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Elevated Serum RBP4 Is Associated with Insulin Resistance in Women with Polycystic Ovary Syndrome

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Retinol binding protein 4 (RBP4) is a novel adipocyte-secreted protein that contributes to systemic insulin resistance. Experiments in mice suggest that elevated RBP4 causes insulin resistance. In the present study, we determined serum RBP4 concentration and evaluated its association with insulin resistance in women with polycystic ovarian syndrome (PCOS); 39 PCOS women and 45 healthy control subjects were enrolled in this study. Serum RBP4, fasting plasma glucose (FPG) and fasting serum insulin (FINS) were measured in all subjects. Furthermore, oral glucose tolerance test (OGTT), Botnia clamp (an intravenous glucose tolerance test followed by an euglycemic hyperinsulinemic clamp), and measurements of sex hormones were performed in 13 control subjects and all the PCOS women. The levels of serum RBP4 were elevated in PCOS women compared with the control (11.69 ± 6.72 versus 7.75 ± 5.96 μg/mL, $p = 0.006$). RBP4 levels were positively correlated with WHR ($r = 0.216$, $p = 0.048$), and intravenous glucose tolerance test β cell index (IVGTT-β index) which reflected β cell function ($r = 0.309$, $p = 0.028$), but were inversely correlated with M value during Botnia clamp, which represented insulin sensitivity ($r = -0.362$, $p = 0.008$). No correlation was found between RBP4 and age, BMI, blood pressure, FPG, FINS, 2-h postprandial glucose, 2-h postprandial insulin, free testosterone, total testosterone, follicle-stimulating hormone (FSH), or luteinizing hormone (LH). In a linear stepwise regression analysis with a model including age, BMI, WHR, free testosterone, IVGTT-β index, and M value as independent variables, only M value showed significant correlation with serum RBP4 levels ($r^2 = 0.105$, $f = 6.640$, $p = 0.012$). In conclusion, serum RBP4 levels are significantly increased in PCOS women and associated with insulin resistance, which indicates that RBP4 may be a contributing factor linking adipose tissue with insulin resistance in PCOS.

Key Words: Retinol binding protein 4 (RBP4); polycystic ovary syndrome; insulin resistance; Botnia clamp.

Introduction

Insulin resistance plays a major role in the pathogenesis of type 2 diabetes. Even in the absence of hyperglycemia or diabetes, insulin resistance constitutes an important risk factor for cardiovascular disease and early death *(1)*. It is well known that adipocyte-secreted adipokines act as an important regulator in insulin resistance *(2,3)*. Recent studies show that retinol binding protein 4 (RBP4) is not only a carrier of retinol, but also a new adipokine. Injection of purified RBP4 or transgenic overexpression of RBP4 in mice decreased insulin sensitivity in muscle and increased gluconeogensis by activation of phosphoenolpyruvate carboxykinase in liver *(4,5)*. Elevated RBP4 levels have been reported in people with type 2 diabetes and obesity *(6)*. Moreover, Graham et al. discovered that serum RBP4 levels were increased even before the development of frank diabetes, and appeared to identify insulin resistance and associated cardiovascular risk factors in human *(7,8)*.

Polycystic ovary syndrome (PCOS) is one of the most common endocrine disorders with uncertain etiology, and affects between 6% and 10% of women at the reproductive age *(9)*. Insulin resistance occurs in 50%–70% of PCOS women, in which over 40% might become the patients with impaired glucose tolerance or type 2 diabetes *(9–11)*. PCOS is characterized by menstrual abnormality, hirsutism, acne, anovalatory infertility, and elevated androgens. It has been confirmed that insulin resistance is a common feature in PCOS *(9–13)*. However, there are few data on serum RBP4 levels and their relation to insulin resistance in PCOS women. The aim of this study was to compare serum RBP4 levels between PCOS women and control subjects, and to investigate the association of RBP4 concentration with insulin sensitivity (or insulin resistance) assessed by Botnia euglycemic hyperinsulinemic clamp.

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Table 1
Physical and Biochemical
Characteristics of Subjects in Control and PCOS Women

Group	Control	PCOS	*p*
No.	45	39	
Age (years)	28.22 ± 3.11	27.21 ± 4.12	0.202
Body mass index (kg/m^2)	21.16 ± 2.18	22.05 ± 2.59	0.088
Waist-to-hip ratio	0.79 ± 0.05	0.81 ± 0.06	0.223
Systolic blood pressure (mmHg)	107 ± 10	104 ± 9	0.118
Diastolic blood pressure (mmHg)	69 ± 8	70 ± 9	0.237
Fasting plasma insulin (mmol/L)	4.78 ± 0.43	5.00 ± 0.81	0.143
Fasting insulin (mIU/L)	9.47 ± 2.27	8.74 ± 2.34	0.129
Retinol binding protein 4 (μg/mL)	7.69 ± 5.89	11.69 ± 6.72	0.005

*Values are mean ± SD. *p* values between groups resulted from independent variable *t* test analysis.

Table 2
Physical and Biochemical Characteristics of Subjects Receiving Clamp Test

		POCS		
Group	Control	Lean	Overweight/obese	*p* ANOVA
No.	13	25	14	
Age (yr)	27.92 ± 2.66	26.92 ± 4.24	27.71 ± 3.79	0.698
Body mass index (kg/m^2)	21.12 ± 2.22	20.45 ± 1.51	24.92 ± 1.25	<0.001
Waist-to-hip ratio	0.76 ± 0.05	0.79 ± 0.06	0.84 ± 0.04	0.001
Systolic blood pressure (mmHg)	106 ± 13	102 ± 11	107 ± 6	0.439
Diastolic blood pressure (mmHg)	65 ± 7	67 ± 7	75 ± 10	0.255
Glucose 0 min (mmol/L)	4.81 ± 0.57	4.95 ± 0.84	5.08 ± 0.78	0.663
Glucose 120 min (mmol/L)	5.85 ± 1.07	6.37 ± 1.46	7.52 ± 1.85	0.015
Insulin 0 min (mIU/L)	7.91 ± 1.90	8.41 ± 2.12	9.32 ± 2.67	0.239
Insulin 120 min (mIU/L)	41.58 ± 21.93	44.61 ± 23.45	97.60 ± 62.06	0.002
Follicle stimulating hormone (mIU/mL)	3.95 ± 2.35	5.80 ± 2.97	7.47 ± 3.68	0.171
Luteinizing hormone (mIU/mL)	6.41 ± 7.78	12.06 ± 9.60	18.08 ± 19.41	0.125
Free testosterone (pg/mL)	1.13 ± 0.28	1.64 ± 0.51	1.75 ± 0.66	0.005
Total testosterone (ng/dL)	34.77 ± 14.40	50.27 ± 22.35	52.83 ± 22.41	0.015
Retinol binding protein 4 (μg/mL)	3.97 ± 3.54	11.06 ± 7.03	12.81 ± 6.21	0.001
M value (mg/kg·min)	12.81 ± 1.76	10.24 ± 2.50	8.63 ± 2.84	<0.001
IVGTT-β index (mIU/L)	388.9 ± 223.5	497.4 ± 499.4	863.6 ± 807.8	0.142

*Values are mean ± SD. *p* values between groups resulted from one-way ANOVA test analysis. M value, glucose disposal rate; IVGTT-β index, intravenous glucose tolerance test β-cell index.

Results

General Characteristics

The clinical and biochemical characteristics of 39 PCOS and 45 control subjects are listed in Table 1. Compared with the control, PCOS women had significantly higher serum RBP4 (11.69 ± 6.72 versus 7.69 ± 5.89 μg/mL, $p = 0.006$); whereas age, BMI, waist-to-hip ratio (WHR), blood pressure, fasting plasma glucose, and fasting serum insulin were not significantly different between the two groups.

The clinical and biochemical characteristics of all the PCOS and the 13 control subjects receiving Botnia clamp (an intravenous glucose tolerance test followed by an euglycemic hyperinsulinemic clamp), are shown in Table 2. Insulin sensitivity in these women was evaluated by glucose disposal rate (M value, mg/kg·min), which was defined as the amount of the glucose supplied to maintain blood glucose level during the last 60 min of the clamp. β-cell function was assessed by intravenous glucose tolerance test β cell index (IVGTT-β index) *(14)*. The PCOS subjects were further divided into two subgroups: lean group and overweight/obese group. Based on the criteria of Asia–Oceania, overweight and obese subjects were defined as BMI greater than or equal to 23 kg/m^2 *(15)*. Compared with the control group, both overweight/obese and lean PCOS groups had significantly higher RBP4, total testosterone, free testosterone, and lower M value. Age, blood pressure, IVGTT-β index, fasting glucose and fasting insulin, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) were similar in the three groups. However, in comparison with controls and

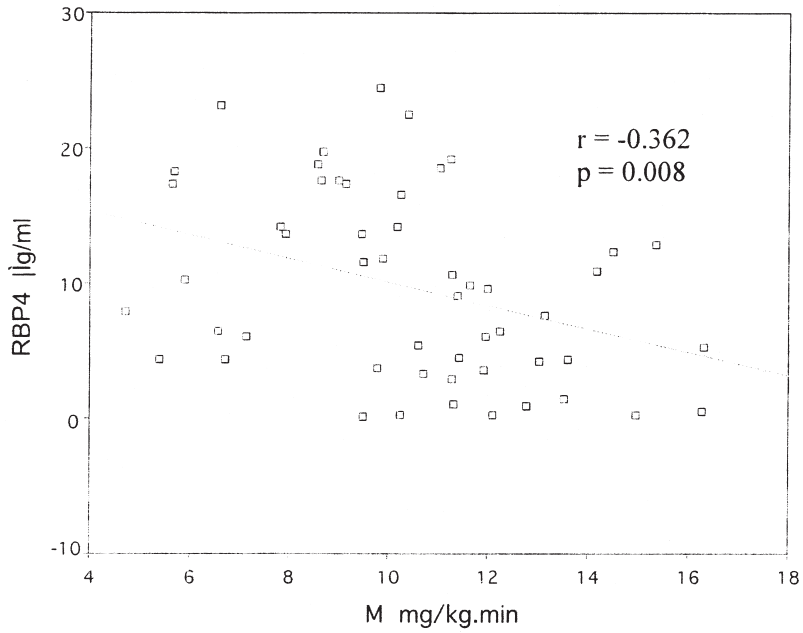

Fig 1. The scatter plot graph showed correlation of serum RBP4 concentration with clamp M value among 52 subjects receiving Botnia clamp.

lean PCOS groups, overweight/obese PCOS group had significantly higher BMI, WHR, 2-h postprandial glucose, and 2-h postprandial insulin.

Univariate Analysis

On the data of all the subjects, univariate correlation analyses showed that serum RBP4 levels were positively correlated with WHR ($r = 0.216$, $p = 0.048$). While in the 52 subjects receiving the clamp, univariate correlation analyses showed that serum RBP4 levels inversely correlated with M value ($r = -0.362$, $p = 0.008$) (Fig. 1), and positively correlated with IVGTT-β index ($r = 0.309$, $p = 0.028$). No correlation was found between RBP4 and age, BMI, blood pressure, fasting glucose, fasting insulin, 2-h postprandial glucose, postprandial insulin, free testosterone, total testosterone, FSH, or LH (data not shown). Univariate analyses of associations on the groups separately were also performed, but no significant correlation between RBP4 and M value was found either in the control or in the PCOS group ($p = 0.82$, $p = 0.23$, respectively).

Adjusted Analysis

In a linear stepwise regression analysis with a model including age, BMI, WHR, free testosterone, IVGTT-β index, and M value as independent variables, only M value showed significant correlation with serum RBP4 levels ($r^2 = 0.105$, $f = 6.640$, $p = 0.012$). The equation model is $Y = 18.802 - 0.351X$. (Y: serum RBP4; X: M value.)

Discussion

Recently, a number of studies have shown that adipose tissue plays an important role in the energy metabolism, and adipocyte-secreted adipokines are important linkages between adipose tissue and other organisms *(2,3,16)*. Increased C-reactive protein, TNFα, and IL-6, but decreased adiponectin, were shown in obesity, type 2 diabetes, and even PCOS. It is well documented that these adipokines were strongly associated with insulin resistance *(17–20)*. RBP4 is a new adipokine mainly expressed in liver and adipose tissue *(4,21)*. Experiments in mice suggest that elevated RBP4 causes insulin resistance, and RBP4 appears to be an elusive link between glucose transport 4 (GLUT4) suppression in adipose tissue and insulin resistance in muscle and liver *(4)*. Graham et al. reported that serum RBP4 levels correlated with the magnitude of insulin resistance in subjects with obesity, impaired glucose tolerance, or type 2 diabetes, and in nonobese, nondiabetic subjects with a strong family history of type 2 diabetes *(7)*. In the present study, we reported first that serum RBP4 concentration was significantly increased in PCOS women, and further linear stepwise regression analysis showed it was significantly associated with M value (insulin sensitivity). These results indicate that RBP4 may be a contributing factor linking adipose tissue with insulin resistance in PCOS.

In order to further analyze the association between serum RBP4 levels and insulin resistance in PCOS, we divided the PCOS group into lean and overweight/obese subgroups. Compared with the control group, both lean and overweight/obese PCOS groups showed significantly lower M value and higher serum RBP4 levels (seen in Table 2), which suggested that the PCOS women had significantly higher levels of insulin resistance and serum RBP4 than the control group, regardless of being obese or not. Graham et al. reported RBP4 was positively associated with BMI and WHR in nondiabetic and diabetic obese subjects *(7)*. In the present study, we found that serum RBP4 had positive correlation with

WHR, but without significant correlation with BMI. However, it should be noted that most PCOS women in this study are non-obese.

In the present study, the correlation analysis also showed the positive relationship between β-cell secretion (which is calculated by IVGTT-β index) and serum RBP4 levels. But a linear stepwise regression did not show a correlation between IVGTT-β index and serum RBP4 levels. This might be explained by increased β-cell compensatory secretion because of insulin resistance in PCOS.

We performed univariate analysis of associations on the groups separately, but no significant correlation was found between RBP4 and M value (insulin sensitivity) either in the control or in PCOS group ($p = 0.82$, $p = 0.23$, respectively). We believed this phenomenon might be due to the small sample. As we know, most PCOS subjects had insulin resistance and low M value, so there was a small degree of variance of M value and RBP4 concentration. In this case, it was difficult to obtain a positive association result between them. This phenomenon might also happen in the control group. Therefore, further research with a larger sample is needed to clarify if there is a correlation between RBP4 and M value in healthy women or PCOS subjects separately.

In conclusion, serum RBP4 concentration is significantly increased in PCOS women and strongly associated with insulin resistance, which indicates that the increased RBP4 may play a role in insulin resistance in PCOS patients.

Materials and Methods

Subjects

Thirty-nine PCOS patients, aged 19–35 yr, were enrolled in the study. The PCOS and control group were matched for age and BMI. PCOS diagnosis was based on the following criteria *(22)*: (1) chronic anovulation: oligomenorrhea or amenorrhea from menarche; (2) clinical and/or increased circulating total or free testosterone, androstenedine; dehydroepiandrosterone levels. And the congenital adrenal hyperplasia, androgen-secreting tumors, Cushing's syndrome, and hyperprolactinemia were ruled out in all the PCOS patients. All PCOS patients did not have obvious hirsutism (hirsutism: Ferriman–Gallwey scores >9). Circulating serum total testosterone (T), free testosterone, follicle-stimulating hormone (FSH), and luteinizing hormone (LH) were determined in all PCOS patients, but we did not measure their serum androstenedine and dehydroepiandrosterone levels. Hyperandrogenemia was defined as T concentration greater than or equal to two times the mean T concentration of the control group. Oligo-ovaluation defined as six or fewer utero vaginal bleeding episodes per year. OGTTs were performed on all PCOS women. The fasting plasma glucose concentrations were within normal range (fasting glucose levels <6.1 mmol/L) except for three PCOS women. Their FPG were 6.43, 7.12, 7.00 mmol/L, respectively, and PPG (2-h postprandial plasma glucose) were 10.2, 6.2, 6.7 mmol/L respectively. The blood pressures of PCOS subjects were less than 140/90 mmHg.

Forty-five healthy volunteers were selected as control group. They had no history of diabetes mellitus in the first-degree relatives. All volunteers had regular menses, with no hirsutism or hyperandrogenemia; 13 control subjects and all PCOS patients received Botnia clamp test.

All subjects were Chinese living in the Chongqing region, and they were given informed consent before the study. The Institutional Review Board of the First Affiliated Hospital, Chongqing University of Medical Sciences approved the study protocol. None of the study participants was taking any medication or instructed on lifestyle modification during recent 3 mo. All the subjects in this study were non-smokers and had no history of thyroid disease.

Methods

The anthropometric measurements were determined by the same physicians from our department. All subjects consumed a diet containing at least 250 g of carbohydrate for at least 3 d before the overnight fasting (12–14 h). Fasting blood samples were taken between d 8 and d 10 of the menstrual cycle or during amenorrhea after excluding pregnancy for measurements of serum RBP4, LH, FSH, total testosterone, and free testosterone. A 75 g oral glucose tolerance test (OGTT) was performed and blood samples were obtained for measurement of plasma glucose and serum insulin at 0 and 120 min. The plasma glucose was measured immediately and the blood samples were frozen at –80°C until assayed.

Botnia Euglycemic Hyperinsulinemic Clamp

Botnia clamp was performed 6–8 d after the OGTT as described previously *(23,24)*. The Botnia clamp was designed to obtain independent measures of insulin secretion and insulin sensitivity during the same test *(14)*. In brief, a small polyethylene catheter was inserted into an antecubital vein for blood sampling. A second catheter was inserted into the contralateral forearm vein for infusion of insulin and glucose solution; 0.3 g/kg body wt of a 50% glucose solution was given at time 0. Blood samples for the measurement of plasma glucose and insulin were obtained at 0, 2, 4, 6, 8, and 10 min (marked as I_x, where I is insulin concentration). The incremental insulin secretion during the first 10 min was called first-phase insulin response. Intravenous glucose tolerance test β-cell index (IVGTT-β index) defined as the sum of I_2, I_4, I_6, and I_8. A 2-h euglycemic hyperinsulinemic clamp begun at 60 min after the glucose bolus. A priming dose of insulin was given followed by an infusion (120 mIU/m^2/min) of short-acting human insulin (Humulin, Lilly) for 120 min, thereby achieving steady-state insulin concentrations during the final 60 min of clamp. A variable infusion of 20% glucose was started at 60 min

to maintain the plasma glucose concentration at 5.2 mmol/L, and blood samples for the measurement of plasma glucose were obtained at 5 min intervals throughout the clamp.

Hormone and Biochemical Assays

Plasma glucose was measured by a glucose oxidase method using a Biosen 5030 Glucose Analyzer (Necar Instruments Inc, Germany). Serum insulin concentrations were measured by RIA (Bei Fang Bioinstitute Inc, China). Serum total testosterone (T), serum follicle-stimulating hormone (FSH), and luteinizing hormone (LH) levels were determined by an automated chemiluminescence system (ACS 180-SE, Bayer, New York, USA). Free testosterone (DSL-4900; DSL) level was measured by RIA using commercial kits. The intraassay and interassay coefficients of variation were: 2.8 and 4.6% for FSH, 4.7 and 6.3% for LH, 11.3 and 13.8% for total testosterone. Serum RBP4 levels were measured by RIA (Phoenix Pharmaceuticals Inc, USA). The sensitivity of the assay for RBP4 were 9.36 ng/mL (IC_{20}), 41.17 ng/mL (IC_{50}), and 224.30 ng/mL (IC_{80}), respectively.

Statistical Analysis

Statistical analysis was performed using the SPSS 11.50 system. Measurements with a skewed distribution were normalized by logarithmic transformation. Comparisons of means and proportions were performed with the Student's *t*-test and one-way ANOVA test, respectively. Correlations were tested by Spearsman correlation coefficients. To allow for covariates and confounders, we performed analysis of covariance and multiple linear regression. p value < 0.05 was considered statistically significant. All the data were presented as means ± SD.

We also did the above statistical analyses after excluding the three PCOS women whose FPG were more than 6.1 mmol/L, and the statistical results were not different (data not shown).

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