

# **Serum Retinol-Binding Protein: A Link Between Obesity, Insulin Resistance, and Type 2 Diabetes**

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*Insulin resistance occurs under conditions of obesity, metabolic syndrome, and type 2 diabetes. It was found to be accompanied by down-regulation of the insulin-responsive glucose transporter GLUT4. Decreased adipocyte GLUT4 caused secretion by adipocytes of the serum retinol-binding protein RBP4. Enhanced levels of serum RBP4 appeared to be the signal for the development of systemic insulin resistance both in experimental animals and in humans. In mice, increased levels of serum RBP4 led to impaired glucose uptake into skeletal muscle and increased glucose production by liver, whereas lowered serum RBP4 levels greatly enhanced insulin sensitivity. Thus, a link has been established between obesity and insulin resistance: RBP4, the vitamin A-transport protein secreted into the circulation by adipocytes.*

**Key words:** GLUT4, insulin resistance, obesity, RBP4, retinol-binding protein, type 2 diabetes

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## **INTRODUCTION**

The body's immediate energy needs are provided by glucose from the blood circulation. The blood level of glucose is maintained within a narrow range. In case of excess, such as after a meal, glucose is taken up by skeletal muscle and adipose tissue; in case of insufficiency, as during starvation, glucose is produced and released by the liver. Glucose uptake is regulated by insulin: in case of a high level of glucose, insulin is secreted and stimulates uptake, storage, and metabolism in skeletal muscle and adipose tissue. Muscle is the

predominant site of glucose uptake upon insulin action; less is taken up by adipose tissue.<sup>1</sup>

## **GLUT4 GLUCOSE TRANSPORTER AND RETINOL BINDING PROTEIN 4 ASSOCIATION**

The glucose transporter GLUT4 facilitates the transport of glucose across plasma membranes into skeletal muscle cells and into adipocytes. This process is the rate-limiting step in glucose uptake into those tissues and is subject to stimulation by insulin. Insulin resistance occurs in the pathogenic conditions of obesity, metabolic syndrome, and type 2 (non-insulin-dependent) diabetes. The expression of GLUT4 is then down-regulated in adipose tissue, resulting in impaired glucose uptake. Unexpectedly, it was found that there was no parallel down-regulation of GLUT4 in skeletal muscle in conditions of insulin resistance, even though normally muscle is the principal tissue for glucose uptake. This observation was made by the laboratory of Kahn<sup>2,3</sup> by generating transgenic mice overexpressing adipose tissue GLUT4 (adip-GLUT4-Tg mice) or mice with reduced expression of adipose GLUT4 (adip-GLUT4<sup>-/-</sup> mice).<sup>3</sup> The mice overexpressing their adipose GLUT4 showed increased insulin sensitivity and greater glucose tolerance than wild-type mice. The mice lacking the adipose glucose transporter (adip-GLUT4<sup>-/-</sup>) were insulin resistant, even though their muscle GLUT4 had remained at the normal level.

That the muscle GLUT4 gene expression remained unaffected by the diabetogenic effect of the reduced adipocyte GLUT4 was unexpected. To explain this effect, Yang et al.<sup>4</sup> proposed the ingenious hypothesis that there must be a factor secreted into the circulation from the affected adipocytes to set off insulin resistance in muscle tissue of these mice. In this regard, adipose tissue serves not merely as an energy store, but also secretes a number of signaling factors into the circulation, such as leptin, resistin, adipokine, and others. To search for the hypothetical diabetogenic factor in adipocytes, the authors<sup>4</sup> used epididymal adipose tissue RNA from adip-GLUT4<sup>-/-</sup> mice and adip-GLUT4-Tg mice in a DNA microarray analysis involving mouse oligonucleotide ar-

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rays containing about 12,000 genes. The authors<sup>4</sup> detected five mRNAs that were increased in adip-GLT4<sup>-/-</sup> mice and decreased in adip-GLUT4-Tg mice. One of these mRNAs coded for a well-known protein, retinol binding protein 4 (RBP4, previously known as RBP), whose only known function hitherto had been the transport of retinol in blood. By reverse transcriptase polymerase chain reaction (RT-PCR), the authors showed that RBP4 mRNA in the adipose tissue of the adip-GLUT4<sup>-/-</sup> mice was increased 2.3 times, while in the adip-GLUT4-Tg mice, it was decreased 54% compared with wild-type controls. This important result showed that RBP4 mRNA expression was inversely related to GLUT4 mRNA expression in adipose tissue. Liver RBP4 mRNA expression remained unchanged.

### **IS RBP4 SECRETED BY ADIPOCYTES A DIABETOGENIC SIGNAL?**

To demonstrate the secreted nature of adipocyte RBP4, serum RBP4 levels were investigated. As expected from their hypothesis, serum RBP4 in adip-GLUT4<sup>-/-</sup> mice was increased 2.5-fold over control mice, both determined by Western blotting. This result suggested that adipocytes may release RBP4 into the circulation as a diabetogenic signal. It had generally been assumed that RBP4 is a protein stored in liver, though it had been shown<sup>7</sup> that RBP4 is stored in adipocytes to the extent of about 20% of that in liver and can be released from adipose tissue into the circulation.

### **SERUM RBP4 IS INCREASED IN OBESE MICE**

Since obesity can be the precursor to type 2 diabetes, the authors<sup>4</sup> fed a high-fat (55% fat) diet to mice. They observed a 2.8-fold rise in serum RBP4 level in obese mice and a 13-fold rise in serum RBP4 in genetically obese (ob/ob) mice. Thus, the serum concentration of RBP4 increases with obesity in mice.

### **DECREASED SERUM RBP4 IMPROVES GLUCOSE TOLERANCE**

The anti-diabetic drug rosiglitazone, an activator of the peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ), which had been shown to reverse insulin resistance and glucose intolerance in adip-GLUT4<sup>-/-</sup> mice,<sup>6</sup> also reversed increased adipocyte RBP4-mRNA expression and completely normalized the elevated serum RBP4 level, prompting the authors to suggest that “this anti-diabetic agent’s [action] raises the possibility that elevation of RBP4 might play a causative role in insulin resistance and type 2 diabetes.”<sup>4</sup>

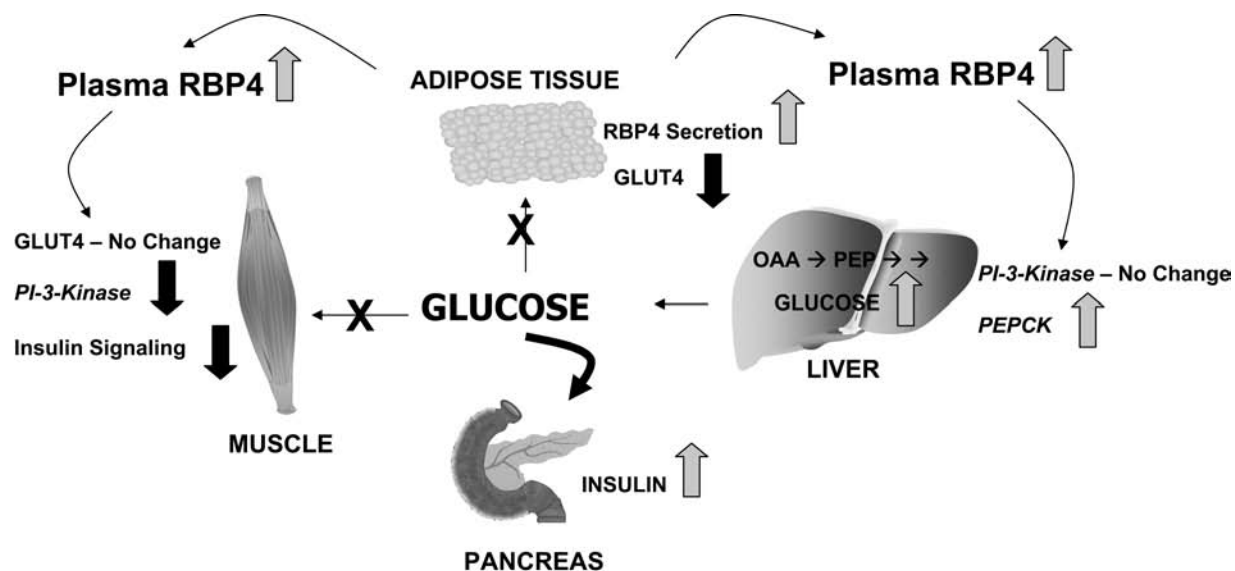
To confirm this hypothesis, the authors<sup>4</sup> generated transgenic mice expressing RBP4 in muscle, resulting in animals with a 3-fold greater serum RBP4 level compared with wild-type controls, a level similar to that found in serum of adip-GLUT4<sup>-/-</sup> mice. These RBP4-overexpressing animals were found to be insulin resistant in insulin tolerance tests. Similarly, injection of recombinant human RBP4 (300  $\mu$ g/d at 8–10 h intervals) into normal wild-type mice, leading to a serum level about 3 times higher than in controls, resulted in insulin resistance and glucose intolerance. These results clearly indicate that RBP4 is a circulating factor involved in the generation of type 2 diabetes.

The inverse of this experiment was to knock out the RBP4 gene. Transgenic mice lacking RBP4 were generated. They were viable and fertile, though with lower-than-normal blood retinol and with defective vision.<sup>7</sup> It was found that their insulin tolerance was greatly enhanced, clearly indicating that the absence of RBP4 improved insulin signaling.

An ingenious experiment lowered the serum level of RBP4 without genetic manipulation by feeding mice fenretinide, a synthetic retinoid. This retinoid binds RBP4 and, because of its bulky side chain, prevents the normal attachment of RBP4 to transthyretin. The unattached RBP4-fenretinide is of such small molecular size that it is excreted in urine, whereas the normally occurring RBP4-transthyretin complex is large enough to be retained in the circulation. Thus, RBP4-fenretinide is lost from the circulation and serum RBP4 declines.<sup>8</sup> The authors<sup>4</sup> made mice obese by feeding them a high-fat diet. The serum RBP4 level of the obese mice was increased and their glucose tolerance was impaired. As expected, administration of fenretinide lowered their raised RBP4 level to that of lean mice. At the same time, their lowered glucose tolerance became normal. The results showed again, in a different way, that RBP4 can directly influence insulin signaling.

### **MOLECULAR MECHANISM OF SERUM RBP4 EFFECTS ON INSULIN SENSITIVITY**

The question then arose: what is the molecular mechanism of the effect of RBP4 on insulin sensitivity (Figure 1)? The investigators<sup>4</sup> focused on the action of insulin on the downstream enzyme from the insulin receptor PI(3)K (phosphoinositide 3-kinase) in mice either overexpressing RBP4 (RBP4-Tg mice) or those lacking RBP4 (RBP4<sup>-/-</sup> mice). It was found that, as expected, injection of insulin into wild-type control mice resulted in a 26-fold increase in PI(3)K activity in muscle, whereas its activity was reduced by 30% in the mice overexpressing RBP4 (RBP4-Tg mice). On the other hand, in RBP4<sup>-/-</sup> adipose tissue-specific knockout mice,



**Figure 1.** Overview of the proposed action of adipose-derived RBP4 on glucose flux in muscle and liver. Recent evidence suggests that, surprisingly, the synthesis and secretion of retinol binding protein 4 (RBP4) by adipose tissue likely plays an important role in glucose homeostasis. Increased secretion of adipose-derived RBP4 into the circulation reduces insulin-dependent glucose uptake by muscle tissue by interrupting insulin signaling in muscle cells by reducing phosphoinositide-3-kinase (PI-3-kinase) activity and subsequent phosphorylation of the insulin receptor substrate-1 (IRS-1), which are necessary components of the insulin signaling pathway. In contrast, in the liver, RBP4 does not affect PI-3-kinase, but instead increases the expression of the enzyme PEPCK, which ultimately results in increased hepatic glucose output that serves to elevate blood glucose.

treatment with insulin caused an increase in PI(3)K activity of only 80%, compared with the 26-fold increase in the wild-type control mice. Confirming these results, the authors<sup>4</sup> found that injection of RBP4 into wild-type mice for 21 days caused a 24% reduction in the insulin-stimulated tyrosine phosphorylation of the insulin receptor substrate-1 (IRS1).

These studies were then extended to obese, insulin-resistant mice with a high level of serum RBP4. As expected, their muscle IRS1 showed a high level of phosphorylation upon insulin injection. However, when these mice were given the synthetic retinoid fenretinide to lower their serum RBP4 level to normal values, the phosphorylation level of their IRS1 was reduced to normal.

Surprisingly, it was found that liver PI(3)K was not affected by RBP4, even though this organ is one of the targets of insulin action. However, the authors<sup>4</sup> found that RBP4 does act on the liver, though by a different mechanism from muscle. Injection of RBP4 into mice resulted in a 41% stimulation of the expression in liver of the mRNA of the gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK). Thus, this glucose-producing enzyme is stimulated by circulating RBP4, and therefore the diabetogenic response to RBP4 by liver was similar to the diabetogenic response by muscle, although the mechanisms were different. To confirm this *in vivo* experiment, the authors<sup>4</sup> treated rat hepatoma cells in culture with RBP4 and found a 94% induction of

the PEPCK protein and greatly increased glucose production. This experiment explained the mechanism of RBP4 action on liver as a direct stimulation of the expression of the gluconeogenic PEPCK.

In summary, it appears from these animal studies that the impairment of the GLUT4 glucose transporter in adipocytes leads to release from the adipocytes of RBP4 into the circulation. RBP4 then causes a decline in glucose uptake into skeletal muscle and a stimulation of glucose production by liver. Tamori et al.<sup>9</sup>, in reviewing this work, suggest that adipose tissue acts as a glucose sensor.

## HUMAN STUDIES

The groundbreaking discoveries of this laboratory<sup>4</sup> in mice were followed by a study of the interaction between obesity, type 2 diabetes, and serum RBP4 in humans in an experiment of astonishingly wide scope.<sup>10</sup> The aims, as outlined by the investigators,<sup>10</sup> were 3-fold: 1) to determine, in humans, whether, as observed in mice, serum RBP4 level correlated with insulin resistance; 2) to determine whether exercise that improves insulin sensitivity would also lower serum RBP4; and 3) to determine whether non-obese persons with normal serum glucose levels, but with first-degree relatives suffering from type 2 diabetes, would show elevated serum RBP4 levels.

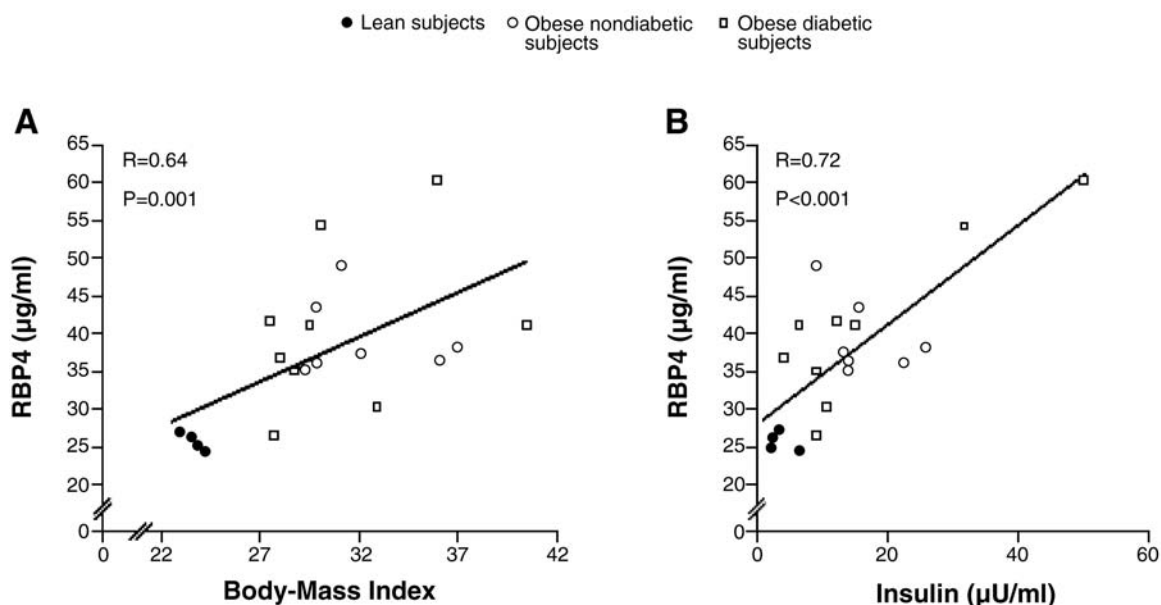
## Serum RBP4 is Related to BMI and Insulin Resistance

For the first aim, a group of 5 lean, healthy men, 7 obese men (BMI > 30), and 9 obese men afflicted with type 2 diabetes were selected. In obese, non-diabetic men, the rate of glucose disposal had dropped by 42%; in obese, diabetic men it had dropped to 56% compared with lean, healthy men. Serum RBP4 had risen significantly from the normal level of  $23.8 \pm 1.0 \mu\text{g/mL}$  to  $39.4 \pm 5.0 \mu\text{g/mL}$  (obese) and  $40.8 \pm 10.8 \mu\text{g/mL}$  (obese, diabetic). Serum RBP4 was correlated inversely with glucose disposal rate (measured by euglycemic-hyperinsulinemic clamp) and positively with fasting blood level of insulin (Figure 2). This study showed that in obesity, even in the absence of diabetes, serum RBP4 was increased. In even slightly obese (overweight) subjects, serum RBP4 was proportional to BMI and insulin resistance. Because there was no difference in RBP4 levels between obese and obese-diabetic subjects, the association of obesity with serum RBP4 could not be due to hyperglycemia, but is likely caused by insulin resistance.<sup>4</sup>

### Effect of Exercise on Serum RBP4

For the second aim, out of a pool of 469 subjects, 20 men and women with normal glucose tolerance, 20

non-obese men and women with newly diagnosed increased rate of glucose disposal, and 20 non-obese men and women suffering from type 2 diabetes were recruited. All subjects spent 60 minutes bicycling or running 3 days a week for 4 weeks. The investigators<sup>10</sup> found that serum RBP4 levels were raised in subjects with insulin resistance (increased rate of glucose disposal) and in diabetics. Serum RBP4 concentration correlated positively with fasting glucose levels, serum insulin levels, systolic blood pressure, and percent body fat. However, the response to exercise was variable. Results from subjects were grouped into thirds according to the magnitude of change in rate of glucose disposal. Serum RBP4 level was reduced by exercise in the third with the greatest drop in the rate of glucose disposal (i.e., greatest magnitude of change from highest to lowest insulin resistance). RBP4 levels remained unchanged by exercise in the third of subjects whose change in rate of glucose disposal after exercise was the lowest. The authors<sup>11</sup> concluded that “a change in the RBP4 level in response to exercise training in a given subject predicted the degree of improvement in insulin sensitivity with greater specificity than did the responses to other adipokines or markers of inflammation [tested by the authors] that are altered in obesity, type 2 diabetes or both.”



**Figure 2.** Relationship of serum RBP4 levels with body-mass index (A) and fasting plasma insulin levels (B) in 5 lean, 7 obese non-diabetic, and 9 obese diabetic subjects. In A, the 95% confidence interval for the Spearman correlation coefficient of 0.64 was 0.30 to 0.84. In B, the 95% confidence interval for the Spearman correlation coefficient of 0.72 was 0.41 to 0.88. All blood was drawn after an overnight fast. (Used with permission from Graham TE, Yang Q, Blüher M, et al. Retinol-binding protein 4 and insulin resistance in lean, obese and diabetic subjects. *N Engl J Med.* 2006;354:2552-2563. Copyright 2006 Massachusetts Medical Society. All rights reserved.)



## **Serum RBP4 and Glucose Disposal in Non-Obese Normal Men with Genetic Predisposition for Type 2 Diabetes**

For the third aim, 26 healthy men with at least one first-degree relative with type 2 diabetes were recruited. They were non-obese, with normal serum glucose concentrations. They showed a great degree of variability in insulin sensitivity, hyperinsulinemia, and hypertension. Serum RBP4 levels were found to be positively correlated with fasting serum insulin, fasting triglyceride levels, and systolic blood pressure. Serum RBP4 concentrations correlated inversely with the rate of glucose disposal independently of body mass and age. It should be noted that increased serum RBP4 levels correlated specifically with cardiovascular risk factors.

## **Adipose RBP4 and GLUT4 Are Inversely Correlated**

In order to determine whether, as in mice, it was the adipocyte GLUT4 expression levels that caused or at least were associated with the rise in serum RBP4, the authors<sup>10</sup> took biopsies of subcutaneous fat from some of their subjects. They determined the concentration of GLUT4 protein in the adipocytes by Western blotting, and found that the GLUT4 level correlated positively with the rate of glucose disposal in the particular person and inversely with the level of serum RBP4. They concluded that these results supported their hypothesis that there is a “mechanistic link between reduced adipocyte GLUT4, elevated RBP4 and insulin resistance.”<sup>10</sup>

## **Is Serum RBP4 a Good Biomarker of Type 2 Diabetes Risk?**

A brief recent report from Japan,<sup>11</sup> which provides an analysis of serum RBP4 levels in 473 Japanese subjects with normal glucose tolerance, found that RBP4 concentrations were correlated with serum triglyceride, uric acid, and somewhat with glycated hemoglobin. However, this study did not find that serum RBP4 correlated with fasting insulin level, BMI, or systolic blood pressure, correlations that were reported previously by Graham et al.<sup>10</sup> Even in 38 of the subjects with family histories of type 2 diabetes, no correlations between serum RBP4 and fasting insulin levels could be detected. Therefore, the authors<sup>11</sup> stated that RBP4 will not be “useful for assessing the risk of type 2 diabetes in Japanese people.” In rebuttal, Graham et al.<sup>10</sup> responded by stating that measurements of fasting insulin levels are not a substitute for measuring insulin resistance, as they did, with the clamp technique.

## **Have Adipocytes Mistaken Obesity for Starvation?**

In a brief review of the paper by Yang et al.<sup>4</sup>, Muoio and Newgard<sup>12</sup> ask the question: what could be the evolutionary origin of the effect of obesity in causing the secretion of a factor, RBP4, that gives rise to insulin resistance and type 2 diabetes? They point to the report by Sivitz et al.<sup>13</sup> that food deprivation, such as in fasting rats, causes a 10-fold decrease of adipose tissue GLUT4-mRNA, an effect similar to that observed by Yang et al.<sup>4</sup> upon induction of obesity. Such an effect of food deprivation on GLUT4 expression in adipose tissue, according to the suggestion of Muoio and Newgard,<sup>13</sup> would have “evolved as a mechanism for restricting glucose uptake by peripheral tissues under famine conditions, thus sparing glucose for the brain.” They propose that the insulin resistance in obesity may result in the same decline in adipocyte GLUT4 as in fasting, “causing the adipocytes in essence to mistake obesity for starvation.”

In summary, the importance of the discoveries of Yang et al.<sup>4</sup> lie in the establishment of a molecular connection between obesity and insulin resistance, the link being RBP4, the vitamin A-transport protein, secreted into the circulation by adipocytes.

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