Plasma Retinol-Binding Protein-4 Concentrations Are Elevated in Human Subjects With Impaired Glucose Tolerance and Type 2 Diabetes

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Editor's comment: This manuscript was in the process of review when another article with similar findings was published (N Engl J Med 354:2552–2563, 2006).

OBJECTIVE — The dysregulation of adipokines is closely associated with the pathogenesis of insulin resistance and type 2 diabetes. Retinol-binding protein-4 (RBP4), a new adipokine, was recently reported to provide a link between obesity and insulin resistance. Here, we examined the relation between plasma RBP4 concentrations and various metabolic parameters in humans.

RESEARCH DESIGN AND METHODS — An enzyme-linked immunosorbent assay was developed to measure human RBP4 plasma concentrations, which were then compared with various parameters related to insulin resistance in subjects with normal glucose tolerance (NGT; $n = 57$), impaired glucose tolerance (IGT; $n = 48$), and type 2 diabetes ($n = 49$).

RESULTS — Plasma RBP4 concentrations were higher in the IGT and type 2 diabetic groups than in the NGT group (median 18.9 [range 11.2–45.8], 20.9 [9.9–48.5], and 18.1 μg/ml [9.3–30.5], respectively). However, no difference was found between plasma RBP4 concentrations in the IGT and type 2 diabetic groups. Plasma RBP4 concentrations were found to be associated with sex, waist circumference, fasting plasma glucose, and insulin resistance. Of these, sex and fasting plasma glucose levels were found to be independent determinants of plasma RBP4 concentration.

CONCLUSIONS — Plasma RBP4 concentrations were found to be elevated in subjects with IGT or type 2 diabetes and to be related to various clinical parameters known to be associated with insulin resistance.

Diabetes Care **29:2457–2461, 2006**

An increased adipose tissue mass is strongly associated with the pathogenesis of insulin resistance and type 2 diabetes (1). Adipose tissue may be viewed as an endocrine organ that secretes many types of adipokines (such as leptin, tumor necrosis factor α, interleukin 6, and adiponectin) that modulate the action of insulin in other tissues (2–5). Moreover, retinol-binding protein-4 (RBP4), a new fat-derived adipokine that specifically binds to retinol (6), has recently been reported to provide a link between obesity and insulin resistance (7). RBP4 was discovered while trying to identify the substance responsible for regulating insulin sensitivity in mice either lacking or overexpressing GLUT4 in adipose tissues (8,9). It is regulated reciprocally in adipose tissue of mice overexpressing or lacking GLUT4. Circulating RBP4 levels were reported to be raised in several different mouse models of obesity and insulin resistance (7). In mice lacking GLUT4, rosiglitazone (a peroxisome proliferator–activated receptor γ agonist) was found to lower circulating levels of RBP4 and to reduce insulin resistance. Increasing the circulating levels of RBP4 leads to glucose intolerance, whereas knock out of the RBP4 gene increases insulin sensitivity. In addition, treatment of mice with fenretinide (which facilitates the excretion of RBP4 into urine) decreased the insulin resistance induced by a high-fat diet (7).

A mechanism whereby RBP4 modulates insulin sensitivity in muscle and liver has been suggested. In skeletal muscle, RBP4 reduces insulin sensitivity by inhibiting both insulin receptor substrate-1 phosphorylation and phosphatidylinositol 3-kinase activation, while increasing hepatic glucose production by increasing PEPCK expression (7).

Although Yang et al. (7) showed, by Western blot, that serum RBP4 levels are elevated in humans with obesity and type 2 diabetes, no report has determined the exact circulating RBP4 concentrations. We therefore developed an enzyme-linked immunosorbent assay (ELISA) for measurement of the serum/plasma concentrations of human RBP4 and compared them with various parameters associated with insulin resistance.

RESEARCH DESIGN AND METHODS

— We enrolled subjects with normal glucose tolerance (NGT; $n = 57$), impaired glucose tolerance (IGT; $n = 48$), or type 2 diabetes ($n = 49$). Assign-

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Received for publication 13 February 2006 and accepted in revised form 3 August 2006.

Abbreviations: ELISA, enzyme-linked immunosorbent assay; HOMA-B, homeostasis model assessment of β-cell function; HOMA-IR, homeostasis model assessment of insulin resistance; IGT, impaired glucose tolerance; NGT, normal glucose tolerance; RBP4, retinol-binding protein-4.

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A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

DOI: 10.2337/dc06-0360

Table 1—*Clinical characteristics of the subjects*

	NGT ($n = 57$)	IGT ($n = 48$)	Type 2 diabetes ($n = 49$)	*P*
Age (years)	52 ± 10	53 ± 12	56 ± 9	0.160
Sex (M/F)	21/36	19/29	20/29	0.911
Systolic blood pressure (mmHg)	123 ± 17	128 ± 17	129 ± 14	0.126
Diastolic blood pressure (mmHg)	78 ± 12	81 ± 14	81 ± 9	0.219
BMI (kg/m^2)	24.4 ± 3.2	24.9 ± 3.1	25.3 ± 2.8	0.283
Waist circumference (cm)	82 ± 9*†	86 ± 7*	87 ± 7†	0.001
Fasting plasma glucose (mmol/l)	4.4 ± 0.8*†	5.3 ± 0.8*‡	6.6 ± 1.0†‡	<0.001
2-h postchallenge plasma glucose (mmol/l)	5.9 ± 1.2*†	9.2 ± 1.0*‡	14.5 ± 2.4†‡	<0.001
Fasting plasma insulin (pmol/l)	62 ± 24†	71 ± 30	79 ± 36†	0.016
Triglycerides (mmol/l)	1.38 (0.43–6.08)	1.53 (0.61–8.38)	1.44 (0.44–5.86)	0.238
Total cholesterol (mmol/l)	4.89 ± 0.92*	5.39 ± 0.97*	5.16 ± 0.79	0.019
LDL cholesterol (mmol/l)	2.90 ± 0.79*	3.30 ± 0.88*	3.18 ± 0.68	0.032
HDL cholesterol (mmol/l)	1.22 (0.78–2.18)	1.26 (0.73–2.67)	1.14 (0.78–2.51)	0.562
Fat mass (kg)	16.8 ± 5.5†	18.3 ± 4.6	19.2 ± 4.9†	0.074
Percent body fat	27.1 ± 6.8	27.8 ± 6.3	29.3 ± 5.8	0.229
A1C (%)	5.6 (5.1–6.7)*†	6.1 (4.6–7.1)*‡	6.4 (5.5–8.4)†‡	<0.001
HOMA-IR	2.0 (0.6–4.0)*†	2.7 (0.2–6.3)*‡	3.3 (1.1–14.0)†‡	<0.001
HOMA-B	206 (46–874)*†	117 (24–769)*‡	82 (31–292)†‡	<0.001

Data are means ± SD (for normal distribution) or median (range). *P* values are by ANOVA. *$P < 0.05$ for NGT vs. IGT; †$P < 0.05$ for NGT vs. type 2 diabetes, ‡$P < 0.05$ for IGT vs. type 2 diabetes.

ment to one of the three groups was determined by a 75-g oral glucose tolerance test, according to the diagnostic criteria of the American Diabetes Association (10). Blood pressure, height, weight, and waist and hip circumferences were measured. Plasma glucose levels were determined using the glucose oxidase method (YSI 2300 STAT; Yellow Springs Instruments, Yellow Springs, OH). Total cholesterol, triglyceride, and HDL cholesterol concentrations were measured enzymatically using an autoanalyzer (Hitachi 747; Hitachi, Tokyo, Japan). HbA_{1c} (A1C) was measured by affinity chromatography using the Bio-Rad Variant II system (Bio-Rad Laboratories, Hercules, CA), and plasma insulin concentrations were measured by radioimmunoassay (BioSource, Nivelles, Belgium). Total fat masses were determined by bioelectrical impedance analysis (Inbody 2.0; Biospace, Seoul, Korea). Homeostasis model assessments of insulin resistance (HOMA-IR) and β-cell function (HOMA-B) were performed as previously described (11). The clinical characteristics of study subjects are shown in Table 1, and all subjects with type 2 diabetes were naïve to antidiabetes therapy. Sixteen of the 154 study subjects were on antihypertensive medications: 1 of 57 NGT, 5 of 48 IGT, and 10 of 49 type 2 diabetes. Ten subjects were on lipid-lowering agents: 2 of 57 NGT, 5 of 48 IGT, and 3 of 49 type 2 diabetes.

The institutional review board of the Clinical Research Institute at Seoul National University Hospital approved the study protocol, and written informed consent was obtained from all subjects.

ELISA for RBP4

The methods used to generate polyclonal anti-human RBP4 antibody and to validate the assay system are available in an online appendix (available at http://care.diabetesjournals.org). Briefly, along with RBP4 standards of concentration 0.001–2.5 μg/ml, 50 μl human plasma at a dilution of 1:100, which was collected from subjects who had fasted overnight using EDTA-containing tubes, was applied to each test well. Then, 50 μl anti-RBP4 was added to each well and incubated at 37°C for 1 h. Each well was then washed three times with PBS containing 0.05% Tween-20. The secondary antibody reaction was performed at 25°C for 1 h, and then each well was washed three times with PBS containing 0.05% Tween-20. Colorimetric reaction was performed for 20 min with the use of horseradish peroxidase–conjugated streptavidin (Zymed, South San Francisco, CA) diluted 1:1,000 in PBS and 2,2′-azino-bis(2-ethylbenzothiazoline-6-sulfonic acid) (Pierce, Rockford, IL) as substrate. Optical densities were measured at 450 nm. The ELISA system had an intra-assay coefficient of variation of 4–8% and an interassay coefficient of

Table 2—*Plasma RBP4 levels in NGT, IGT, and type 2 diabetic groups*

	Plasma RBP4 concentrations (μg/ml)			*P*			
	NGT ($n = 57$)	IGT ($n = 48$)	Type 2 diabetes ($n = 49$)	ANOVA	NGT vs. IGT	NGT vs. type 2 diabetes	IGT vs. type 2 diabetes
Total	18.1 (9.3–30.5)	18.9 (11.2–45.8)	20.9 (9.9–48.5)	0.002	0.023	0.0004	0.308
Male	19.4 (10.7–30.5)	22.5 (12.3–45.8)	23.8 (13.4–48.5)	0.040	0.067	0.008	0.650
Female	16.2 (9.3–28.2)	18.0 (11.2–34.6)	20.1 (9.9–31.5)	0.049	0.166	0.016	0.322

Data are median (range). Plasma RBP4 concentrations were log transformed before analysis.

Table 3—*Correlation between plasma RBP4 levels and various metabolic parameters in subjects with NGT (n = 57)*

	Pearson's coefficient	*P*
Age	0.11	0.432
BMI	0.19	0.162
Waist circumference	0.32	0.016
Systolic blood pressure	0.14	0.306
Diastolic blood pressure	0.06	0.641
Fat amount	0.16	0.248
Percent body fat	0.09	0.533
Fasting plasma glucose	0.37	0.005
2-h postchallenge plasma glucose	0.17	0.198
Fasting plasma insulin	0.15	0.264
Triglyceride*	0.26	0.053
Total cholesterol	0.18	0.189
LDL cholesterol	0.10	0.458
HDL cholesterol*	−0.08	0.555
A1C*	0.21	0.113
HOMA-IR*	0.28	0.038
HOMA-B*	−0.18	0.226

*These variables were log transformed before analyses.

variation of 5–10% (see online appendix for validation details).

Statistical analyses

All normally distributed continuous variables are expressed as means ± SD, whereas variables with a skewed distribution are expressed as medians and ranges. Student's *t* test, ANOVA, Pearson's correlation analyses, and multiple linear regression analyses were performed using SPSS software (SPSS, Chicago, IL). *P* values <0.05 were considered statistically significant.

RESULTS

Plasma RBP4 concentrations in patients with NGT, IGT, and type 2 diabetes

As shown in Table 2, plasma RBP4 concentrations were higher in the IGT and type 2 diabetic groups than in the NGT group. However, the difference between the IGT and NGT groups was attenuated when divided into each sex group. No difference in plasma RBP4 levels was observed between the IGT and type 2 diabetic groups.

Plasma RBP4 concentrations were modestly correlated with waist circumference, fasting plasma glucose, and HOMA-IR in the NGT group (Table 3). Moreover, fasting plasma glucose levels were found to be an independent determinant for plasma RBP4 concentrations (online appendix Table 5).

Differences in plasma RBP4 concentrations according to sex

The median (range) for RBP4 in plasma was 21.0 μg/ml (10.7–48.5) for men and 18.1 μg/ml (9.3–34.6) for women ($P = 0.001$). Since data regarding menopausal status were not available, we arbitrarily subdivided sex groups at 50 years of age (Table 4). Plasma RBP4 levels in women over the age of 50 were found to be significantly higher than those in women under the age of 50. However, no such age-associated difference in RBP4 plasma levels was observed in men. Moreover, women over the age of 50 had significantly lower plasma RBP4 levels than men.

Clinical characteristics according to plasma RBP4 quartiles

To examine the association between plasma RBP4 concentrations and other parameters related to insulin resistance, we divided our study subjects into plasma RBP4 concentration quartiles (Table 5). The proportion of IGT and type 2 diabetes increased on increasing RBP4 concentration. Also, the male proportion, waist circumference, fasting and postchallenge plasma glucose levels, triglyceride levels, and HOMA-IR values increased on increasing RBP4. Although plasma RBP4 concentrations were found to be associated with waist circumference, they showed no significant correlation with BMI, fat mass, or percent body fat. Multivariate analysis revealed that sex and fasting plasma glucose levels were independently associated with plasma RBP4 concentrations (Table 6).

CONCLUSIONS

— In this study, we found that plasma RBP levels, measured by competitive ELISA (see online appendix for validation details), are elevated in subjects with IGT or type 2 diabetes. It was interesting to note that RBP4 was abundant in human plasma (9.3–30.5 μg/ml in normal subjects), which concurred with findings in rodents (6).

Contrary to the previous finding that obese subjects have higher serum RBP4 levels than lean subjects (7), we found no association between plasma RBP4 levels and the amount or percentage of body fat. However, *ob/ob* mice were found to have a 13-fold–higher serum RBP4 level than control mice, and, according to Yang et al. (7), obese human subjects with elevated serum RBP4 levels had unequivocally higher BMIs than lean control subjects (32.4 ± 1.2 vs. 23.8 ± 0.2 kg/m^2). Thus, the relatively narrow BMI distribution of our study subjects might result in no relation between RBP4 levels and BMI. However, we found that waist circumferences increased with plasma RBP4 quartiles. It is possible that visceral fat is more important than total-body fat in determining circulating RBP4 levels.

Although Yang et al. (7) showed an

Table 4—*Plasma RBP4 concentrations according to age and sex*

		Age <50 years	Age ≥50 years	*P*
Plasma RBP4 concentrations (μg/ml)	Male	20.3 (10.7–35.0) (27)	21.9 (12.4–48.5) (33)	0.172
	Female	15.4 (9.3–21.4) (24)	19.7 (9.9–34.6) (70)	<0.001

Data are median (range) (*n*). $P = 0.04$, total male vs. female ≥50 years of age.

Table 5—*Clinical characteristics according to the quartiles of plasma RBP4 concentrations*

	First quartile	Second quartile	Third quartile	Fourth quartile	*P* for trend
n	38 (100)	39 (100)	38 (100)	39 (100)	
Plasma RBP4 (μg/ml)	13.4 (9.3–15.8)	17.7 (15.9–19.4)	21.1 (19.5–22.5)	26.6 (22.6–48.5)	<0.001
NGT (%)	22 (57.9)	14 (35.9)	13 (34.2)	8 (20.5)	
IGT (%)	11 (28.9)	13 (33.3)	10 (26.3)	14 (35.9)	<0.001
Type 2 diabetes (%)	5 (13.2)	12 (30.8)	15 (39.5)	17 (43.6)	
Sex (M/F)	10/28	12/27	16/22	22/17	0.004
Age (years)	51 ± 12	53 ± 11	57 ± 10	53 ± 10	0.055
SBP (mmHg)	123 ± 19	127 ± 15	127 ± 15	130 ± 17	0.313
DBP (mmHg)	78 ± 12	79 ± 10	80 ± 11	82 ± 13	0.304
BMI (kg/m^2)	24.2 ± 3.7	24.6 ± 2.9	24.9 ± 3.2	25.5 ± 2.3	0.304
Waist (cm)	81 ± 9	84 ± 8	87 ± 8	87 ± 6	0.005
Fat mass (kg)	17.4 ± 6.5	18.3 ± 4.5	18.0 ± 5.3	18.3 ± 3.7	0.847
Percent body fat	27.7 ± 7.9	29.0 ± 5.5	27.8 ± 6.4	27.1 ± 5.5	0.653
FPG (mmol/l)	4.8 ± 1.0	5.1 ± 1.1	5.5 ± 1.3	6.1 ± 1.1	<0.001
2-h postchallenge plasma glucose (mmol/l)	7.8 ± 3.0	9.8 ± 4.0	10.2 ± 4.0	11.0 ± 3.9	0.003
Fasting plasma insulin (pmol/l)	63 ± 37	71 ± 25	72 ± 27	75 ± 30	0.331
Triglycerides (mmol/l)*	1.40 (0.45–4.00)	1.11 (0.44–6.60)	1.41 (0.71–8.38)	1.70 (0.43–5.86)	0.005
Total cholesterol (mmol/l)	4.96 ± 0.80	4.95 ± 1.00	5.20 ± 0.72	5.42 ± 1.06	0.074
LDL cholesterol (mmol/l)	3.06 ± 0.80	2.96 ± 0.72	3.17 ± 0.71	3.28 ± 0.93	0.340
HDL cholesterol (mmol/l)*	1.24 (0.73–2.18)	1.24 (0.85–1.99)	1.18 (0.75–1.99)	1.15 (0.83–2.67)	0.905
A1C (%)*	5.8 (5.2–7.6)	5.8 (5.1–8.1)	6.2 (5.2–8.1)	6.3 (4.6–8.4)	0.050
HOMA-IR*	1.9 (0.2–8.8)	2.5 (0.6–5.4)	2.6 (0.9–7.5)	3.0 (1.1–14.0)	<0.001
HOMA-B*	149 (44–661)	126 (24–874)	135 (31–769)	92 (47–356)	0.063

Data are *n* (%) or median (range). *These variables were log transformed before analyses.

unequivocal difference between normal and obese subjects, with or without diabetes, in terms of circulating RBP4 concentrations, we found that glucose tolerance status had only a small effect on plasma RBP4 concentrations. This is probably attributable to the narrow BMI range shown by our study subjects. However, we cannot rule out the possibility that this discrepancy was caused by the different measuring systems used (i.e., band intensity by Western blotting vs. ELISA optical density) or by the different affinities of the antibodies used. Even though differences in the plasma RBP4 concentrations among glucose tolerance groups were small, as shown in Table 5, various clinical parameters were found to be correlated with plasma RBP4. Furthermore, there was a step up in fasting insulin and HOMA-IR between the first and second quartiles, which suggests that small differences in plasma RBP4 concentrations might be physiologically relevant.

Plasma RBP4 concentrations were found to be sexually dimorphic, which had previously been reported for adipokines, such as leptin and adiponectin, and explained on the basis of different fat amounts and the influences of sex hormones (12–14). As described previously, no difference was found in plasma RBP4 levels with respect to fat amounts or body fat percentages in the present study. Moreover, although data regarding menopausal status was unavailable in our female subjects, plasma RBP4 levels in women over the age of 50 years were found to be significantly higher than those of women under the age of 50, while levels in men were higher than both. Thus, it appears that differences in sex hormone status might affect RBP4 plasma levels.

Fasting plasma glucose levels were found to increase with plasma RBP4 quartile and were found to be an independent factor determining plasma RBP4 levels. Yang et al. (7) initially reported that obesity, but not hyperglycemia, is an important determinator of circulating RBP4 levels. However, they could not examine the quantitative relation between plasma RBP4 and blood glucose levels. The possible mechanism underlying increased fasting plasma glucose levels in subjects with higher plasma RBP4 levels probably concerns increased hepatic glucose output, as RBP4 has been reported to upregulate the expression of PEPCK, a key enzyme in hepatic gluconeogenesis, in the liver (7).

Although the sample size in the present study was small, plasma RBP4 showed no significant correlation with leptin or adiponectin concentrations

Table 6—*Multivariate analysis for the relationship between metabolic parameters and plasma RBP4 level* (n = *154*)

	Unstandardized coefficients		Standardized coefficients	
	β	SE	β	*P*
Constant	2.172	0.278		<0.001
Sex	0.135	0.050	0.216	0.007
Waist circumference	0.003	0.003	0.083	0.345
Fasting plasma glucose	0.005	0.001	0.337	<0.001
HOMA-IR	0.047	0.126	0.037	0.712

In this multivariate analysis, variables in Table 5 with a *P* value <0.05 were included.

(data not shown). It was reported that the serum levels of most adipokines were normal in mice with adipocyte-specific GLUT4 ablation (7) and suggested that the signals that regulate adipokine secretion may differ among different adipokines (15).

This study shows that plasma RBP4 concentrations are elevated in patients with IGT or type 2 diabetes and are associated with various clinical parameters associated with insulin resistance. It should be determined whether plasma concentration of RBP4 changes under various physiological conditions. In regard to this point, we observed that there were no significant changes in plasma RBP4 levels during oral glucose tolerance test ($n = 6$; online appendix Fig. 2). It is also important to investigate whether plasma RBP4 concentration can predict future development of type 2 diabetes.

Acknowledgments— This study was supported by grant FPR05C1-110 of the 21C Frontier Functional Proteomics Project from the Korean Ministry of Science & Technology.

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