Retinol-Binding Protein 4 in Twins

Regulatory Mechanisms and Impact of Circulating and Tissue Expression Levels on Insulin Secretion and Action

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OBJECTIVE—Retinol-binding protein (RBP) 4 is an adipokine of which plasma levels are elevated in obesity and type 2 diabetes. The aims of the study were to identify determinants of plasma RBP4 and *RBP4* mRNA expression in subcutaneous adipose tissue (SAT) and skeletal muscle and to investigate the association between RBP4 and in vivo measures of glucose metabolism.

RESEARCH DESIGN AND METHODS—The study population included 298 elderly twins (aged 62–83 years), with glucose tolerance ranging from normal to overt type 2 diabetes, and 178 young (aged 25–32 years) and elderly (aged 58–66 years) nondiabetic twins. Peripheral and hepatic insulin sensitivity was assessed by a euglycemic-hyperinsulinemic clamp, and β-cell function was estimated from an intravenous glucose tolerance test.

RESULTS—The influence of environmental versus genetic factors in the regulation of plasma RBP4 increased with age. Plasma RBP4 was elevated in type 2 diabetes and increased with duration of disease. Plasma RBP4 correlated inversely with peripheral, but not hepatic, insulin sensitivity. However, the association disappeared after correction for covariates, including plasma adiponectin. Plasma retinol, and not RBP4, was inversely associated with insulin secretion. SAT *RBP4* expression correlated positively with *GLUT4* expression and inversely with glucose tolerance. Skeletal muscle *RBP4* expression reflected intramuscular fat, and although it was suppressed by insulin, no association with insulin sensitivity was evident. *RBP4* expression was not associated with circulatory RBP4.

CONCLUSIONS—In conclusion, our data indicate that RBP4 levels in plasma, skeletal muscle, and fat may be linked to insulin resistance and type 2 diabetes in a secondary and noncausal manner. *Diabetes* 58:54–60, 2009

Retinol-binding protein (RBP) 4 is a transport protein for retinoids such as vitamin A in the blood (1). It is mainly produced in the liver but has recently been identified as an adipokine (2,3). The adipose tissue source, which secretes RBP4 into the circulation, may predominantly be visceral fat, including tissue (i.e., hepatic) fat depots (4,5). Recently, *RBP4* expression has been detected in skeletal muscle, and RBP4 was accordingly suggested to be a myokine (6,7).

Plasma RBP4 concentration may be under the influence of age and obesity (8,9). In addition, genes could play a role in determination of plasma RBP4, since certain single nucleotide polymorphisms in the *RBP4* gene promoter have been shown to be associated with elevated plasma RBP4 (10). Nevertheless, little is known about the relative importance of genes versus environmental factors in determination of plasma RBP4.

Elevated plasma RBP4 levels have been observed in subjects with insulin resistance and type 2 diabetes (4,5,8,9,11,12). Downregulation of adipocyte GLUT4, causing impaired glucose uptake, is thought to represent a mechanism for upregulation of RBP4 secretion (3,13). An intervention study (3) in rodents has demonstrated that lowering of plasma RBP4 ameliorates insulin resistance. Thus, RBP4 may be a putative drug target for treatment of type 2 diabetes. The cellular mechanisms of action of RBP4 are largely unknown and may be mediated by the protein itself as well as its carried retinoids. It has long been known that vitamin A is involved in the regulation of metabolism. Vitamin A depletion may enhance hepatic oxidation of citric acid cycle intermediates (14), whereas administration of 13-*cis*-retinoic acid to healthy humans has been shown to induce reversible insulin resistance, including reduced glucose oxidation (15). Retinoids act as regulators of gene transcription through a series of nuclear receptors (16). Among others, the gene for PEPCK is controlled by retinoids (17), and it has been demonstrated that injection of RBP4 causes an upregulation of hepatic *PEPCK* mRNA (3). In the present study, we aimed to investigate determinants of plasma RBP4 as well as *RBP4* mRNA expression in adipose tissue and skeletal muscle tissue and their influences on in vivo glucose metabolism.

RESEARCH DESIGN AND METHODS

Population 1 consisted of 298 monozygotic (MZ) ($n = 126$, 49 pairs and 28 single twins) and same-sex dizygotic (DZ) ($n = 172$, 56 pairs and 60 single twins) elderly twins aged 62–83 years (18,19). Oral glucose tolerance test (OGTT) data were obtained in 295 subjects for which glucose tolerance status ranged from normal ($n = 170$) over impaired glucose tolerance ($n = 83$) to overt type 2 diabetes ($n = 42$). A total of 22 subjects had known type 2 diabetes and were treated with diet or glucose-lowering medication. Population 2 included 178 MZ ($n = 97$, 48 pairs and 1 single twin) and same-sex DZ ($n = 81$, 40 pairs and 1 single twin) twins without type 2 diabetes divided into two age-groups (aged 25–32 and 58–66 years) (20). Zygosity was determined by a questionnaire concerning phenotypic similarities (18) and, in population 2, additionally by polymorphic genetic markers (21). The study was approved by the regional ethical committees and conducted in accordance with the Helsinki Declaration.

Clinical examination. Both populations underwent measures of height and weight for calculation of BMI and a 75-g OGTT. In addition, subjects in

TABLE 1
Characteristics including age, anthropometry, and plasma RBP4 and retinol of subjects from population 1

Variable	MZ twins	DZ twins	P
n (male/female)	126 (71/55)	172 (75/97)	—
Age (years)	74.2 ± 4.8	73.4 ± 5.4	0.3
BMI	26.3 ± 4.7	26.1 ± 3.9	0.9
Waist-to-hip ratio	0.92 ± 0.09	0.89 ± 0.10	0.05
RBP4 (μg/ml)	56.4 ± 17.0	55.1 ± 16.7	0.4
Retinol (μmol/l)	2.25 ± 0.67	2.21 ± 0.55	0.5

Data are means ± SD unless otherwise indicated.

population 2 underwent a dual-energy X-ray absorptiometry scan with measurement of total body fat percentage (22). Peripheral insulin sensitivity was determined by a 2-h (40 mU/m² per min) euglycemic-hyperinsulinemic clamp, which included 3-³H-glucose infusion for measurement of hepatic glucose production (HGP), and indirect calorimetry for measurements of glucose oxidation (GOX) and fat oxidation (FOX) rates (20,23,24). Insulin-stimulated glucose disposal rate (R_d) and HGP were calculated by Steele's equations for non–steady state (25). Hepatic insulin sensitivity was assessed as the absolute difference in HGP between the basal state and upon insulin infusion (ΔHGP) and as hepatic insulin sensitivity index (i.e., basal HGP multiplied by fasting plasma insulin). β-Cell function was estimated from an intravenous glucose tolerance test by calculation of the first-phase disposition index (D_{i1}) (20). Finally, physical fitness was estimated as Vo_{2max} calculated from the maximal load on an ergometer bicycle (26).

Tissue biopsies. Subcutaneous adipose tissue (SAT) biopsies were collected from the abdomen in a subgroup of population 1 ($n = 196$). Skeletal muscle biopsies were obtained from the vastus lateralis muscle before and during insulin stimulation in a subgroup of population 2 ($n = 96$) (23). The tissue specimens were taken under local anesthesia (lidocaine) using a Bergström needle with suction applied and were quickly blotted on filter paper and frozen in liquid nitrogen.

Analytical methods. Plasma glucose was measured by the glucose dehydrogenase oxidation method (20). Fasting levels of plasma RBP4 were determined by enzyme-linked immunosorbent assay (ELISA) (27). The ELISA results were validated by Western blotting. A total amount of 0.2 μl plasma was diluted 1:100 and loaded in 10 μl of loading buffer (LDS sample buffer; Invitrogen, Carlsbad, CA) and 1 mol/l dithiothreitol mixed 5:2 on 10% Bis-Tris gels (Invitrogen). Electrophoresis in NuPAGE MES running buffer (Invitrogen) and blotting in NuPAGE transfer buffer were conducted according to the manufacturer's recommendations. The membrane was incubated overnight in polyclonal anti-RBP4 antibody (cat. no. A0040; Dako, Glostrup, Denmark) diluted 1:500. Detection and membrane development were performed with a polyclonal horseradish peroxidase–linked anti-rabbit antibody (Cell Signaling, Danvers, MA) diluted 1:2,000 and LumiGLO reagent (Cell Signaling). Imaging and protein quantification were carried out by means of an LAS-3000 Image Reader (FujiFilm, Tokyo, Japan) and MultiGauge 2.0 software (FujiFilm). The agreement between plasma RBP4 quantities measured by ELISA and Western blotting was good ($r = 0.76$, $P < 0.001$). Fasting plasma retinol was measured by high-pressure liquid chromatography (28). In all 468 plasma samples analyzed, mean RBP4 was 2.58 ± 0.70 μmol/l, whereas mean retinol was 2.19 ± 0.58 μmol/l. RBP4 and retinol concentrations correlated tightly ($r = 0.93$, $P < 0.001$). Fasting plasma interleukin-6 and tumor necrosis factor-α levels were measured by Quantikine ELISA kits (R&D systems, Minneapolis, MN), and fasting plasma adiponectin was measured by a human adiponectin ELISA kit (Linco Research, St. Charles, MO) (29,30).

Total RNA was isolated from skeletal muscle and SAT with TRI reagent (Sigma-Aldrich, St. Louis, MO). Random-hexamer primed reverse-transcription reactions were performed from 1 μg of total RNA in 100 μl of water using a RevertAid first-strand cDNA synthesis kit (Fermentas Life Sciences, Ontario, Canada). *RBP4*, *GLUT4*, and *ADIPOQ* (adiponectin) cDNA was quantified by TaqMan real-time PCR on an ABI Prism 7900 HT system (Applied Biosystems, Foster City, CA) using gene expression assays for *RBP4* (Hs00198830_m1), *GLUT4* (Hs00168966_m1), and *ADIPOQ* (Hs00605917_m1). The mRNA quantities of target genes were normalized to the mRNA level of *PPIA* (cyclophilin A) (4326316E) and expressed in arbitrary units (AU). The reactions were performed with 20–80 ng of cDNA in 4.5 μl of water, 0.5 μl of gene expression assay, and 5 μl of Universal PCR Master Mix (Applied Biosystems).

Statistical methods

Mixed ANOVA. Uni- and multivariate analyses were conducted using the PROC MIXED procedure in SAS (version 9.1; SAS Institute, Cary, NC), with adjustment for zygosity and intra–twin pair relationships (23). Furthermore, adjustment for sex, age, obesity, and physical fitness were performed. Before analysis, the response variable was transformed by natural logarithm.

Correlation analyses. Pearson or Spearman correlation coefficients were calculated. The total phenotypic variance is the sum of the variance attributable to effects of both genetic and environmental factors. The heritability (h^2) expresses the proportion of variance attributable to genetic variance and was calculated from intraclass correlations in MZ and DZ twins [$h^2 = (r_{MZ} - r_{DZ}) \times 2$] (31).

Data presentation. Data are presented as means ± SD. Parameter estimates are shown in percentage with 95% CIs. Statistical significance was defined as $P < 0.05$.

RESULTS

Tables 1 and 2 summarize the characteristics including age, anthropometry, and plasma RBP4 and retinol of subjects from populations 1 and 2.

Factors regulating circulating RBP4

Age. In population 2, plasma RBP4 levels were significantly lower in young (45.8 ± 11.6 μg/ml) compared with elderly (51.2 ± 9.8 μg/ml) subjects ($P = 0.009$). When adjusted for sex and body fat percentage, age was an independent predictor of plasma RBP4 ($P = 0.006$).

Sex. Among elderly subjects in both populations 1 and 2, plasma RBP4 was slightly (though not significantly) higher in male subjects (population 1: 57.9 ± 16.5 μg/ml; population 2: 52.9 ± 9.4 μg/ml) than in female subjects (population 1: 54.0 ± 13.6 μg/ml; population 2: 49.7 ± 10.1 μg/ml; $P = 0.09$ and $P = 0.2$, respectively). Among young subjects, the differences in plasma RBP4 between male and female subjects reached the level of significance (male subjects: 50.1 ± 10.8 μg/ml; female subjects: 39.8 ± 10.1 μg/ml; $P < 0.001$). In addition, when adjusted for age and body fat percentage, sex was an independent predictor of plasma RBP4 in population 2 ($P < 0.001$).

Heredity. In population 1, the intraclass correlation coefficients were alike in MZ and DZ twins ($r_{MZ} = 0.52$, $r_{DZ} = 0.35$; $P = 0.3$). Similarly, in population 2, there were no statistically significant differences in intraclass correlations, neither among young ($r_{MZ} = 0.75$, $r_{DZ} = 0.44$; $P =$

TABLE 2
Characteristics including age, anthropometry, and plasma RBP4 and retinol of subjects from population 2

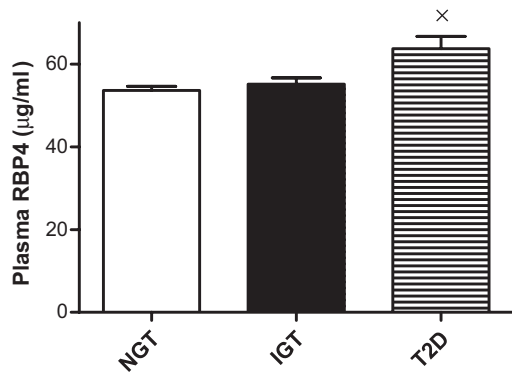| Variable	Young MZ twins	Young DZ twins	P	Elderly MZ twins	Elderly DZ twins	P
n (male/female)	57 (34/23)	38 (22/16)	—	40 (22/18)	43 (16/27)	—
Age (years)	28.2 ± 1.9	27.4 ± 2.0	0.06	61.5 ± 2.7	62.0 ± 2.0	0.3
BMI	24.3 ± 3.0	24.1 ± 3.3	0.9	26.4 ± 3.6	25.8 ± 4.9	0.7
Waist-to-hip ratio	0.85 ± 0.09	0.84 ± 0.07	0.8	0.91 ± 0.09	0.87 ± 0.11	0.9
Body fat (%)	22.3 ± 6.5	21.2 ± 6.8	0.3	27.6 ± 10.0	27.7 ± 10.0	0.3
RBP4 (μg/ml)	46.2 ± 11.7	45.3 ± 11.7	0.9	51.3 ± 10.4	51.0 ± 9.4	0.9
Retinol (μmol/l)	1.92 ± 0.50	1.87 ± 0.48	0.9	2.20 ± 0.44	2.19 ± 0.42	0.9

Data are means ± SD unless otherwise indicated.

FIG. 1. Plasma RBP4 and glucose tolerance. Plasma RBP4 concentration (means ± SE) in subjects with normal glucose tolerance (NGT; n = 170), impaired glucose tolerance (IGT; n = 83), and type 2 diabetes (T2D; n = 42). P values are adjusted for twin pair and zygosity status. ×P < 0.001 for normal glucose tolerance vs. type 2 diabetes.
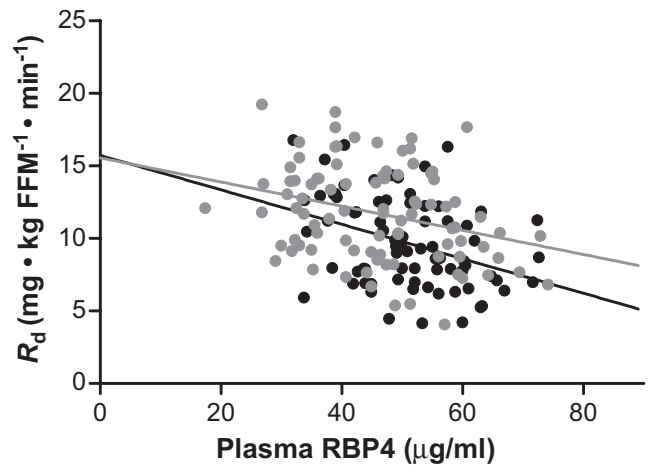

FIG. 2. Plasma RBP4 and R_d. Insulin-stimulated glucose disposal rate (R_d) as a function of plasma RBP4 in 90 young (gray circles) twins (r = −0.30, P = 0.004) and 77 elderly (black circles) twins (r = −0.37, P = 0.001).

0.09) nor elderly (r_{MZ} = 0.40, r_{DZ} = 0.53; P = 0.6) twins. The heritability estimates for plasma RBP4 were h^2 = 0.63 for young twins from population 2 and h^2 = 0.35 for elderly twins from population 1. A heritability estimate was not calculable for elderly twins from population 2 because of a higher intraclass correlation in DZ twins.

Obesity. Subjects were stratified according to BMI (lean: BMI <25 kg/m^2, overweight: 25 ≤ BMI < 30 kg/m^2, and obese: BMI ≥30 kg/m^2). In population 1, plasma RBP4 in lean (n = 119), overweight (n = 134), and obese (n = 43) subjects was 52.4 ± 14.8, 57.7 ± 14.6, and 58.3 ± 16.6 μg/ml, respectively, with a significant difference between lean and obese subjects (P = 0.003). In population 2, plasma RBP4 differed between lean and obese subjects (P = 0.004) within the young age-group (lean [n = 63]: 43.9 ± 11.0 μg/ml; overweight [n = 25]: 48.5 ± 12.0 μg/ml; obese [n = 4]: 58.9 ± 10.8 μg/ml), whereas no difference in plasma RBP4 was seen in the elderly subjects stratified for BMI (lean [n = 26]: 50.5 ± 10.5 μg/ml; overweight [n = 37]: 51.0 ± 10.7 μg/ml; obese [n = 15]: 52.6 ± 6.5 μg/ml). When adjusted for sex and age, BMI was a significant predictor of plasma RBP4 in both populations 1 and 2 (P = 0.02 and P = 0.005, respectively).

Metabolic effects of circulating RBP4 and retinol

Glucose tolerance. Mean plasma RBP4 concentrations were higher in subjects with type 2 diabetes (63.7 ± 19.4 μg/ml) and impaired glucose tolerance (55.1 ± 14.2 μg/ml) compared with subjects with normal glucose tolerance (53.6 ± 13.3 μg/ml) (Fig. 1), and the difference between subjects with normal glucose tolerance and type 2 diabetes achieved statistical significance (P < 0.001). Subjects with known type 2 diabetes (n = 22, 71.2 ± 21.9 μg/ml) had significantly higher plasma RBP4 than subjects with newly diagnosed type 2 diabetes (n = 26, 58.2 ± 16.2 μg/ml; P = 0.02). Additionally, RBP4 was positively associated with glucose tolerance (i.e., 2-h post OGTT plasma glucose levels), independent of sex, age, and BMI (P = 0.01).

Peripheral insulin sensitivity. In both young and elderly twins, plasma RBP4 correlated inversely with R_d (r = −0.30, P = 0.004 and r = −0.37, P = 0.001, respectively) (Fig. 2). When adjusted for sex, age, body fat percentage, and $V_{O_{2max}}$, RBP4 was independently associated with R_d with an effect of −4.8% (−9.6 to −0.04) for a 1-SD increase in RBP4. The corresponding effect of retinol on R_d was −5.0% (−9.2 to −0.4) (P = 0.04). When plasma concentrations of tumor necrosis factor-α, interleukin-6,

and adiponectin were introduced into the multivariate model, the independent association between R_d and plasma RBP4 or retinol disappeared (P = 0.3 and P = 0.07, respectively). In contrast, plasma adiponectin was positively associated with R_d, independent of sex, age, body fat percentage, plasma interleukin-6, plasma tumor necrosis factor-α, and plasma RBP4 (P < 0.001).

Plasma RBP4 correlated with insulin-stimulated rates of glucose and fat oxidation in young (r = −0.36, P < 0.001; and r = 0.24, P = 0.02, respectively) but not in elderly (r = −0.20, P = 0.08; and r = 0.14, P = 0.2, respectively) subjects. Nevertheless, after adjustment for sex, age, body fat percentage, and $V_{O_{2max}}$, plasma RBP4 was a significant predictor of GOX and FOX (P = 0.02 and P = 0.007, respectively), with a −6.0% decrease (−11.0 to –1.0) in GOX and a 20.7% increase (5.8–35.7) in FOX for a 1-SD increase in RBP4. The similar effects of retinol on GOX and FOX were −4.9% (−9.4 to 0.01; P = 0.05) and 22.7% (3.0–49.9; P = 0.01), respectively.

Hepatic insulin sensitivity. ΔHGP correlated inversely with plasma RBP4 in both young (r = −0.27, P = 0.01) and elderly (r = −0.25, P = 0.03) twins. However, when adjusted for sex, age, and body fat percentage, plasma RBP4 was not an independent predictor of ΔHGP (P = 0.5). There was no significant correlation between plasma RBP4 and hepatic insulin sensitivity index (young: r = −0.041, P = 0.7; elderly: r = 0.15, P = 0.2). Furthermore, plasma retinol was not associated with hepatic insulin sensitivity (data not shown).

β-Cell function. D_{i1} correlated with plasma RBP4 in elderly (r = −0.30, P = 0.01) but not in young (r = −0.057, P = 0.6) subjects. However, the association disappeared upon adjustment for sex, age, and body fat percentage (P = 0.1). On the other hand, when replacing RBP4 with retinol in the multivariate analysis, retinol was independently and inversely associated with D_{i1} (P = 0.04). The effect of a 1-SD increase in plasma retinol was associated with a −9.2% decrease (−16.3 to −0.7) of D_{i1}.

Tissue *RBP4* mRNA expression

SAT. *RBP4* mRNA expression was significantly higher in female subjects (n = 94, 1.09 ± 0.50 AU) compared with male subjects (n = 78, 0.84 ± 0.42; P < 0.001). Subjects with normal glucose tolerance (n = 94, 1.06 ± 0.53 AU) had significantly higher *RBP4* mRNA expression than subjects with impaired glucose tolerance (n = 49, 0.93 ±
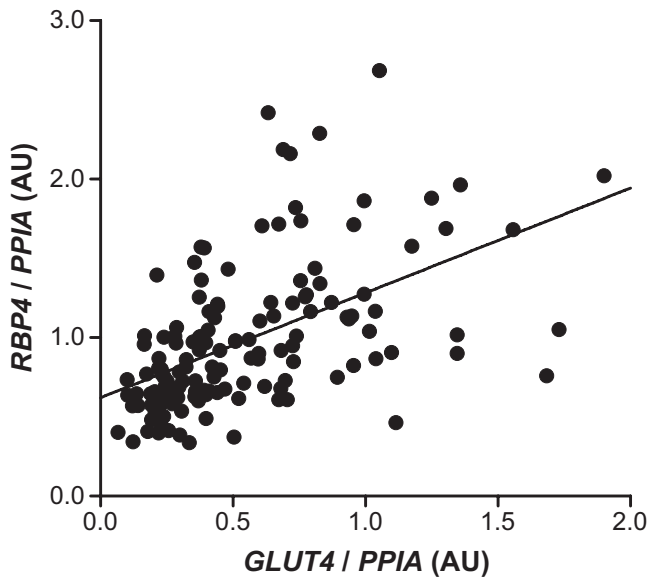
FIG. 3. Subcutaneous adipose tissue *GLUT4* versus *RBP4* expression. Paired *GLUT4* and *RBP4* mRNA expression values, normalized to *PPIA* (cyclophilin A) mRNA, in 141 twins. $r = 0.61$, $P < 0.001$.

FIG. 5. Skeletal muscle adiponectin versus *RBP4* expression. Paired log basal expression values for *ADIPOQ* (adiponectin) and *RBP4* mRNA normalized to *PPIA* (cyclophilin A) mRNA expression in 96 twins ($r = 0.86$, $P < 0.001$).

0.44 AU) and type 2 diabetes ($n = 28$, 0.79 ± 0.31 AU; $P = 0.01$ and $P < 0.001$, respectively) (Fig. 3). There was a positive correlation between *GLUT4* and *RBP4* expression ($r = 0.61$, $P < 0.001$) (Fig. 4). *GLUT4* expression was inversely associated with BMI ($r = -0.40$, $P < 0.001$) and with 2-h post-OGTT plasma glucose ($r = -0.24$, $P = 0.002$), whereas *RBP4* expression did not correlate with BMI ($r = -0.052$, $P = 0.5$). No correlation was seen between *RBP4* expression and plasma RBP4 ($r = -0.12$, $P = 0.1$).

Skeletal muscle. *RBP4* mRNA expression in skeletal muscle was significantly lower in male subjects ($n = 51$, 8.12 ± 10.02 AU) than in female subjects ($n = 45$, 38.75 ± 55.69 AU; $P < 0.001$) and in young ($n = 34$, 9.94 ± 12.21 AU) compared with elderly ($n = 62$, 29.91 ± 49.47 AU) subjects ($P = 0.02$). In both young and elderly subjects, *RBP4* expression correlated positively with body fat percentage ($r = 0.37$, $P = 0.03$; and $r = 0.41$, $P < 0.001$, respectively) and with skeletal muscle adiponectin expression ($r = 0.86$, $P < 0.001$) (Fig. 5). No significant correlation was found between *GLUT4* and *RBP4* expression in
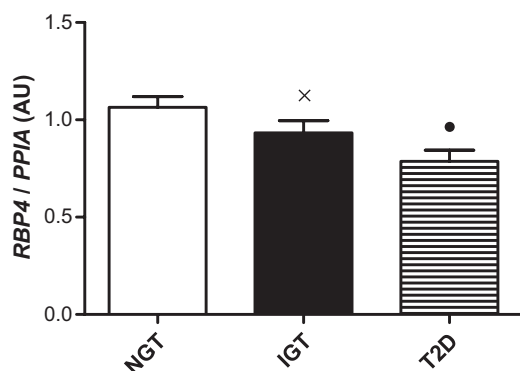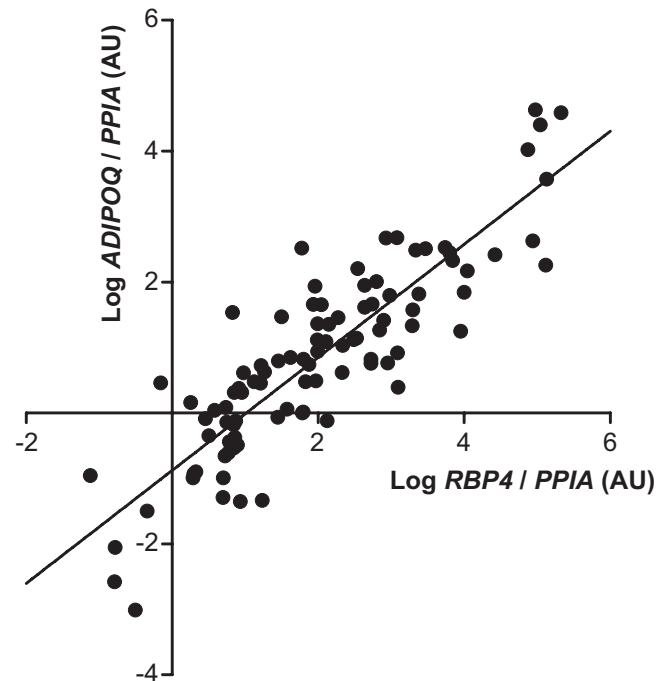
young and elderly subjects ($r = -0.32$, $P = 0.08$; and $r = -0.074$, $P = 0.6$, respectively). *RBP4* expression was significantly reduced upon insulin stimulation (basal: 22.48 ± 41.53 AU, insulin: 11.68 ± 18.54 AU; $P = 0.004$) (Fig. 6). However, when adjusted for sex, age, body fat percentage, and $V_{O_{2max}}$, insulin-stimulated *RBP4* expression was not an independent predictor of R_d ($P = 0.4$). No correlation was observed between basal *RBP4* expression and plasma RBP4 concentration ($r = 0.0090$, $P = 0.9$).

DISCUSSION

Elevated plasma RBP4 has been found to be associated with insulin resistance and type 2 diabetes in some (4,5,8,9,11,12), but not all (32–35), studies. The present study takes a step further in the investigation of the role of RBP4 as a mediator of insulin resistance. The unique twin study design combined with detailed metabolic characterization as well as eligible tissue specimens made it possi-

FIG. 4. Subcutaneous adipose tissue *RBP4* expression and glucose tolerance. Means ± SE for *RBP4* mRNA expression normalized to *PPIA* (cyclophilin A) mRNA expression is shown for subjects with normal glucose tolerance (NGT; $n = 94$), impaired glucose tolerance (IGT; $n = 49$), and type 2 diabetes (T2D; $n = 28$). P values are adjusted for twin pair and zygosity status. $\times P = 0.01$ for normal glucose tolerance vs. impaired glucose tolerance; ●$P < 0.001$ for normal glucose tolerance vs. type 2 diabetes.
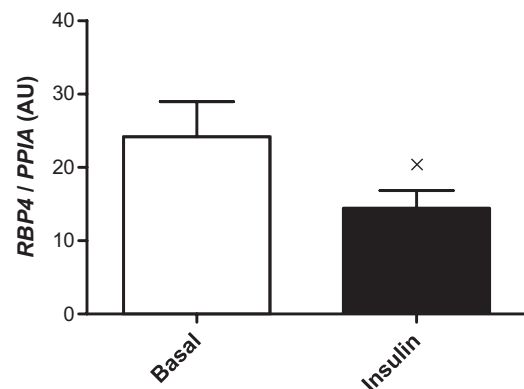

FIG. 6. Insulin stimulation and muscle *RBP4* expression. Means ± SE for basal and insulin-stimulated skeletal muscle *RBP4* mRNA expression normalized to *PPIA* (cyclophilin A) mRNA expression in 79 twins. $\times P = 0.004$.

ble to explore areas that have not been fully covered previously. These include the relative influence of genes versus environment on circulating RBP4, the putative role of RBP4 as a myokine, and, finally, the influence of RBP4 on distinct in vivo measures of glucose metabolism.

Regulating factors of plasma RBP4. The relative importance of genetic versus environmental etiological factors was determined by an estimate of heritability, which is the proportion of variance attributed to genetic factors. In the young twins, the heritability for plasma RBP4 was 63%, indicating a genetic component, which is in accordance with previous findings of an association between variants of the *RBP4* gene and plasma RBP4 levels (10). Interestingly, the genetic influence declined with advancing age, suggesting that environmental factors may be the predominant regulator of plasma RBP4 in the elderly.

Male subjects had higher plasma RBP4 levels than female subjects, which is consistent with previous findings (4,8). The sex-specific difference in plasma RBP4 concentration seemed to decrease with age. Thus, among young twins, the plasma RBP4 concentration was 26% higher in male than in female subjects, whereas in elderly subjects, the difference was only 6–7%. It may be speculated that the greater difference in plasma RBP4 between male and female subjects in young compared with elderly subjects could be due to an influence of, and possibly regulation by, sex hormones, although oral contraception has been demonstrated to elevate plasma RBP4 (36). A relatively larger age-related increase in plasma RBP4 in female subjects than in male subjects could also be due to an alteration in body fat distribution toward android shape in aging female subjects. Elderly subjects had 12% higher plasma RBP4 concentration than young subjects. The fact that aging seemed to play a role in determination of plasma RBP4 is in agreement with a previous study (8).

Obesity, as expressed by BMI, has previously been shown to be positively associated with plasma RBP4 (9,37). In accordance, we demonstrated 4–34% higher plasma RBP4 in obese compared with lean subjects, depending on twin population. Importantly, the association between overall obesity and plasma RBP4 was independent of sex and age. It has been demonstrated that particularly hepatic fat accumulation may lead to elevation of plasma RBP4 (5,38). Supporting this theory, we found a positive association between plasma levels of alanine amminotransferase and RBP4 (data not shown). In addition to genes, sex, and obesity, factors such as lipid metabolism (32), inflammation (39), hepatic function (40), and renal function (41) may influence plasma RBP4 levels. These areas were not investigated in the present study.

Plasma RBP4 and in vivo glucose metabolism. Molar plasma concentrations of RBP4 and retinol correlated tightly, and therefore associations to metabolic rates were almost identical for RBP4 and retinol. When stratifying according to glucose tolerance, we found that diabetic subjects had 19% higher plasma RBP4 concentration than subjects with normal glucose tolerance, and the multivariate analysis confirmed that plasma RBP4 predicted glucose tolerance independently of sex, age, and obesity. Plasma RBP4 may therefore represent a biomarker for impairment of glucose tolerance. Interestingly, plasma RBP4 was significantly higher in subjects with known type 2 diabetes (i.e., a longer duration of disease) than in subjects with newly diagnosed type 2 diabetes, possibly related to the higher plasma glucose levels in the former

group. Hence, an upregulation of plasma RBP4 could be a secondary event to hyperglycemia.

Previous studies (4,5,9,12) have demonstrated an inverse association between RBP4 and peripheral insulin sensitivity as determined by the euglycemic-hyperinsulinemic clamp technique. In the present study, we confirm this association, independent of the known influencing factors, age, body fat percentage, and physical fitness. However, the association with R_d was markedly surpassed by plasma adiponectin, making it less likely that plasma RBP4 is a key determinant of peripheral glucose uptake. In contrast, the effect of plasma RBP4 on insulin-stimulated fat oxidation rate was noteworthy. It has been demonstrated that treatment of adipocytes with retinoic acid, a major retinol metabolite, led to decreased adipogenesis and increased fat oxidation (42). Thus, plasma RBP4 could, via delivery of retinol to the cells, stimulate fat oxidation, thereby decreasing glucose oxidation according to Randle's cycle (43).

It has been shown in rodents that elevation of plasma RBP4 was associated with an upregulation of HGP, which was ascribed to an increased expression of *PEPCK* (3). To our knowledge, the present study is the first to describe the relationship between plasma RBP4 and hepatic insulin sensitivity measured by the gold standard euglycemic-hyperinsulinemic clamp in human subjects. Importantly, these data do not support a role of plasma RBP4 or retinol in regulation of hepatic insulin sensitivity.

Plasma retinol, but not RBP4, was an independent, negative predictor of D_{i1}. It has previously been demonstrated that plasma RBP4 was associated with impaired β-cell function in elderly subjects (34). Our data indicate that the observed association could be mediated by retinol. However, the fact that no association was found between plasma retinol and D_{i1} in young subjects contradicts a causal connection between plasma retinol and β-cell failure.

Skeletal muscle and adipose tissue *RBP4* mRNA expression. In accordance with a previous study (6), *RBP4* expression in skeletal muscle was low compared with that in SAT (data not shown). Female subjects had significantly higher *RBP4* expression than male subjects, and mRNA levels correlated with total body fat percentage and with skeletal muscle adiponectin mRNA expression. Female sex is associated with increased body fat percentage, including intramuscular fat depots (44), and adiponectin is almost exclusively expressed in adipocytes (45), hence representing a surrogate for adipose tissue. Therefore, it is plausible that the *RBP4* expression level in skeletal muscle reflects the amount of intramuscular fat rather than RBP4 being a true myokine synthesized by the muscle fibers. The lack of correlation between skeletal muscle *GLUT4* and *RBP4* expression supports this theory. It has previously been demonstrated that intramuscular fat content is not associated with plasma RBP4 (5). Accordingly, we did not find a correlation between muscle *RBP4* expression and plasma RBP4. The suppression of skeletal muscle *RBP4* expression to 55% of the basal level upon insulin infusion suggested a connection between glucose metabolism and skeletal muscle *RBP4* expression. However, we did not demonstrate a relationship between skeletal muscle *RBP4* expression and peripheral insulin sensitivity.

Adipocyte *GLUT4* mRNA and GLUT4 protein have been shown to be downregulated in obese and type 2 diabetic subjects (46). In the present study, these data were confirmed at mRNA level. It has been hypothesized that the

impaired glucose uptake in GLUT4-deficient adipocytes could lead to a compensatory upregulation of *RBP4* (13). Accordingly, *RBP4* expression has been shown to correlate inversely with *GLUT4* expression in visceral fat (3,47). In the present study we demonstrated a relatively strong positive correlation between SAT *GLUT4* and *RBP4* expression, which is in line with findings from two previous studies (6,33). Furthermore, we observed significantly lower SAT *RBP4* expression in diabetic subjects compared with subjects with normal glucose tolerance. This downregulation of *RBP4* expression could be due to the hyperinsulinemia characterizing the diabetic and/or insulin-resistant state, parallel to our finding of an insulin-induced suppression of *RBP4* expression in skeletal muscle. Female subjects had higher SAT *RBP4* expression than male subjects, though the sex-specific difference was less pronounced than in skeletal muscle. Differential expression of adipokines in SAT and visceral adipose tissue has been demonstrated in several studies (48,49), hence their metabolic activity is thought to be different depending on fat depot. Accordingly, higher *RBP4* expression has been observed in visceral compared with SAT, and the difference was reflected by a stronger association of visceral fat *RBP4* expression than of SAT *RBP4* expression with plasma RBP4 (47). In the present study, we did not find a correlation between SAT *RBP4* expression and plasma RBP4 as previously reported (33).

In conclusion, our data indicate that elevated plasma RBP4 in type 2 diabetes is a secondary and predominantly nongenetic phenomenon and that plasma RBP4, per se, plays only a minor, if any, role in the development of insulin resistance in humans. Plasma glucose and insulin may be involved in the regulation of *RBP4* expression in SAT and skeletal muscle; however, these tissues do not contribute to a noteworthy degree to production of plasma RBP4.

ACKNOWLEDGMENTS

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