hsp70, and Hsp90 mRNA expressions, respectively, whereas in the 3T3-L1, it was 5.3%, 12%, and 6.3%. In addition, immunostaining

Figure 5 Immunofluorescence in C2C12 and 3T3-L1cells. Cells grown on glass coverslips in 6-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 Hsp70 antibody for 12 h at 4°C in PBS-1% BSA. Cells were incubated with FITC-conjugated antibody. The coverslips were mounted on fluorescent mounting medium and visualized on fluorescence microscope. *Green* Hsp70. *Group I* control C2C12 and 3T3-L1 cells (mono-culture). *Group II* mono-cultured C2C12 and 3T3-L1 cells with cortisol. *Group III* co-cultured C2C12 and 3T3-L1 cells with cortisol.

Group I (C2C12): Hsp70 | Group II (C2C12): Hsp70 | Group III (C2C12): Hsp70

Group I (3T3-L1): Hsp27 | Group II (3T3-L1): Hsp70 | Group III (3T3-L1): Hsp70

Green: Hsp70

Figure 6 Immunofluorescence in C2C12 and 3T3-L1cells. Cells grown on glass coverslips in 6-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 Hsp90 antibody for 12 h at 4°C in PBS-1% BSA. Cells were incubated with FITC-conjugated antibody. The coverslips were mounted on fluorescent mounting medium and visualized on fluorescence microscope. *Green* Hsp90. *Group I* control C2C12 and 3T3-L1 cells (mono-culture). *Group II* mono-cultured C2C12 and 3T3-L1 cells with cortisol. *Group III* co-cultured C2C12 and 3T3-L1 cells with cortisol.

Group I (C2C12): Hsp90   Group II (C2C12): Hsp90   Group III (C2C12): Hsp90

Group I (3T3-L1): Hsp90   Group II (3T3-L1): Hsp90   Group III (3T3-L1): Hsp90

Green: Hsp90

Discussion

Hsps are molecular chaperones that participate in the damaged protein repair process and protein folding. Hsps are ubiquitous in every organism and its level elevates when exposed to high temperature and causes oxidative damage (Feder and Hofmann 1999; Kregel 2002). Increased protein damage will trigger the Hsps mRNA and protein expressions (Anatham et al. 1986; Morimoto 1998). Stress hormones, such as cortisol, secrete due to low blood sugar and other stimuli. Cortisol provides energy by accelerating protein degradation (protein damage). Cortisol increases Hsps mRNA and protein expression by accelerating transcription and translation in mammals, fish, and invertebrates (Udelsman et al. 1993; Murphy et al. 1996; Paroo and Noble 1999; Lacoste et al. 2001; Currie et al. 2008).

Even though some recent studies have reported that Hsps are expressed under physiologic conditions, this plays a very important role in cellular functions (Lindquist and Craig 1988). Consumption of caffeine activates sympathetic activation which in turn leads to increased Hsp72 due to increased production of catecholamines. Increased content of epinephrine found in the caffeine was associated with increased Hsp72 (Whitham et al. 2006). Caffeine is known to stimulate cortisol secretion, which results in the release of Hsp72 in humans (Whitham et al. 2006). Aortic Hsp70 mRNA induction occurs as a direct and specific response to alpha 1-adrenergic receptor stimulation (Udelsman et al. 1994). Oxidative stress produced by an accelerating rate of protein degradation by cortisol and epinephrine and there is a report that oxidative stress increases Hsp70 mRNA and protein levels in C2C12 cells (Jiang et al. 2011).

Cell growth and developments are artificial and unnatural process in vitro condition. Generally, cells are removed from animals and are cultured. Vitamins, minerals, and serum growth factors are provided for the cell viability and growth. Living tissues are complex networks with multiple cellular interactions. The one-dimensional mono-culture technique may not be reliable to mimic the in vivo cell physiology. Recently, the three-dimensional co-culture system has been come into research to evaluate the cellular functions (Dodson et al. 1997).

Co-cultures denote the growth of two different cell types in shared medium, where physical contact between cell types might have influence on cell function. This may be a strong reason for the variation in Hsps expression between mono and co-culture experiments. Our previous research reports also showed that the variation in the calpains expression and, myogenic and adipogenic marker gene expression between mono and co-culture experiments (Muthuraman 2014a, b). The novel co-culture technique may be the most reliable method to mimic the cell physiology compared to mono-culture method. Our co-culture experimental results

intensity differed between the mono and co-culture experiments.

significantly varied from the mono-culture results, which imply more accuracy and importance of co-culture system in the research.

Conclusion

Our co-culture experimental data demonstrates that cortisol increases the Hsps expression in the C2C12 and 3T3-L1 cells. Furthermore, the co-culture experimental results differed from the mono-culture result. In conclusion, the stress hormone augments Hsps expression, may be through the accelerating protein degradation. Furthermore, the three-dimensional co-culture could be considered a more reliable method in the biological research.

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