Scientific Research and Essays Vol. 5(13), pp. 1632-1639, 4 July, 2010
Available online at http://www.academicjournals.org/SRE
ISSN 1992-2248 ©2010 Academic Journals

Full Length Research Paper

Relationship between insulin resistance and adiponectin expression in a rat model of polycystic ovary syndrome

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Accepted 4 June, 2010

Our study investigated the relationship between insulin resistance (IR) and adiponectin expression in the adipose tissue of rat polycystic ovarian syndrome (PCOS) model. Female rats were divided into two equal groups according to their age, size and vitality. In Group 1, rats received subcutaneous injections of DHEA (once daily) for 20 consecutive days to induce PCOS, and those in Group 2 were injected with oil at the same period. Ovary weight, serum insulin and sex hormone levels were determined. Oral glucose tolerance test, light and electron microscopy were also performed. The adiponectin mRNA and protein in the adipose tissue were also measured. The ovary weight in Group 1 was higher than that in Group 2 ($p < 0.05$). Numbers of follicular cysts and corpora lutea in Group 1 were also significantly increased. The levels of serum testosterone and estradiol, fasting serum glucose and insulin were dramatically elevated when compared with those in Group 2 ($p < 0.05$). Additionally, the expression of adiponectin mRNA and protein was markedly down-regulated in the white adipose tissues of Group 1. The absence of adiponectin may play an important role in the pathogenesis of IR in PCOS, which makes adiponectin a promising therapeutic strategy in patients with IR induced by PCOS.

Key words: Polycystic ovarian syndrome, adiponectin, insulin resistance, sex hormone, animal model.

INTRODUCTION

Polycystic ovary syndrome (PCOS), one of the most common endocrine and metabolic disorders of premenopausal women, is characterized by menstrual disturbance, infertility, polycystic ovaries, hirsutism and hyper-androgenism. 50–70% of PCOS patients exhibit some degree of insulin resistance (IR); a leading risk factor for the development of type 2 diabetes mellitus and cardiovascular disease (Lobo and Carmina, 2000; DeUgarte et al., 2005). Obesity appears to be closely associated with PCOS, and more than half of the patients with PCOS are overweight or obese (Azziz et al., 2004). However, the severity of IR accompanied with obesity varies considerably in patients with PCOS, and the correlation between obesity and IR remains not well understood.

Obesity is defined as an increase of adipose tissue mass. However, the etiology of IR and the primary factors involved in the uptake and metabolism of glucose by skeletal muscles are still unclear (DeFronzo et al., 1981). In respect of PCOS, increasing attention has been paid to numerous products secreted by adipocytes. The term "adipokines" has been used to describe those factors produced by adipocytes, including tumor necrosis factor (TNF), interleukin-6 (IL-6), leptin, resistin, and adiponectin (Trayhurn et al., 2006; Burks et al., 2000; Kong et al., 2006). There is growing evidence that these factors are the important determinants of IR through exerting effects on circulating hormones or adipocytes. But data concerning the relationship between adiponectin and IR in PCOS patients are still controversial (Toulis et al., 2009; Gulcelik et al., 2008).

Some studies indicated that low adiponectin level in PCOS women was closely correlated with obesity (Orio

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et al., 2003; Panidis et al., 2003; Carmina et al., 2005), while others found a relationship between adiponectin and IR which was independent of obesity (Spranger et al., 2004; Ducluzeau et al., 2003; Sieminska et al., 2004; Ardawi and Rouzi, 2005).

A variety of studies have examined serum adiponectin in human beings and indicated decreased level in obese and diabetic individuals, which was inversely associated with some parameters altered in the IR (Weyer et al., 2001). In addition, a few studies have been conducted to determine the relationship between adiponectin, obesity and IR in human beings. However, the expression of adiponectin has not been thoroughly examined in trails and the correlation between adiponectin and other cytokines has not been well understood.

It is difficult to evaluate the etiology and development of human PCOS due to different ethnic backgrounds (Diamanti-Kandarakis et al., 2008; Sáez et al., 2008), but the similarities in key steps of mammalian reproduction make animal models attractive for studying the pathogenesis of this syndrome. On the basis of a successfully established rat PCOS model, this study was performed to detect the expression of adiponectin in adipose tissue of rat model. It was evaluated that there was an inverse association between adipose tissue adiponectin and insulin resistance. These data suggest that adiponectin plays an important role in obesity-associated insulin resistance of PCOS.

MATERIALS AND METHODS

Animals and experimental protocols

The experimental design as well as animal care and use was approved by the ethics committee of Jinan University and conformed to the National Institute of Health guidelines on the ethical use of animals. Twenty one-day-old Sprague-Dawley (SD) female rats were purchased from the Medical Experimental Animal Center of Guangdong Province. These rats were divided into two equal groups according to their age, size and vitality (n = 19, each group). In Group 1, rats were subcutaneously injected with dehydroepiandrosterone (DHEA) (6 mg/100 g body weight dissolved in 0.20 ml of sesame oil; once daily) for 20 consecutive days. Rats in Group 2 (Control Group) were injected with 0.2 ml of sesame oil daily for 20 consecutive days (Henmi et al., 2001).

The rats were fasted overnight before tail-vein blood samples were obtained, followed by glucose gavage (1.5 g/ml/kg of body weight). Then, anti-coagulated blood was collected at 30, 60 and 120 min after gavage, followed by centrifugation. The blood samples were stored at -20℃ for assay. All protocols were proved by the Ethics Committee of Ji'nan University.

Tissue collection

Rats were sacrificed by decapitation, and blood was collected for measurement of serum sex hormones. The serum and red blood cells were separated by centrifugation. The serum was stored at -20℃ for subsequent assay. Ovaries were removed and weighed. A fraction of ovaries was fixed in 4% formaldehyde for hematoxylin and eosin (HE) staining for light microscopy. Parametrial adipose tissues were rapidly removed and stored at -80℃ until use.

Detection of plasma glucose and insulin

Serum glucose level of different time points was detected by JINGDU glucometer (GT-1640 automatic analyzer, Japan). Serum insulin was detected by RIA analysis with a RIA kit (Beckman Company, America).

Hormone assay

After collection, culture medium and rat serum samples were stored at -20℃ for hormone measurement. Serum sex hormones were detected by chemiluminescence analysis with a CLIA kit (Beckman Company, USA). The detection limit of the assay was 0.05 - 10 ng/ml. Testosterone and progesterone levels were measured using electrochemiluminescence immunoassays on an Elecsys E-170 analyzer (Roche Diagnostics Systems, Basel, Switzerland). The intra- and inter-assay coefficients of variation were 5 and 8.0%, respectively. The intra-assay coefficient of variation for estradiol was 14%. The intra-assay coefficient of variation for LH was 5.6%, and the interassay coefficient was 13.9%. For FSH, the intra-assay and interassay coefficients of variation were 3.0 and 5.0%, respectively. The sensitivity of the method defined as the detectable concentration was 0.15 pg/ml.

RNA extraction and RT-PCR

Total RNA was extracted from the isolated adipose tissues of each rat with a Trizol Reagent kit (Roche Company, Switzerland). The integrity of the extracted total RNA was displayed by 1% agarose gel electrophoresis and the RNA concentration was deter-mined under ultraviolet (UV) at 260 nm. One microgram of RNA was used for reverse transcription polymerase chain reaction (RT-PCR) with a RT-PCR kit (MBI Company, China).

The cDNA was amplified by PCR using specific primers for rat adiponectin cDNA, previously reported by Kreier et al. (2002). The primers were as follows: for adiponectin, forward: 5'-ACAAGGCCGTTCTCTTCACCTA-3'; reverse: 5'-GGTCCACATTCTTTTCCTGATACTG-3'. The expected size of amplification was 52 bp. To normalize the mRNA in each sample, cDNA was also amplified using primers specific to β-actin(forward: 5'-CCTAAGGCCAACCGTAAAG-3'; reverse: 5'-TCTTCATGGTGCTAGGAGCCA-3'). The expected size of amplification was 623 bp.

Thirty five cycles of amplification were performed and each cycle consisted of denaturation at 95℃ for 30 s, annealing at 60℃ for 1 min, and extension at 70℃ for 1 min, with an additional extension at 72℃ for 10 min. Five microliters of RT-PCR products were loaded onto 1.5% agarose gel, and subsequently visualized and quantified using GDS-8000 Gel Scientific Image System and Image Quanta Analysis Software (Amersham Pharmacia Biotech, Hong Kong). The relative levels of adiponectin and β-actin mRNA in the original total RNA extracted from the adipose tissue were obtained. Adiponectin gene expression was normalized by the β-actin expression.

Western blot

Total protein was extracted from adipose tissues. Twenty micrograms of protein was subject to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The samples were electrophoretically transferred to a PVDF membrane using transfer buffer. The membrane was then incubated with rabbit

adiponectin antibody (1:1000), and then incubated with streptavidin sheep-radish peroxidase conjugate (diluted 1:5000). The membrane was rinsed with TBST four times and the protein was visualized using an enhanced chemiluminescence system (ECL) and X-ray film, followed by analysis with the Gel Images Analysis System (UVP, USA).

Statistical analysis

All data were presented as means ± SE. Student *t* test was performed using SPSS, Version 10.0 Software (Qinghua University, China). A value of $p < 0.05$ was considered statistically significant.

RESULTS

Morphological changes of ovaries and serum sex hormone levels

As described in former essays (Wang et al., 2004), average ovary weight in DHEA-treated rats was significantly higher than that in control group. The ovaries in the DHEA group showed multiple follicular cysts, and the ratio of follicular cysts to normal follicles was significantly increased. Histological examination indicated 8 - 9 layers of granulosa cells were observed in the follicular cysts of the control group which was more than that in the DHEA treated group (2 - 3 layers). The proportion of corpus luteum in ovaries was significantly increased in the control group when compared with DHEA group (Figures 1 and 2). There was a decrease trend in the number of linear granulosa, canaliculus, rough and smooth endoplasmic reticulum and fat droplet in the granulosa cells of control group, compared with those in DHEA group (Figure 3 and 4).

As shown in the same essay, the serum testosterone was significantly elevated in the DHEA group ($p < 0.001$). Compared with control group, the serum estrogen (E2) and luteotropic hormone (LH) were also markedly increased in the DHEA-treated rats ($p < 0.05$). The serum follicle-stimulating hormone (FSH) and progesterone (P) in the DHEA group were slightly decreased ($p > 0.05$) and the PRL was slightly elevated ($p > 0.05$) when compared with those in control group.

Oral glucose tolerance test and serum insulin levels

As also shown in the same essay, the level of fasting serum glucose in the DHEA Group was significantly higher than that in Control Group ($p < 0.001$) which was corresponding to the increased level of fasting and 120-min insulin ($p < 0.05$). The fasting glucose value of insulin sensitivity index (ISI=1/INS×GC, but the value means the calculated natural logarithm, and we get the positive one) was significantly increased in the DHEA Group compared with Control Group ($p < 0.001$), and significant difference was also found in the 120-min glucose value of 1/INS×GC between DHEA Group and Control Group ($p < 0.05$).

Expression of adiponectin mRNA in adipose tissues

Adiponectin mRNA was investigated in adipose tissues of the two groups. Adiponectin mRNA expression in DHEA Group was significantly down regulated as compared with Control Group ($p < 0.05$) (Figure 5a). The level of adiponectin mRNA expression was 12% lower in DHEA Group than in Controlled Group (15.97 ± 3.88 vs 18.11 ± 4.01; $p < 0.05$) (Figure 5b).

Expression of adiponectin protein in adipose tissues

As seen in the Western blot analysis of the levels of adiponectin in adipose tissues of the two groups, DHEA administration to young female rats resulted in a significant decrease in adiponectin protein compared with untreated young female rats ($p < 0.05$) (Figures 6a and b).

DISCUSSION

Adiponectin is one of the adipokines secreted by adipocytes. It was first isolated from mice adipocytes by Scherer et al. (1995). Since it has high homology to the complement component C1q and a relative molecular weight of 30 kD, it was initially named ACRP30. The cDNA of adiponectin was cloned from mouse adipocytes and named AdipQ, when Meada et al. (1996) identified and cloned the homolog of ACRP30 and analog of AdipoQ from human adipocytes and called it the adipose most abundant gene transcript-1 (apM1), because it was the most abundantly transcribed gene in adipose tissue. Meanwhile, Nakano et al. (1996) purified the protein product encoded by the apM-1 gene from human plasma and referred to it as gelatin-binding protein 28 (GBP28) due to its gelatin-binding activity with a molecular weight of 28 kD. Arita et al. (1999) named it the hormone adiponectin and established a method to measure it in human serum.

Recent studies showed that the serum adiponectin level in PCOS patients was significantly lower than in normal individuals (Yilmaz et al., 2009). More recently, it was demonstrated that reduced adiponectin levels in circulation related to oligo-ovulation or anovulation and hyperandrogenemia in women with PCOS accompanied by obesity and IR (Katsikis et al., 2009). But the exact molecular mechanism by which adiponectin functions is still not known, and its role in the PCOS is unclear either.

The pathogenesis of PCOS is not well understood, but a variety of studies have shown that IR plays a role in the occurrence and development of PCOS. Moreover, mouse and human adiponectins share high sequence and

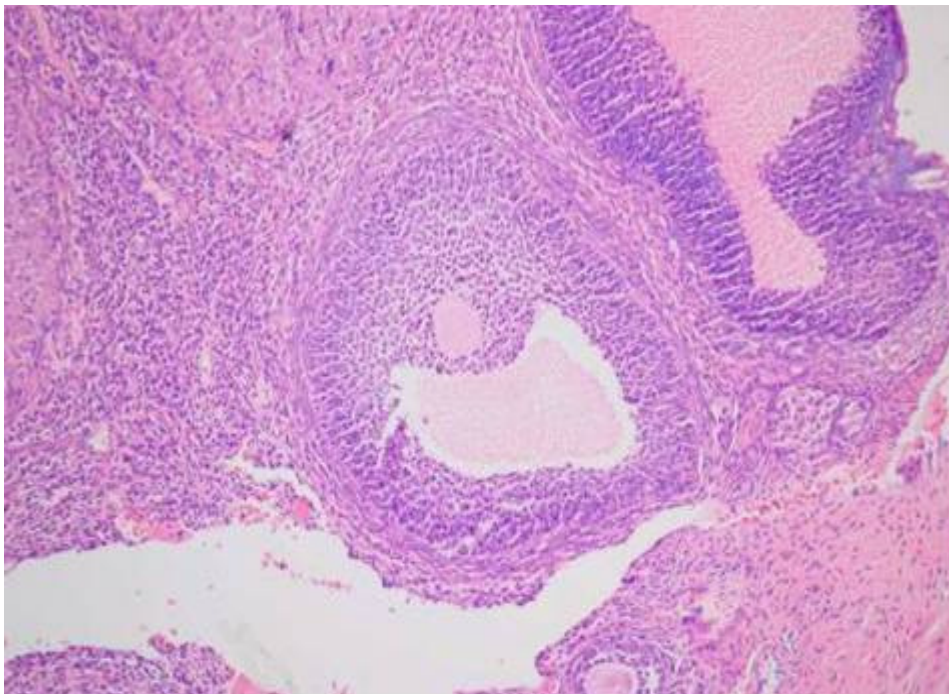

Figure 1. Granulosa cells of follicular cysts in Control Group were divided into 8~9 layers, as determined by light microscopy, which was more than that in DHEA Group. The proportion of corpus luteum was significantly increased as compared with the DHEA Group (HE×200).

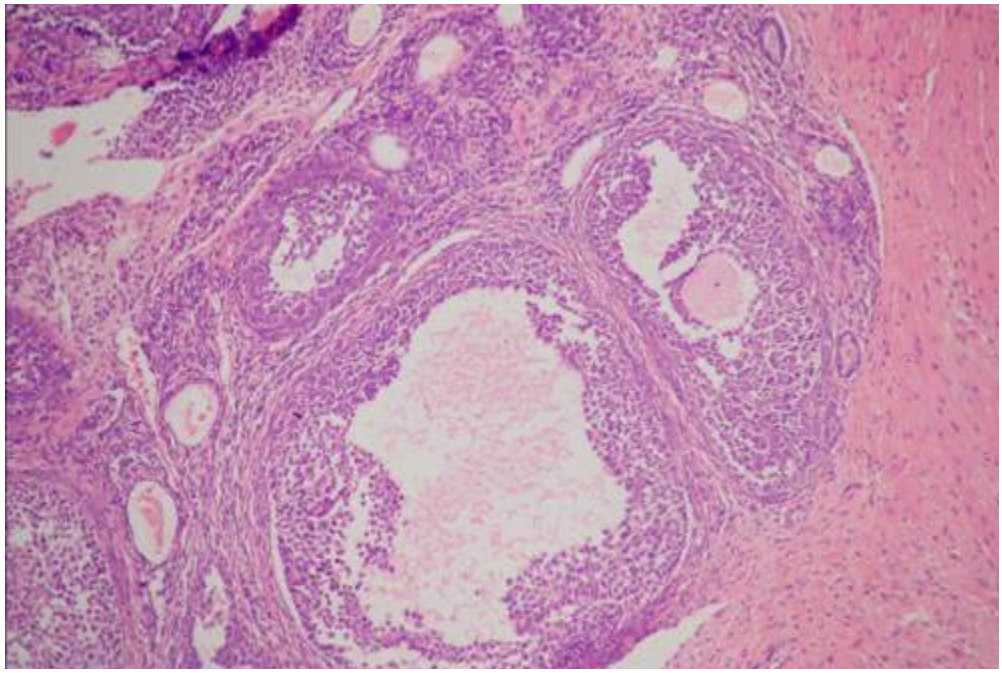

Figure 2. There were multiple follicular cysts in the DHEA Group, as determined by light microscopy, and granulosa cells of follicular cysts were only divided into 2~3 layers (HE×200).

structural homology; therefore, our results from the rat PCOS model may elucidate the potential roles of adiponectin in PCOS and IR to a certain extent. The main criteria for determining the validity of an animal PCOS model should maximize the anatomic and physiological (pathopgyological) similarities between animal PCOS and

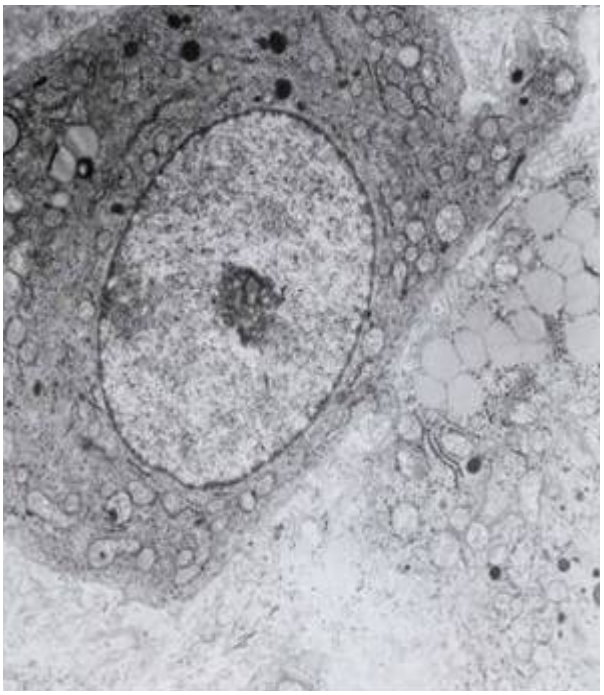

Figure 3. There was a decrease trend in the number of linear granulosa, canaliculus, rough and smooth endoplasmic reticulum and fatty deposition in the granulosa cells of Control Group, as determined by electron microscopy (×6300).

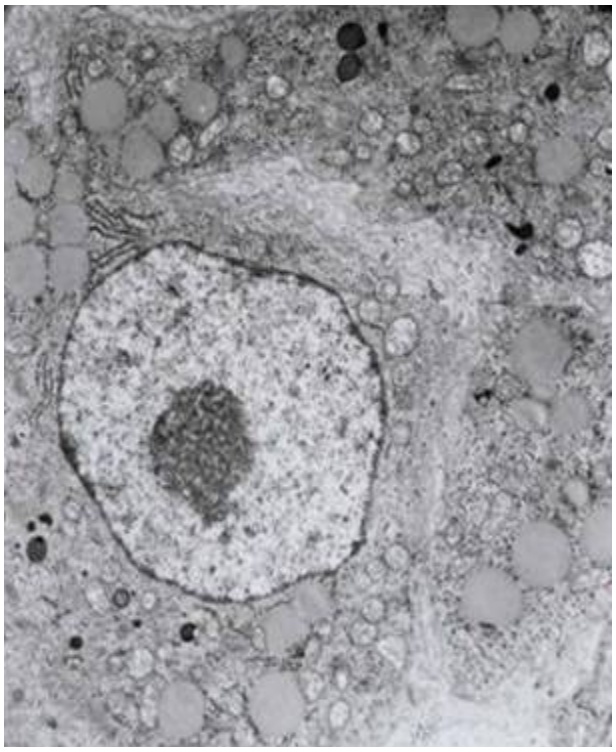

Figure 4. There was an increase trend in the number of linear granulosa, canaliculus, rough and smooth endoplasmic reticulum and fatty deposition in the granulosa cells of DHEA Group, as determined by electron microscopy (×6300).

human PCOS. The rats treated with DHEA in the present study had some of the salient features of human PCOS. Follicular cysts as the main manifestation of PCOS were observed in DHEA-treated rats. In our study, the morphological changes and serum sex hormone levels in PCOS rats induced by DHEA were similar to that seen in patients with PCOS, and meanwhile the PCOS rats had evident hyperinsulinemia. This study also suggested that the expression of adiponectin mRNA in adipose tissues was significantly lowered in rat PCOS model.

It has been shown that obesity and IR are the common characteristics of many diseases, and the direct consequence of obesity and IR is the occurrence of diabetes (Moran et al., 2003). So, in order to explore the relationship between adiponectin and IR, the roles of adiponectin in the occurrence and development of diabetes should be preferentially determined. The main physiological role of adiponectin is to increase glucose uptake in mature adipocytes. Usually, adiponectin signal does not involve the phosphorylation of insulin-like growth factor receptor-1 (IGF-1R) and insulin receptor substrate-1 (IRS-1), which are activated by insulin. Adiponectin can stimulate the serine-phosphorylation of 5'-AMP-activated protein kinase (AMPK), thus resulting in its activation and promoting the phosphorylation of AMPK down-stream rate-limiting enzyme acetyl-Co A carboxylase (ACC). AMPK inhibitor can completely abolish adiponectin-induced glucose uptake, indicating that AMPK activation mediates the glucose uptake. In addition, adiponectin can also increase fatty acid oxidation and glucose transportation in muscle, and activate AMPK to down-regulate the expression of phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6P) in the liver (Chandrasekar et al., 2008). These findings allowed Nayak et al. (2009) to propose that adiponectin was a hormone that linked obesity to IR and type II diabetes.

Although the adipose tissue enlargement is suspected to result from an impaired glucose metabolism, the correlation between adiposity and IR in PCOS remains unclear. Adipocytes play an essential role in storing triglycerides, which provide energy in the form of free fatty acids (FFA), and the released FFA may contribute to the IR of peripheral tissues (Mook et al., 2004). In addition, adipocytes can secrete numerous peptides and cytokines, including resistin, TNF-α, adiponectin, leptin, etc. Adipocytes may confer effects on the systemic metabolism through these products, consequentially resulting in IR. The interaction between these products constitutes a complex regulatory net affecting the endocrine function of adipose tissues.

Although the etiology of PCOS has not yet been elucidated, and the relationship between hyerandro-genemia and hyperinsulinemia remains unsettled, accumulating evidence suggests that insulin is capable of stimulating ovarian androgen synthesis (Cenk et al., 2003). In this study, it was verified that the concentrations of serum testosterone in the DHEA-treated rats were higher than in

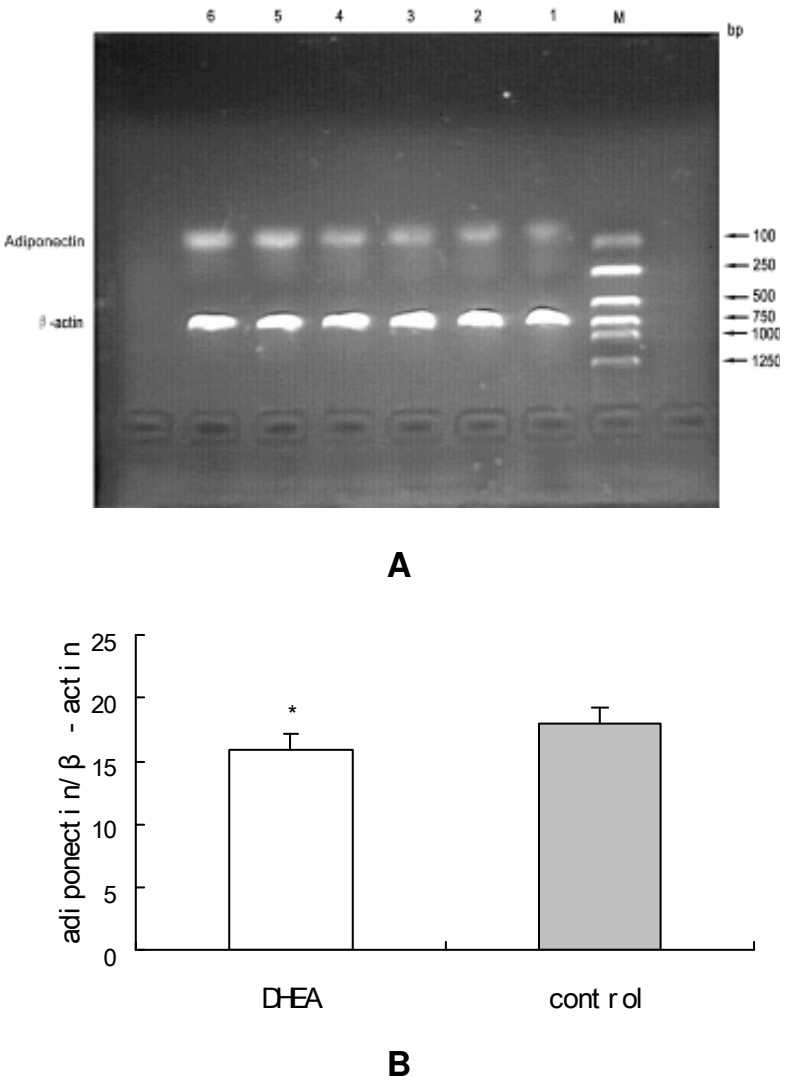

Figure 5. Adiponectin mRNA expression in two groups (A) RT-PCR result (M: molecular weight standard; 1-4: DHEA Group; 5 - 6: Control Group), (B) Gray value of adiponectin and β-actin, *p < 0.05.

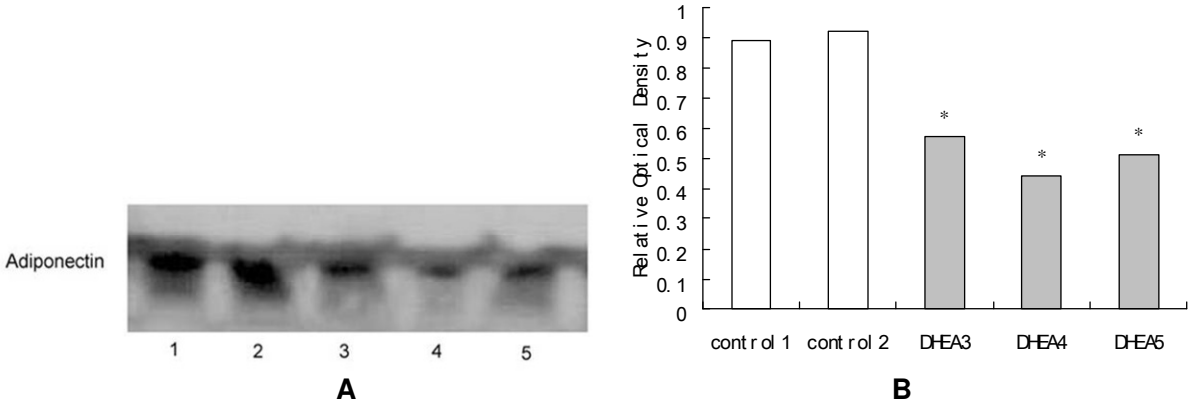

Figure 6. Adiponectin protein expression in two groups, A: Western blot result (1 – 2: control group; 3 - 5: DHEA Group); (B) Comparison on OD value of adiponectin between two groups, *p < 0.001

control rats ($p < 0.001$). It appeared that elevated testosterone was an important characteristic of PCOS. To clarify the potential role of adiponectin produced by adipose tissues in PCOS rats with IR which was confirmed by high fasting serum glucose, insulin and INS/GC, we detected the expression of adiponectin mRNA in adipose tissues of two groups. The results showed the expression of adiponectin mRNA in DHEA-treated Group was significantly lowered as compared with that in Control Group ($p < 0.05$). These findings suggested that reduced expression of adiponectin mRNA in adipose tissues might be one of the important pathogenic factors of IR in women with PCOS.

In conclusion, the relationship between adiponectin and insulin resistance suggests that adiponectin potentially could serve as a marker for disease risk in women with PCOS and provide opportunity for earlier intervention if knowledge is successfully translated from laboratory to clinical practice. However, further investigation of the relationship between adiponectin and PCOS is required before there can be direct application to clinical practice. Understanding the endocrine function of adipose tissues and the regulatory pathways with involvement of adiponectin will be helpful for the development of new therapeutic strategies for PCOS.

ACKNOWLEDGEMENTS

This project was supported by a grant from Department of Health of Guangdong Province (No. A2009370).

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