An ELISA for plasma retinol-binding protein using monoclonal and polyclonal antibodies: Plasma variation in normal and insulin resistant subjects

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Abstract

Objectives: Plasma retinol-binding protein (RBP) has been linked to insulin resistance and cardiovascular risk, yet little is know of its natural variation in plasma. We examined this in normal subjects and compared plasma levels and variability in lean subjects and subjects with the metabolic syndrome.

Methods: We established an “in house” ELISA for plasma RBP and measured levels in 20 normal subjects over daylight hours and 2 subject groups, either lean or classified with the metabolic syndrome.

Results: Plasma RBP in normal subjects did not vary over the day with no differences between males and females. There was also no difference in plasma RBP levels and between the age- and sex-matched lean subjects compared to the metabolic syndrome group.

Conclusion: The lack of variation in plasma RBP in normal subjects and the lack of difference between plasma RBP in normal and metabolic syndrome subjects suggest the link between plasma RBP and insulin resistance is tenuous. Investigating a large cohort over the diabetic non-diabetic spectrum may clarify this issue.

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Keywords: Retinol-binding protein; Insulin resistance; Biovariation; Plasma; Diurnal variation

Introduction

Retinol-binding protein (RBP) is a 21 kDa protein and is the major carrier of retinol in the circulation and exists as a 1:1 molar complex bound stoichiometrically to transthyretin (prealbumin). This large complex, with a molecular weigh of 76 kDa, is preserved in the circulation and serves to deliver retinol from hepatic storage to target tissues. The protein also maintains a low level of free retinol in plasma, thereby protecting cell membranes from hydrophobic toxicity [1–3]. Following delivery of retinol to target tissues, RBP losses affinity for transthyretin, and returns to the plasma as apo-RBP and is then subjected to glomerular filtration. On the basis of these properties, there has been clinical interest in the potential of the measurement of RBP as a nutritional marker, an acute phase reactant [4] and an indicator for alcohol-induced liver disease [5]. In addition, urinary RBP has been used as a marker of proximal tubular damage, as virtually all filtered RBP is resorbed, via megalin receptor mediated uptake [6], with increased RBP excretion being indicative of tubular dysfunction [7,8].

Retinol-binding protein is a member of the lipocalin superfamily of proteins that bind hydrophobic ligands [9]. The influx of retinol into target cells involves a specific membrane-bound receptor for RBP which facilitates the cellular uptake of retinol alone or retinol bound to RBP. Although the RBP receptor has not been characterised, a novel receptor for a related lipocalin has been cloned [10]. This receptor family and related genes may represent a widespread new biological mechanism whose significance has been highlighted by the suggestion that RBP,
via its receptor, may play a role in type 2 diabetes [11]. Recent interest in RBP was catalysed by the observation that the expression of RBP is elevated in GLUT4 glucose transporter knockout mice and in humans with obesity and type 2 diabetes [12]. Plasma levels of RBP in these insulin resistant mice normalised following treatment with rosiglitazone, an insulin-sensitising drug [12]. Furthermore, the transgenic overexpression of human RBP or injection of recombinant RBP in normal mice caused insulin resistance. A subsequent study in humans reported that serum RBP levels correlated with the degree of insulin resistance in subjects with obesity, impaired glucose tolerance and type 2 diabetics and was associated with components of the metabolic syndrome. Plasma RBP levels also correlated with markers of the metabolic syndrome in non-obese and non-diabetic subjects with a strong family history of type 2 diabetes [13]. Also of interest was the observation that exercise training induced improvements in insulin resistance and was associated with a reduction in serum RBP levels. Adipocyte GLUT4 protein levels and serum RBP levels have also been shown to be inversely correlated [12].

Given the emerging interest in plasma RBP, rather than relying on an existing ELISA format for RBP in plasma [14], we decided to establish our own “in house” sandwich ELISA to RBP, using polyclonal and monoclonal antibodies, and to further investigate its role as a marker of insulin resistance. In this paper we report the development of a RBP ELISA using monoclonal and polyclonal antibodies. We also document the diurnal variation of RBP in plasma as well as its biovariation in normal lean subjects and in matched subjects with components of the metabolic syndrome and report no difference in plasma RBP between these two groups.

Materials

Human RBP (>98% pure) was obtained from Calbiochem, La Jolla, CA, USA). Rabbit antiserum to human RBP intact and peroxidase labelled was from Dakopatts, Denmark (Cat. No. A0040 and P0304, respectively). Antimouse Ig-peroxidase and isotyping kits were from Life Sciences, Amersham, Bucks., UK.

Methods

Immunisation and cell fusion

Female RBF-DN mice were immunised with 10 μg of RBP in complete Freunds adjuvant at 4-week intervals. One week after the 3rd injection, the spleens were excised and the splenic lymphocytes cells fused with FOXY-NY myeloma cells at a ratio of 5:1, using 50% polyethylene glycol. After fusion, the cells were resuspended to 10⁶/mL in RPMI containing 10% fetal calf serum (v/v), 2 mM L-glutamine, and AAT (adeno/aminopterin/thymidine; 75: 0.08: 16 × 10⁻⁶ M, respectively). They were plated at 10⁵ cells/well in 96-well culture plates (16 plates in total) and grown under standard conditions with regular media changes. Positive hybridomas were cloned twice by limiting dilution using RBF/DN spleen cells as a feeder layer (100 × 10⁵/well). After cloning, the cells were grown in 50–200 mL culture flasks and supernatants centrifuged prior to use [15]. Thiomersal (0.01%) was added to the supernatants, as a preservative, which were then aliquoted and stored at either 4 °C, in the short term, or –20 °C for longer term storage. Immunoglobulin class and subclass were determined using the isotyping kit.

Screening of supernatants

ELISA plates (Falcon 3912 microtest III; Beckton Dickinson, Oxnard, CA, USA) were coated with 100 μL/well of rabbit anti-RBP serum, diluted in phosphate-buffered saline (PBS), 20 μL of antibody in 10 mL PBS. Phosphate-buffered saline was 0.05 M NaH₂PO₄, 0.15 M NaCl, adjusted to pH 7.4 with 5 M NaOH. Coated plates were covered and stored at least overnight and up to 7 days at 4 °C. Following coating the plates were washed four times, with PBS containing 0.1% Tween-20 and then “blocked” with 150 μL/well of assay buffer (PBS containing 0.1% Tween-20 and 0.1% gelatin (w/v)) for 5–10 min at 20 °C. After the plates were emptied, 100 μL of diluted human urine (1:10 in assay buffer) from a kidney failure patient containing high levels of RBP (2 mg/L) was added to each well for a 2 h incubation at 20 °C. The plates were washed again and then 50 μL of assay buffer added to each well, followed by 50 μL of supernatant for a further 2 h incubation at 20 °C. The plates were washed 4 times and sheep antimouse Ig-peroxidase added (100 μL/well) at 1:1000 in assay buffer) for 1 h at 20 °C, after which the plates were washed and 100 μL/well of substrate added. The substrate was prepared by the addition of 600 mL aqueous solution containing 8.2 g anhydrous sodium acetate and 3.6 g citric acid to 400 mL of methanol containing 270 mg of tetramethyl benzidine. 500 μL of 30% H₂O₂ was finally added and the substrate stored in a dark bottle at room temperature. Colour development was terminated by the addition of 100 μL of 0.9 M HCl per well and the absorbance read at 450 nm on a BMG Fluostar Galaxy (BMG, Technologies GmbH, Germany). To avoid evaporation losses the plates were covered during all the steps preceding the addition of substrate. The use of urine containing high levels of RBP for screening purposes conserved purified RBP which was used for relative epitope mapping. The screening and cloning protocols produced 3 hybridomas secreting RBP antibodies into culture supernatants. These were designated 17G2, 17G10 and 23C2.

Relative mapping of antigenic determinants

Experiments were carried out to determine whether RBP monoclonal antibody 17G2 (isotype IgG2aκ) recognised a different determinant from those of supernatants 17G10 and 23C2 (both isotype IgG1κ). ELISA plates were coated with Dako polyclonal RBP antibody (A0040), blocked and authentic RBP standards added. Competition experiments were then carried out to determine whether the IgG1s RBP antibody supernatants (17G10 and 23C2) blocked the subsequent binding of 17G2 supernatant (isotype IgG2aκ) and vice versa, together with appropriate controls. These experiments were carried...
out using sequential incubations at room temperature, each of 30 min, with washing steps between each antibody incubation. The bound monoclonal RBP antibodies were finally detected with antimouse IgG1-peroxidase and/or antimouse IgG2a-peroxidase (Cortex Biochem, San Leandro, CA, U.S.A., 1:1000 in assay buffer) using similar conditions to those described above.

**RBP ELISA**

Briefly, microtitre plates were coated, as described previously, with rabbit anti-RBP serum diluted in PBS (20 μL in 10 mL PBS), 100 μL per well. Following coating the plates were washed and blocked with assay buffer (150 μL per well) for 1 h at 20 °C. The plates were emptied by inversion and either 100 μL of RBP standard dilution (95 μL assay buffer followed by 5 μL RBP standard) or patient plasma (1:20,000 dilution in assay buffer) added to each well for a 2 h incubation at 20 °C. Standards were authentic RBP, serially diluted from 1000 μg/L to generate a series of RBP standards from 0 to 250 μg/L. Following the 2 h incubation the plates were washed and supernatant 17G10 (diluted 1:50 in assay buffer) was added at 100 μL per well for a further incubation for 1 h at 20 °C. The plates were washed again and 100 μL of sheep antimouse IgG-peroxidase (1:1000 in assay buffer) was added for 30 min at 20 °C after which the plates were finally washed, substrate added, colour developed and then terminated prior to spectrophotometry. Results were expressed in mg/L after correction for plasma dilution. As part of the ELISA validation, the results were compared with samples which were also analysed by detection of antibody plate-bound RBP using a peroxidase labelled rabbit polyclonal RBP antibody (P0304, 1:500 in assay buffer and 1 h at 20 °C), followed by washing and the addition of substrate.

Intra-assay variation was determined by measuring the RBP level in 3 different commercial quality control plasma based samples (Lyphochek, Immunoassay Plus, Bio-Rad Laboratories, Irvine, CA, USA) each 5 times in duplicate. Inter-assay variation was determined using the same quality control samples over 5 consecutive assays. Results were expressed as mean ± S.D.

**Subjects**

Written informed consent was obtained from all subjects and the studies were approved by the Canterbury Ethics Committee, Christchurch, New Zealand.

**Diurnal study subjects**

Normal subjects (10 males and 10 females, aged between 20 and 40 years) were recruited from hospital staff and were apparently fit and healthy. These subjects had no oral, inhaled or parenteral glucocorticoid exposure within the preceding 12 months, were not acutely ill and avoided exercise or alcohol on the study day. Blood samples were collected, into EDTA tubes, from an indwelling catheter inserted in an arm vein, the plasma separated and samples stored at −20 °C until analysed. Sampling commenced at 0800 h and a minimum of 12 samples were collected half hourly until 1030 h and then hourly until 1730 h.

**Biovariation study subjects**

Volunteers without diabetes (5 males and 5 females) were recruited from a clinical database of subjects with the metabolic syndrome classified according to the ATP III criteria [16]. The subjects were selected if they possessed 3 or more of these criteria, with at least one subject from each of the 4 decades of life from 20 to 60 years being included in the study. Five age-matched males and five age-matched females with one or less of
the APT III criteria were recruited as paired lean controls for the study. Fasting blood samples were collected between 0800 and 0900 h on days 1, 10, 20 and 30 in EDTA tubes. The plasma was separated and stored at −30 °C until batch analysed, with each sample being assayed in duplicate. In this group, plasma glucose was determined using an Aeroset analyser (Abbott Laboratories, IL, USA) and native insulin using an immunoassay analyser (IMX, Abbott Laboratories, IL, USA).

Statistical analysis

Analysis of data was carried out using the statistical package SPSS version 11.5 (SPSS Inc. Chicago, IL, USA) and Sigmastat 3.1 (Systat Software Inc. Point Richmond, CA, USA). Diurnal RBP data were analysed by repeated measures of analysis of variance followed by paired t-tests. Biological variation in the healthy controls and metabolic syndrome subjects was calculated from analytical, within individual, and between individual components of variation (CV_a, CV_i and CV_G respectively) calculated by nested ANOVA [17]. The reference change value (RCV) for significant changes in serial results (p<0.05) in an individual subject was calculated using the formula \(2.77(CV_a^2 + CV_i^2)^{1/2}\). The concept of biological individuality is expressed as the index of individuality (IoI) and is derived from the ratio of intra- and inter-individual variation. Low IoI values (<0.6) indicate that the dispersion of values for any individual will span only a small part of the reference interval and hence conventional population-based reference ranges would be inadequate for detecting whether a significant change has occurred [18]. Conversely, when the IoI is 1.4 or greater, the variation in an individual will fit population reference limits more closely and hence be suitable for a screening test. Retinol-binding protein levels, fasting insulin, HOMA and BMI were compared between metabolic syndrome and lean groups using paired t-tests or the Wilcoxon signed rank test as appropriate.

Insulin resistance was determined using the homeostasis model assessment (HOMA) computer model from fasting plas-

<table>
<thead>
<tr>
<th>RBP added (mg/L)</th>
<th>RBP recovered (mg/L)</th>
<th>RBP expected (mg/L)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>9</td>
<td>9</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>21</td>
<td>19</td>
<td>111</td>
</tr>
<tr>
<td>20</td>
<td>31</td>
<td>29</td>
<td>107</td>
</tr>
<tr>
<td>40</td>
<td>49</td>
<td>49</td>
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</tr>
<tr>
<td>80</td>
<td>90</td>
<td>89</td>
<td>101</td>
</tr>
</tbody>
</table>

ma glucose and insulin measurements with results expressed as percentage sensitivity [19]. Low values indicate insulin resistance.

Results

The fusion, screening and subsequent cloning protocols identified three hybridoma cell lines secreting antibodies to RBP. The hybridomas were identified as 17G2, 17G10 and 23C2 and were isotyped as IgG2a, IgG1κ and IgG1κ respectively, with these differences being exploited for mapping experiments.

The relative antigenic determinant mapping experiments show that 17G2 monoclonal antibody (isotype IgG2aκ) is unlikely to share antigenic determinants with the other two IgG1 monoclonal antibodies (17G10 and 23C2) (Fig. 1a). Comparisons in the upper panel of Fig. 1 with respect to antibody 17G10 show that the dose–response curve generated when Dako antibody bound SHBG is probed with antibody 17G10 followed by 17G2 and finally antimouse IgG1-peroxidase, is identical to that with 17G10 followed by antimouse IgG1-peroxidase. This suggests that there was no interference by antibody 17G2. This was confirmed by the reverse incubation (17G2 followed by 17G10 and finally antimouse IgG1-peroxidase) with the dose–response curve being identical to that of 17G10 followed by antimouse IgG1-peroxidase (data not shown). On the other hand, while not identical, the determinants may be proximal, with 17G10 exhibiting higher affinity as the curves

Fig. 2. Typical ELISA 2 site standard curve using plates coated with polyclonal RBP antibody and standards, with bound RBP being detected with mouse monoclonal antibody 17G10, followed by antimouse IgG Fc-peroxidase.

Fig. 3. Plasma RBP levels (mg/L) measured by either rabbit polyclonal antibodies or immobilised rabbit polyclonal antibody followed by RBP monoclonal antibody 17G10. The line \(x = y\) is indicated.
controls are shown in Table 4. The RCV for RBP was 119.5 mg/L in subjects with the metabolic syndrome and matched BMI (kg/m²) were 35.8±6.5 and 22.7±2.6, respectively. The higher RCV in the metabolic group is also reflected by a greater index of individuality (IoI). This implies that, for any subject with the metabolic syndrome, individual variation in plasma RBP levels over the day is greater than the age- and sex-matched normal cohort. Interestingly the RCV for the metabolic syndrome cohort was greater than the age- and sex-matched normal cohort which implies that the metabolic syndrome cohort is less stable, over the 30 day study period, than matched normal lean controls. The higher RCV in the metabolic group is also reflected by a greater index of individuality (IoI). This implies that, for any subject with the metabolic syndrome, individual variation in plasma RBP levels over the day is greater than the age- and sex-matched normal cohort.

### Table 2
Parallelism of plasma RBP ELISA

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
<th>Patient 4</th>
<th>Patient 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:10,000</td>
<td>105</td>
<td>58</td>
<td>61</td>
<td>54</td>
<td>31</td>
</tr>
<tr>
<td>1:20,000</td>
<td>42</td>
<td>27</td>
<td>28</td>
<td>29</td>
<td>16</td>
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<tr>
<td>1:40,000</td>
<td>17</td>
<td>15</td>
<td>14</td>
<td>15</td>
<td>9</td>
</tr>
<tr>
<td>1:80,000</td>
<td>11</td>
<td>11</td>
<td>10</td>
<td>11</td>
<td>7</td>
</tr>
</tbody>
</table>

Five plasma samples serially diluted and assayed for RBP. Values are expressed as mg/L.

A typical standard curve is shown in Fig. 2, with recovery of authentic purified RBP from intact plasma shown in Table 1. Fig. 3 shows plasma RBP levels measured using either monoclonal antibody detection (17G10) or detection using peroxidase labelled rabbit RBP polyclonal antibody. Parallelism of diluted plasma samples is indicated in Table 2 and within- and between-assay variation is listed in Table 3. Fig. 4 shows the normal ranges, mean ±1 SD for plasma RBP at various times of the day. Circulating RBP did not change significantly over the 30 day study period, than matched normal lean controls. The mean 0800 h RBP levels±SD (range) were 37.1±14.6 mg/L (18–63) for males and 36.4±9.6 (25–50) for females (p=0.92). The sensitivity of the plasma RBP assay at two standard deviations from zero was less than 4 mg/L and the useful working range was 8 to 125 mg/L. The results for the biovariability study on RBP in subjects with the metabolic syndrome and matched controls are shown in Table 4. The RCV for RBP was 119.5 mg/L in the metabolic syndrome group and 22.5 mg/L in the control group and the IoI was 2.4 and 0.13 respectively.

### Discussion

In this study we show an absence of any significant diurnal variation in plasma RBP levels in normal subjects over the daytime course studied and similar levels in adult males and females. Our rationale for using the 0800 h to 1730 h sampling schedule was that it spanned the times when blood samples are usually taken in a clinical or outpatient setting. We also showed agreement between RBP levels measured in the diurnal study and the biovariability study of both normal and metabolic subjects. Interestingly the RCV for the metabolic syndrome cohort was greater than the age- and sex-matched normal cohort which implies that the metabolic syndrome cohort is less stable, over the 30 day study period, than matched normal lean controls. The higher RCV in the metabolic group is also reflected by a greater index of individuality (IoI). This implies that, for any subject with the metabolic syndrome, individual variation in plasma RBP levels over the day is greater than the age- and sex-matched normal cohort which implies that the metabolic syndrome cohort is less stable, over the 30 day study period, than matched normal lean controls. The higher RCV in the metabolic group is also reflected by a greater index of individuality (IoI). This implies that, for any subject with the metabolic syndrome, individual variation

### Table 4
Biovariability of plasma RBP in metabolic syndrome and lean control subjects matched for age and gender

<table>
<thead>
<tr>
<th></th>
<th>Metabolic syndrome (n=10)</th>
<th>Lean (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting insulin (pmol/L)</td>
<td>144±116</td>
<td>35±18 (p&lt;0.001)</td>
</tr>
<tr>
<td>HOMA (% sensitivity)</td>
<td>46.4±21.9</td>
<td>174.0±104.8 (p&lt;0.001*)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>35.8±6.5</td>
<td>22.7±2.6 (p&lt;0.001)</td>
</tr>
<tr>
<td>RBP (mg/L)</td>
<td>36.6±6.7</td>
<td>32.8±7.7 (p&lt;0.20)</td>
</tr>
<tr>
<td>RBP RCV (mg/L)</td>
<td>119.5</td>
<td>22.5</td>
</tr>
<tr>
<td>RBP IoI</td>
<td>2.4</td>
<td>0.13</td>
</tr>
<tr>
<td>Within-subject C.V. %</td>
<td>21.4</td>
<td>8.3</td>
</tr>
<tr>
<td>Between-subject C.V. %</td>
<td>13.7</td>
<td>22.7</td>
</tr>
<tr>
<td>Within-assay C.V. %</td>
<td>16.8</td>
<td>10.2</td>
</tr>
</tbody>
</table>

Shown is the mean ±SD, fasting insulin, homeostasis model assessment (HOMA) (% sensitivity), body mass index (BMI), reference change values (RCV) and index of individuality (IoI). Significant comparisons (p<0.05) are indicated using the paired t-test or Wilcoxon’s signed rank test for non-normal distribution, *.
will fit population reference limits more closely than normal subjects in whom identification of abnormal plasma RBP levels may be more difficult. Importantly, there was no significant difference in plasma RBP levels between these age- and sex-matched groups, despite the highly significant differences in insulin sensitivity, fasting insulin and BMI.

This contrasts with the findings of Graham et al. [13] where elevated serum RBP 4 was associated with components of the metabolic syndrome, including elevated BMI and fasting insulin levels. Although they measured what was termed “plasma RBP 4” it would appear that plasma RBP 4 and plasma RBP are immunologically identical, as in their studies “RBP 4” was measured using the same polyclonal RBP antibody from Dako as used in our capture ELISA. This together with the demonstration that plasma RBP levels are similar whether measured by either the Dako pairing of RBP polyclonal antibodies or the Dako polyclonal paired with monoclonal antibody 17G10 supports this conclusion. We suggest that the nomenclature, retinol-binding protein 4, is confusing and that this protein should simply be referred to as retinol-binding protein.

It seems difficult to rationalise why insulin resistant subjects should have elevated plasma levels of RBP compared to normal subjects. The contention is that RBP synthesis is upregulated in adipose tissue [12], presumably overriding hepatic production, resulting in a net increase in circulating RBP. However, although adipocytes contain significant amounts of RBP mRNA [20], hepatocytes have, by far, the greatest abundance of RBP mRNA, compared to other tissues, [1,21] and the hepatic production of RBP is predominantly regulated by vitamin A and nutritional status [4]. Furthermore, RBP is deemed a negative acute phase reactant with hepatic production reduced in inflammation [4]. These factors would be expected to confound the relationship between circulating RBP and insulin resistance.

Although the circulating levels of RBP reported here are similar to the levels reported by Graham et al. [13] we did not discern a significant difference in RBP levels between normal age- and sex-matched cohorts of normal and metabolic syndrome subjects, despite the significant difference in the prevalence of metabolic markers. It is possible that a difference could emerge using a much larger study group. However, our study had sufficient power (>90%) to detect at least a 33% difference between the groups which is less than the differences reported by Graham et al. [13]. It is therefore important that a large study is carried out to fully test the hypothesis that plasma RBP is indeed a marker of insulin resistance. Now that we have established and validated an assay for plasma RBP, demonstrated the absence of diurnal variation and quantified its biovariation, we are in a position to test the hypothesis that plasma RBP is a marker of insulin resistance. This assumes additional importance as profound differences between rodents and humans in the regulation of adipose or circulating RBP challenge the notion that RBP has a significant role in glucose homeostasis [22].

To our knowledge this is the also the first report of monoclonal antibodies, and their characterisation, to RBP. Given that some recognise different determinants suggests they may prove useful analytical tools as well as potentially useful additional biological probes for studies of RBP. At least one of these antibodies, 17G2, is unlikely to share antigenic determinants with either 17G10 or 23C2 and, as such, increases the panel of antibodies available for studies of RBP.

References