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Retinol-binding Protein 4 (RBP4) Protein Expression Is Increased in Omental Adipose Tissue of Severely Obese Patients

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Abstract

Visceral fat has been linked to insulin resistance and type 2 diabetes mellitus (T2DM); and emerging data links *RBP4* gene expression in adipose tissue with insulin resistance. In this study, we examined RBP4 protein expression in omental adipose tissue obtained from 24 severely obese patients undergoing bariatric surgery, and 10 lean controls (4 males/6 females, BMI = 23.2 ± 1.5 kg/m²) undergoing elective abdominal surgeries. Twelve of the obese patients had T2DM (2 males/10 females, BMI: 44.7 ± 1.5 kg/m²) and 12 had normal glucose tolerance (NGT: 4 males/8 females, BMI: 47.6 ± 1.9 kg/m²). Adipose RBP4, glucose transport protein-4 (GLUT4), and p85 protein expression were determined by western blot. Blood samples from the bariatric patients were analyzed for serum RBP4, total cholesterol, triglycerides, and glucose. Adipose RBP4 protein expression (NGT: 11.0 ± 0.6 ; T2DM: 11.8 ± 0.7 ; lean: 8.7 ± 0.8 arbitrary units) was significantly increased in both NGT ($P = 0.03$) and T2DM ($P = 0.005$), compared to lean controls. GLUT4 protein was decreased in both NGT ($P = 0.02$) and T2DM ($P = 0.03$), and p85 expression was increased in T2DM subjects, compared to NGT ($P = 0.03$) and lean controls ($P = 0.003$). Regression analysis showed a strong correlation between adipose RBP4 protein and BMI for all subjects, as well as between adipose RBP4 and fasting glucose levels in T2DM subjects ($r = 0.76$, $P = 0.004$). Further, in T2DM, serum RBP4 was correlated with p85 expression ($r = 0.68$, $P = 0.01$), and adipose RBP4 protein trended toward an association with p85 protein ($r = 0.55$, $P = 0.06$). These data suggest that RBP4 may regulate adiposity, and p85 expression in obese-T2DM, thus providing a link to impaired insulin signaling and diabetes in severely obese patients.

INTRODUCTION

Obesity is strongly associated with insulin resistance and impaired glucose-mediated insulin secretion, and adds considerably to overall cardiovascular morbidity and mortality (1,2). Accumulation of abdominal fat may be even more problematic (3). It is now well established that adipose tissue is an active endocrine organ, and that adipocytes secrete a multitude of biologically active proteins and adipokines that mediate insulin resistance, including retinol-binding protein 4 (RBP4) (ref. 4). Yang *et al.* were the first to report that elevated serum RBP4 was associated with insulin resistance, and that ablation of RBP4 in mice improved insulin sensitivity (4). In humans, serum RBP4 levels are increased in obesity, impaired glucose tolerance, and type 2 diabetes mellitus (T2DM) (5). It is also known that *RBP4* gene expression is increased in visceral as compared to subcutaneous adipose tissue in obese persons (6). However, there are no data showing an increase in RBP4 protein expression in visceral adipose tissue in humans who are obese or have T2DM.

Insulin resistance is partially characterized by impaired insulin signaling through the insulin receptor substrate-1/phosphoinositol 3-kinase signaling pathway (7). Phosphoinositol 3-kinase is comprised of a p110 catalytic subunit, and a p85 regulatory subunit (8), and is activated upon phosphorylation by insulin receptor substrate-1. Contrary to what one would expect, elevations of p85 protein have been reported to impair insulin signaling (8,9). Further, adipose tissue expression of glucose transport protein 4 (GLUT4) is downregulated in obesity and insulin resistance (10), resulting in impaired insulin-stimulated glucose transport into adipocytes. The relationship between RBP4 protein levels and insulin signaling and GLUT4 protein in visceral adipose tissue is not known.

Given that alterations in serum and RBP4 mRNA, as well as changes in GLUT4 and p85 protein expression, have all been reported in insulin resistance states, we sought to determine whether RBP4 protein expression was increased in visceral adipose tissue of obese humans with T2DM compared to obese-normal glucose tolerance (NGT), and if so whether there was also increased expression of p85 protein together with decreased expression of GLUT4 protein. We hypothesized that RBP4 would be more highly expressed in omental adipose tissue from obese-T2DM, as compared to obese-NGT and lean controls, and that RBP4 would be associated with p85 and GLUT4 expression.

METHODS AND PROCEDURES

Participants

Omental fat samples were obtained from 24 patients with Grade 3 obesity, classified into obese-NGT or obese-T2DM (NGT: 4 males/8 females, BMI: 47.6 ± 1.9 kg/m²; T2DM: 2 males/10 females, BMI: 44.7 ± 1.5 kg/m²), undergoing Roux-en-Y gastric bypass surgery, and from 10 lean subjects (4 males/6 females, BMI= 23.2 ± 1.5 kg/m²) undergoing elective abdominal surgery. Samples were taken from the omental fat depot adjacent to the greater curvature of the stomach during laparoscopic surgery using endoscopic scissors. No cautery was used and samples were immediately placed in saline and were lysed in preparation for western blot analysis.

Analytical determinations

A fasting blood sample was drawn from bariatric surgery patients within 3 days before surgery and analyzed for plasma glucose, total cholesterol, and triglycerides by the clinical pathology lab at the Cleveland Clinic. A separate nonfasting blood draw was performed during the preoperative assessment visit, 1 week before surgery, and serum RBP4 was measured via sandwich enzyme-linked immunosorbent assay kit (ALPCO Diagnostics,

Salem, NH) with interassay variations at two levels of 7.7% (1.2 ± 0.1) and 10.8% (2.4 ± 0.3).

Western blot analysis

Samples were lysed (20 mmol/l Tris; 1% NP-40; 137 mmol/l NaCl; 1 mmol/l CaCl₂; 1 mmol/l MgCl₂; 10% (v/v) glycerol; 1 mmol/l dithiothreitol; 1 mmol/l phenylmethanesulfonyl fluoride; 2 mmol/l Na₃VO₄) and immediately stored at -80°C . Total protein was determined via bicinchoninic acid method (Pierce, Rockford, IL). A 20- μg protein sample was loaded on 4–12% bis-tris gels and adjusted for volume with deionized water. Gels were transferred to a nitrocellulose membrane and blocked for 1 h at room temperature in 5% nonfat milk in 1% Tween-Tris-buffered saline. Membranes were washed and incubated in primary RBP4 antibody (1:1,000) (cat. no. HPA001641; Sigma, St Louis, MO) in Tween-Tris-buffered saline for 1.5 h at room temperature, anti-GLUT4 (1:1,000) (Cell Signaling Technologies, Beverly, MA), or p85 (1:1,000) (Cell Signaling Technologies) in 5% bovine serum albumin-Tween-Tris-buffered saline overnight at 4°C . Membranes were washed and incubated with secondary antibody horseradish peroxidase goat anti-rabbit (1:2,000) (Pierce, Rockford, IL) and washed in Tween-Tris-buffered saline. The membranes were developed via enhanced chemiluminescent substrate (Super Signal West Pico; Pierce), followed by exposure to Kodak X-OMAT film (Rochester, NY). Membranes were stripped (0.5 mol/l NaOH), washed, and re-probed for β -actin (cat. no. A4700; Sigma). Films were scanned and quantitated using Image J (NIH, Bethesda, MD). A 20- μg sample of rat liver and adipose tissue were run as a reference control.

Data analysis

Statistical differences between groups were determined by ANOVA and paired two-tailed *t*-tests where appropriate. Data are presented as the mean \pm s.e. The level of statistical significance was set *a priori* at $P < 0.05$.

RESULTS

Clinical characteristics and blood analysis

There was no difference between obese-NGT and obese-T2DM subjects for BMI, total cholesterol, low-density lipoprotein, or high-density lipoprotein. As expected, obese-T2DM subjects had significantly higher fasting blood glucose (NGT = 89.1 ± 3.3 mg/dl; T2DM: 147.9 ± 16.6 mg/dl, $p = 0.0006$). Further, there was no difference between groups for serum RBP4 (NGT = 48.5 ± 6.4 $\mu\text{g/ml}$; T2DM: 46.1 ± 4.2 $\mu\text{g/ml}$, $P = 0.76$).

Protein expression in omental fat

Adipose RBP4 protein expression was significantly elevated in both the obese-NGT ($P = 0.03$) and obese-T2DM ($P = 0.005$) groups compared to lean controls (Figure 1a,b). There was no difference between obese-NGT and obese-T2DM ($P = 0.42$). Total GLUT4 protein was significantly reduced in the obese patients (NGT: 7.4 ± 0.6 arbitrary units, $P = 0.02$; T2DM: 7.5 ± 0.6 arbitrary units, $P = 0.03$) compared to lean controls (9.7 ± 0.9 arbitrary units) (Figure 1c). There was no difference in GLUT4 expression between the obese subgroups (NGT vs. T2DM, $P = 0.94$). Total p85 protein was significantly elevated in obese-T2DM as compared to lean controls ($P = 0.003$) and obese-NGT ($P = 0.03$) (Figure 1d).

Correlation analyses

The combined data from all subjects revealed that omental RBP4 protein was significantly correlated with BMI ($r = 0.37$, $P = 0.04$). Strong association trends were noted between total

GLUT4 protein and BMI ($r = -0.35$, $P = 0.08$), and p85 protein and BMI ($r = 0.31$, $P = 0.08$). Fasting blood glucose levels were significantly correlated with both adipose RBP4 ($r = 0.76$, $P = 0.004$) and p85 total protein ($r = 0.63$, $P = 0.03$) for the T2DM subjects. Further, in the obese-T2DM group, there was a strong association between serum RBP4 levels and p85 protein ($r = 0.68$, $P = 0.01$), while total adipose RBP4 protein and p85 protein trended toward a significant correlation ($r = 0.55$, $P = 0.06$).

DISCUSSION

The strong relationship between adipose RBP4 protein and adiposity as reflected by BMI suggests that adipose tissue RBP4 expression may play a role in adipogenesis. Retinaldehyde, which is an oxidized derivative of retinol is expressed in adipose tissue and has been shown to inhibit adipogenesis, possibly through suppression of nuclear receptor responses (11). Although speculative on our part, there is the possibility that increased RBP4 in adipose tissue binds and holds retinol, thus reducing retinaldehyde formation and the inhibition of adipogenesis in the cell. We also found a strong association between adipose RBP4 expression and fasting glucose for the T2DM subjects, suggesting that upregulation of tissue RBP4 may be secondary to hyperglycemia.

RBP4 has been linked to insulin resistance (4,5). Insulin resistance is characterized by decreased insulin-stimulated glucose uptake and defective signaling in the phosphoinositol 3-kinase pathway in metabolically active tissues including adipose tissue (1,12). Herein, we report an increase in p85 protein expression in severely obese-T2DM subjects. The strong correlation between p85 and serum RBP4 protein, and the trend toward an association between p85 and adipose RBP4 protein, suggests that elevations in RBP4, both systemically and locally, may upregulate expression of p85 and this may contribute to impaired insulin signaling and decreased glucose uptake resulting in hyperglycemia (8,13). This possibility is supported by the observation that p85 protein expression in adipose tissue is associated with fasting glucose levels in the obese-T2DM subjects.

Insulin-facilitated glucose transport via GLUT4 is a critical component of insulin action in adipose tissue. Similar to previously published data (10,14), we find that GLUT4 protein is decreased in adipose tissue from obese patients. It has been suggested that RBP4 is produced and secreted in response to downregulation of GLUT4 in adipose tissue and this in turn acts to mediate insulin resistance in muscle and glucose production in the liver (10). However, we found no correlation between adipose RBP4 protein expression and GLUT4 protein in adipose tissue in this study. Associations between serum RBP4, as well as RBP4 mRNA, and adipose tissue GLUT4 expression have been inconsistent. Some studies report an inverse correlation (5,6), others a positive correlation (14–16). Our lack of association suggests that suppression of GLUT4 might be independent of adipose RBP4 expression and might be due to other factors such as macrophage migration into adipose tissue (17), or overproduction of macrophage-derived cytokines, which result in reduced GLUT4 protein (18). Alternatively, resistin is an adipocyte-secreted hormone that is elevated in obesity (19) and similar to RBP4 is abundant in the liver (20) and involved in adipogenesis. Thus, it may be that RBP4 expression is regulated via resistin or another adipokine in a paracrine manner. However, further studies are needed to evaluate these possibilities.

In summary, our data show for the first time that total RBP4 protein is elevated in omental adipose tissue in severely obese patients. We found a strong correlation between BMI and adipose RBP4, suggesting that RBP4 may be linked in some way to adipogenesis. Further, our data agree with previously published reports that adipose GLUT4 protein is downregulated in obese subjects, and that p85 is upregulated. We also observed correlations between serum RBP4 and p85 protein, and fasting blood glucose and adipose RBP4

expression, and fasting blood glucose and p85 expression in obese-T2DM subjects. Collectively, these data suggest that RBP4 may be involved in adipogenesis in severely obese patients, and in the downregulation of insulin signaling and subsequent hyperglycemia observed in severely obese-T2DM patients.

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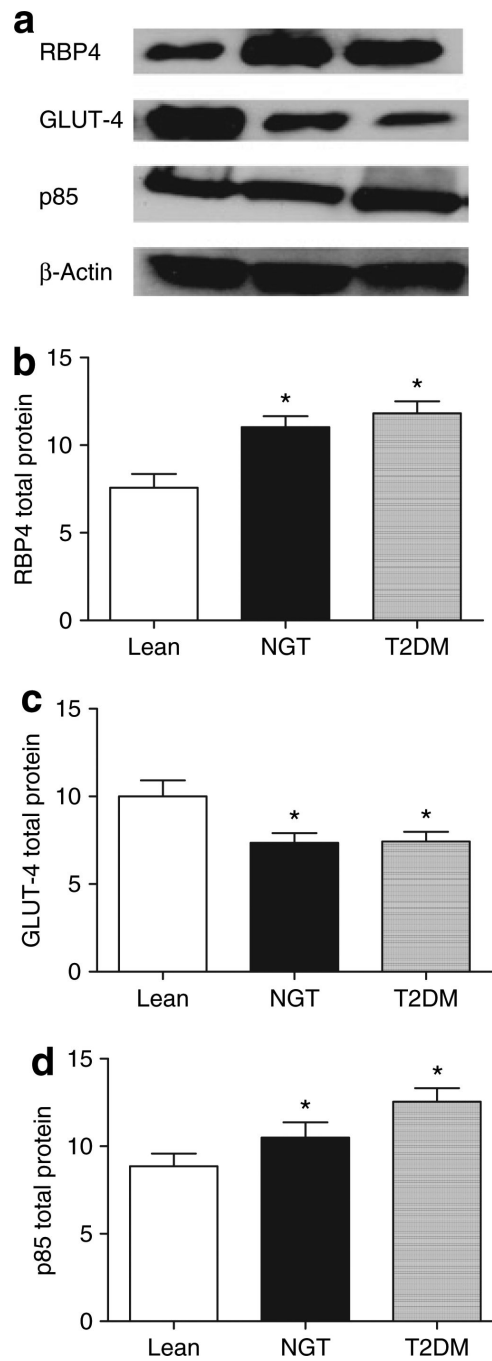


Figure 1.

Omental fat samples were obtained from lean, obese-normal glucose tolerant (NGT), and obese-type 2 diabetic patients (T2DM) and 20 μ g of protein was run on 4–12% bis-tris gels. Blots were probed for (b) RBP4, (c) GLUT-4, and (d) p85 total protein and were normalized to β -actin. Representative blots are shown in a. * $P < 0.05$, significantly different from lean control.