

Insulin Resistance Is Unrelated to Circulating Retinol Binding Protein and Protein C Inhibitor

Miriam Promintzer, Michael Krebs, Jelena Todoric, Anton Luger, Martin Georg Bischof, Peter Nowotny, Oswald Wagner, Harald Esterbauer, and Christian Anderwald

Department of Internal Medicine III (M.P., M.K., A.L., M.G.B., P.N., C.A.), Division of Endocrinology and Metabolism, Clinical Institute of Medical and Chemical Laboratory Diagnostics (J.T., O.W., H.E.), and Department of Clinical Pharmacology (C.A.), Medical University of Vienna, A-1090 Vienna, Austria

Context: Recent data suggest that circulating retinol-binding protein (RBP) might be involved in the pathogenesis of insulin resistance. Moreover, protein C inhibitor (PCI), which specifically binds retinoic acid, was found to be increased in myocardial infarction survivors who are also insulin resistant.

Objective: The objective of this study was to investigate the association of insulin resistance with RBP factors and PCI active antigen.

Design and Setting: This was a clinical study.

Patients: Nondiabetic humans with high (IS; $n = 20$, 14 females, six males, aged 47.2 ± 1.9 yr, body mass index 26 ± 1 kg/m²) and low (IR; $n = 20$, 14 females, six males, aged 45.5 ± 1.7 yr, body mass index 28 ± 1 kg/m²) insulin-stimulated glucose-disposal (M) participated in this study.

Main Outcome Measures: M was measured by 2-h hyperinsulinemic (40 mU·min⁻¹·m⁻²)-isoglycemic clamp tests. Measurements of

RBP were performed using a nephelometric method and validated using quantitative Western blotting.

Results: M (80 – 120 min) was higher in IS (10.9 ± 0.6 mg·min⁻¹·kg⁻¹) than IR (4.0 ± 0.2 ; $P < 10^{-12}$). Fasting plasma RBP concentrations were comparable between IS and IR measured by both nephelometry (IS: 4.4 ± 0.3 ; IR: 4.6 ± 0.3 mg/dl, $P = 0.6$) and quantitative Western blot (IS 7.9 ± 0.5 , IR 8.3 ± 0.6 mg/dl; $P = 0.6$). Fasting plasma PCI active antigen was similar in both groups. Plasma RBP and PCI were not significantly related to M. RBP was positively correlated with uric acid ($r = 0.488$, $P = 0.003$), triglycerides ($r = 0.592$, $P < 0.001$), prealbumin ($r = 0.63$, $P < 0.0001$), and vitamin A ($r = 0.75$, $P < 10^{-6}$).

Conclusions: Our data demonstrate that healthy, insulin-resistant humans do not show altered plasma retinol binding factors, such as RBP and PCI. Both do not significantly correlate with insulin sensitivity. Thus, our findings do not support the hypothesis of insulin sensitivity modulation by proteins involved in retinol transport. (*J Clin Endocrinol Metab* 92: 4306–4312, 2007)

INSULIN RESISTANCE is characterized by reduced insulin action in insulin-sensitive tissues. Adipose tissue might modulate insulin sensitivity by not only lipid release (1) but also secretion of adipokines (2, 3). Retinol-binding protein (RBP) is currently regarded as an adipokine because it is secreted by both the liver and adipose tissue and stored in adipose tissue (4). So far, one plasma RBP has been described as a protein that is essential for retinoid transport in human plasma (5, 6). Its plasma concentration reflects liver function and nutritional state (6).

A recent report by Yang *et al.* (7) suggests a direct association of elevated serum RBP concentrations with insulin resistance in rodents. Adipose-specific glucose transporter-4 knockout mice were insulin resistant in muscle and liver and also exhibited elevated expression of the RBP4 gene.

Overexpression of RBP or injection of RBP in mice impaired insulin signal transduction in skeletal muscle and

increased hepatic glucose production, whereas deletion of the RBP4 gene increased insulin sensitivity (7). Thus, it has been suggested that alterations of retinol transport proteins might contribute to insulin resistance.

However, contrasting results have been reported on the impact of circulating RBP concentrations on insulin resistance in humans: RBP levels were described to be increased in overweight/obese, insulin-resistant humans when compared with normal-weight controls (8) and to be correlated to insulin-mediated glucose uptake (9–11). Cho *et al.* (12) reported elevated RBP levels in patients with impaired glucose tolerance and type 2 diabetes when compared with a body mass index (BMI)-matched, glucose-tolerant control group. In contrast Janke *et al.* (13) found similar circulating RBP levels among normal-weight, overweight, and obese nondiabetic women and reported no significant correlation of RBP and insulin resistance as did most recent studies (14–16).

Protein C inhibitor (PCI), a novel protein involved in retinoid transport (17), interacts with retinoic acid and other retinoids, in addition to inhibiting anticoagulant protein C in blood (18, 19). In male survivors of myocardial infarction who were most likely insulin resistant (20), plasma PCI active antigen was found to be increased and related to the number of acute coronary events suffered (21).

Therefore, we tested the hypothesis that circulating proteins involved in retinol transport, such as RBP and PCI

First Published Online August 28, 2007

Abbreviations: BMI, Body mass index; EGP, endogenous glucose production; FFA, free fatty acid; γ GT, γ -glutamyl transferase; HDL, high-density lipoprotein; IR, insulin-resistant; IS, insulin-sensitive; M value, insulin-mediated glucose uptake; OGIS, OGTT-based index of insulin sensitivity; OGTT, oral glucose tolerance test; PCI, protein C inhibitor; RBP, retinol-binding protein.

JCEM is published monthly by The Endocrine Society (<http://www.endo-society.org>), the foremost professional society serving the endocrine community.

active antigen, might interfere with whole-body insulin sensitivity in nondiabetic humans. To exclude direct effects of body weight, age, and gender on insulin resistance, we compared insulin-sensitive and -resistant humans that were well matched for age, BMI, and gender.

Subjects and Methods

Study participants

Forty nondiabetic humans were recruited by local advertising (Table 1). The subjects were healthy and not treated with medications for diabetes, hypertension, or dyslipidemia or other medication known to influence glucose homeostasis. None of the women were taking oral contraceptives, eight women were postmenopausal, and one woman was receiving postmenopausal estrogen therapy. All participants had been instructed to refrain from excessive physical exercise and ingest an isocaloric carbohydrate-rich diet 3 d before the examinations. The protocol was approved by the institutional ethics board, and all study participants gave informed consent after the nature and the possible consequences of the procedures had been explained.

Study d 1

After a 12-h overnight fast, study participants underwent a complete medical history, taken with routine laboratory, physical check, and blood sampling for measurement of RBP and PCI. Urinary protein excretion was measured by Multistix 10 SG (Bayer, Newbury, UK) and was negative in all subjects. Body weight and fat mass were measured by Tanita bioimpedance balance (Yiewsley, UK). Thereafter an oral glucose (75 g) tolerance test (OGTT) was performed.

Study d 2

After a 12-h overnight fast, two catheters (Vasofix; Braun, Melsungen, Germany) were inserted in the left and right antecubital vein for blood sampling and infusions, respectively. In 21 subjects, a primed-continuous infusion (5 min: 4 mg/kg lean body weight; 0.04 mg/min per lean body weight) of D-[6,6-²H₂]glucose (98% enriched; Cambridge Isotope Laboratories, Andover, MA) was started at –120 min for measurement of endogenous glucose production (EGP) (22). The clamp goal was determined from the mean of three basal plasma glucose measurements.

TABLE 1. Anthropometric and clinical characteristics including (serum) routine laboratory measurements of the IS and IR study participants

	IS	IR
n	20	20
Age (yr)	47.2 ± 1.9	45.5 ± 1.7
Gender (female/male)	14/6	14/6
BMI (kg/m ²)	25.6 ± 0.8	27.9 ± 1.1
Body weight (kg)	74.7 ± 2.8	80.5 ± 3.9
Height (cm)	170.7 ± 2.0	169.6 ± 2.2
Waist to hip ratio	0.85 ± 0.02	0.90 ± 0.02
Fat mass (kg)	21.5 ± 1.9	27.0 ± 2.2
Fat-free mass (kg)	53.2 ± 2.8	53.6 ± 2.9
RR syst/dia (mm Hg)	119 ± 3/78 ± 2	121 ± 3/79 ± 1
HbA1c (%)	5.5 ± 0.1	5.7 ± 0.1
Serum triglycerides (mg/dl)	90.0 ± 7.3	103.5 ± 14.8
Serum total cholesterol (mg/dl)	220.0 ± 7.6	209.8 ± 7.8
Serum HDL cholesterol (mg/dl)	65.2 ± 2.7 ^a	55.7 ± 2.4
Serum LDL cholesterol (mg/dl)	136.7 ± 7.6	133.7 ± 7.7
ASAT (U/liter)	24.0 ± 1.5	24.4 ± 2.2
ALAT (U/liter)	21.3 ± 1.5	26.5 ± 4.5
γGT (U/liter)	15.0 ± 1.1 ^a	22.1 ± 3.1
Serum creatinine (mg/dl)	0.90 ± 0.03	0.80 ± 0.04
Serum uric acid (mg/dl)	4.6 ± 0.2	4.8 ± 0.2

Student's *t* test IS vs. IR. ASAT, Aspartate aminotransaminase; ALAT, alanine aminotransaminase; RR syst/dia, systolic and diastolic blood pressure; HbA1c, hemoglobin A1c.

^a *P* < 0.05 IS vs. IR.

Whenever the calculated clamp goal was outside 80 and 100 mg/dl, 80 and 100 mg/dl, respectively, were taken as the clamp goal. The hyperinsulinemic-isoglycemic clamp test was started with primed-continuous insulin (Actrapid; Novo Nordisk, Bagsvaerd, Denmark) infusion (40 mU insulin per minute per square meter body surface area). Plasma glucose was maintained at the clamp goal by infusing variable amounts of D-glucose, enriched with D-[6,6-²H₂]glucose. Blood was collected in EDTA-containing tubes, centrifuged, and plasma was stored at –20 C.

According to the insulin-stimulated glucose use, we divided the participants into two groups, an insulin-resistant [IR; insulin-mediated glucose uptake (M value) of 5.1 mg/kg·min or below] and an insulin-sensitive (IS; M value > 5.1 mg/kg·min) group (Table 1).

Plasma metabolites and proteins

Plasma concentrations of glucose, insulin, C-peptide, and free fatty acids (FFAs) were measured as described (23, 24). Serum concentrations of triglycerides and total and high-density lipoprotein (HDL) cholesterol were measured by routine laboratory techniques (www.kimclat).

Plasma RBP concentration was measured by three independent methods: nephelometry, ELISA, and quantitative Western blot.

For measurement of RBP by nephelometry, N Antiserum to human plasma RBP (code OUVO; Dade Behring Inc., Deerfield, IL) was used. Measurements were done with the Dade Behring nephelometer BN II. Intra- and interassay coefficients of variation were 1.9 and 2.2%, respectively. The assay was approved for *in vitro* diagnostic use in the United States and Europe and demonstrated to show excellent correlations with results obtained from radial immunodiffusion.

Because the majority of previous publications analyzed RBP in serum (8, 13), we also measured RBP in serum and plasma in 15 humans by using the nephelometric method. RBP in plasma (2.9 ± 0.4 mg/dl) was not different from that in serum (3.2 ± 0.4 mg/dl) (*P* = 0.6), and serum and plasma values closely correlated with each other (*r* = 0.996, *P* < 10^{–14}).

For the determination of RBP by quantitative Western blotting, we precisely followed detailed protocols published by Graham *et al.* (25). In brief, full-length recombinant human RBP4 (AdipoGen, Seoul, South Korea) was used to prepare two different series of RBP4 standard solutions: 1, 0.5, 0.25, and 0.13 and 0.63, 0.31, 0.16, and 0.12 μg/ml, respectively. Sera were diluted 1:200 or 1:400, separated by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (GE Healthcare Biosciences, Munich, Germany). After blocking nonspecific binding, blots were incubated overnight at 4 C with a polyclonal rabbit antihuman RBP4 antibody (Dako Cytomation, Hamburg, Germany) followed by 1 h incubation at room temperature with horseradish peroxidase-conjugated donkey antirabbit IgG secondary antibody (GE Healthcare Biosciences). Chemiluminescence generated by the ECL Plus Western blotting detection system (GE Healthcare Biosciences) was detected and quantified using the Lumi-Imager F1 and the Lumi Analyst 3.0 software, respectively (Roche Diagnostics, Mannheim, Germany). Second-order polynomial curves were used to fit the purified RBP4 standards (range *r*² = 0.9747–0.9934; mean *r*² = 0.9873) for each individual gel and calculate concentrations in serum samples. Samples exceeding upper or lower limits of the standard curve were rerun at appropriate dilutions.

The RBP-ELISA was performed following the detailed instructions of the manufacturer (AdipoGen Inc., Seoul, South Korea).

Unless otherwise noted, all statements in this article refer to results obtained by the nephelometric method.

Because RBP concentrations are also governed by vitamin A status and the carrier protein prealbumin, vitamin A in plasma was measured using a commercially available, Communauté Européenne-marked HPLC assay (Chromsystems, Munich, Germany), and prealbumin in plasma was determined by nephelometry (Dade Behring; catalog no. OUIF).

Plasma PCI active antigen was measured with PCI Actbind ELISA kit (Technoclone, Vienna, Austria) and is given as percent of normal human plasma.

Gas chromatography/mass spectrometry

Mole percent excess of plasma and infusate D-[6,6-²H₂]glucose was measured by gas-chromatography/mass spectrometry as described (1).

Calculations

EGP and M values, both given in milligrams glucose per minute per kilogram, were calculated as described (24, 26, 27). Hepatic insulin resistance index was calculated by multiplying fasting insulin concentrations with fasting EGP as described by Gastaldelli *et al.* (28). OGTT-based index of insulin sensitivity (OGIS) was determined from OGTT plasma glucose/insulin concentrations as described (29).

Statistical analyses

All data are given as means \pm SE. Comparisons within each group were analyzed with the two-tailed paired Student's *t* test. Comparisons between both groups were done with the two-tailed unpaired Student's *t* test. Linear correlations are Pearson product-moment correlations. Differences were considered statistically significant at $P < 0.05$.

Results

Participants' characteristics

The anthropometric and clinical characteristics of the IS and IR participants are given in Table 1. The groups were matched for age, gender, and BMI. HDL cholesterol was higher in IS, whereas γ -glutamyl transferase (γ GT) was elevated in IR (each $P < 0.05$). Plasma vitamin A concentrations were comparable between IS and IR study participants (IS: 2.9 ± 0.2 ; IR: 2.8 ± 0.2 μ mol/liter; $P = 0.7$). Plasma vitamin A concentrations were associated with fasting RBP concentrations ($r = 0.75$; $P < 10^{-6}$). Plasma prealbumin which carries RBP in the circulation was not different between the groups (IS: 25.7 ± 0.2 ; IR: 24.9 ± 0.2 mg/dl; $P = 0.6$) and was positively correlated to fasting RBP concentrations ($r = 0.63$; $P < 10^{-4}$).

OGTT (Fig. 1)

During the OGTT, plasma glucose between 30 and 120 min, plasma insulin between 20 and 150 min, and FFAs between 60 and 90 min were higher in IR (each $P < 0.05$ vs. IS) (Fig. 1, A–C).

IS, when determined from the OGTT (OGIS), was lower in IR at 120 min (397 ± 16 ml/min $^{-1}$ ·m $^{-2}$ vs. IS: 448 ± 13 , $P < 0.02$) and 180 min (411 ± 17 ml/min $^{-1}$ ·m $^{-2}$ vs. IS: 455 ± 10 , $P < 0.04$).

RBP during OGTT. Plasma RBP concentrations were not different between both groups before and 90 and 180 min after glucose load (Fig. 1D). At 180 min, IS showed lower plasma RBP when compared with basal values (Fig. 1D) ($P < 0.03$). RBP values were lower in females (3.7 ± 0.2 mg/dl) than males before (5.8 ± 0.5 mg/dl, $P < 0.001$) and 90 (3.6 ± 0.2 vs. 5.1 ± 0.2 mg/dl, $P < 0.001$) and 180 min (3.6 ± 0.2 vs. 5.0 ± 0.3 mg/dl, $P = 0.002$) after ingestion of glucose.

Clamp test

Metabolites and hormones. During the clamp tests, plasma glucose was different only between 20 and 50 min ($P < 0.05$) (Fig. 2A). Clamp insulin infusion resulted in a similar increase in plasma insulin in both groups (Fig. 2B). During clamp, plasma FFAs were up to 195% higher in IR between 60 and 120 min ($P < 0.001$) (Fig. 2C).

Glucose metabolism. During standardized hyperinsulinemia, whole-body glucose use [M (80–120 min)] was lower in IR

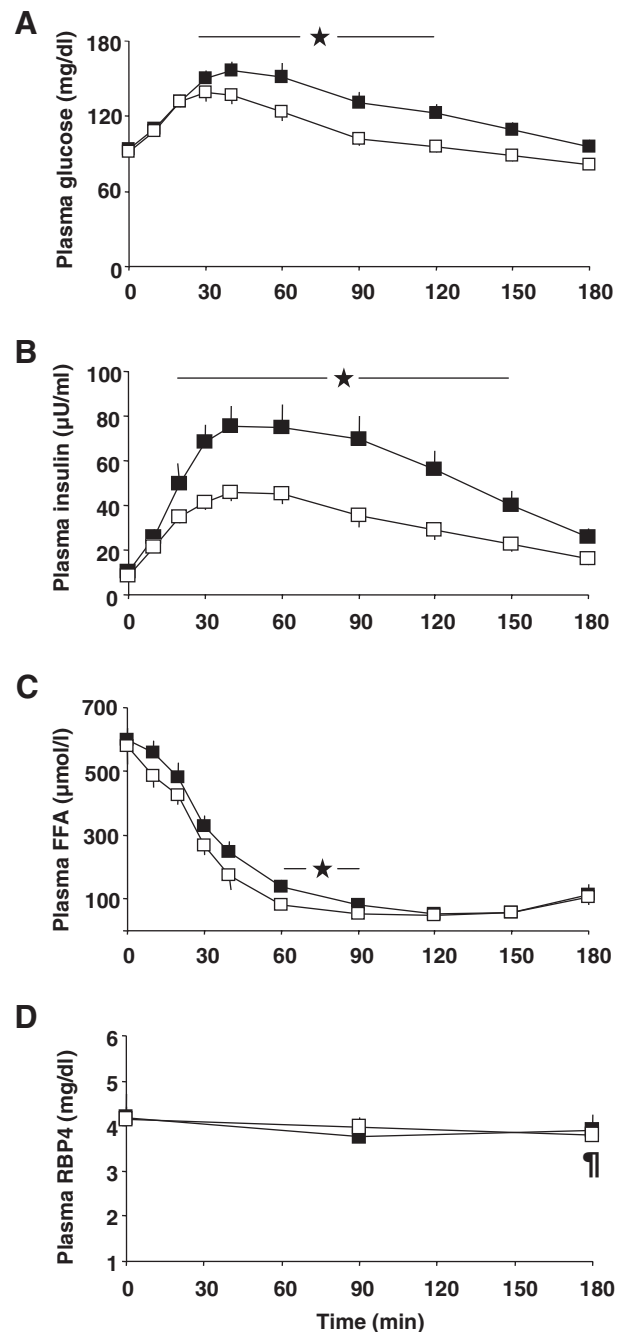


FIG. 1. Plasma concentrations of glucose (A), insulin (B), FFAs (C), and RBP (nephelometry) (D) in IS (\square , $n = 20$) and IR (\blacksquare , $n = 20$) study participants during an OGTT. Data are given as means \pm SE. \star , $P < 0.05$ for IS vs. IR (Student's *t* test); \parallel , $P < 0.03$ for IS 180 min vs. basal (paired *t* test).

(4.0 ± 0.2 mg glucose per minute per kilogram) when compared with IS (10.9 ± 0.6 ; $P < 10^{-12}$) (Fig. 2D). EGP was comparable before and during the clamp in both groups (Fig. 2E). Hepatic insulin resistance index tended to be higher in IR (13.3 ± 2.3), compared with IS (8.2 ± 0.9 ; $P = 0.059$).

RBP during clamp. Fasting levels of plasma RBP were not different between IS and IR (IS: 4.4 ± 0.3 ; IR: 4.6 ± 0.3 mg/dl) (Fig. 2F). Moreover, plasma RBP was comparable between

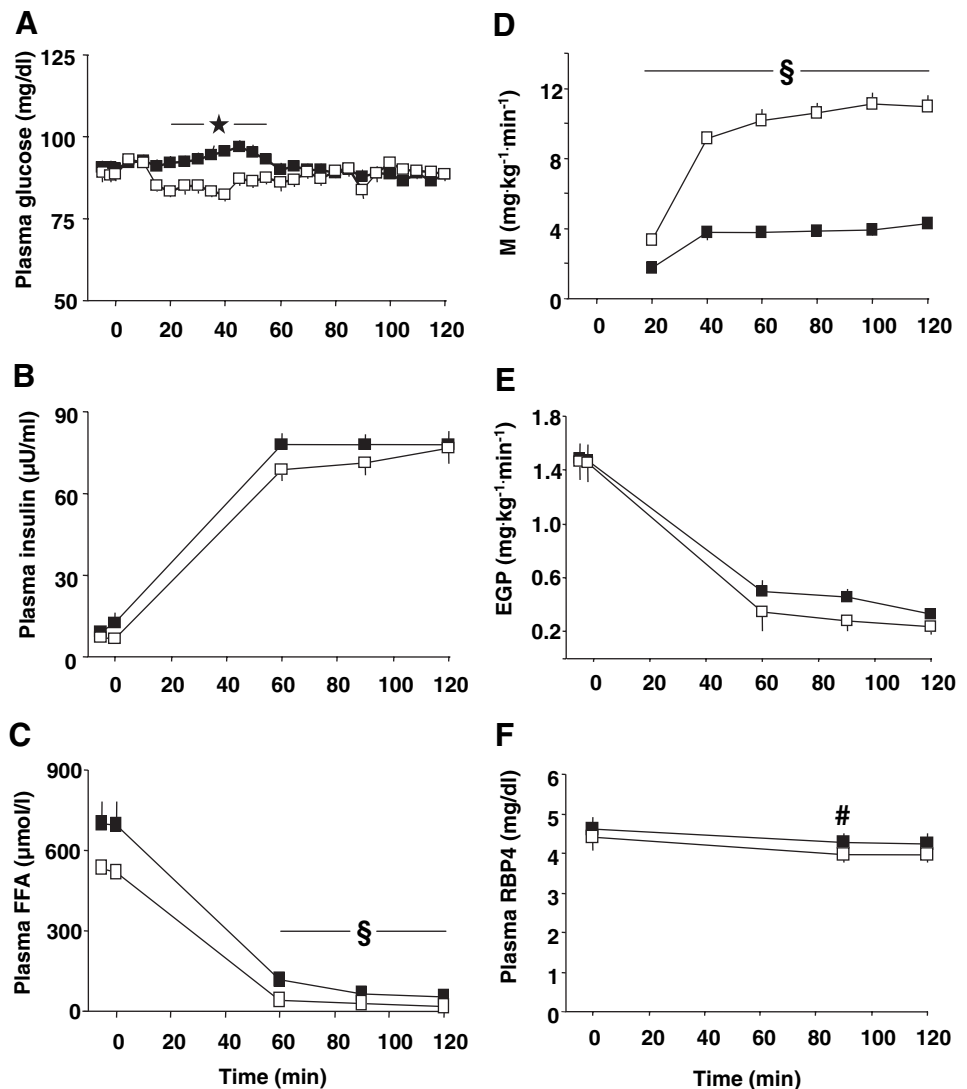


FIG. 2. Time course of plasma glucose (A), plasma insulin (B), plasma FFAs (C), M (D), EGP (E), and plasma RBP (nephelometry) (F) in IS (□) and IR (■) study participants during hyperinsulinemic isoglycemic clamp test (40 mU insulin per minute per square meter body surface area). Data are given as means \pm SE. *, $P < 0.05$ for IS vs. IR (Student's *t* test); §, $P < 0.001$ for IS vs. IR (Student's *t* test); #, $P < 0.005$ for IS and IR vs. basal (paired *t* test).

both groups during the clamp test (Fig. 2F). After 90 min hyperinsulinemia, plasma RBP decreased by 9 and 7% in IS and IR, respectively ($P < 0.005$) (Fig. 2F). RBP values were lower in female than male study participants at fasting (female: 4.1 ± 0.2 /male: 5.4 ± 0.4 mg/dl; $P = 0.004$) as well as after 90 (female: 3.7 ± 0.2 /male: 5.0 ± 0.2 mg/dl; $P < 0.001$) and 120 min (female: 3.8 ± 0.2 /male: 5.0 ± 0.2 mg/dl; $P = 0.001$) of hyperinsulinemia. RBP was not different between pre- and postmenopausal women (data not shown). RBP did not correlate with EGP at any time of the clamp (basal: $r = 0.15$, $P = 0.49$; 90 min: $r = -0.081$, $P = 0.7$; 120 min: $r = 0.3$, $P = 0.2$).

RBP determined by quantitative Western blotting. Fasting plasma concentrations of RBP were comparable between IS (7.9 ± 0.5 mg/dl, $n = 17$) and IR (8.3 ± 0.6 mg/dl, $n = 20$; $P = 0.63$).

RBP determined by ELISA. Fasting plasma RBP concentrations were not different between IS (4.9 ± 0.3 mg/dl, $n = 18$) and IR (5.0 ± 0.4 mg/dl, $n = 20$; $P = 0.84$) study participants.

Results of nephelometry, ELISA, and quantitative Western

blot were strongly associated with the highest correlation coefficients between nephelometry and quantitative Western blot (Table 2).

PCI

Fasting plasma PCI active antigen was similar between both groups (IS: $107 \pm 16\%$ vs. IR: $95 \pm 4\%$) (Fig. 3A).

TABLE 2. Pearson's correlation of RBP concentrations obtained by nephelometry, ELISA, and quantitative Western blot

	RBP nephelometry	RBP ELISA	RBP Western blot
RBP nephelometry		$r = 0.623$ $P < 10^{-4}$	$r = 0.736$ $P < 10^{-6}$
RBP ELISA	$r = 0.623$ $P < 10^{-4}$		$r = 0.669$ $P < 10^{-5}$
RBP Western blot	$r = 0.736$ $P < 10^{-6}$	$r = 0.669$ $P < 10^{-5}$	

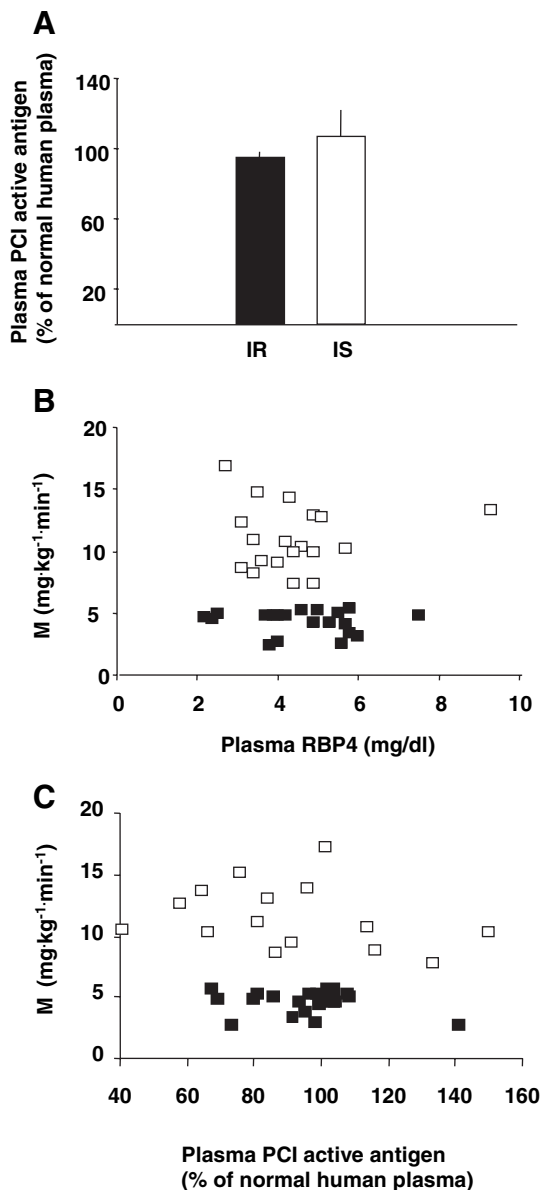


FIG. 3. A, Fasting plasma PCI active antigen (percent of normal human plasma) in IS and IR study participants (means \pm SE). Correlation analyses of M during the 80- to 120-min interval with plasma RBP4 (nephelometry) ($r = -0.076$, $P = 0.6$) (B) and plasma PCI active antigen ($r = -0.039$, $P = 0.8$) (C) in IS (\square) and IR (\blacksquare) study participants.

Correlation analyses

RBP. Plasma RBP did not significantly correlate with M (Fig. 3B) or OGIS 120/180 min (data not shown). When the relationship between RBP and M was determined separately for males and females, no difference was evident on the basis of gender (males: $r = 0.094$, $P = 0.77$; females: $r = -0.184$, $P = 0.36$). Also, fasting plasma RBP concentrations, determined by ELISA ($r = -0.27$, $P = 0.87$) and quantitative Western blotting ($r = -0.13$, $P = 0.44$) did not correlate with M.

RBP was positively correlated with body weight ($r = 0.407$, $P = 0.02$), height ($r = 0.415$, $P = 0.02$), body surface area ($r = 0.453$, $P = 0.007$), fat-free mass ($r = 0.494$, $P = 0.003$), tri-

glycerides ($r = 0.592$, $P < 0.001$), and serum activity of aspartate aminotransaminase ($r = 0.581$, $P < 0.001$), alanine aminotransaminase ($r = 0.631$, $P < 0.001$), and γ GT ($r = 0.639$, $P < 0.001$). RBP was correlated positively with serum concentrations of creatinine ($r = 0.439$, $P = 0.009$) and uric acid ($r = 0.488$, $P = 0.003$) in females only and correlated negatively with low-density lipoprotein-cholesterol in males ($r = -0.591$, $P = 0.043$). No correlation with RBP was found for total cholesterol, HDL cholesterol, hemoglobin A1c, and FFAs as well as fat mass; thigh, waist, and hip circumference; waist to hip ratio; BMI; and age.

PCI. Plasma PCI active antigen did not significantly correlate with M (Fig. 3C) or OGIS 120/180 min (data not shown), and correlation with M did not differ between males and females (males: $r = -0.364$, $P = 0.272$; females: $r = -0.052$, $P = 0.804$). PCI was positively related to waist to hip ratio in females ($r = 0.454$, $P = 0.022$).

Discussion

We investigated the potential association of insulin-resistance and retinol-binding factors (RBP and PCI) in two groups of nondiabetic humans who were markedly different concerning insulin sensitivity but well matched for age, gender, and BMI. In this study, plasma RBP concentrations were measured with three independent methods including quantitative Western blotting, which is currently regarded as the gold standard in measuring RBP (25). We did not find any difference in plasma RBP levels between IR and IS humans. During elevated plasma insulin concentrations (OGTT and clamp test), plasma RBP was in part decreased when compared with respective basal values. Fasting PCI active antigen was not different between both groups. Both plasma RBP concentration and PCI active antigen were not related to insulin sensitivity.

Study participants

Our study population was healthy, middle aged, and moderately overweight. In IR, insulin resistance was certainly present in skeletal muscle, fat tissue, but, interestingly, not in liver, which suggests an early stage of insulin resistance. Previous reports from our laboratory indicate that hepatic insulin resistance seems to be related to chronic hyperglycemia, which was not found in any of our study participants (29, 30–32). In particular, these insulin-resistant humans appeared to be at high risk for developing the metabolic syndrome, whose definition and treatment are currently under debate (33). Thus, an early circulating marker of insulin resistance, as RBP is proposed to be (25), would certainly be helpful for patients' identification and future treatment strategies.

Plasma RBP

Fasting plasma RBP concentrations measured by a highly sensitive nephelometric assay that has been certified and approved for clinical *in vitro* diagnostics were in the normal range (3–6 mg/dl according to the manufacturer) and were comparable with RBP concentrations measured by a recently developed ELISA (12). Furthermore, we verified our

results using quantitative Western blotting, which consistently showed approximately 2-fold higher RBP4 values than those measured by nephelometry when using a commercially available full-length recombinant standard for human RBP4. Interestingly, this difference was entirely dependent on the standard used in Western blotting because inclusion of and normalization to the nephelometric standard resulted in values that were highly comparable with nephelometric measurements. Of note, in contrast to recent observations, plasma RBP measured by three different methods including quantitative Western blotting was neither different between the groups nor associated with insulin resistance, measured by both clamp and OGIS. Differences in the study populations might have contributed to these divergent results. In these previous studies, elevated serum RBP concentrations were observed in insulin-resistant patients with obesity (8) and/or impaired glucose metabolism (12).

In contrast, in the present study, healthy nondiabetic, and only slightly overweight participants were included. Furthermore, insulin-sensitive and -resistant participants were well matched for gender, age, and BMI as well as fat and fat-free mass, with similar fasting insulin concentrations. Plasma concentrations of vitamin A and prealbumin were determined to exclude possible confounding effects on RBP concentration. Our results confirm the close association of vitamin A and prealbumin with RBP.

In our participants, plasma RBP levels were not related to insulin sensitivity but directly associated with anthropometric parameters such as body weight, height, fat-free mass, and body surface area and circulating molecules involved in liver, fat, and kidney metabolism such as serum creatinine, triglycerides, and transaminases/ γ GT.

From this it follows that plasma RBP in nondiabetic humans is not involved in insulin-mediated glucose disposal but rather depends on anthropometric properties and/or its release and elimination, the latter of which predominantly occurs in the kidney (34).

On the other hand, our data support a recent report from Janke *et al.* (13) that found no changes of serum RBP concentrations among lean, overweight, and obese women. Those women showed differences in insulin sensitivity, which was, however, measured by a mathematical model (homeostasis model assessment), which is correlated with but cannot substitute the hyperinsulinemic clamp (29).

Although circulating RBP levels were reported to be elevated in diabetic subjects (8) and RBP is produced from and stored in insulin-responsive tissues, the possible effect of hyperinsulinemia on RBP was up to now only marginally investigated. Fasting plasma insulin was positively correlated to fasting serum RBP (8). From this positive association, one might assume that a rise in plasma insulin would induce RBP release. However, whereas plasma insulin was increased during OGTT and clamp test in the present study, plasma RBP was either unchanged or reduced but did not increase. This finding argues against a direct, acute insulin effect on circulating RBP levels.

In accordance with previous studies (9, 10, 12), female study participants had lower RBP concentrations than males. Because plasma RBP was positively correlated with fat-free mass and body weight, both of which are higher in men,

these gender-related differences of body composition might account for this observation.

Plasma PCI active antigen

The complete biological function of PCI, in analogy to RBP, also a retinol-binding factor, has not been elucidated in previous studies (17). Initially described as an inhibitor of the anticoagulant protein C (19), it was also reported to be elevated in male survivors of myocardial infarction. Because patients with myocardial infarction become insulin resistant (20), we investigated the potential association of PCI and insulin sensitivity. We found that PCI was not different between the groups and did not correlate with insulin resistance. This indicates that PCI does not play a major role in the pathogenesis of insulin resistance.

In conclusion, our data demonstrate that healthy, nondiabetic, insulin-resistant humans do not show altered retinol-binding factors in plasma, such as RBP and PCI, both of which do not significantly correlate with insulin sensitivity. From this it follows that RBP does not appear to be a suitable marker for early insulin resistance as suggested (25). Moreover, plasma RBP is positively correlated to body weight and height as well as triglycerides, creatinine, uric acid, and liver enzymes, all of which are metabolites of organs that are responsible for the release and elimination of RBP. Furthermore, a short-term rise in plasma insulin does not appear to modulate circulating RBP concentrations. Thus, our findings do not support the hypothesis of insulin sensitivity modulation by proteins involved in retinol transport.

Acknowledgments

The authors are grateful to all volunteers for participation and to H. Lentner and A. Hofer (the Metabolic Unit) for skillful care of the study participants. After giving informed consent, the study participants were simultaneously included in the EGIR-RISC project (www.egir.org); head, Professor Dr. E. Ferrannini, University of Pisa, Pisa, Italy).

Received November 15, 2006. Accepted August 17, 2007.

Address all correspondence and requests for reprints to: Michael Krebs, M.D., Division of Endocrinology and Metabolism, Department of Internal Medicine III, Medical University of Vienna, Waehringer Guertel 18-20, A-1090 Vienna, Austria. E-mail: michael.krebs@meduniwien.ac.at; or Harald Esterbauer, M.D., Ph.D., Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University Vienna, Waehringer Guertel 18-20, A-1090 Vienna, Austria. E-mail: harald.esterbauer@meduniwien.ac.at.

The work was supported by a grant from the Austrian Diabetes Association (to C.A.) and an unrestricted research grant from Novo Nordisk, which is gratefully acknowledged. M.P. was employed as Post-Doc on a grant from the Austrian Society of Internal Medicine (Josef Skoda Award), which was competitively awarded (to M.K.). RISC is supported by European Union Grant QLGI-CT-2001-01252 and Astra-Zeneca (Vienna subcontractors 2001–2005; Professor Dr. W. Waldhäusl; since 2005: A.L. and C.A.).

Disclosure Statement: M.P., M.K., J.T., M.G.B., P.N., O.W., H.E., and C.A. have nothing to declare. A.L. received consulting fees from Pfizer, Novo Nordisk, Aventis, Lilly, and Takeda and lecture fees from Pfizer, Novo Nordisk, and Merck.

References

1. Krebs M, Krssak M, Nowotny P, Weghuber D, Gruber S, Mlynarik V, Bischof M, Stingl H, Fornsinn C, Waldhausl W, Roden M 2001 Free fatty acids inhibit the glucose-stimulated increase of intramuscular glucose-6-phosphate concentration in humans. *J Clin Endocrinol Metab* 86:2153–2160

2. **Anderwald C, Muller G, Koca G, Furnsinn C, Waldhausl W, Roden M** 2002 Short-term leptin-dependent inhibition of hepatic gluconeogenesis is mediated by insulin receptor substrate-2. *Mol Endocrinol* 16:1612–1628
3. **Anderwald C, Brabant G, Bernroider E, Horn R, Brehm A, Waldhausl W, Roden M** 2003 Insulin-dependent modulation of plasma ghrelin and leptin concentrations is less pronounced in type 2 diabetic patients. *Diabetes* 52:1792–1798
4. **Tsutsumi C, Okuno M, Tannous L, Piantadosi R, Allan M, Goodman DS, Blaner WS** 1992 Retinoids and retinoid-binding protein expression in rat adipocytes. *J Biol Chem* 267:1805–1810
5. **Kanai M, Raz A, Goodman DS** 1968 Retinol-binding protein: the transport protein for vitamin A in human plasma. *J Clin Invest* 47:2025–2044
6. **Redondo C, Burke BJ, Findlay JB** 2006 The retinol-binding protein system: a potential paradigm for steroid-binding globulins? *Horm Metab Res* 38:269–278
7. **Yang Q, Graham TE, Mody N, Preitner F, Peroni OD, Zabolotny JM, Kotani K, Quadro L, Kahn BB** 2005 Serum retinol binding protein 4 contributes to insulin resistance in obesity and type 2 diabetes. *Nature* 436:356–362
8. **Graham TE, Yang Q, Bluher M, Hammarstedt A, Ciaraldi TP, Henry RR, Wason CJ, Oberbach A, Jansson PA, Smith U, Kahn BB** 2006 Retinol-binding protein 4 and insulin resistance in lean, obese, and diabetic subjects. *N Engl J Med* 354:2552–2563
9. **Lee DC, Lee JW, Im JA** 2007 Association of serum retinol binding protein 4 and insulin resistance in apparently healthy adolescents. *Metabolism* 56:327–331
10. **Gavi S, Stuart LM, Kelly P, Melendez MM, Mynarcik DC, Gelato MC, McNurlan MA** 2007 Retinol-binding protein 4 is associated with insulin resistance and body fat distribution in non-obese subjects without type 2 diabetes. *J Clin Endocrinol Metab* 92:1886–1890
11. **Stefan N, Hennige AM, Staiger H, Machann J, Schick F, Schleicher E, Fritsche A, Haring HU** 2007 High circulating retinol-binding protein 4 is associated with elevated liver fat, but not with total-, subcutaneous-, visceral-, or intramyocellular fat in humans. *Diabetes Care* 30:1173–1178
12. **Cho YM, Youn BS, Lee H, Lee N, Min SS, Kwak SH, Lee HK, Park KS** 2006 Plasma retinol-binding protein-4 concentrations are elevated in human subjects with impaired glucose tolerance and type 2 diabetes. *Diabetes Care* 29:2457–2461
13. **Janke J, Engeli S, Boschmann M, Adams F, Bohnke J, Luft FC, Sharma AM, Jordan J** 2006 Retinol-binding protein 4 in human obesity. *Diabetes* 55:2805–2810
14. **von Eynatten M, Lepper PM, Liu D, Lang K, Baumann M, Nawroth PP, Bierhaus A, Dugi KA, Heemann U, Alolio B, Humpert PM** 2007 Retinol-binding protein 4 is associated with components of the metabolic syndrome, but not with insulin resistance, in men with type 2 diabetes or coronary artery disease. *Diabetologia* 50:1930–1937
15. **Yao-Borengasser A, Varma V, Bodles AM, Rasouli N, Phanavanh B, Lee MJ, Starks T, Kern LM, Spencer 3rd HJ, Rashidi AA, McGehee Jr RE, Fried SK, Kern PA** 2007 Retinol binding protein 4 expression in humans: relationship to insulin resistance, inflammation, and response to pioglitazone. *J Clin Endocrinol Metab* 92:2590–2597
16. **Ziegelmeier M, Bachmann A, Seeger J, Lossner U, Kratzsch J, Bluher M, Stumvoll M, Fasshauer M**, Serum levels of the adipokine RBP-4 in relation to renal function. *Diabetes Care*, in press
17. **Jerabek I, Zechmeister-Machhart M, Binder BR, Geiger M** 2001 Binding of retinoic acid by the inhibitory serpin protein C inhibitor. *Eur J Biochem* 268:5989–5996
18. **Suzuki K, Deyashiki Y, Nishioka J, Toma K** 1989 Protein C inhibitor: structure and function. *Thromb Haemost* 61:337–342
19. **Marlar RA, Griffin JH** 1980 Deficiency of protein C inhibitor in combined factor V/VIII deficiency disease. *J Clin Invest* 66:1186–1189
20. **Smit JW, Romijn JA** 2006 Acute insulin resistance in myocardial ischemia: causes and consequences. *Semin Cardiothorac Vasc Anesth* 10:215–219
21. **Carroll VA, Griffiths MR, Geiger M, Merlo C, Furlan M, Lammle B, Binder BR** 1997 Plasma protein C inhibitor is elevated in survivors of myocardial infarction. *Arterioscler Thromb Vasc Biol* 17:114–118
22. **Hother-Nielsen O, Henriksen JE, Holst JJ, Beck-Nielsen H** 1996 Effects of insulin on glucose turnover rates in vivo: isotope dilution versus constant specific activity technique. *Metabolism* 45:82–91
23. **Krebs M, Krssak M, Bernroider E, Anderwald C, Brehm A, Meyerspeer M, Nowotny P, Roth E, Waldhausl W, Roden M** 2002 Mechanism of amino acid-induced skeletal muscle insulin resistance in humans. *Diabetes* 51:599–605
24. **Anderwald C, Bernroider E, Krssak M, Stingl H, Brehm A, Bischof MG, Nowotny P, Roden M, Waldhäusl W** 2002 Effects of insulin treatment in type 2 diabetic patients on intracellular lipid content in liver and skeletal muscle. *Diabetes* 51:3025–3032
25. **Graham TE, Wason CJ, Bluher M, Kahn BB** 2007 Shortcomings in methodology complicate measurements of serum retinol binding protein (RBP4) in insulin-resistant human subjects. *Diabetologia* 50:814–823
26. **Inzucchi SE, Maggs DG, Spollett GR, Page SL, Rife FS, Walton V, Shulman GI** 1998 Efficacy and metabolic effects of metformin and troglitazone in type II diabetes mellitus. *N Engl J Med* 338:867–872
27. **DeFronzo R, Tobin J, Andres R** 1979 Glucose clamp technique: a method for quantifying insulin secretion and resistance. *Am J Physiol* 237:E214–E223
28. **Gastaldelli A, Miyazaki Y, Pettiti M, Buzzigoli E, Mahankali S, Ferrannini E, DeFronzo RA** 2004 Separate contribution of diabetes, total fat mass, and fat topography to glucose production, gluconeogenesis, and glycogenolysis. *J Clin Endocrinol Metab* 89:3914–3921
29. **Mari A, Pacini G, Brazzale AR, Ahren B** 2005 Comparative evaluation of simple insulin sensitivity methods based on the oral glucose tolerance test. *Diabetologia* 48:748–751
30. **Fasching P, Ratheiser K, Damjancic P, Schneider B, Nowotny P, Vierhapper H, Waldhäusl W** 1993 Both acute and chronic near-normoglycemia are required to improve insulin resistance in type 1 (insulin-dependent) diabetes mellitus. *Diabetologia* 36:346–351
31. **Bischof MG, Krssak M, Krebs M, Bernroider E, Stingl H, Waldhausl W, Roden M** 2001 Effects of short-term improvement of insulin treatment and glycemia on hepatic glycogen metabolism in type 1 diabetes. *Diabetes* 50:392–398
32. **Bischof MG, Bernroider E, Krssak M, Krebs M, Stingl H, Nowotny P, Yu C, Shulman GI, Waldhausl W, Roden M** 2002 Hepatic glycogen metabolism in type 1 diabetes after long-term near normoglycemia. *Diabetes* 51:49–54
33. **Daskalopoulou SS, Athyros VG, Kolovou GD, Anagnostopoulou KK, Mikhailidis DP** 2006 Definitions of metabolic syndrome: where are we now? *Curr Vasc Pharmacol* 4:185–197
34. **Jaconi S, Saurat JH, Siegenthaler G** 1996 Analysis of normal and truncated holo- and apo-retinol-binding protein (RBP) in human serum: altered ratios in chronic renal failure. *Eur J Endocrinol* 134:576–582

JCEM is published monthly by The Endocrine Society (<http://www.endo-society.org>), the foremost professional society serving the endocrine community.