Circulating retinol binding protein 4, insulin sensitivity, insulin secretion and insulin disposition index in obese and nonobese subjects

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Abstract

OBJECTIVE- Recent investigations disclosed an upregulation of retinol binding protein-4 (RBP4) in the adipose tissue of several insulin-resistant mouse models and increased serum RBP4 concentration in subjects with obesity and type 2 diabetes in association with insulin resistance. There is some experimental evidence that RBP4 could also been linked to insulin secretion.

RESEARCH DESIGN AND METHODS- We aimed to evaluate insulin secretion, insulin sensitivity, insulin disposition index (minimal model analysis) and circulating RBP4 (ELISA) in nondiabetic men with a wide range of obesity (n=107).

RESULTS- Serum RBP4 concentration was nonsignificantly different among lean, overweight and obese subjects. Circulating RBP4 was not associated with age, body mass index, waist-to-hip ratio or metabolic parameters, including insulin sensitivity (r=-0.03, p=0.6). On the contrary, circulating RBP4 was negatively associated with insulin secretion, especially in obese subjects (r=-0.48, p=0.007), in whom RBP4 was also linked to insulin disposition index (r=-0.44, p=0.01). On multiple regression analyses to predict insulin secretion (AIRg), insulin sensitivity was the only factor that contributed to 17% of AIRg variance in nonobese subjects. In obese subjects, however, RBP4 emerged as an independent factor that contributed independently to AIRg variance (23%).

CONCLUSIONS- Our results suggest that over-secretion of RBP4 may negatively affect β-cell function directly or by preventing the binding of transthyretin to its receptor. These mechanisms could be behind the association between increased circulating RBP4 and type-2 diabetes. RBP4 could be one signal from insulin-resistant tissues that impacts on β-cell secretion.
Serum retinol binding protein 4 (RBP4) has recently found to be increased in insulin-resistant subjects (1). Graham et al. (1) reported increased serum RBP4 concentration in subjects with obesity or type 2 diabetes compared with lean subjects. Insulin resistance was positively associated with serum RBP4 concentration and invoked to be causally related with type 2 diabetes. In fact, RBP4 is upregulated in the adipose tissue of several insulin-resistant mouse models (1,2). Transgenic expression or injections of RBP4 caused insulin resistance in mice, whereas experimentally decreased RBP4 levels ameliorated insulin resistance in diet-induced obesity. RBP4 augmented hepatic gluconeogenesis and attenuated insulin signaling in skeletal muscle (2). RBP4 was established as a rodent adipokine several years ago (3,4) and confirmed recently (5).

Recent findings in humans suggest that the increase in systemic RBP4 concentrations in insulin-resistant subjects or subjects with type 2 diabetes is not explained by increased RBP4 production in adipose tissue (5). In this study, the authors did not see a relationship between adipose tissue RBP4 expression and serum RBP4 levels in postmenopausal women. In fact, RBP4 mRNA was downregulated in subcutaneous abdominal adipose tissue, and circulating RBP4 concentrations were similar in the normal weight, overweight, and obese groups (5). Five-percent weight loss improved the HOMA index by 20%, while this change was associated with only a small decrease of adipose RBP4 expression and no significant change in RBP4 serum levels. In addition, a relationship between the HOMA index and adipose RBP4 expression or with circulating RBP4 concentrations was not observed (5). On the other hand, it is well known that retinol is pathophysiologically linked to β-cell function (6). Because abnormalities in insulin secretion contribute also to the development of the metabolic abnormalities observed in type 2 diabetes, we hypothesized that, besides insulin sensitivity, serum RBP4 levels could also be related to insulin secretion and the insulin disposition index.

RESEARCH DESIGN AND METHODS
We studied one hundred and seven non-diabetic men were consecutively enrolled in a prospective study of cardiovascular risk factors as described in reference 7. Inclusion and Exclusion Criteria
All subjects reported that their body weight had been stable for at least three months before the study. A food frequency questionnaire was obtained from all subjects. None of the subjects was taking any medication or had any evidence of metabolic disease other than obesity. Inclusion criteria were: 1) Body mass index (BMI, weight in kilograms divided by the square of height in meters) less than <40 kg/m^2; 2) absence of any systemic disease, 3) absence of clinical symptoms and signs of infection in the previous month by structured questionnaire to the patient. Informed consent was obtained from all subjects. Local Ethics Committee approved the study.

Measurements
BMI was calculated as weight (in kilograms) divided by height (in meters) squared. The subjects’ waist was measured with a soft tape midway between the lowest rib and the iliac crest. The hip circumference was measured at the widest part of the gluteus region. The waist-to-hip ratio (WHR) was then calculated. Blood pressure was measured in the supine
position on the right arm after a 10-min rest; a standard sphygmomanometer of appropriate cuff size was used and the first and fifth phases were recorded. Values used in the analysis are the average of three readings taken at 5-min intervals. Patients were requested to withhold alcohol and caffeine during at least 12 h prior to the different tests.

**Insulin sensitivity and secretion**

All subjects had fasting plasma glucose < 7.0 mM. Type 2 diabetes was ruled out by an oral glucose tolerance test (OGTT) according to criteria from the American Diabetes Association (8). **Insulin sensitivity** was measured using the frequently sampled intravenous glucose tolerance test (FSIVGTT). **Insulin secretion** was calculated as the insulin area during the first 10 minutes of the FSIVGTT. This test also provides the insulin disposition index, a parameter emerging from the model that represents the ability of the pancreatic islets to compensate for insulin resistance. The disposition index is useful in the search of the causes of the failure of adequate beta-cell compensation in type 2 diabetes, and in the recognition of the nature of the signal(s) from insulin-resistant tissues that fail to elicit the appropriate beta-cell increment in sensitivity to glucose and other stimuli (9).

In brief, the experimental protocol started between 8:00 and 8:30 AM after an overnight fast. A butterfly needle was inserted into an antecubital vein, and patency was maintained with a slow saline drip. Basal blood samples were drawn at -30, -10 and -5 minutes, after which glucose (300 mg/Kg body weight) was injected over 1 minute starting at time 0, and insulin (Actrapid, Novo, Denmark; 0.03 U/kg) was administered at time 20. Additional samples were obtained from a contra-lateral antecubital vein up to 180 minutes, as previously described (7).

**Analytical methods**

Blood samples were drawn from each subject after an overnight fasting period. Serum was centrifuged at 4000g for 10 minutes, immediately divided into aliquots, and frozen at -80°C until analysis. Serum glucose concentrations were measured in duplicate by the glucose oxidase method with the use of a Beckman Glucose Analyser II (Beckman Instruments, Brea, CA). The coefficient of variation was 1.9%. Serum insulin concentrations were measured in duplicate by a monoclonal immuno-radiometric assay (IRMA, Medgenix Diagnostics, Fleunes, Belgium). Intraassay and interassay coefficients of variation (CV) were less than 7% (7,10). HbA1c was measured by high performance liquid chromatography by means of a fully automated glycated hemoglobin analyser system (Hitachi L-9100, USA). Total serum cholesterol was measured through the reaction of cholesterol esterase/cholesterol oxidase/peroxidase. Total serum triglycerides were measured through the reaction of glycerol-phosphate-oxidase and peroxidase.

Serum RBP4 concentrations were measured by nephelometry (Dade Behring Inc., Marburg, Germany). Sensitivity of the method is 0.01 mg/dl. The intra-interassay coefficients of variation were 3.1% and 2.2% respectively.

**Statistical methods**

Descriptive results of continuous variables are expressed as mean (SD) if normally distributed, or as median and interquartile range. Before statistical analysis, normal distribution and homogeneity of the variances were evaluated using Kolmogorov -Smirnov’ s test and Levene’s
test and then variables were given a log-
transformation if necessary. These pa-
rameters (insulin sensitivity, insulin sec-
retion, insulin disposition index, triglyc-
erides) were analysed on a log scale
and tested for significance on that scale.
The anti-log transformed values of the
means are reported in the Tables. Di-
ferences between groups were tested by
ANOVA’s test for continuous variables.
Relation between variables was tested
using Pearson’s test. Multivariate linear
regression analysis was performed in a
stepwise manner. A value of \( p < 0.05 \)
was considered significant. Given this value of
\( p = 0.05 \), the study had an 85% power to
detect significant correlations (Pearson’s
coefficient of at least 0.3) between param-
eters in bilateral tests. The study also had a 67% power to detect significant
differences of at least 1 SD in serum RBP4
concentration between obese and nonobese
subjects. Computations were carried out
with SPSS version 11.0.

RESULTS
Main characteristics of study subjects are
shown in Table 1. We studied 25 lean
subjects (BMI \( \leq 25 \); mean BMI 23.5 \( \pm 1.04
Kg/m^2 \), mean age 46.2 \( \pm 10.3 \) years), 52
subjects with overweight (BMI \( \geq 25 \) and
<30; mean BMI 27.2 \( \pm 1.4 \) Kg/m\(^2\), mean
age 51.3 \( \pm 10.8 \) years) and 30 obese
subjects (BMI \( \geq 30 \); mean BMI 33.5 \( \pm 2.4
Kg/m^2 \), mean age 52.3 \( \pm 11.6 \) years). Age
was not significantly different among these
groups (ANOVA \( p = 0.1 \)).
In all subjects as a whole, circulating RBP4 was not found to be associated with age, body mass
index, waist-to-hip ratio, blood pressure or
circulating lipids. Serum RBP4 concentration was found to be similar among lean (3.6 \( \pm 0.7 \) mg/dl), overweight (3.9 \( \pm 0.9 \) mg/dl) and obese subjects (4.02
\( \pm 1.1 \) mg/dl) \( p = 0.4 \).

Five lean subjects, 9 overweight and 12
obese subjects showed glucose intolerance
(2 h OGTT serum glucose between 7.8 and
11.1 mmol/l). After excluding these
subjects from the analyses, the results
remained essentially the same. Serum
RBP4 concentration was not significantly
different between subjects with normal or
impaired glucose tolerance, both in
nonobese subjects (3.8 \( \pm 0.8 \) vs. 3.8 \( \pm 1.08
mg/dl, p = 0.6, \) in normal and glucose
intolerant subjects, respectively) and obese
subjects (3.9 \( \pm 1 \) vs. 4.06 \( \pm 1.2 \) mg/dl,
\( p = 0.3 \), in normal and glucose intolerant
subjects, respectively).
Circulating RBP4 tended to be positively
linked to glycated hemoglobin \( (r = 0.16,
p = 0.08) \) and did not show association with
insulin sensitivity \( (r = -0.03, p = 0.6) \). On the
contrary, circulating RBP4 was negatively
associated with insulin secretion \( (r = -0.27,
p = 0.006) \) and tended to be associated with
insulin disposition index \( (r = -0.18, p = 0.06) \).
When we evaluated these associations
separately in obese and nonobese subjects,
the relationships of RBP4 with insulin
secretion and insulin disposition index
were especially significant among obese
subjects (Figure 1). In these subjects,
RBP4 was also negatively associated with
insulin at 30 min after oral glucose load, a
surrogate of insulin secretion \( (r = -0.39,
p = 0.03) \).
Circulating RBP4 concentrations were not
associated with \( S_G \) (glucose effectiveness)
in all subjects as a whole, or in obese and
nonobese subjects separately \( (r \) coefficients
from 0.0005 to 0.02, \( p = NS \)).
On multiple regression analyses to predict
insulin secretion (AIRg), insulin sensitivity
was the only factor that contributed to 17%
of AIRg variance in nonobese subjects
(Table 2). In obese subjects, however,
RBP4 emerged as an independent factor
that contributed independently to AIRg
variance (23%) after controlling for BMI,
age and insulin sensitivity (Table 2, lower panel).

**DISCUSSION**

In this article we describe that circulating RBP4 was not significantly associated with insulin sensitivity or obesity status in middle aged men. These findings appear to contradict a previous publication (1). This could be related to differences concerning the relatively small population studied. Graham et al. (1) evaluated 7 obese men and 9 obese men with type 2 diabetes compared with 5 lean subjects in study 1. The differences could also be due to the RBP4 assay. Western blotting and an ELISA sandwich assay produced RBP4 measurements that distinguished normal individuals from insulin-resistant individuals and correlated with magnitude of insulin resistance (1). However, that ELISA assay is no longer available (11). These authors compared different methods measuring plasma RBP4 in a very recent study (11). One of the conclusions was that competitive enzyme-linked immunoassays may selectively underestimate serum RBP4 levels in the setting of insulin resistance due to assay saturation. In addition, commercially available sandwich ELISA reports RBP4 concentrations that inversely correlate with insulin resistance, but values in normal subjects are higher than expected (11). The authors also stated that other assay methods, especially nephelometry, deserve testing (11). This was precisely the method we used.

In contrast to this recent observation (1), Janke et al. also found that RBP4 was not associated with insulin resistance evaluated using HOMA (5). The authors argued that “detection of RBP4-mediated changes in insulin sensitivity may require more accurate measurements of insulin sensitivity and patients with a wider range of insulin sensitivities than used in our study”. We have used a relatively strong measure of insulin sensitivity in subjects with a wide range of adiposity and we also did not observe associations between RBP4 and insulin sensitivity. In agreement with the findings by Janke et al. in postmenopausal women, we did not detect differences in serum RBP4 concentration according to obese status in middle-aged men.

In contrast, we observed a significant association between circulating RBP4 and insulin secretion in all subjects as a whole. This association was especially remarkable in obese subjects in whom RBP4 was also negatively linked to insulin disposition index. The insulin disposition index represents the ability of the pancreatic islets to compensate for insulin resistance. The disposition index is useful in the search for the causes of the failure of adequate beta-cell compensation in type 2 diabetes, and in the recognition of the nature of the signal(s) from insulin-resistant tissues that fail to elicit the appropriate beta-cell increment in sensitivity to glucose and other stimuli. Importantly, circulating RBP4 was independently associated with insulin secretion even after accounting for insulin sensitivity, BMI and age among obese subjects. Our data suggest that obese subjects with increased circulating RBP4 show incapacity to adapt to low insulin sensitivity by enhancing insulin secretion (Figure 1) and, in keeping with this, they also show a reduced insulin disposition index.

We should point out that the negative relationship between RBP4 and insulin secretion was observed after an oral glucose bolus and also after an intravenous load, indicating that the associations detected do not depend on the route of glucose administration.
It is well known that retinol is pathophysiologically linked to β-cell function (6). On the other hand, retinol-binding protein circulates in serum forming a complex with transthyretin (TTR), a transport protein for thyroxine. A recent investigation disclosed that TTR constitutes a functional component in pancreatic β-cell stimulus-secretion coupling (12). TTR inhibits the binding of RBP to the receptor (13). It is thus possible that increased serum RBP4 prevents transthyretin from exerting its β-cell stimulus-secretion effects.

In fact, circulating RBP4 is highly bound to TTR in a 1:1 stoichiometric ratio, and there is little or no ‘free RBP4’ in circulation (14). In a very recent study using gel filtration chromatography to analyse the RBP4-TTR complex, the authors found that increased serum RBP4 remains bound to TTR in insulin-resistant states (11). Because the affinity of RBP4-TTR binding is very strong (14), the relative stoichiometry and affinity of the two proteins in serum could conceivably influence kinetics of RBP4-antibody binding.

In summary, our results suggest that over-secretion of RBP4 may negatively affect β-cell function directly or by preventing the binding of TTR to its receptor. These mechanisms could be behind the association between increased circulating RBP4 and type-2 diabetes (1). To our knowledge, this is the first report of an association between serum RBP4, insulin secretion and insulin disposition index.

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REFERENCES


# TABLE 1.

## CLINICAL AND LABORATORY VARIABLES IN STUDY SUBJECTS

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>(n=107)</th>
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<tbody>
<tr>
<td>Age (years)</td>
<td>50.4 (11.3)</td>
</tr>
<tr>
<td>BMI (kg/m²) (range)</td>
<td>27.8 (3.6) (19.4-39.8)</td>
</tr>
<tr>
<td>WHR</td>
<td>0.93 (0.06)</td>
</tr>
<tr>
<td>SBP (mm Hg)</td>
<td>126 (15)</td>
</tr>
<tr>
<td>DBP (mm Hg)</td>
<td>80 (10)</td>
</tr>
<tr>
<td>HDL-cholesterol (mg/dl)</td>
<td>46 (13)</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>102 (71.5-142.5)</td>
</tr>
<tr>
<td>Fasting glucose (mg/dl)</td>
<td>97 (10)</td>
</tr>
<tr>
<td>Fasting insulin (mIU/L)</td>
<td>8.7 (6.1-11.9)</td>
</tr>
<tr>
<td>$S_I$ (min⁻¹·mIU/L·10⁻⁴)</td>
<td>2.1 (1.1-3.3)</td>
</tr>
<tr>
<td>$S_G$ (min⁻¹)</td>
<td>0.019 (0.006)</td>
</tr>
<tr>
<td>AIRg (min·mIU/L)</td>
<td>342 (176-536)</td>
</tr>
<tr>
<td>RBP4 (mg/dL)</td>
<td>3.8 (1.03)</td>
</tr>
</tbody>
</table>

BMI: body mass index; WHR: waist-to-hip ratio; SBP and DBP: systolic and diastolic blood pressure; $S_I$, $S_G$ and AIRg: insulin sensitivity, glucose effectiveness and acute insulin response to glucose, respectively (from frequently-sampled intravenous glucose tolerance tests). Data are mean (SD) for Gaussian variables and median and interquartile range for non-Gaussian variables.
**TABLE 2.** Linear Multivariate Regression Analysis With Acute Insulin Response To Glucose (Airg) As Dependent Variable

**Non-obese subjects**

<table>
<thead>
<tr>
<th>Independent Variables</th>
<th>Unstandardized Coefficients</th>
<th>Standardized Coefficients</th>
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<tr>
<td></td>
<td>B</td>
<td>Std. Error</td>
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<tr>
<td>(Constant)</td>
<td>2.297</td>
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<tr>
<td>Insulin sensitivity</td>
<td>-0.522</td>
<td>0.231</td>
</tr>
<tr>
<td>Body mass index</td>
<td>0.023</td>
<td>0.020</td>
</tr>
<tr>
<td>Age</td>
<td>0.000</td>
<td>0.004</td>
</tr>
<tr>
<td>RBP4</td>
<td>-0.023</td>
<td>0.038</td>
</tr>
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</table>

**Obese Subjects**

<table>
<thead>
<tr>
<th>Independent Variables</th>
<th>Unstandardized Coefficients</th>
<th>Standardized Coefficients</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>B</td>
<td>Std. Error</td>
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<tr>
<td>(Constant)</td>
<td>2.644</td>
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<tr>
<td>Insulin sensitivity</td>
<td>0.539</td>
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<tr>
<td>Body mass index</td>
<td>0.026</td>
<td>0.033</td>
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<tr>
<td>Age</td>
<td>-0.011</td>
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</tr>
<tr>
<td>RBP4</td>
<td>-0.175</td>
<td>0.073</td>
</tr>
</tbody>
</table>
FIGURE LEGEND

Figure 1. Correlation graph of serum RBP4 and log$_{10}$-transformed AIRg (from FSIGT studies) (upper panel) and insulin disposition index (lower panel) in obese men. $r$ and $p$ values are shown from Pearson’s analysis (non-adjusted data).
Figure 1