CLINICAL STUDY

Retinol-binding protein-4 is not strongly associated with insulin sensitivity in normal pregnancies

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Abstract

Objective: Recently, experimental and clinical studies suggest that retinol-binding protein-4 (RBP4) may provide a link between obesity and insulin resistance. However, no previous studies have investigated the impact of circulating RBP4 on measures of insulin resistance in normal pregnant women, and the objective of this study is to measure serum RBP4 in early and late pregnancy and relate these to measures of insulin resistance and secretion controlling for changes in fat mass.

Design and methods: Samples were obtained during oral glucose tolerance test (OGTT) from 44 normal pregnancies at weeks 14–16 and 30–32. Measures of fat mass were body mass index (BMI) and leptin while insulin sensitivity and secretion were predicted from OGTT. Leptin and RBP4 were measured by immunoassay.

Results: Insulin sensitivity decreased during the course of pregnancy. Insulin sensitivity and secretion were best explained by BMI and circulating leptin, but not RBP4, both in early and late pregnancy. However, a marked increase in fasting RBP4 from early to late pregnancy was observed, and this change was associated with a decline in insulin sensitivity. A marked increase in RBP4 was found during OGTT at weeks 14–16 with an opposite temporal course at weeks 30–32.

Conclusion: The increased fat mass and insulin resistance during normal pregnancy was best explained by measures of fat mass. However, the increase in RBP4 from early to late pregnancy, associated with a decline in insulin sensitivity, potentially indicates interactions with glucose metabolism.

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Introduction

Increased adipose tissue mass is strongly associated with the pathogenesis of insulin resistance and diabetes mellitus (DM)2 (1). Pregnancy is associated with increased maternal fat mass and decreased insulin sensitivity. Overweight among pregnant women is a strong predictor of gestational diabetes and preeclampsia. Adipose tissue secretes many types of adipokines that modulate the action of insulin in other tissues (2). Recently, experimental models demonstrated that retinol-binding protein-4 (RBP4) may provide a link between obesity and insulin resistance by reducing peripheral and hepatic insulin sensitivity and increasing hepatic gluconeogenesis (3). Accordingly, several clinical studies reported that circulating RBP4 is elevated in human subjects with impaired glucose tolerance and DM2 (4, 5). Furthermore, RBP4 levels were recently found to be elevated in women with gestational diabetes mellitus (GDM) in the second trimester (6).

Although RBP4 has received recent attention due to its potential role in regulating insulin sensitivity, it has been widely studied in relation to mobilization and transport of

retinol from liver to target tissues (7). In the target tissues, metabolic enzymes convert retinol to retinoic acid, which then controls vitamin A signaling. Vitamin A is essential for maintaining pregnancy and morphogenesis of most developing organs and tissues. Thus, the mammalian fetus acquires vitamin A from the maternal circulation, in which the bulk is in the form of retinol bound RBP4, primarily synthesized in the liver. When studying associations between RBP4 and insulin resistance during pregnancy this may be important to bear in mind.

To our knowledge, no previous studies have investigated the impact of circulating RBP4 on measures of insulin resistance in pregnant women. In this study, we measured plasma RBP4 and measures of fat mass early and late in normal pregnancy and investigated its influence on insulin sensitivity and secretion.

Methods

Study population

The present study is a pilot study of a larger prospective cohort study (STORK) aimed at investigating factors of

importance for the increase in macrosomia frequency. Pregnant Scandinavian women referred to Rikshospitalet University Hospital, and already included in STORK, were included in this study. Women with arterial hypertension, renal disease, liver disease, diabetes mellitus, thyroid disorders or other endocrine or chronic diseases, smokers, earlier premature births, twin pregnancies, or GDM in a previous pregnancy were excluded. Fifty women were originally included. During the investigation period, six women were excluded; two women had early premature birth, one woman lost her baby, and three women did not show up on the second visit.

Study protocol

The pregnant women came in the fasting state and the first visit was in weeks 14–16 and the second in weeks 30–32 of the pregnancy. They underwent a 75 g oral glucose tolerance test (OGTT) at both visits. During the tests, samples were collected at the following times: 0, 30, 60, 90, and 120 min. The glucose solution was given after the baseline sample and it was consumed within ~5 min. In addition to the blood samples, the subjects' body mass index (BMI) and s.c. fat mass were determined at both visits. Weight was measured on a digital scale without heavy clothes and shoes. The gestational age at delivery varied, and therefore birth weight corresponding to 40 complete weeks was estimated as described (8). Briefly, the birth weight corresponding to 40 complete weeks was estimated using standardized birth weights based on data from the Medical Birth Registry of Norway (8). Two independent researchers estimated the birth weight by looking up the gestational age and birth weight and projecting the corresponding value at 40 weeks on the birth weight by gestational age percentiles plot. There were separate plots for male and female. The mean estimated birth weight of the two researchers was used. Insulin sensitivity (ISI_{OGTT}) was predicted using the index of Matsuda & DeFronzo (9). Insulin secretion was determined by the Stumvoll first- and second-phase measure of insulin secretion (10). These are defined under Table 1. The study was approved by the local ethical committee and conducted according to the Helsinki Declaration. The subjects were given oral information at the first visit and gave their informed consent to participate.

Blood measurements

Blood samples were collected into 7 ml tubes containing EDTA on ice, centrifuged at 4 °C at 3000 RCF for 10 min, and plasma aliquoted and stored at –80 °C until assayed. Leptin (Linco Research Inc., St Charles, MO, USA) and insulin (DPC, Los Angeles, CA, USA) were analyzed by RIA. RBP4 was measured by a competitive enzyme immunoassay (Biosource International,

Table 1 Mean (±s.d.) maternal characteristics at 14–16 weeks, 30–32 weeks, and change in this period.

	Baseline (weeks 14–16)	Visit 2 (weeks 30–32)	Change
Age, (years)	31 ± 5	–	–
Parity, <i>n</i> (0,1,2,3)	22,18,3,1	–	–
Weight, (kg)	72.2 ± 13.3	79.6 ± 13.0	7.4 ± 3.8 [‡]
BMI (kg/m ²)	25.2 ± 4.2	28.1 ± 4.2	2.6 ± 1.5 [‡]
Glucose (mM)	3.9 ± 0.4	4.6 ± 0.4	0.7 ± 0.4 [‡]
Insulin (ng/ml)	26.6 ± 26.3	31.9 ± 23.8	5.3 ± 27.6 [*]
Leptin (ng/ml)	20.9 ± 12.5	25.3 ± 15.3	4.5 ± 9.6 [†]
ISI_{OGTT}	7.8 ± 4.6	4.3 ± 2.9	–3.5 ± 2.9 [‡]
Stumvoll first phase	1110 ± 252	1070 ± 350	–35 ± 279
Stumvoll second phase	288 ± 60	286 ± 84	0 ± 68

BMI, body mass index. ^{*}*P* < 0.05, [†]*P* < 0.01, and [‡]*P* < 0.001. ISI_{OGTT} : $10\,000/\sqrt{Glucose_{0\,min} \times Insulin_{0\,min} \times mean\,Glucose_{0-120\,min} \times mean\,Insulin_{0-120\,min}}$. Stumvoll first phase: $1283 + 1.829 \times Insulin_{30\,min} - 138.7 \times Glucose_{30\,min} + 3.772 \times Insulin_{0\,min}$; Stumvoll second phase: $287 + 0.4164 \times Insulin_{30\,min} - 26.07 \times Glucose_{30\,min} + 0.9226 \times Insulin_{0\,min}$.

Camarillo, CA, USA) in one run. In our hands, the intra-assay variation was 13.1%. The mean recovery of two samples spiked with different concentrations of recombinant RBP4 was 92%, range 68–108%. The sensitivity, defined as the reading of the mean optical density ± 3 s.d. of the zero standard, was calculated to be 0.02 µg/ml. Serial dilution of two samples 1:1–1:8 gave a mean recovery of 89%.

Statistical analysis

A change score was calculated for all variables defined as value at 30–32 weeks minus value at 14–16 weeks. Unpaired and paired data were analyzed using appropriate parametric tests on log-transformed data if necessary. To examine the relationship between maternal insulin sensitivity, secretion, and independent predictors, we used simple linear regression followed by multiple linear regression analysis with stepwise addition of variables; BMI, leptin, age, and RBP4. *P* values are two sided and considered significant when <0.05.

Results

Maternal characteristics

Table 1 shows the baseline maternal characteristics at weeks 14–16, weeks 30–32, and the change in this period. Significant increases during pregnancy were observed for BMI, fasting glucose, insulin, and leptin. Insulin sensitivity decreased significantly, while no significant changes were observed for Stumvoll first- and second-phase insulin secretion.

Biochemistry

Figure 1A shows a marked increase in baseline levels of RBP4 from 14–16 to 30–32 weeks of pregnancy. The response to OGTT at 14–16 and 30–32 weeks for RBP4, glucose, and insulin is shown in Fig. 1B. At 14–16 weeks, a continuous increase was seen in RBP4 throughout the OGTT ($P < 0.001$ repeated measures ANOVA), in contrast to glucose and insulin that flatten out or decreased after 30 min. Surprisingly, while the response to OGTT at 30–32 weeks was markedly enhanced for glucose and insulin, the temporal course was opposite for RBP4 with a steady decline during OGTT ($P < 0.001$ repeated measures ANOVA), almost the mirror image of the response at 30–32 weeks (Fig. 1B). To further investigate the associations between the responses to OGTT for these measures, we correlated the area under the curve (AUC). No significant associations were found between the AUC for RBP4 and glucose, insulin, or leptin at either time point (14–16 or 30–32 weeks, data not shown). Finally, no associations were observed at any time point between baseline serum RBP4 at 14–16 weeks, 30–32 weeks, or change in this period and baseline glucose and insulin, age, or BMI.

Correlations

Figure 2 shows that insulin sensitivity was associated with BMI and leptin at both time points, while insulin secretion was associated with BMI and leptin in early and late pregnancy respectively (Fig. 3). Finally, the decrease in insulin sensitivity from 14–16 to 30–32 weeks was associated with an increase in RBP4 in the same period. No association between insulin sensitivity/secretion and age was found. Multiple regression as described in statistics did not reveal any additional information than the univariate associations (data not shown). Thus, the strongest independents (Figs 2 and 3) remained in the model (e.g. leptin was the sole predictor for insulin sensitivity at 14–16 weeks, BMI at 30–32 weeks).

Discussion

In normal pregnant women, glucose homeostasis is maintained by an exaggerated rate of insulin release, which accompanies a decreased sensitivity to insulin as demonstrated in our study by a decreased ISI_{OGTT} and response in insulin to OGTT during the course of pregnancy. However, the mechanisms responsible for these alterations of glucose metabolism in pregnancy are not well characterized, but insulin resistance and deficits of insulin secretion are considered main factors (11, 12). Defects in the ability of fat cells to transport glucose are linked to insulin resistance in muscle and liver, and increased adipose tissue mass is strongly associated with the pathogenesis of insulin resistance and DM2 (1). Accordingly, BMI and serum leptin levels in both early and late pregnancy were associated with insulin sensitivity and secretion in our study. This is in agreement with previous studies in women with GDM (13, 14) as well as normal pregnancies (14) showing correlations between measures of insulin sensitivity and circulating leptin. The increase in leptin during the course of pregnancy, correlated with increased BMI, point toward the possibility that the increasing fat mass may induce changes in leptin. Still, associations independent of fat mass have been demonstrated for leptin on insulin resistance, although this matter is of debate (15).

Recently, RBP4 was reported to provide a link between obesity and insulin resistance (3) and based on the longitudinal changes in glucose metabolism and fat mass in the present study, it could be implicated in the increase in insulin resistance observed during pregnancy. Indeed, a marked increase in serum RBP4 was observed with advancing gestation, compatible with a detrimental effect on insulin sensitivity. However, serum RBP4 was poorly associated with insulin sensitivity and secretion at 14–16 or 30–32 weeks suggesting no apparent role in mediating insulin resistance in the pregnant state. By contrast, Chan *et al.* recently demonstrated no changes in circulating RBP4 during the course of pregnancy (6). There are

Figure 1 (A) Change in fasting RBP4 from 14–16 to 30–32 weeks of pregnancy. (B) Response to OGTT at 14–16 and 30–32 weeks for RBP4, glucose, and insulin. The response to OGTT was $P < 0.001$ for RBP4, glucose, and insulin at both weeks 14–16 and 30–32 as measured by repeated measures ANOVA and further $P < 0.001$ compared with baseline for time points 30–120.

Figure 2 Correlations between ISI_{OGTT} and BMI, leptin, and RBP4 at weeks 14–16 and 30–32 during normal pregnancy. The right panel shows the correlation between changes in these parameters from weeks 14–16 to 30–32. *See Table 1 for formula.

several issues that may help explain these discordant results. We wished to investigate the association between changes in fat mass, insulin resistance, and RBP4, and all samples were taken in the fasting state in the morning. In the study by Chan *et al.* blood for the last time point was collected immediately after delivery making this time point questionable for representing changes during pregnancy (6). Immediately after delivery, a marked rise in stress-related hormones that may have anti-insulinic actions could possibly influence circulating RBP4 levels (16). Also, some differences have been reported that are dependent on what type of

assay is used for determining RBP4 (17). Still, our results are not incompatible with the study by Chan *et al.* who found that women with GDM were characterized by increased RBP4, although they did not correlate circulating levels with measures of insulin resistance (6). Thus, the decrease in insulin sensitivity from 14–16 to 30–32 weeks, correlated with the increase in serum RBP4 in this period, may suggest a potential effect on insulin resistance not reflected at the individual time points. This was a pilot study and possibly similar measurements in the total cohort will be more revealing.

Figure 3 Correlations between Stumvoll first-phase insulin secretion and BMI, leptin, and RBP4 at weeks 14–16 and 30–32 during normal pregnancy. The right panel shows the correlation between changes in these parameters from weeks 14–16 to 30–32.

Little is known about how RBP4 may affect insulin signaling. Indeed, the RBP4 treatment of human adipocytes increased, rather than decreased, the amount of glucose transporter (GLUT)4 at the plasma membrane and thereby increased basal glucose uptake (18). This is further supported by reports demonstrating a positive relationship between adipose tissue RBP4 and GLUT4 mRNA levels in adipose tissue from obese subjects (19). Thus, an increased circulating RBP4 would potentially increase glucose disposal, although we were unable to demonstrate an association with the temporal courses of glucose or insulin in our study. Many binding proteins derived mainly from the liver increase during pregnancy under the influence of estrogen due to hormonal requirements by the fetus. However, *in vitro*, at least in rats, estrogen increases RBP mRNA in the kidney but not liver (20). This does not exclude the RBP4 production in the liver during pregnancy but suggests that the sources for increasing levels may be many including release from fat, kidney, and liver cells as well as placenta. The transport of retinol (bound to RBP4) from mother to fetus occurs mainly via the placenta and is processed by receptors for RBP and cellular RBP, and mRNA for RBP4 has been detected in various placental cells (21) and small amounts of the protein have been detected in placental microsomes (22). Hence, an increase in RBP4 from early to late pregnancy could reflect increased synthesis from an expanding placenta. Studies investigating the possible contribution of placental RBP4 to maternal circulating levels or looking at the association between placental mRNA levels of RBP4 and presence/absence of GDM would be informative.

The study is limited by the relatively small number of subjects investigated. This limits the amount of variables that can be included as independents in the regression model. Also, we studied women without a history of diabetes mellitus or GDM limiting the interpretation of our results to concern normal pregnancies. Indeed, the associations between insulin kinetics and RBP4 may be different in women with more severe insulin resistance. We did not use the glucose clamp technique, the reference standard for directly determining metabolic insulin sensitivity in humans, although the ISI_{OGTT} as determined in the present study correlates reasonably well with the estimates of whole-body insulin sensitivity determined by the glucose clamp. Most measurements of fat mass are based on a constant and known composition of hydrated and dry tissue. However, in pregnancy, a relative excess of body water can be observed and consequently the usual assumptions made for the fat-free mass do not hold (23). Importantly, with regard to insulin resistance, central visceral fat, in particular, is strongly associated with insulin resistance and type 2 diabetes. However, computed tomography at abdominal level is the best estimation of visceral fat but cannot be used during pregnancy because of its potential harmful effects on the fetus. Nonetheless,

BMI at index pregnancy have been associated with long-term risk of diabetes (24). Furthermore, we did try an estimation of fat mass based on skinfold thickness but do not present these data since BMI correlated more strongly with insulin resistance/secretion and finally, these formulas have only been validated in late pregnancy (25).

In conclusion, the increased fat mass and increased insulin resistance during normal pregnancy is not associated with RBP4 at any time points investigated. However, a marked increase in RBP4 was observed from early to late pregnancy and this change was associated with a decline in insulin sensitivity, potentially indicating interactions with glucose metabolism. Studies investigating associations between placental RBP4 expression and insulin resistance could be informative.

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