Effects of Rosiglitazone on Adiponectin Expression in 3T3-L1 Adipocytes at High Levels of Both Testosterone and Insulin

Yu-xia WANG¹, Wei-jie ZHU², Hai-yan ZHANG²

1. Department of Obstetrics and Gynecology, the First Affiliated Hospital, Jinan University, Guangzhou 510630, China
2. Institute of Reproductive Immunology, Jinan University, Guangzhou 510632, China

Objective To evaluate effects of rosiglitazone (RSG) on the expression of adiponectin in mature adipocytes at high levels of both testosterone (T) and insulin in vitro culture.

Methods Mouse 3T3-L1 preadipocytes were induced to be mature adipocytes and used in this study. According to RSG concentrations, the cells added with T (10⁻⁵ mol/L) and insulin (10⁻⁴ mol/L) were divided into 4 groups: free-RSG group (0 mol/L RSG, FR-TI), low-dose group (10⁻⁹ mol/L RSG, LR-TI), middle-dose group (10⁻⁷ mol/L RSG, MR-TI) and high-dose group (10⁻⁶ mol/L RSG, HR-TI). Besides, the cells added with RSG without T and insulin were also divided into 4 groups: FR, LR, MR, and HR. These 8 groups were incubated for 42 h. Cell viability was determined by MTT assay. Expression of adiponectin was detected by Western blotting.

Results The maximum viability in FR-TI group was observed at point of 42 h. The growth of the adipocytes was significantly inhibited in MR-TI group compared with FR-TI (P<0.01). The level of adiponectin in MR-TI group was higher than that in LR-TI group (P<0.01). However, with RSG increasing, HR-TI group showed the lowest level of adiponectin among three treatment groups (P<0.01). In addition, adiponectin expression in MR-TI group was significantly higher than that in MR group (P<0.01).

Conclusion RSG could increase the expression of adiponectin in 3T3-L1 adipocytes under high levels of both T and insulin, but it acts in a narrow concentration range.

Key words: adiponectin; rosiglitazone (RSG); 3T3-L1 adipocyte; polycystic ovary syndrome (PCOS)
Adiponectin is one of the most abundant plasma protein adipocytokines, secreted specifically from adipose tissue, that displays protective actions in obese and diabetic humans\(^1\). Previous studies show that adiponectin has roles on anti-inflammatory, anti-atherogenic and insulin-sensitizing properties, which have many health benefits, such as the improvement of insulin sensitivity\(^2,3\). Increasing evidences indicate that adiponectin levels have an inverse relationship with insulin resistance (IR) in obesity, type II diabetes, dyslipidemia, hypertension and cardiovascular disease, as well as in polycystic ovary syndrome (PCOS)\(^4,5\). A Meta-analysis has suggested that there are lower circulating levels of adiponectin in women with PCOS\(^6\). However, the metabolic mechanism of adiponectin has not been fully understood, and it is unclear so far whether adiponectin is a primary cause or secondary to or in parallel with IR. PCOS is a common endocrine disorder in women of reproductive age that is characterized by chronic anovulation, hyperandrogenism and polycystic ovaries\(^7\). Frequently, IR accompanied by compensatory hyperinsulinaemia plays a central role in the pathophysiology of PCOS\(^8\). In recent years, there has been increased focus on adipocytokines such as leptin, adiponectin, visfatin, resistin, interleukin-6 (IL-6), and tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)), which may mediate the lipid metabolism disturbance in PCOS, among which the association between adiponectin levels and PCOS remains controversial\(^9\).

The nuclear receptor peroxisome proliferator-activated receptor-\(\gamma\) (PPAR\(\gamma\)), expressed predominantly in adipose tissue, is a main regulator of insulin sensitivity at a cellular level in adipocytes\(^10\). It is necessary and sufficient for adipogenesis and is also critical for functions of mature adipocytes, including lipid metabolism, adipokine secretion, and insulin sensitivity\(^11\). Rosiglitazone (RSG), one of high-affinity ligands for PPAR\(\gamma\), acts in vivo as potent insulin sensitizers, which could improve IR of PCOS in clinic\(^12\). However, the underlying molecular mechanism on RSG treatment associated with adipocyte-specific genes in PCOS has not been fully clarified.

Because hyperandrogenism and hyperinsulinaemia play important roles in both endocrine and lipid metabolism disorder in PCOS, in the present study, we selected 3T3-L1 adipocytes as the model with high levels of testosterone (T) and insulin to form the external environment of PCOS, aiming to clarify effects of RSG on the expression of adiponectin in mature adipocytes under such conditions and provide better understanding on the etiology of PCOS related to the mechanism of lipid metabolism.

**Materials & Methods**

**Cell culture and differentiation**

Mouse fibroblast line 3T3-L1 preadipocytes were obtained from Shanghai Cell Bank (Chinese Academy of Sciences), and maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Sigma, USA),
100 IU/ml penicillin and 0.1 μg/ml streptomycin. After cell confluence, 3T3-L1 preadipocytes were differentiated using a protocol similar to those described previously[13]. Cells were induced to differentiate by treatment with an induction medium containing 0.5 mmol/L isobutylmethylxanthine (IBMX, Sigma-Aldrich, USA), 10 μg/ml insulin (Sigma, USA) and 1 μmol/L dexamethasone (DEX) for 2 d. The cells were then incubated for 2 d with DMEM supplemented with 10% FBS and 10 μg/ml insulin, and the cells were then fed every 2 d with DMEM containing 10% FBS. Cell differentiation was monitored by evaluating cell morphology under phase-contrast microscopy. Cells were considered to be adipocytes when numerous lipid droplets were observed in the cytoplasm.

Cell treatment

According to RSG concentrations, mature adipocytes added with T (10^-5 mol/L) and insulin (10^-4 mol/L) were divided into 4 groups[14,15]: free-RSG group (0 mol/L RSG, FR-TI), low-dose group (10^-9 mol/L RSG, LR-TI), middle-dose group (10^-7 mol/L RSG, MR-TI) and high-dose group (10^-6 mol/L RSG, HR-TI). Besides, the cells added with RSG without T and insulin also were divided into 4 groups: FR, LR, MR and HR (the concentrations of low-, middle- and high-dose RSG were the same as the above mentioned concentrations). The above 8 groups were incubated for RSG stimulating the cells for 42 h[16]. After the indicated treatment times, the cells were used for later experimental uses.

MTT assay

3T3-L1 adipocytes were cultured in 96-well plates. For the 0 h, 12 h, 24 h, 42 h, 48 h, and 72 h experimental time-points, the cells received 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, USA) and were further incubated for 4 h. The purple formazan crystals were then dissolved with DMSO for 10 min before their absorbance values were read on a microplate reader at 490 nm.

Western blotting

Western blotting was performed according to our laboratory method[17]. Briefly, total protein was extracted from the adipocytes using a cell lysis buffer and protease inhibitor cocktail. Following centrifugation at 10 000×g and 4 °C for 15 min, the protein concentration was assessed using the Bradford protein assay kit (Bio Rad Laboratories, USA). Total samples (20 μg) were loaded onto a 12% SDS PAGE gel and transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences, USA). The membranes were blocked using 5% non fat dry milk in a Tris buffered sodium chloride Tween 20 (TBST) solution (20 mmol/L Tris, pH 7.6, 137 mmol/L sodium chloride and 0.1% Tween 20) at room temperature for 1 h. The PVDF membranes were subsequently incubated with monoclonal antibodies against mouse adiponectin (1 : 1 000; Abcam, USA) and anti-GAPDH antibody diluted 1 : 500 overnight at 4 °C. Following this, the membranes were incubated with secondary antibody goat anti rabbit HRP (1 : 2 000; Abcam, USA) at room temperature for
2 h, following 10 min wash for three times in TBST. The signals were displayed by an enhanced chemiluminescence kit. Antigens were visualized by imaging acquisition and quantified by densitometry.

**Statistical analysis**

Results were expressed as the mean ± standard deviation (\( \bar{x} \pm s \)) of three independent experiments. Groups were compared by one-way ANOVA followed by LSD and Duncan. A \( P \)-value <0.05 was considered to be significant. Statistics were analyzed by using SPSS 16.0 software.

**Results**

**Cell viability of mature adipocytes**

Cell viability of mature adipocytes in FR and FR-TI groups during incubating period is showed in Figure 1. At the point of 42 h, maximal viability was observed in both FR and FR-TI groups. As shown in Figure 2, after treated with RSG, the growth of the cells was significantly inhibited in HR, MR, and LR groups when compared with FR group (\( P<0.01 \)). However, RSG did not affect the viability of cells in HR-TI group. RSG decreased cell viability in MR-TI and LR-TI groups compared with FR group (\( P<0.01 \)). The maximal inhibition for cell viability was observed in MR group among treatment groups (\( P<0.01 \)).

**Adiponectin protein expression**

Levels of adiponectin protein expression for 8 groups are shown in Figure 3.

Adiponectin in LR group was significantly higher than that in FR group (\( P<0.01 \)). But with the concentration of RSG increased, the levels of adiponectin in MR and HR groups were significantly lower than that in FR group (\( P<0.01 \)).

MR-TI group had the maximum level of adiponectin among treatment groups (\( P<0.01 \)). However, at high level of RSG, the expression of adiponectin in HR-TI group decreased and reached the lowest level, even lower than that in FR-TI group (\( P<0.01 \)).

![Figure 1 Cell viability of mature adipocytes in FR and FR-TI groups during 72 h incubation (n=3)](image)
Figure 2  Cell viability of mature adipocytes after RSG treatment ($n=3$)

*: $P < 0.01$, compared with FR group; #: $P < 0.01$, compared with MR-TI group
&: $P < 0.01$, compared with others; $\$: $P < 0.01$, compared with LR group

Figure 3  Levels of adiponectin protein expression in mature adipocytes ($n=3$)

*: $P < 0.01$, compared with FR group; #: $P < 0.01$, compared with MR-TI group
&: $P < 0.01$, compared with others; $\$: $P < 0.01$, compared with LR group
Compared between MR and MR-TI groups, the latter had higher adiponectin expression ($P<0.01$). On the other hand, at low and high doses of RSG, lower adiponectin expressions in LR-TI and HR-TI groups were determined than those in LR group and HR group, respectively ($P<0.01$).

**Discussion**

As one of adipocytokines in PCOS, adiponectin plays an important role in the pathogenesis of the metabolic syndrome\cite{18}. In the present study, both hyperandrogenism and hyperinsulinism that was similar to the environment of PCOS were imitated to culture 3T3-L1 cells for investigating effects of RSG on the expression of adiponectin. The results demonstrated that LR-TI and MR-TI groups had increased adiponectin levels, which indicated that RSG could promote the synthesis of adiponectin protein under high levels of T and insulin. Because RSG is a PPAR$\gamma$ ligand\cite{10}, changes of levels of adiponectin suggested that RSG might affect the secretion of adiponectin through PPAR$\gamma$ pathway, thereafter ameliorate IR of mature adipocytes at high levels of T and insulin. On the other hand, when RSG was increased to the high dose, adiponectin protein was significantly reduced. This phenomenon indicated that RSG enhanced protein synthesis of adiponectin would be in a very narrow range.

PPAR$\gamma$ ligand promotes adipocyte differentiation by regulating the secretion of adipocytokines\cite{19}. In this research, it was observed that RSG had a weak effect on mature adipocytes to increase the level of adiponectin. Moreover, when the cells were interfered by T and insulin, there was a trend that adiponectin expression increased after RSG treatment. This further explained that RSG may up-regulate the expression of PPAR$\gamma$, through which several adipocytokines would be increased or decreased in mature adipocytes. Thus, combined with the existing literature and this study, we could speculate that hyperandrogenism and hyperinsulinism might induce or at certain degree deteriorate IR of PCOS.

In this study, it was not proved that the RSG enhanced adiponectin in a dose-dependent manner, which manifested in previous report for other adipocytokine\cite{20}. RSG increased the viability of 3T3-L1 adipocytes in high concentration, whereas increased adiponectin level in mid-concentration of RSG. In PCOS patients, PPAR$\gamma$ ligand also may lead to abnormal biological significance when excessively high dose was used. The results from this study indicated that maybe there is an endogenous molecule mediates the effect of RSG on PPAR$\gamma$, and multiple mechanisms would exist for inhibition of adiponectin or even differentiation of adipocytes.

In summary, this study revealed that RSG could increase expression of adiponectin in 3T3-L1 adipocytes at high levels of both T and insulin, but it acts in a narrow concentration range. Further researches should clarify the relationship on the regulation of IR and hyperandrogenism involved in PCOS between adiponectin and other adipocytokines.
References


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Conference Information

3rd Annual Mayo Clinic Collaborative Symposium: Update in Minimally Invasive Gynecologic Surgery
February 5th to 7th Arizona / Scottsdale Obstetrics / Gynecology
Contact: Gloria Cadden, CME Department, Mayo Clinic Scottsdale
Phone: 480-301-4580
Fax: 480-301-9176
E-mail: cadden.gloria@mayo.edu
Website: http://www.mayo.edu/cme/surgical-specialties-2015s978

International Symposium on Cancer in Young Women: Pregnancy & Fertility
February 5th to 6th Belgium / Leuven Obstetrics / Gynecology, Oncology
Contact: Liesbeth Leemans, Gynaecologische Oncologie, KU Leuven
Phone: 011-32-1-634-2876
E-mail: liesbeth.leemans@uzleuven.be
Website: http://kuleuviancongres.be/cancerinyoungwomen

56th Annual Obstetrics & Gynecology Update
February 8th to 11th Utah / Park City Obstetrics / Gynecology
Contact: Natalie Moore, University of Utah
Phone: 801-581-5501
Fax: 801-585-5146
E-mail: natalie.moore@hsc.utah.edu
Website: http://cmetracker.net/UUHSC/Catalog?sessionType=C

20th Annual Multidisciplinary Symposium on Breast Disease
February 12th to 15th Florida / Amelia Island Obstetrics / Gynecology, Oncology
Contact: Continuing Medical Education, University of Florida College of Medicine
Phone: 352-733-0064
Fax: 352-733-0007
E-mail: cme-mail@ufl.edu
Website: http://msbd.cme.ufl.edu/

Expert Fetal Medicine
February 12th to 13th United Kingdom / London Obstetrics / Gynecology
Contact: The Symposium Office, Imperial College London
Phone: 011-44-20-7594-2150
Fax: 011-44-20-7594-2155
E-mail: sympreg@imperial.ac.uk
Website: http://www.symposia.org.uk/main/eventprog.asp?evcd=15.01