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Retinol-binding protein levels are increased in association with gonadotropin levels in healthy women

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Abstract

Recent studies have demonstrated an association between retinol-binding protein (RBP4) and insulin resistance. Retinol-binding protein is decreased in women and elevated in polycystic ovary syndrome. However, prior studies have not investigated the relationship between RBP4, gonadal steroids, and gonadotropins in healthy women. The aim of this study was to determine the RBP4 levels in a cohort of healthy women with a range of body mass indices and glucose tolerances to investigate the relationship between RBP4, gonadotropin levels, and menopausal status. Serum RBP4 levels were measured by enzyme-linked immunosorbent assay and quantitative Western blot in 88 healthy women (aged 24-59 years) from the general community in a cross-sectional study. Retinol-binding protein was higher in postmenopausal compared with premenopausal women (26.1 ± 2.1 vs 19.3 ± 0.5 μg/mL, $P = .001$). In univariate analysis, RBP4 was associated with follicle-stimulating hormone ($r = 0.37$, $P = .0004$), luteinizing hormone ($r = 0.3$, $P = .005$), and sex hormone–binding globulin ($r = -0.24$, $P = .03$) and trended to significance with estradiol ($P = .09$) but not with free testosterone or dehydroepiandrosterone sulfate. Retinol-binding protein was also associated with insulin at 2 hours during an oral glucose tolerance test ($r = 0.24$, $P = .03$) and the area under the curve for insulin during the oral glucose tolerance test ($r = 0.26$, $P = .02$). In multivariate regression modeling, both follicle-stimulating hormone ($P = .03$) and luteinizing hormone ($P = .04$) remained significantly associated with RBP4 after controlling for estradiol, sex hormone–binding globulin, insulin area under the curve, cholesterol, triglycerides, waist-to-hip ratio, and tumor necrosis factor α. Retinol-binding protein was not associated with inflammatory markers or with carotid intima-media thickness. Therefore, RBP4 is higher in postmenopausal women and is associated with gonadotropin concentrations in healthy women.

1. Introduction

Recent studies have identified a novel adipokine, retinol-binding protein (RBP4), as a putative mediator of insulin resistance [1]. Both transgenic and pharmacologic increases in RBP4 result in development of insulin resistance. Conversely, reductions in RBP4 levels by knockout or pharmacologic means decrease insulin resistance in mice [1]. The effects of RBP4 are thought to be mediated by alterations in insulin signaling through insulin receptor substrate–1 and phosphoinositide 3-kinase activation [1]. In contrast to the data in mice, the role of RBP4 in humans is less clear. Although most studies support a role for RBP4 in mediating insulin resistance [2-8], several studies do not support such an effect [9-11]. In addition, an association between RBP4 and abdominal obesity has been shown in some [2,7,8] but not all studies [3,5,6,9,11].

Relatively little is known regarding the potential regulation of RBP4 by sex steroids and related gonadotropins, and the primary purpose of our study was to investigate the relationship of RBP4 with these parameters. Although 2 studies have reported a lack of sexual dimorphism in RBP4 [5,10], 3 other studies have demonstrated lower RBP4 levels in female subjects [3,6,8]. Moreover, serum RBP4 levels are elevated in polycystic ovary syndrome (PCOS) and associated with insulin resistance [12]. Tan et al [13] confirmed the elevated RBP4 levels in PCOS and further demonstrated an increase in both messenger RNA (mRNA) and protein levels of RBP4 in adipose tissue explants from PCOS subjects after treatment

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with 17-β-estradiol in a dose-dependent manner. This effect was specific to estradiol, as testosterone, androstenedione, and dehydroepiandrosterone sulfate (DHEA-S) did not have any effects on RBP4 mRNA or protein levels in human adipose tissue explants [13].

Adipocytes express estrogen receptor α and β [14] as well as androgen receptors [15]. Higher RBP4 levels in men as well as in PCOS suggest that androgens may increase levels of RBP4 and/or that estrogen may lower levels of RBP4. In contrast, in vitro data demonstrate that 17-β-estradiol directly stimulates both mRNA and protein levels of RBP4 in adipose tissue explants of PCOS subjects [13]. Interestingly, adipocytes also express mRNA for pituitary hormone receptors [16] and functionally respond with activation of various signaling cascades in response to pituitary hormones [17]. More recently, adipocytes were found to express receptors for luteinizing hormone (LH) and respond to human chorionic gonadotropin with increased growth and differentiation [18]. This raises the alternative hypothesis that RBP4 is regulated directly by the gonadotropins, LH, and follicle-stimulating hormone (FSH), independently and in addition to gonadal steroids.

We had previously demonstrated an association of another adipokine, adiponectin, to carotid intima-media thickness (IMT), a measure of atherosclerosis, in otherwise healthy women [19]. Adiponectin is inversely associated with obesity [20], insulin resistance, and diabetes [21]. Interestingly, it is also lower in patients with coronary artery disease [22]. Adiponectin is therefore thought to have an antiatherosclerotic and anti-inflammatory effect [23,24]. Similarly, 2 previous studies have demonstrated positive correlations between C-reactive protein (CRP) and RBP4 [7,8], also suggesting a role for RBP4 in mediating inflammation and atherosclerosis. However, no study has evaluated the relationship between RBP4 and atherosclerosis directly with carotid IMT. A secondary end point of the study was therefore to determine the relationship between RBP4, inflammatory indices, and carotid IMT in women.

We measured serum RBP4 levels in a previously well-characterized cohort of female subjects and compared RBP4 levels by menopausal status, serum FSH, LH, estradiol, testosterone, and DHEA-S levels to determine the relationship between RBP4, sex steroids, and gonadotropins. In addition, RBP4 levels were analyzed for association with indices of insulin sensitivity, fat distribution, and carotid IMT. We hypothesized that RBP4 would increase in association with gonadotropin levels and be associated with carotid IMT in otherwise healthy women.

2. Subjects and methods

2.1. Subjects

Subjects were recruited from the community at large through advertisement and primary care referrals between October 2000 and May 2004. Inclusion criteria included age between 18 and 60 years, female sex, body mass index (BMI) greater than 20 kg/m^2 and less than 35 kg/m^2, and healthy, without known acute or chronic illnesses (including not receiving medications for diabetes, hypertension, or dyslipidemia or acute infections within 3 months of study). Exclusion criteria included use of steroids, growth hormone, oral contraception pills, or any anabolic agents within 3 months of study. Other exclusion criteria included substance abuse, pregnancy or breast-feeding within the past year, or history of bilateral oophorectomy. One postmenopausal subject was taking hormone replacement therapy and had an estradiol of 36.6 pg/mL and an FSH of 21 IU/L. Data were analyzed with and without this subject and were not found to be any different. Therefore, the final results presented here include data from this subject. Data from this cohort establishing the relationship between adiponectin and IMT were previously published [19]. The study was approved by the Massachusetts General Hospital and Massachusetts Institute of Technology institutional review committees where they were seen. All subjects provided written consent.

2.2. Reproductive function

Menstrual history was obtained in all subjects. History of hysterectomy or oophorectomy was obtained. Based on history, subjects were categorized as eumenorrheic (normal menstrual function), oligomenorrheic (less than 3 menstrual periods in the 3 months before enrollment), or amenorrheic.

2.3. Hormonal measurements

All testing was performed in the early follicular phase when appropriate (for premenopausal eumenorrheic subjects) after a 12-hour overnight fast. Measurements were obtained in duplicate, and average values are reported. Serum RBP4 was measured using an enzyme-linked immunosorbent assay (ELISA) (ALPCO Diagnostics, Salem, NH) and quantitative Western blots. Intraassay coefficient of variation (CV) for ELISA was 5%, and interassay CV ranged from 9.7% to 9.8%. Details on the Western blot methodology are presented below. Serum FSH was measured using a solid-phase immunoradiometric assay (Diagnostic Products, Los Angeles, CA) with an intraassay CV of 2.2% to 3.8%. Serum LH was also measured using a solid-phase immunoradiometric assay (Diagnostic Products) with an intraassay CV of 1.0% to 1.6%. Serum estradiol was measured by radioimmunoassay (RIA) (Diagnostic Systems Laboratories, Webster, TX) with an intraassay CV of 6.5% to 8.9%. Serum free testosterone concentration was determined as the product of the percentage of free testosterone concentration, measured by equilibrium dialysis, and the total testosterone concentration (Endocrine Sciences, Calabasas Hills, CA). The intraassay CV of free testosterone was 6.9%, and that of total testosterone was less than 8.1%. The sensitivity of the percentage of free testosterone assay was 0.1%, and the

sensitivity of the total testosterone assay was 3 ng/dL. Sex hormone–binding globulin (SHBG) levels were measured by immunoradiometric assay (Esoterix, Austin, TX) with an intraassay CV of less than 4% and an interassay CV of 7.8% to 10.6%. Serum DHEA-S was measured by RIA (Siemens Medical Solutions Diagnostics, Los Angeles, CA) with an intraassay CV range of 3.8% to 5.3% and interassay CV range of 6.3% to 11%. Insulin and glucose were measured at 0, 30, 60, 90, and 120 minutes after a standard 75-g oral glucose tolerance test (OGTT). Serum insulin levels were measured using an RIA (Diagnostic Products) with intraassay and interassay CV range from 3.1% to 9.3% and 4.9% to 10%, respectively. Adiponectin was measured using an RIA (Linco Research, St Charles, MO) with an intraassay and interassay CV range of 1.8% to 6.2% and 6.9% to 9.3%, respectively. C-reactive protein was measured using an ultrasensitive ELISA (Diagnostic System Laboratories) with an intraassay and interassay CV range of 1.7% to 3.9% and 2.8% to 5.1%, respectively. The sensitivity (minimum detection limit) of the assay is 1.6 ng/mL. Homocysteine was measured by recombinant enzymatic cycling assay (Carolina Liquid Chemistries, Brea, CA) with an intraassay and interassay CV range of 1.8% to 3% and 1% to 3%, respectively. Levels of both tumor necrosis factor α (TNFα) and interleukin-6 (IL-6) were measured using ELISA kits from R&D Systems (Minneapolis, MN). The ELISA for TNFα has an intraassay and interassay CV range of 4.2% to 5.6% and 4.6% to 7.4%, respectively, whereas the ELISA for IL-6 has an intraassay and interassay CV range of 6.9% to 7.8% and 6.5% to 9.6%, respectively.

2.4. Western blot

Quantitative Western blotting using full-length recombinant RBP4 (rRBP4) (3378-LC, R&D Systems) was validated, and calibration curve using rRBP4 (standard solution) was performed in each Western blot along with samples. Samples were analyzed in duplicate, and average optical density values were used to determine the quantity. Standard solutions of 15, 30, 60, or 120 μg/mL rRBP4 were prepared in Tris-buffered saline containing 1% NP-40. Standards and sera were diluted 1:100 in sodium dodecyl sulfate polyacrylamide gel electrophoresis buffer and boiled for 5 minutes. Fifteen microliters of diluted standards or sera and molecular weight markers were electrophoresed on 18% Tris-glycine sodium dodecyl sulfate polyacrylamide gel electrophoresis gels (Criterion brand; BioRad, Hercules, CA) and transferred to nitrocellulose. Western blotting was performed with nonfat milk (BioRad) as a blocking agent. Blots were incubated overnight at 4°C with primary antibody directed against RBP4 (A0040, DAKOCytomation, DAKO USA, Carpinteria, CA) diluted 1:1000 and for 1 hour at room temperature with horseradish peroxidase–conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA; diluted 1:2000). Bands were detected by enzymatic chemiluminescence (GE Healthcare Bio-Sciences, Piscataway, NJ) and quantified with ImageJ software (National Institutes of Health, Bethesda, MD). A single band for rRBP4 migrated at about 21 kd. Linear curve was used to fit the purified RBP4 standards (r^2 >0.98) for each individual gel and to calculate concentrations in serum samples. This method provides absolute measurements of RBP4 concentrations with minimal gel to gel variability (±10% CV for the same sample from duplicate gels). To avoid irregularities from gel quality or protein transfer, RBP4 standards were placed in the same position on each gel. To monitor assay quality, serum reference samples were included on every gel.

2.5. Carotid IMT

Carotid IMT measurements were performed as previously described [19,25]. Briefly, imaging was conducted using a high-resolution 7.5-MHz phased-array transducer (Hewlett Packard SONOS 2000/2500, Palo Alto, CA). Digital images were captured directly to a Windows NT workstation using a high-quality, high-speed frame capture card made by Data Translation (Marlboro, MA). Either the 90° or the 45° image in diastole was selected as the best view for image quality. Edge detection and mean IMT calculation were accomplished with an in-house computer program. The published reproducibility of the technique is excellent, with a standard deviation of 0.007 mm [26]. The average IMT over the length of the measured segments on the right is reported.

2.6. Body composition

Weight and anthropometric measurements were determined in the morning, before breakfast. Anthropometric measurements were obtained using an inelastic tape measure by the Bionutrition Staff of the Massachusetts Institute of Technology General Clinical Research Center. Cross-sectional abdominal computed tomographic scans at the level of the L4 pedicle were performed to assess abdominal visceral adipose tissue (VAT) and subcutaneous adipose tissue area as previously reported [19,25].

2.7. Statistical methods

Continuous variables were tested for normality of distribution with the use of the Wilk-Shapiro test and examination of the histogram distribution. Variables that were normally distributed were compared using the Student *t* test, whereas those variables that were not normally distributed were compared using the nonparametric Wilcoxon rank sum test. Statistical significance was assumed when the null hypothesis could be rejected at *P* less than .05. Univariate regression analysis was performed comparing RBP4 and other variables using the Pearson correlation coefficient. A standard least-squares multivariate regression analysis was performed. All variables that were significant on univariate analysis with a *P* less than .1 were included in the analysis. In addition, a stepwise forward selection

multivariate regression analysis was performed, with P less than .1, to enter, to confirm the results of the multivariate analysis. Luteinizing hormone and FSH were entered as continuous variables in these analyses. All statistical analyses were performed using SAS JMP statistical software (version 5.1; SAS Institute, Cary, NC).

3. Results

3.1. Characteristics of the study subjects

Eighty-eight otherwise healthy women aged 24 to 59 years were enrolled in this study, and their characteristics are described in Table 1. Eleven subjects were postmenopausal

Table 1
Baseline demographic and clinical characteristics of the study population

	All subjects (n = 88)	Premenopausal (n = 77)	Postmenopausal (n = 11)	P
Demographics				
Age (y)	40.8 ± 0.7	39.6 ± 0.7	49.3 ± 1.6	<.0001[a]
Race (%)				.22[c]
White	33 (38)	27 (35)	6 (55)	
African American	37 (42)	33 (43)	4 (36)	
Hispanic	12 (14)	12 (16)	0 (0)	
Asian	4 (4)	4 (5)	0 (0)	
Other	2 (2)	1 (1)	1 (9)	
Anthropometric parameters				
Weight (kg)	73.2 ± 1.6	73.9 ± 1.7	68.2 ± 3.9	.23
BMI (kg/m^2)	27.1 ± 0.5	27.3 ± 0.5	26.1 ± 1.1	.42
Iliac waist (cm)	87.8 ± 1.3	88.1 ± 1.4	85.9 ± 3.3	.64
Hip (cm)	105.9 ± 1.0	106.1 ± 1.1	104.3 ± 2.7	.37
Waist-to-hip ratio	0.83 ± 0.01	0.83 ± 0.01	0.82 ± 0.02	.90
SAT (cm^2)	284.3 ± 13.6	286.8 ± 14.3	267.0 ± 43.2	.52
VAT (cm^2)	72.3 ± 4.9	72.9 ± 5.5	68.5 ± 9.6	.76
% Body fat	34.0 ± 0.7	33.9 ± 0.7	35.2 ± 1.8	.53
Hemodynamic parameters				
Systolic blood pressure (mm Hg)	110.9 ± 1.5	111.2 ± 1.6	108.6 ± 3.7	.57
Diastolic blood pressure (mm Hg)	68.5 ± 1.1	68.5 ± 1.2	68.0 ± 2.2	.87
Endocrine parameters				
FSH (IU/L)	15.9 ± 3.3	6.2 ± 0.4	83.6 ± 15.6	<.0001[b]
LH (IU/L)	7.9 ± 1.1	5.1 ± 0.6	27.1 ± 4.7	<.0001[b]
Estradiol (pg/mL)	53.8 ± 5.0	58.6 ± 5.4	20.3 ± 5.4	.0004[b]
SHBG (nmol/dL)	83.0 ± 4.4	83.2 ± 4.8	81.5 ± 12.3	.76
DHEAS (μg/dL)	128.6 ± 10.7	135.3 ± 11.9	84.7 ± 17.1	.04[b]
Serum free testosterone (pg/mL)	2.8 ± 0.2	3.0 ± 0.2	1.5 ± 0.3	.002[b]
Metabolic parameters				
Fasting glucose on OGTT (mg/dL)	83.8 ± 1.2	83.9 ± 1.4	82.9 ± 2.0	1.00
2-h glucose on OGTT (mg/dL)	106.9 ± 3.6	107.3 ± 4.0	104.2 ± 7.1	.88
Fasting insulin on OGTT (μU/mL)	6.6 ± 0.3	6.6 ± 0.4	7.0 ± 0.4	.10
2-h insulin on OGTT (μU/mL)	42.8 ± 3.6	40.8 ± 3.7	57.3 ± 12.7	.13
Insulin AUC during OGTT	5306.3 ± 256.7	5201.8 ± 279.4	6153.7 ± 527.3	.04[b]
RBP4 (μg/mL)	20.1 ± 0.6	19.3 ± 0.5	26.1 ± 2.1	.001[b]
Lipid panel				
Total cholesterol (mg/dL)	176.7 ± 3.5	173.4 ± 3.4	199.9 ± 13.4	.03[b]
HDL cholesterol (mg/dL)	57.5 ± 1.6	55.6 ± 1.6	70.3 ± 4.0	.002[b]
LDL cholesterol (mg/dL)	104.4 ± 3.3	103.1 ± 3.3	113.5 ± 13.0	.63
Triglycerides (mg/dL)	75.4 ± 3.9	74.7 ± 4.3	80.3 ± 9.3	.38
Inflammatory parameters				
TNFα (pg/mL)	23.2 ± 4.0	24.3 ± 4.4	14.9 ± 7.1	.30
IL-6 (pg/mL)	1.9 ± 0.1	1.8 ± 0.2	2.2 ± 0.5	.63
Homocysteine (μmol/L)	7.2 ± 0.2	7.1 ± 0.2	7.8 ± 0.9	.54
CRP (mg/L)	2.4 ± 0.4	2.1 ± 0.3	4.4 ± 3.1	.52
Adiponectin (μg/mL)	8.4 ± 0.6	8.1 ± 0.7	10.2 ± 1.2	.03[b]
REE (kcal)	1393.0 ± 20.1	1410.3 ± 21.4	1271.8 ± 45.7	.02[a]
Carotid IMT (mm)	0.64 ± 0.01	0.63 ± 0.01	0.70 ± 0.03	.007[b]

Data are presented as mean ± standard error of the mean. REE indicates resting energy expenditure.

[a] P value obtained from Student t test comparison between pre- and postmenopausal women in normally distributed samples.

[b] P value obtained from Wilcoxon rank sum test comparison between pre- and postmenopausal women in samples that were not normally distributed.

[c] Race was evaluated as a dichotomous variable (white or not) using a χ^2 test.

as determined by follicular phase FSH greater than 20 IU/L, and 77 were categorized as premenopausal. None of the subjects with FSH greater than 20 IU/L were younger than 40 years.

3.2. RBP4 concentrations in menopausal women

Menopausal subjects were older compared with premenopausal subjects (49.3 ± 1.6 vs 39.6 ± 0.7 years, $P < .0001$). Both FSH (83.6 ± 15.6 vs 6.2 ± 0.4 IU/L, $P < .0001$) and LH (27.1 ± 4.7 vs 5.1 ± 0.6 IU/L, $P < .0001$) were higher and estradiol (20.3 ± 5.4 vs 58.6 ± 5.4 pg/mL, $P = .0004$), free testosterone levels as determined by equilibrium dialysis technique (1.5 ± 0.3 vs 3 ± 0.2 pg/mL, $P = .002$), and DHEA-S (84.7 ± 17.1 vs 135.3 ± 11.9 μg/dL, $P = .04$) were lower in the postmenopausal women. Insulin area under the curve (AUC) during the OGTT was higher in the postmenopausal subjects (6153.7 ± 527.3 vs 5201.8 ± 279.4, $P = .04$); however, fasting insulin and 2-hour insulin during the OGTT were not significantly different. There were no significant differences between body weight, indices of body fat distribution, or inflammatory markers (TNFα, IL-6, homocysteine, CRP). Total cholesterol (199.9 ± 13.4 vs 173.4 ± 3.4 mg/dL, $P = .03$) and high-density lipoprotein (HDL) cholesterol (70.3 ± 4 vs 55.6 ± 1.6 mg/dL, $P = .002$) were higher in the postmenopausal group, whereas triglycerides and low-density lipoprotein (LDL) cholesterol were not different. Resting energy expenditure was lower (1271.8 ± 45.7 vs 1410.3 ± 21.4 kcal, $P = .02$) in the postmenopausal group. Both adiponectin (10.2 ± 1.2 vs 8.1 ± 0.7 μg/mL, $P = .03$) and carotid IMT were higher (0.7 ± 0.03 vs 0.63 ± 0.01 mm, $P = .007$) in the postmenopausal group (Table 1). Serum RBP4 levels were 35% higher in postmenopausal women (26.1 ± 2.1 vs 19.3 ± 0.5 μg/mL, $P = .001$) (Fig. 1).

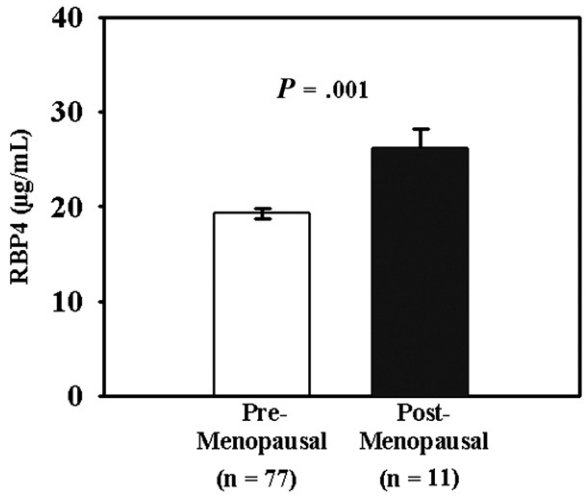

Fig. 1. Serum RBP4 levels in pre- and postmenopausal women. The white bar denotes premenopausal women (n = 77), and the black bar denotes postmenopausal women (n = 11). Data are expressed as mean ± SEM in micrograms per milliliter. P is equal to .001 by Wilcoxon rank sum test.

Table 2
Univariate analysis of correlation with serum RBP4 levels (in micrograms per milliliter) expressed as a Pearson correlation coefficient (r) in all subjects including pre- and postmenopausal women (N = 88)

Variable	Pearson correlation coefficient (r)	P value
Age (y)	0.12	.26
Waist-to-hip ratio	0.18	.09
Endocrine parameters		
FSH (IU/L)	0.37	.0004
LH (IU/L)	0.30	.005
SHBG (nmol/dL)	−0.24	.03
Estradiol (pg/mL)	−0.18	.09
Serum free testosterone (pg/mL)	−0.02	.88
DHEAS (μg/dL)	−0.01	.90
Metabolic parameters		
Fasting insulin on OGTT (μU/mL)	0.2	.07
2-h insulin on OGTT (μU/mL)	0.24	.03
Insulin AUC	0.26	.02
Fasting glucose on OGTT (mg/dL)	−0.08	.44
2-h glucose on OGTT (mg/dL)	−0.06	.60
Lipid panel		
Total cholesterol (mg/dL)	0.24	.02
HDL (mg/dL)	0.04	.73
LDL (mg/dL)	0.18	.10
Triglycerides (mg/dL)	0.28	.008
Inflammatory markers		
TNFα (pg/mL)	−0.21	.08
CRP (μg/mL)	−0.15	.22
Adiponectin (μg/mL)	−0.07	.54
Carotid IMT (mm)	0.17	.12

3.3. Confirmation of RBP4 results by quantitative Western blot

Results for RBP4 were recapitulated using quantitative Western blot analysis. The RBP4 concentrations assessed by ELISA correlated significantly with RBP4 by Western blot ($r = 0.42$, $P < .0001$). Moreover, differences by menstrual status in RBP4 concentrations were also confirmed using Western blot; for example, RBP4 levels were higher in postmenopausal than premenopausal subjects (96.5 ± 12.8 vs 75.9 ± 4.8 μg/mL, $P < .05$).

3.4. Univariate and multivariate regression analyses

The results of the univariate and multivariate regression analyses are summarized in Tables 2 and 3, respectively. Among the entire group, including pre- and postmenopausal subjects, RBP4 was positively associated with FSH ($r = 0.37$, $P = .0004$) and LH ($r = 0.3$, $P = .005$) and negatively associated with SHBG ($r = −0.24$, $P = .03$). The association between RBP4 and estradiol trended toward significance ($r = −0.18$, $P = .09$). In contrast, RBP4 was not associated with free testosterone or DHEA-S. Retinol-binding protein was positively associated with 2-hour insulin ($r = 0.24$, $P = .03$) and AUC for insulin during OGTT ($r = 0.26$, $P = .02$) and trended to significance with fasting insulin ($r = 0.2$, $P = .07$). In contrast, RBP4 was not associated with fasting

Table 3
Relationships between adjusted covariates and serum RBP4 levels (in micrograms per milliliter) in multivariate regression modeling in all subjects including pre- and postmenopausal women (N = 88)

Parameter	Estimate (β)	Standard error	P value
A			
FSH (IU/L)	0.04	0.02	.03
Estradiol (pg/mL)	−0.02	0.02	.21
SHBG (nmol/dL)	−0.02	0.02	.20
Insulin AUC	0.0004	0.0003	.23
Waist-to-hip ratio	6.18	9.94	.54
Total cholesterol (mg/dL)	0.02	0.02	.28
Triglycerides (mg/dL)	0.007	0.02	.69
TNFα (pg/mL)	−0.002	0.02	.93

Parameters with a P less than .1 in univariate analysis were included in the multivariate regression model. The overall model attained an R^2 of 0.30 and was highly significant with a P less than .01.

B			
LH (IU/L)	0.12	0.06	.04
Estradiol (pg/mL)	−0.03	0.02	.10
SHBG (nmol/dL)	−0.02	0.02	.21
Insulin AUC	0.0004	0.0003	.22
Waist-to-hip ratio	4.16	9.89	.68
Total cholesterol (mg/dL)	0.02	0.02	.22
Triglycerides (mg/dL)	0.008	0.02	.65
TNFα (pg/mL)	0.003	0.02	.90

Parameters with a P less than .1 in univariate analysis were included in the multivariate regression model. The overall model attained an R^2 of 0.29 and was highly significant with a P equal to .01.

glucose, 2-hour glucose during OGTT, age, body weight, or indices of body fat distribution. Retinol-binding protein was not associated with adiponectin, IL-6, homocysteine, or CRP and trended to significance with TNFα ($r = -0.21$, $P = .08$). Retinol-binding protein was not associated with carotid IMT (Table 2).

A standard least-squares multiple regression analysis was performed with variables that reached a P less than .1 on univariate analysis. These variables included FSH, estradiol, SHBG, insulin AUC, waist-to-hip ratio, total cholesterol, triglycerides, and TNFα. Luteinizing hormone was initially excluded because it was colinear with FSH and less strongly related to RBP4 on univariate analysis. Similarly, fasting insulin and 2-hour insulin during OGTT were excluded because they were colinear with and less strongly related to RBP4 then insulin AUC in the univariate analysis. The total R^2 for the model was 0.3 with a P less than .01. Follicle-stimulating hormone remained significantly related to RBP4 ($\beta = .04$, $P = .03$) after controlling for these parameters (Table 3A). Stepwise forward regression modeling also identified FSH as most robustly related to RBP4.

Creation of a standard least-squares model with LH instead of FSH was also significant with a total R^2 for the model of 0.29 and a P equal to .01. Luteinizing hormone remained significantly related to RBP4 ($\beta = 0.12$, $P = .04$) after controlling for the parameters as above (Table 3B). Stepwise forward regression modeling also identified LH as most robustly related to RBP4. However, the addition of both LH and FSH to the model resulted in loss of significance between RBP4 and either gonadotropin, confirming colinearity.

As RBP4 is made in adipocytes, models adjusting for body weight and indices of body fat distribution were also tested. None of the more specific indices of body fat, for example, VAT, were significant in the multivariate regression modeling. A model was also created with age given its association with menopause but was also not significant. In addition, further modeling controlling for physical activity, alcohol use, and tobacco use, separately and together, was also created without significant change in the overall findings. The significant relationship between RBP4 and gonadotropins seen in the primary model was confirmed in all subsequent models, none of which resulted in a significantly greater R^2 than the primary model.

4. Discussion

In this study, we demonstrate a novel relationship between gonadotropins and RBP4, a putative mediator of insulin resistance, in healthy women. Serum RBP4 levels were higher in postmenopausal subjects compared with premenopausal subjects and were significantly related to FSH and LH on univariate analysis in our cohort of healthy women. The relationship between gonadotropins and RBP4 remained significant on multivariate regression analysis controlling for estradiol, body weight, body fat distribution, and measures of insulin sensitivity as well as physical activity, tobacco use, and alcohol use, suggesting an independent relationship between gonadotropins and RBP4. In addition, RBP4 was not associated with any inflammatory markers including TNFα, IL-6, homocysteine, CRP, or adiponectin; and furthermore, RBP4 was not associated with carotid IMT, although our study may have been underpowered to detect these secondary end points.

Serum RBP4 concentrations determined in our study were within the previously published range for subjects without glucose intolerance or type 2 diabetes mellitus [2,5]. Consistent with its putative role as a mediator of insulin resistance [2-6], we observed a significant relationship between RBP4 and 2-hour insulin as well as insulin AUC during OGTT; and thus, our results extend this observation to healthy women. Consistent with previous data [3,5,6,9,11], we were unable to detect a significant effect of body weight, BMI, percentage of body fat, or body fat distribution on RBP4 levels in healthy women, not selected for obesity or insulin resistance. As RBP4 is primarily made in the liver [27], the degree of liver fat may be a more prominent predictor of RBP4 levels rather than measures of generalized adiposity as demonstrated by Stefan et al [5]. We did not assess for other conditions, including sleep apnea, which might also affect insulin resistance and RBP4 concentrations.

The most striking finding of this study is the relationship between RBP4 and gonadotropin concentrations. Retinol-binding protein was found to be significantly elevated in

postmenopausal women compared with premenopausal women. In univariate regression analysis of both pre- and postmenopausal women, both FSH and LH were significantly associated with RBP4; and this association remained significant after controlling for other variables including age, physical activity, tobacco use, alcohol use, body weight, indices of body fat distribution and insulin sensitivity, cholesterol, SHBG, and estradiol in multivariate regression analysis. Our data therefore suggest an independent relationship between gonadotropins and RBP4. A potential role for gonadal steroids in the regulation of RBP4 was recently illustrated by Tan et al [13]. Tan et al treated omental and subcutaneous adipose tissue explants from PCOS subjects with 17-β-estradiol and noted an increase in RBP4 mRNA at 4 hours and in RBP4 protein levels at 24 hours. No effects were seen on RBP4 mRNA or protein levels after treatment with testosterone, androstenedione, DHEA-S, or insulin. These findings are consistent with a direct role for 17-β-estradiol in the regulation of RBP4. However, in our postmenopausal women, estradiol levels are lower, suggesting that the increase in RBP4 demonstrated in postmenopausal women is unlikely to be due to estradiol. In contrast, our data suggest a potential role for gonadotropins, independent of gonadal steroids, in the regulation of RBP4.

The physiologic significance of a 35% difference in RBP4 between pre- and postmenopausal women is not clear. In animal models, such as heterozygous Rbp4 knockout ($Rbp4^{+/-}$) mice, physiologic sequelae including improvement in insulin sensitivity with increased phosphoinositide 3-kinase activity were noted with a 50% reduction in serum levels of RBP4 [1]. In previously performed cross-sectional studies of humans, other studies have found even smaller differences between groups, ranging from a 15% increase in diabetic subjects compared with normal glucose-tolerant subjects [3] to a 23% increase in serum RBP4 levels in obese compared with nonobese adolescents [8]. In an interventional study of human subjects, a 43% reduction in RBP4 levels was associated with improved insulin sensitivity by exercise training [2]. Therefore, a 35% difference in an abundantly circulating protein may be clinically significant.

The strong relationship seen between RBP4 and gonadotropins may also contribute to the changes in insulin sensitivity associated with menopause [28]; for example, RBP4 may mediate the insulin resistance seen with aging in women. Insulin AUC during the OGTT was higher in the postmenopausal subjects, and RBP4 did correlate with insulin parameters among the entire group of pre- and postmenopausal women in our study. These data obtained in women support the relationship of RBP4 to glucose metabolism previously reported in men [2,4] and suggest that this relationship exists in women, despite sexual dimorphism in circulating RBP4 concentrations.

It is not clear from our study whether FSH, LH, or both might potentially regulate RBP4. In our analysis, FSH had a slightly stronger statistical association with RBP4; but the association with both FSH and LH was very strong. Thus, it is not clear if one or both might potentially regulate RBP4. To date, only LH receptors have been identified on adipocytes [18], suggesting that LH may be the physiologic regulator of RBP4 rather than FSH. Alternatively, RBP4 may be regulated by covariates or downstream elements of LH/FSH. Conversely, RBP4 may modulate gonadotropin secretion through effects on gonadal steroid regulation or an as yet unknown mechanism. Causality cannot be determined from a cross-sectional study; and it will be important to determine the direct effects of FSH, LH, and estradiol on RBP4 in prospective studies of women.

The hypothesis that RBP4 is regulated by gonadotropins and/or gonadal steroids is not necessarily contrary to the sexual dimorphism noted in previous reports and may in fact explain some of the heterogeneity in the previous data. Both gonadotropins and gonadal steroid levels vary through the menstrual cycle and after menopause. Therefore, the timing of sampling from female subjects may have biased the previously reported RBP4 values in the female population. In addition, as menopausal status is associated with age, changes in body composition and body fat distribution [29,30], insulin resistance [28], and the metabolic syndrome [31], it is not inconceivable that previous studies of RBP4 may have been confounded by this relationship. Lastly, RBP4 is known to be made primarily in the liver followed by adipocytes [27]; and hepatic and extrahepatic production of RBP4 may also be confounding the sexual dimorphism of RBP4.

Retinol-binding protein was not associated with adiponectin in this cohort of healthy women, consistent with findings from previous studies [5,10]. The lack of association is suggestive of a different physiologic regulatory mechanism and function. Inflammation has been postulated to play a role in mediating the manifestation of metabolic complications of obesity and particularly that of insulin resistance [32]. However, the association between inflammatory indices and RBP4 has yet to be clearly demonstrated. Two studies have demonstrated positive correlations between CRP and RBP4 [7,8], and 1 study has failed to detect this association [10]. We did not detect a significant correlation with inflammatory markers including IL-6, homocysteine, or CRP and RBP4, although a trend toward significance with TNFα was noted. Moreover, there was no relationship between RBP4 and carotid IMT, suggesting that RBP4 is not involved in the proinflammatory, proatherosclerotic state associated with obesity, contrary to adiponectin [23,24]. However, these results should be interpreted with some caution given the lack of sufficient power inherent in our unequal sample size and will need to be confirmed in future studies.

We confirmed our results from ELISA using quantitative Western blotting, as Graham et al [33] suggest that quantitative Western blotting is the most reliable method of assaying serum RBP4. The values reported by ELISA were on the average lower than those by Western blot, consistent with prior reports in the literature demonstrating a greater dynamic range for the Western blot [33]. The ELISA and Western blot assays demonstrated a strong positive correla-

tion ($P < .0001$). Moreover, we confirmed the primary results, for example, that RBP4 levels are higher in postmenopausal women using the Western blot.

Our study has a number of limitations. Causality or the directionality of the relationship between gonadotropins and RBP4 cannot be determined from a cross-sectional study; nor can we exclude effects of a parameter that may covary with FSH or LH, for example, inhibin. Our sample is also admittedly skewed with more premenopausal subjects than postmenopausal subjects, which could lead to lack of sufficient power to detect true differences. We were nonetheless able to detect a significant difference between RBP4 in pre- and postmenopausal subjects, and this difference is supported by the strong relationship between RBP4 and gonadotropins seen in the regression analyses.

In summary, we conclude that RBP4 is higher in postmenopausal women and associated with gonadotropins in healthy women. The strong association with insulin during OGTT confirms prior work suggesting a relationship between RBP4 and insulin resistance and extends this observation to healthy women. In addition, RBP4 was not associated with any indices of inflammation or with carotid IMT. Further studies are necessary to delineate the physiologic relationship between gonadotropins and RBP4 and to confirm the lack of relationship between RBP4, inflammatory markers, and cardiovascular disease risk in women.

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