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Effect of the Crude Polysaccharides from *Poria cocos* on mRNA Expression of Adipogenic Factors During 3T3-L1 Preadipocyte Differentiation

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Abstract: To study the effect of the crude polysaccharides from *Poria cocos* (PCCP) on mRNA expression of adipogenic factors during 3T3-L1 preadipocyte differentiation. 3T3-L1 preadipocytes were induced to differentiate with MDI (3-isobutyl-1-Methylxanthine, Dexamethasone and Insulin) induction media contained PCCP (50 μg mL^{-1}) and cultured for 8 days. The extent of the differentiation was assessed by Nile Red staining and GPDH activity. In addition, researchers used real-time quantitative PCR to analyzed the expression of genes related to adipocyte differentiation. The results showed that treatment of differentiating 3T3-L1 preadipocytes with PCCP (50 μg mL^{-1}) resulted in an increase in the expression of the adipocyte genes such as Fatty Acid System Synthase (FAS), adipocyte fatty acid binding Protein (aP2), insulin-responsive Glucose Transporter (GLUT4) and transcriptional factor genes such as CCAAT/Enhancer Binding Protein α (C/EBPα), peoxisome Proliferator-Activated Receptor gamma (PPARγ) and Sterol Regulatory Element-Binding Protein 1c (SREBP-1c) to some degree. Overall, the data suggested that there was the tendency of increasing the expression of genes related to adipocyte differentiation in PCCP treated cells.

Key words: *Poria cocos*, polysaccharides, 3T3-L1 preadipocytes, aP2, FAS, C/EBPα, PPARγ, Srebf1, GLUT4

INTRODUCTION

Diabetes mellitus is a life-threatening chronic metabolic disease which is caused by a defect in insulin production, insulin action or both (Laakso, 2001). The worldwide increase in diabetes mellitus is becoming a major health concern. Extensive research has revealed that adipose tissue has an important role in controlling whole-body glucose homeostasis in both normal and disease states (Guilherme *et al*., 2008). Most importantly, adipocytes of type 2 diabetes patients are insulin-resistant and are not capable of accumulating lipids to their full capacity (Anand and Chada, 2000; Okuno *et al*., 1998; Yang *et al*., 2004). Accordingly, adipocyte differentiation in cell culture has been used as a model of insulin insensitivity to study novel antidiabetic drugs (Maeda *et al*., 2001; Staels and Fruchart, 2005). Before the discovery of insulin and the invention of conventional antidiabetic drugs, Chinese people had been using herbal medicine for the treatment of diabetes for a long time (Lau *et al*., 2008). Hundreds of herbs and traditional Chinese medicine formulas have been reported to have been used for the treatment of diabetes mellitus (Jia *et al*., 2003). *Poria cocos*, Bai Fu Ling in Chinese is a medicinal fungus of the family Polyporaceae that grows on the roots of old dead pine trees (Lu *et al*., 2010). It alone or in combination with other herbs is often used to treat diabetes as well as other disorders. Earlier research showed that the antidiabete activity of poria cocos was associated with improvement in insulin sensitivity (Li *et al*., 2011). Polysaccharides as one of the most important components of *Poria cocos* has been demenstrated that it has active role in promoting differentiation 3T3-L1 fibroblasts (Wang *et al*., 2011). In the present study, researcher further evaluated the effect of the PCCP on the expression of genes related to adipocyte differentiation during 3T3-L1 preadipocyte differentiation.

MATERIALS AND METHODS

Cell culture: The 3T3-L1 cell lines were obtained from the Center of Cell Culture Collection of Academia Sinica (Shanghai, China) and the cells were cultured by high glucose Dulbecco's Modified Eagle's Medium (DMEM, Hyclone) supplemented with 10% newborn calf serum (NBS, Gibco) and antibiotics (100 IU mL^{-1} penicillin and 100 μg mL^{-1} streptomycin) in a 5% CO_2 humidified atmosphere at 37°C. Differentitaion was induced by treating the postconfluent 3T3-L1 preadipocytes with a

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cocktail of 170 nM insulin, 1 μM dexamethasone (Sigma) and 0.25 mM isobutylmethylxanthine (Sigma) in DMEM supplemented with 10% NBS for 48 h. The cells were then cultured with DMEM supplemented with 170 nM insulin (Sigma) and 10% NBS for the following 48 h and changed every 2 days. To study effect of PCCP on the expression of adipogenic factors during 3T3-L1 preadipocyte differentiation, the PCCP was kindly provided by pharmacology lab of Sichuan Agricultural University. The total carbohydratic content of the PCCP is 81.3% and the PCCP were added to the culture media at the concentration of 50 μg mL for 8 days. Undifferentiation control was only treated with DMEM supplemented with 10% NBS and medium was changed every other day for 8 days.

Nile Red staining: To visualize intracellular lipids, cells treated with induction media containing PCCP at 50 μg mL^{-1} for 8 days in 96 well culture plate were stained with Nile Red dye (AdipoRed, Lonza). In brief, culture media was removed and washed with PBS (pH 7.4). Then, each well was added with 200 μL of PBS and 5 μL of the Nile Red dye (AdipoRed, Lonza). After 10 min, fluorescent images were captured with an inverted fluorescence microscope (Olympus IX71) equiped with CCD video camera.

GPDH activity assay: After 8 days of culture with PCCP (50 μg mL^{-1}) in the differentiation medium, the media were discarded and cells were rinsed twice with PBS. Cells were then lysed in buffer containing 50 mM Tris buffer (pH 7.5) containing 1 mM EDTA and 1 mM β-mercaptothanol. The cell suspensions were then centrifuged at 12,000 rpm for 20 min at 4°C and the supernatant was used for the estimation of protein concentration and cytosolic GPDH activity. Protein concentration was estimated by the method of Bradford. GPDH activity was measured by the method of Wise and Green (1979).

Total RNA extraction: To test the effects of PCCP on genes related to adipocyte differentiation, 3T3-L1 preadipocytes in 6 well culture plate were induced to differentiation with culture media containing PCCP (50 μg mL^{-1}) for 8 days. Total RNA was extracted using Trizol Reagent (TaKaRa) according to the manufacturer's instructions.

Real time RT-PCR a nalysis: Total RNA was reverse-transcribed by using PrimeScipt™ RT reagent kit (TaKaRa), according to the manufacturer's instructions. Each RT-reaction served as a template in a 25 μL PCR reaction mixture containing 12.5 μL SYBR Premix Ex Taq™ (TaKaRa), 0.5 μL of each primer (10 μM) and 9.5 μL H_2O. The PCR reactions was performed by using the 4Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). SYBR green fluorescence was detected at the end of each cycle to monitor the amount of PCR product. At the end of each run, melting curve profiles were recorded. The primer sequences were listed in Table 1. To normalize each sample for RNA content, ACTB a house-keeping gene was used.

Table 1: Primer sequences

Genes	Orientation	Primer sequence (5'-3')	Amplicon size (bp)
*ACTB**	Forward	agccatgtacgtagccatcc	228
	Reverse	ctctcagctgtggtggtgaa	
PPARγ	Forward	ctgtgagaccaacagcctga	244
	Reverse	aatgcgagtggtcttccatc	
C/EBPα	Forward	gcagtgtgcacgtctatgct	266
	Reverse	aagtcttagccggaggaagc	
Srebf1	Forward	tacttcttgtggcccgtacc	129
	Reverse	tcaggtcatgttggaaacca	
FAS	Forward	tgggttctagccagcagagt	158
	Reverse	accaccagagaccgttatgc	
GLUT4	Forward	actcttgccacacaggctct	174
	Reverse	aatggagactgatgcgctct	
aP2	Forward	tcacctggaagacagctcct	182
	Reverse	aatccccatttacgctgatg	

Data analysis: Results are expressed as the means±SEM of triplicate determinations. The data was analyzed by ANOVA procedure of Statistical Analysis System (SAS Institute Inc., Cary, NC). Statistical significance was established at the $p<0.05$ level.

RESULTS

Nile Red staining: Nile Red is a fluorescent dye which has also been shown to be highly selective and specific for neutral lipids (Greenspan *et al*., 1985). Figure 1a shows the staining of undifferentiated 3T3-L1 cells and there is no clearly visible lipid droplets in 3T3-L1 cells. Figure 1b and c, respectively shows the staining of 3T3-L1 differentiating cells untreated with PCCP (control group) and treated with PCCP (50 μg mL^{-1}). And there are clearly visible lipid droplets in 3T3-L1 cells. However, 3T3-L1 cells treated with PCCP at the concentration of 50 μg mL^{-1} have larger lipid droplets in size than control group in representative images.

GPDH activity assay: The extent of the differentiation was also assessed by determination of GPDH activity on day 8 of culture. GPDH was used as a marker of late adipocyte differentiation which can reflect the extent of fat cell adipogenesis and differentiation. As shown in Fig. 2, PCCP at the concentration of 50 μg mL^{-1} increased GPDH

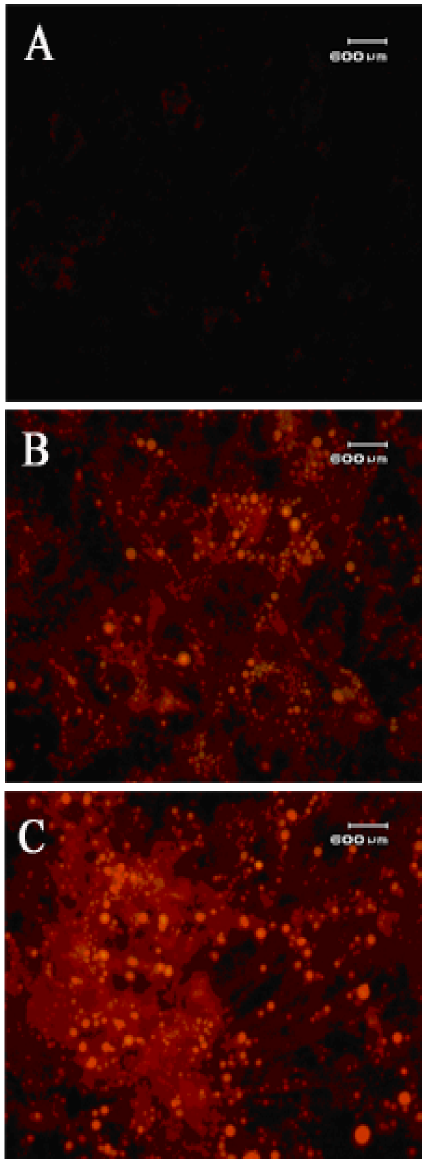

Fig. 1: Nile Red staining of 3T3-L1 cells treated with induction medium contained the PCCP (50 μg mL^{-1}). A) Undifferentation control cells. B) Cells treated with PCCP (0 μg mL^{-1}). C) Cells treated with PCCP (50 μg mL^{-1})

activity in differentiating preadipocytes compared with control group ($p<0.05$). It is consistent with changes of the morphology.

Effect of PCCP on mRNA expression of adipogenic factors: Adipogenesis is accompanied by increased expression of various transcriptional factors and adipocyte specific genes. On day 8, researchers measured the expression level of mRNA of adipogenic factors,

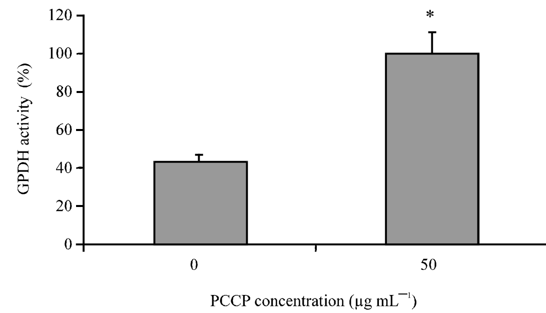

Fig. 2: Effect of PCCP on GPDH activity in 3T3-L1 cells. 3T3-L1 cells were treated with PCCP (50 μg mL^{-1}) in differentiation media for 8 days. Assays were performed in three replicates for each treatment. *Denoted that the difference is significant compared with control, $p<0.05$

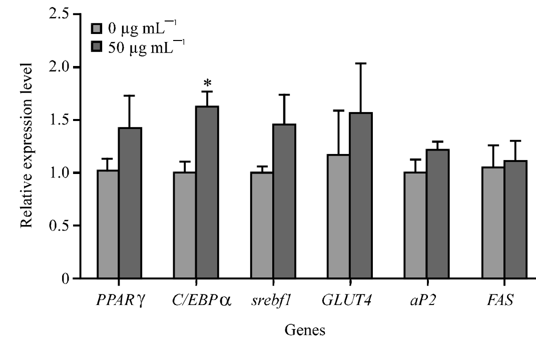

Fig. 3: mRNA expression of PPARγ, C/EBPα, Srebf1, GLUT4, FAS and aP2 in 3T3-L1 cells treated with PCCP (50 μg mL^{-1}). *Denoted that the difference is significant compared with control, $p<0.05$

PPARγ, C/EBPα, Srebf1, GLUT4, FAS and aP2, in 3T3-L1 cells untreated with PCCP (control group) and treated with PCCP (50 μg mL^{-1}) by real-time RT-PCR. As shown in Fig. 3, PCCP increased the expression of PPARγ, C/EBPα and Srebf1 mRNA in 3T3-L1 cells compared with controls, by approximately 39.25, 60.80 and 44.43%, respectively. In addition as shown in Fig. 3, GLUT4, FAS and aP2 mRNA increased in 3T3-L1 cells compared with controls by approximately 33.70, 5.72 and 19.96%, respectively. Thus, there was a trend toward increased expression of adipogenic factors in PCCP treated cells.

DISCUSSION

Besides polysaccharides exhibit various bioactivies such as antitumor, antibacterial and immunological activities (Kim *et al*., 2008; Mizuno *et al*., 1996; Zhu *et al*., 2012) several polysaccharides have been revealed the

antidiabete activity (Yanga *et al*., 2008). Previous research showed that polysaccharides exerted antidiabete effect through increasing insulin release and insulin sensitivity (Cho *et al*., 2007). Furthermore, antioxidation activity of polysaccharides is helpful to protect Β-cells in the pancreas against damaging effect of free radicals (Zhao *et al*., 2007). The preliminary research has demonstrated that PCCP (50 μg mL^{-1}) have active role in promoting differentiation of 3T3-L1 cells (Wang *et al*., 2011). The results of GPDH activity assay further confirmed it again. Thus, researcher postulate that PCCP have potential antidiabete activity. In the present study, researchers further explored the effect of PCCP on mRNA expression of adipogenic factors during 3T3-L1 preadipocyte differentiation.

During preadipocytes differentiation, many transcription factors including PPARγ and C/EBPα are sequentially activated to regulate the adipogenic gene expression cooperatively (Lin *et al*., 2007). C/EBPα is an important adipocyte transcription factor which binds to and transcritionally activates a number of adipocyte specific genes including *aP2*, *SCD1*, *GLUT4* and *ob* genes (Qian *et al*., 1998). The results revealed that PCCP significantly increased C/EBPα mRNA expression compared with control group ($p<0.05$). The effect of PCCP on C/EBPα mRNA expression was consistent with Astragalus polysaccharides (Liu *et al*., 2007). The PPARα is a member of the nuclear receptor superfamily of transcription factors and was considered as a master transcriptional regulators of adipogenesis and drive adipocyte-specific gene expression (Ji *et al*., 2010). PPARγ mRNA was up-regulated with PCCP treatment in 3T3-L1 cells to some degree but there was no significant difference compared with control group ($p>0.05$). SREBP-1c belongs to the SREBP family of Basic Helix-Loop-Helix Leucine-Zipper (bHLH-LZ) transcription factors which is involved in adipocyte differentiation. SREBP-1c was encoded by *Srebf1* gene (Fujimori *et al*., 2007). As same as PPARγ, PCCP induced an increase in the abundance of Srebf1 mRNA in 3T3-L1 cells but there was no significant difference compared with control group ($p>0.05$).

GLUT4 was a member of the facilitative sugar transporter family and was responsible for facilitating glucose uptake into adipocytes which is expressed during the differentiation of 3T3-L1 cells from preadipocytes to adipocytes (Yokomori *et al*., 1999). In the present study, GLUT4 were higher in PCCP treated cells than in control group but the difference was not statistically significant ($p>0.05$). As the another component of the *Poria cocos*, pachymic acid significantly up-regulated the GLUT4 mRNA (Huang *et al*., 2010). The aP2 is a key mediator of intracellular transport and metabolism of fatty acids (Sun *et al*., 2003). FAS plays a central role in *de novo* lipogenesis in mammals. This study showed PCCP mildly increased FAS an aP2 mRNA expression ($p>0.05$).

In the present study, researchers just researched the effect of PCCP on the mRNA expression of adipogenic factors at transcriptional level and a post-transcriptional research was needed to further explore.

CONCLUSION

In summary, there was the tendency to increase the expression of genes related to adipocyte differentiation in PCCP treated cells.

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