An obese brain and an inflamed body: Central and peripheral consequences of obesity

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Serum Retinol Binding Protein–4 Is Inversely Associated with Insulin Action in Adipose Tissue, Skeletal Muscle and Liver in Obese Women


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Submitted
Abstract

**Background:** Adipose-tissue expression of retinol binding protein 4 (RBP4) is associated with obesity-induced insulin resistance and impairs insulin action in skeletal muscle, but less is known about its effects on insulin sensitivity in adipose tissue and liver in humans. We aimed to explore the relation between serum and tissue RBP4 and peripheral and hepatic insulin sensitivity as well as insulin sensitivity of adipose tissue in obese humans.

**Methods:** We performed a 2-step hyperinsulinemic-euglycemic clamp using a stable glucose and a stable glycerol isotope tracer to assess hepatic and peripheral as well as adipose-tissue insulin sensitivity respectively in 20 morbidly obese women undergoing Roux-en-Y gastric bypass surgery. To assess RBP4 expression levels, tissue biopsies were obtained during surgery from liver, subcutaneous and visceral adipose tissue in the obese women as well as in 6 lean healthy controls undergoing elective laparoscopic cholecystectomy.

**Results:** RBP4 expression was increased in both liver and subcutaneous and visceral adipose tissue in the obese subjects. Serum RBP4, but not tissue RBP4 expression, inversely correlated with peripheral glucose uptake and insulin-mediated suppression of lipolysis, free fatty acids and endogenous glucose production.

**Conclusions:** Gene expression of RBP4 is increased in liver and adipose tissue of morbidly obese subjects compared to lean controls. While tissue RBP4 expression does not correlate with insulin sensitivity, serum RBP4 correlates inversely with insulin action in adipose tissue, skeletal muscle and liver. These data indicate that in obesity RBP4 signals as a hormone affecting insulin sensitivity of multiple metabolic pathways.
Introduction

The last several decades the prevalence of obesity has increased worldwide. Obesity increases the risk for, among others, type 2 diabetes mellitus (T2DM), dyslipidemia and cardiovascular disease. As a result, obesity is associated with a higher mortality risk (1). The major underlying cause of the metabolic complications of obesity is the development of insulin resistance, which is defined as impaired insulin action in insulin-sensitive tissues. Insulin resistance in skeletal muscle results in reduced glucose uptake whereas in adipose tissue reduced insulin action results both in reduced glucose uptake and reduced suppression of lipolysis. Finally insulin resistance in the liver leads to reduced suppression of endogenous glucose production (EGP) (2). Obesity-induced insulin resistance is characterized by a downregulation of the glucose transporter-4 (GLUT4) in adipocytes (3), leading to whole-body insulin resistance (4) and elevated expression of retinol binding protein 4 (RBP4) (5, 6). RBP4 increases the expression of the major gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK) in liver and impairs insulin signaling in skeletal muscle (5). In obese and T2DM subjects, adipose expression and serum levels of RBP4 are increased and are associated with insulin resistance in most, but not all studies (7). RBP4 increases basal EGP and reduces insulin-mediated EGP suppression in rodents (5), but whether serum RBP4 is associated with impaired hepatic insulin sensitivity in humans is unknown. The liver is the main production site of RBP4 (8, 9) and while hepatic RBP4 expression is increased in obese rodents (10), the effect of obesity on hepatic RBP4 expression in humans is unclear. Next to disturbed glucose handling in states of insulin resistance, dysfunctional adipose tissue in obesity is characterized by increased release of free fatty acids (FFA), resulting in whole-body insulin resistance (15). Whether RBP4 interacts with the lipolytic pathway is unknown.

We studied the relation between circulating and tissue RBP4 and insulin action in adipose tissue, skeletal muscle and liver and show that adipose-tissue and hepatic RBP4 expression are both increased in obesity and that serum RBP4, but not tissue RBP4, is inversely correlated with insulin action in adipose tissue, skeletal muscle and liver.

Methods

Subjects

Twenty morbidly obese female subjects scheduled for laparoscopic Roux-en-Y gastric bypass (RYGB) surgery were included (16). Six lean healthy women undergoing elective laparoscopic cholecystectomy served as control subjects for the measurement of tissue RBP4 only. The control subjects were eligible for the study if they were older than 18 years, understood the objective of the study, were competent to give informed consent, had a body mass index (BMI) between 20-25 kg/m² with a stable weight and had a normal glucose tolerance test according to the criteria of the ADA (17). Exclusion criteria were performance of vigorous exercise, family history of T2DM, a recent history (6 months or less) of substantial alcohol or drug abuse and the use of any medication. The study was approved by the Medical Ethical
Committee of the Academic Medical Center (AMC) of the University of Amsterdam. After a complete description of the study had been given, written informed consent was obtained.

**2-step hyperinsulinemic euglycemic clamp**

Glucose metabolism and lipolysis were assessed in the obese subjects only (16). The subjects were admitted to the Metabolic Clinical Research Unit of the AMC at 08:00 a.m. after an overnight fast. A catheter was inserted into an antecubital vein for infusion of \([6,6-\text{H}_2]\)glucose, [\(1,1,2,3,3-\text{H}_5\)]glycerol (>99% enriched; Cambridge Isotopes, Andover, MA, USA), glucose 20%, and insulin. Another catheter was inserted into a contralateral hand vein and kept in a thermoregulated (60°C) plexiglas box for sampling of arterialized venous blood. Saline was infused as NaCl 0.9% at a rate of 50 ml/h to sustain catheter patency. [\(6,6-\text{H}_2\)]-glucose and [\(1,1,2,3,3-\text{H}_5\)]glycerol were infused as tracers to study glucose kinetics and lipolysis (total triacylglycerol hydrolysis), respectively. At 09:00 a.m. (T = -2h), after drawing a blood sample for background enrichment of plasma glucose and glycerol, a primed-continuous infusion of [\(6,6-\text{H}_2\)]glucose and of [\(1,1,2,3,3-\text{H}_5\)]glycerol were started at a rate of 0.11 µmol/kg/min after a priming dose equivalent to 120 min infusion. After 110, 115 and 120 min, blood samples were drawn for determination of glucose and glycerol enrichments, glucoregulatory hormones and FFA. Subsequently, at 11:05 a.m. (T = 0), a continuous infusion of insulin (Actrapid 100U/ml; Novo Nordisk Farma, Alphen a/d Rijn, the Netherlands) was started for 2h at a rate of 20 mU/m² body surface area/min. At T = 2h, the infusion rate of insulin was increased to 60mU/m² body surface area. Plasma glucose was measured every 10 min and glucose 20% was infused at a variable rate to maintain plasma glucose at 5.0 mmol/L. [\(6,6-\text{H}_2\)]glucose was added to the 20% glucose solution to achieve glucose enrichments of 1% to minimize changes in isotopic enrichment due to changes in the infusion rate of exogenous glucose. At T = 2h and T = 4h, 5 blood samples with a 5 min interval were drawn to measure glucose and glycerol enrichments and 2 samples were drawn to measure glucoregulatory hormones and FFA. During the study, the participants were allowed to drink water only.

**Biopsies**

RYGB surgery was performed by experienced bariatric surgeons in two medical centers (Rijnstate Hospital, Arnhem and Slotervaart Hospital, Amsterdam, the Netherlands). The laparoscopic cholecystectomies were performed by an experienced surgeon in one medical center (Alkmaar Medical Centre, Alkmaar, the Netherlands). Before starting the Roux-en-Y procedure, tissue biopsies were taken from the liver and visceral and subcutaneous abdominal adipose tissue. Local hemostasis was checked directly after the biopsies and at the end of the surgical procedure. Tissue samples were obtained after a comparable fasting period (10-12 hours). The samples were immediately frozen in liquid nitrogen and stored at -80 °C until further analyses.
Gene expression

Total RNA was isolated using TRIzol reagent (Invitrogen, Breda, the Netherlands), followed by
the NucleoSpin II extraction kit (Macherey & Nagel GmbH, Duren, Germany) according to the
manufacturer’s recommendations. Briefly, 1 ml of TRIzol and glass beads (Biospec Products
Inc., Bartlesville, OK, USA) were added to the tissue. After vigorous shaking using a Fast Prep-24
machine for 20 sec. at 4.5 m/s (MP Biomedicals, Santa Ana, CA, USA), the homogenate was
centrifuged (10 min at 12,000 x g at 4 °C). The non-lipid containing fraction was transferred
and 200 μl of chloroform was added. The mixture was subsequently vortexed and centrifuged
(15 min. at 16,100 x g at 4 °C). The aqueous phase was transferred to a new tube and an equal
volume of 70% ethanol was added. Afterwards, we continued with the NucleoSpin II extraction
kit according to the manufacturer’s instructions. RNA concentrations were measured using
the Nanodrop Spectrophotometer 1 (Nanodrop Technologies, Wilmington, NC, USA).
cDNA was synthesized using Superscript II (Invitrogen) according to the manufacturer’s
instructions. After synthesis, the cDNA was diluted 20 times. Expression of RBP4 and GLUT4
were normalized to the housekeeping gene Acidic Ribosomal Phosphoprotein P0 (RPLP0 or
36B4).

Protein isolation

Cell lysates were prepared in RIPA buffer (150mM NaCl, 50mM Tris–HCl pH 7.4, 2mM EDTA,
0.5% deoxycholaat, 1mM Na3VO4, 20mM NaF, 0.5% Triton X-100), supplemented with
protease inhibitor cocktail (Roche, Basel, Switzerland) and PMSF.

RBP4 protein

Serum was diluted 30 times in a standard lysis buffer. Adipose tissue was lysed in standard
lysis buffer. 1 μl equivalent of human serum and 50 μg equivalent of human adipose tissue
were separated by 18% SDS-PAGE and transferred to nitrocellulose membranes. Human
RBP4 proteins were detected using anti-human RBP4 polyclonal antisera (Dako, Glostrup,
Denmark, catalog #A0040).

Analytical procedures

Glucose was measured using a Biosen C-line plus glucose analyzer (EKF Diagnostics, Barleben/
Magdeburg, Germany). Insulin was determined using an Immulite 2000 system (Diagnostic
Products Corporation, Los Angeles, CA, USA), with a chemiluminiscent immunometric assay
(intra-assay variation 4-5%; inter-assay variation 5%; detection limit 15 pmol/l). FFA were
measured using an enzymatic method (NEFA-Cc, Wako Chemicals, Neuss, Germany; intra-
assay variation 1%; inter-assay variation 4-15%; detection limit 0.02 mmol/l). [6,6-2H 2]glucose
and [1,1,2,3,3-2H 5]glycerol enrichment were measured as described previously (16).
**Body composition**

Fat-free mass was assessed using bioelectrical impedance analysis (Maltron BF-906, Rayleigh, UK).

**Calculations**

EGP, lipolysis rate and peripheral glucose uptake (rate of disappearance \([R_d]\)) were calculated using modified versions of the Steele equations as described previously (18, 19). EGP is expressed as \(\mu\text{mol/(kg fat-free mass)/min}\). Lipolysis and \(R_d\) are expressed as \(\mu\text{mol/kg/min}\). Insulin-mediated suppression of lipolysis, FFA and EGP are expressed as the % suppression of the basal values and was assessed during the first step of the hyperinsulinemic-euglycemic clamp. Insulin-mediated suppression of lipolysis and FFA represent a measure of adipose tissue insulin sensitivity. As approximately 80% of insulin-mediated glucose uptake occurs in skeletal muscle, Rd represents a measure of skeletal-muscle insulin sensitivity. Finally insulin-mediated suppression of EGP is a measure of hepatic insulin sensitivity. The quantitative insulin sensitivity check index (QUICKI) was used as an indirect measure of insulin sensitivity to compare the lean versus obese subjects and was calculated as described previously (20).

**Statistical analysis**

Data are presented as mean and range. Due to the small sample size and non-normal distribution of study parameters, nonparametric tests were used. Between-group differences were tested using the Mann-Whitney U test. Correlations were calculated using the Spearman correlation test. All statistical analyses were run on IBM SPSS version 21 (SPSS, Chicago, IL, USA). A p-value < 0.1 was considered a trend and a p-value < 0.05 was considered statistically significant.

**Results**

**Demographic and metabolic characteristics**

Table 1 summarizes demographic and metabolic characteristics. The subjects were all female and similar in age. The obese subjects were insulin resistant, reflected in a lower QUICKI than the control group. Since an adipose-tissue specific decreased GLUT4 expression is a central feature of whole-body insulin resistance (3), we measured GLUT4 expression in the adipose tissue compartments of both groups. GLUT4 expression was lower in the obese subjects in both the subcutaneous (\(P < 0.001\)) and visceral (\(P = 0.019\)) adipose tissue compartments (fig. 1a). Both, subcutaneous and visceral adipose GLUT4 expression, were inversely correlated with BMI (\(r_s = -0.601, P = 0.001; r_s = -0.502, P = 0.011, \text{resp.}\)). Subcutaneous but not visceral adipose GLUT4 expression correlated with QUICKI (\(r_s = 0.551, P = 0.004\) and \(r_s = 0.307, P = 0.136\) respectively). Similarly, in the obese subjects subcutaneous GLUT4 expression correlated with \(R_d\) (\(r_s = 0.591, P = 0.013\)) while visceral GLUT4 did not (\(r_s = 0.397, P = 0.115\)) (fig. 1b).
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SERUM RETINOL BINDING PROTEIN-4 IS INVERSELY ASSOCIATED WITH INSULIN ACTION IN ADIPOSE TISSUE, SKELETAL MUSCLE AND LIVER IN OBESE WOMEN

*Serum RBP4 is inversely correlated with insulin sensitivity*

We performed flux measurements in the obese in order to distinguish the relation of RBP4 and insulin action in different tissues. Because circulating RBP4 interferes with insulin signaling (5), we hypothesized that elevated circulating RBP4 would be inversely associated with insulin action in insulin-sensitive tissues. Indeed, serum RBP4 correlated inversely with $R_d$, which primarily reflects insulin action in skeletal muscle ($r_s = -0.551$, $P = 0.022$) and correlated strongly inversely with insulin-mediated suppression of lipolysis ($r_s = -0.635$, $P = 0.008$) and FFA ($r_s = -0.672$, $P = 0.03$), reflective of insulin action in adipose tissue. In addition, serum RBP4 correlated inversely with insulin-mediated suppression of EGP, indicating that in obesity RBP4 interacts with insulin action in liver ($r_s = -0.637$, $P = 0.006$) (fig. 2). Adipose and hepatic RBP4 expression did not correlate with any of the metabolic fluxes.

*Adipose and hepatic RBP4 is increased in obese women*

Subcutaneous adipose RBP4 expression was increased in the obese subjects compared to the lean controls ($P = 0.015$) (fig. 3a). Visceral-adipose RBP4 expression showed a trend toward an increase ($P = 0.062$) (fig. 2b), therefore we measured RBP4 protein in that adipose compartment and show VAT RBP4 protein levels were significantly increased in the obese ($P = 0.009$) (fig. 2c). Hepatic RBP4 expression was also significantly increased in the obese subjects ($P < 0.001$) (fig. 2d). In obese subjects, neither hepatic nor SAT or VAT expression of RBP4 correlated significantly with serum levels.

**Table 1. Demographic and metabolic characteristics**

<table>
<thead>
<tr>
<th>CHARACTERISTIC</th>
<th>LEAN (N=6)</th>
<th>OBESE (N=20)</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td>AGE (YEARS)</td>
<td>36 [26-45]</td>
<td>41 [26-58]</td>
<td>0.120</td>
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<tr>
<td>BMI (KG/M²)</td>
<td>22.1 [20-24.3]</td>
<td>45.3 [38.7-61.3]</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>FASTING GLUCOSE (MMOL/L)</td>
<td>5.0 [4.5-5.6]</td>
<td>5.7 [4.4-8.8]</td>
<td>0.200</td>
</tr>
<tr>
<td>FASTING INSULIN (PMOL/L)</td>
<td>31.1 [14-63]</td>
<td>83.1 [20-142]</td>
<td>0.002</td>
</tr>
<tr>
<td>QUICKI</td>
<td>0.39 [0.34-0.44]</td>
<td>0.33 [0.30-0.41]</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Data are presented as mean [range]; QUICKI: quantitative insulin sensitivity check index with a higher index indicating higher insulin sensitivity.
Figure 1. Relative GLUT4 mRNA expression in subcutaneous and visceral adipose tissue. Data presented as mean ± standard error. Adipose GLUT4 mRNA is increased in obese subjects versus lean controls (* p < 0.001 and # p = 0.019) (A). Subcutaneous adipose GLUT4 expression is correlated with glucose disappearance rate (Rd) in obese subjects (B). SAT = subcutaneous adipose tissue, VAT = visceral adipose tissue.
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Figure 2. Serum RBP4 is inversely correlated with glucose disappearance rate ($R_d$) and insulin-mediated suppression of basal lipolysis, free fatty acids and endogenous glucose production (EGP).
**Discussion**

As shown previously (5, 7), expression of RBP4 was increased while GLUT4 was decreased in subcutaneous and visceral adipose tissue in obese subjects. In addition to adipose tissue, we show that hepatic RBP4 expression was increased in our obese subjects compared to the lean controls. The regulation of tissue RBP4 is only partly understood. In liver, RBP4 is synthesized primarily in hepatocytes (22) and serves as the main carrier of vitamin A that is stored as retinyl esters in hepatic stellate cells (21). Retinyl esters can be hydrolyzed by retinyl ester hydrolases and transferred to hepatocytes where they are bound to RBP4 and released into the circulation (23). In addition to retinol itself (23, 24), other factors can also induce RBP4 expression, such as glucagon (25).

While many studies have shown a relation between circulating RBP4 and glucose infusion rates, indicative of whole body insulin sensitivity, we aimed to further elucidate which insulin target tissues are affected by increased levels of circulating and tissue RBP4. In obese subjects, tissue RBP4 did not correlate with any of the insulin-sensitive fluxes while serum
RBP4 showed a strong inverse correlation with insulin sensitivity of adipose tissue, reflected in suppression of lipolysis and FFA, skeletal muscle, reflected in glucose uptake, and liver, reflected in suppression of EGP.

Whether there is a specific role for RBP4 in insulin-mediated suppression of lipolysis is unknown. Intracellular lipolysis within adipose tissue is inhibited by insulin through modulation of the major lipases adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) (26). Activated HSL-induced hydrolysis of retinyl esters increases cytosolic retinol (27, 28), which could induce RBP4 expression (23, 24), thereby providing a link between lipolysis and retinol metabolism in adipocytes. Conversely, RBP4 could impair insulin-mediated lipolysis suppression in adipose tissue. Previous studies have shown that RBP4 activates antigen-presenting cells resulting in activation of T cells and macrophages within adipose tissue. This further induces a proinflammatory phenotype with production of cytokines such as TNFα and is associated with impaired insulin signaling in adipocytes (11, 12). As a result, systemic insulin resistance and glucose intolerance develop (12). Next to its effects on glucose handling, TNFα also stimulates lipolysis (29) and therefore RBP4 may indirectly inhibit lipolysis suppression by activating proinflammatory immune cells. However, further studies are needed to elucidate the role of RBP4 in insulin-mediated suppression of adipose-tissue lipolysis.

In addition, in the obese subjects serum RBP4 correlated inversely with insulin-mediated peripheral glucose uptake, indicating an effect of circulating RBP4 on insulin action primarily in skeletal muscle, since the majority of infused glucose is disposed in muscle under hyperinsulinemic conditions (30). The mechanism of RBP4-induced skeletal muscle insulin resistance is partly understood, e.g. it might act through binding to its high affinity receptor STRA6 and activating pathways that attenuate insulin signaling, such as SOCS3 (13), which inhibits insulin-mediated tyrosine phosphorylation of insulin receptor substrate 1 (31), ultimately blocking downstream insulin signaling. Alternatively, data from twin studies suggested that RBP4 might contribute to insulin resistance in a secondary manner (ref 32).

In our obese subjects, serum RBP4 was inversely correlated with insulin-mediated suppression of EGP, consistent with previous findings in rodent and in-vitro studies (5) showing that RBP4 induces hepatic expression of PEPCK, the major gluconeogenic enzyme and directly impairs insulin-mediated suppression of glucose production by hepatocytes (5, 10). RBP4 might also act by a retinol-dependent mechanism as retinoids induce the expression of gluconeogenic enzymes such as PEPCK and glucose-6-phosphatase in a forkhead box protein 1-dependent manner (33).

In conclusion, although hepatic and visceral and subcutaneous adipose RBP4 expressions all are increased in morbidly obese, circulating but not tissue RBP4 is inversely correlated to insulin sensitive metabolic fluxes, suggesting that RBP4 also signals as a circulating hormone. We conclude that in obesity, serum RBP4 is inversely correlated with insulin action in adipose tissue, liver and muscle.
References


