Impact of stress hormone on adipogenesis in the 3T3-L1 adipocytes

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Abstract Stress hormone is known to play a vital role in lipolysis and adipogenesis in fat cells. The present study was carried out to evaluate the effect of epinephrine on adipogenesis in the 3T3-L1 cells. The investigation on adipogenesis was done in both mono and co-cultured 3T3-L1 cells. 3T3-L1 preadipocytes and C2C12 cells were grown independently on transwell plates and transferred to differentiation medium. Following differentiation, C2C12 cells transferred to 3T3-L1 plate and treated with medium containing 10 µg/ml of epinephrine. Adipogenic markers such as fatty acid binding protein 4, peroxisome proliferator activating receptor, CCAAT/enhancer-binding protein, adiponectin, lipoprotein lipase and fatty acid synthase mRNA expressions were evaluated in the 3T3-L1 cells. Epinephrine treatment reduced adipogenesis, evidenced by reducing adipogenic marker mRNA expression in the 3T3-L1 cells. In addition, glycerol accumulation and oil red-O staining supported the reduced rate of adipogenesis. Taking all together, it is concluded that the stress hormone, epinephrine reduces the rate of adipogenesis in the mono and co-cultured 3T3-L1

cells. In addition, the rate of adipogenesis is much reduced in the co-cultured 3T3-L1 cells compared monocultured 3T3-L1 cells.

Keywords Adipogenesis · 3T3-L1 · Stress hormone · CEBPA

Introduction

Growth, development and differentiation of fat cells are regulated by several factors such as endocrine, paracrine and autocrine molecules. Fat and muscle cell interaction has been implicated to play a vital role in the process of adipogenesis (Dodson et al. 1997). Adipogenesis is regarded as a tightly regulated cellular differentiation process. Differentiated adipocytes reveal morphological change, growth arrest and enhanced expression of lipogenic genes. Ligand-dependent activation of peroxisome proliferator activating receptor (PPAR γ) regulates the complex transcriptional process of adipogenesis (Barak et al. 1999; Kubota et al. 1999; Rosen et al. 1999). In animals, adipose tissue is dynamic which is expanding and shrinking due to hormonal, neurogenic and nutritional signaling (Gesta et al. 2007; Shi and Clegg 2009; Spalding et al. 2008). Adipogenesis is a dynamic developmental process through the conversion of preadipocytes to mature adipocytes. Adipogenic differentiation has been studied at terminal stage

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using preadipocytes (Couture et al. 2009). Adipocytes come from mesenchymal stem cells a common precursor for myocytes, chondrocytes and osteocytes (Billon et al. 2007).

In animals, stress induces secretion of cortisol and epinephrine into the circulation from the adrenal gland (Tai et al. 2007). After sympathetic nerve activation, epinephrine activates various physiological processes (Lundberg et al. 1989). Adipogenesis is the reverse process of lipolysis. Adipose tissue stimulates lipolysis when incubated with epinephrine (Leboeuf et al. 1959). Epinephrine, nor-epinephrine, glucagon, ghrelin, growth hormone, testosterone and cortisol trigger 7TM receptors which activate adenylate cyclase. Adenylate cyclase activation leads to increased production of cAMP, which activates protein kinase A, which activates lipases found in adipose tissue (Macfarlane et al. 2008). The present study was designed to examine the effect of stress hormone on adipogenesis in the 3T3-L1 adipocytes.

Materials and methods

Materials

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

Differentiation

C2C12 and 3T3-L1 preadipocytes (ATCC® CL-173™, Manassas, VA, USA) were incubated at a density of 7,000 cells/cm² and grown in DMEM containing 10 % FBS 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, 0.5 µg insulin and 1 % penicillin/streptomycin. C2C12 cells were grown to 90 % confluence and changed into differentiation medium and fed with fresh differentiation medium every day.

Co-culture

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. Following cell differentiation, inserts containing C2C12 cells were transferred to the 3T3-L1 cell plates (Sun and Zemel 2008).

Treatment of cells

Epinephrine was freshly diluted in the medium before treatment. The cultures were incubated with medium containing with 10 µg/ml epinephrine for 72 h at 37 °C in 5 % CO₂ prior to harvesting.

Experimental groups

The experimental groups were designated as follows: Control 3T3-L1, monocultured 3T3-L1 with epinephrine and co-cultured 3T3-L1 with epinephrine.

Cell viability

Cell viability was measured by 2 % trypan blue staining. The number of viable cells was estimated in each group.

RT-PCR

Cells were lysed in Trizol reagent and total RNA was extracted from each sample 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. RT-PCR was performed using a cDNA equivalent of 10 ng of total RNA from each sample with primers specific for mouse. Fatty acid binding protein 4 (FABP4) (forward: 5'-AGATGAAGGTGCTCTGGT-3', reverse: 5'-CTCATAAACTCTGGTGGC-3'), PPARγ (forward: 5'-CCACCGTTGACTTCTCCA-3', reverse: 5'-AGGC TCCACTTTGATTGC-3'), CCAAT/enhancer-binding protein (CEBPA) (forward: 5'-GCGCAAAGCCAAG AAGTCC-3', reverse: GCGGCTCAGTTGTTCCACC C-3'), adiponectin (forward: 5'-AACATGCCCATTTCG CTTTAC-3', reverse: 5'-ATTACGCTCTCCTTCCCC AT-3'), lipoprotein lipase (forward: 5'-AGGGCTCTGC CTGAGTTGTA-3', reverse: 5'-AGAAATTTTCAAG GCCTGGT-3'), fatty acid synthase (forward: 5'-GTGA GGCTGAGGCTGAGAC-3', reverse: 5'-GGCACGCA GCTTGTAAGTAGA-3') and a housekeeping gene GAPDH (forward: 5'-CACCCCTCAAGATTGTCAGC-3', reverse: 5'-TAAGTCCCTCCACGATGC-3'). The reac

tion was carried out in 10 μ l using SYBR Green Master Mix (Invitrogen) 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).

Determination of glycerol accumulation

3T3-L1 cell differentiation was determined by measuring glycerol accumulation using Adipolysis Assay Kit. Differentiated 3T3-L1 cells were washed two times with PBS and incubated with an incubation solution with 2 % BSA for 1 h. Supernatant was collected and glycerol content was determined by measuring at 540 nm in spectrophotometer.

Oil red O staining

3T3-L1 cells were stained by oil-red O following treatment with epinephrine for 72-h. 3T3-L1 cells were fixed in 10 % formalin for 30 min, washed once with 60 % isopropanol, twice with distilled water and incubated with oil red-O solution for 5 min at room temperature. The cells were stained with hematoxylin, washed with distilled water and photographed using an optical microscope.

Statistical analysis

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

Results

Cell viability

3T3-L1 cell viability was determined by 2 % trypan blue exclusion method and counting in a Neubauer chamber in each group. 3T3-L1 cell viability was determined before and after treatment. The mean percentage of viable 3T3-L1 cells was 98, 97 and 97 % in the control 3T3-L1, monocultured 3T3-L1 with

Fig. 1 RT-PCR of FABP4 in 3T3-L1 adipocytes. The cultures were incubated with medium containing 10 μ g/ml epinephrine. Total RNA was isolated from the control and treated cells with the Trizol reagent. The first-strand cDNA was synthesized from 1 μ g 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 PPAR γ in 3T3-L1 adipocytes. The cultures were incubated with medium containing 10 μ g/ml epinephrine. Total RNA was isolated from the control and treated cells with the Trizol reagent. The first-strand cDNA was synthesized from 1 μ g 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 ** $p < 0.01$

epinephrine and co-cultured 3T3-L1 with epinephrine, respectively.

mRNA expression

FABP4, PPAR γ , CEBPA, adiponectin, lipoprotein lipase and fatty acid synthase mRNA expressions were quantitated by RT-PCR. FABP4 mRNA expression was significantly reduced by 27 and 34 % in the monocultured 3T3-L1 cells with epinephrine and

Fig. 3 RT-PCR of CEBPA in 3T3-L1 adipocytes. The cultures were incubated with medium containing 10 µg/ml epinephrine. Total RNA was isolated from the control and treated cells with the 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. * $p < 0.05$

Fig. 5 RT-PCR of lipoprotein lipase in 3T3-L1 adipocytes. The cultures were incubated with medium containing 10 µg/ml epinephrine. Total RNA was isolated from the control and treated cells with the 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 *** $p < 0.001$

Fig. 4 RT-PCR of adiponectin in 3T3-L1 adipocytes. The cultures were incubated with medium containing 10 µg/ml epinephrine. Total RNA was isolated from the control and treated cells with the 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 *** $p < 0.001$

Fig. 6 RT-PCR of fatty acid synthase in 3T3-L1 adipocytes. The cultures were incubated with medium containing 10 µg/ml epinephrine. Total RNA was isolated from the control and treated cells with the 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. ** $p < 0.01$ and *** $p < 0.001$

co-cultured 3T3-L1 cells with epinephrine group, respectively (Fig. 1). PPAR γ mRNA expression was significantly reduced by 19 and 27 % in the monocultured 3T3-L1 cells with epinephrine and co-cultured 3T3-L1 cells with epinephrine group, respectively (Fig. 2). CEBPA mRNA expression was significantly reduced by 18 and 19 % in the monocultured 3T3-L1 cells with epinephrine and co-cultured 3T3-L1 cells with epinephrine group, respectively (Fig. 3).

Adiponectin mRNA was significantly reduced by 13 and 34 % in the monocultured 3T3-L1 cells with epinephrine and co-cultured 3T3-L1 cells with epinephrine group, respectively (Fig. 4). Lipoprotein lipase mRNA expression was significantly reduced by 32 and 64 % in the monocultured 3T3-L1 cells with epinephrine and co-cultured 3T3-L1 cells with epinephrine group, respectively (Fig. 5). Fatty acid synthase mRNA expression was significantly reduced by 16 and 28 % in the

Fig. 7 Measurement of glycerol accumulation in 3T3-L1 cells following epinephrine treatment. *** $p < 0.001$

monocultured 3T3-L1 cells with epinephrine and co-cultured 3T3-L1 cells with epinephrine group, respectively (Fig. 6).

Glycerol accumulation

Glycerol accumulation in 3T3-L1 cells was determined by measuring the accumulation of glycerol using an Adipolysis Assay Kit. Glycerol accumulation significantly increased 1.2 and 1.4 fold in the monocultured 3T3-L1 cells with epinephrine and co-cultured 3T3-L1 cells with epinephrine group, respectively (Fig. 7).

Oil red O staining

We measured mature adipocytes by oil red-O staining. Mono and co-cultured 3T3-L1 cells had less mature adipocytes compared to control 3T3-L1 (Fig. 8).

Discussion

Muscle mass of animals depends on the rate of protein synthesis and degradation (Higgins et al. 1988). Several factors can produce stress in animals. When an animal experiences stress, hormones, such as adrenaline and cortisol, are released into the body of the animal. Epinephrine and nor-epinephrine belong to adrenaline, responsible for glucose-energy metabolism. Adipogenesis is the reversal of lipolysis. Lipolysis is the breakdown of lipids and involves the hydrolysis of triglycerides into free fatty acids followed by further degradation into acetyl units by

Fig. 8 Differentiated 3T3-L1 preadipocyte stained by *oil-red O* following treatment with epinephrine. (Color figure online)

beta oxidation for energy requirements. Stress hormone trigger 7TM receptors, which activate adenylate cyclase. Activation of adenylate cyclase increased production of cAMP, which activates protein kinase A, activates lipases found in adipose tissue (Macfarlane et al. 2008). Lipolysis occurs abundantly in the fat cells during stress.

Epinephrine which is released from the adrenal medulla is an important mediator of physiological processes during stress. Epinephrine increases protein degradation by increasing calpain proteolytic system. The increased protein degradation and calpain activity may affect the rate of adipogenesis. Adipose tissue

stimulates lipolysis when incubated with epinephrine (Leboeuf et al. 1959). In the present study, epinephrine significantly reduced adipogenic marker gene mRNA expression. In our previous experiment, epinephrine increases calpain activities in C2C12 muscle cells. Thus, treatment of 3T3-L1 cells with epinephrine significantly reduces adipogenesis through lipolytic activity. This is the first study to investigate the effect of epinephrine on adipogenesis in 3T3-L1 adipocytes using a co-culture of C2C12 and 3T3-L1 cells.

Conclusion

In conclusion, the stress hormone (epinephrine) reduces mRNA expression of adipogenic markers in the mono and co-cultured 3T3-L1 cells. In addition, glycerol accumulation and Oil red-O staining data supported the reduced rate of adipogenesis in mono and co-cultured 3T3-L1 cells. Furthermore, the rate of adipogenesis was much reduced in the co-cultured 3T3-L1 cells compared to mono-cultured 3T3-L1 cells. Co-cultured data significantly differed from those obtained with mono-cultured 3T3-L1 cells. Co-culturing is believed to be a more reliable model to study the cellular and physiological functions.

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