Decreased clearance of serum retinol-binding protein and elevated levels of transthyretin in insulin-resistant ob/ob mice

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Mody N, Graham TE, Tsuji Y, Yang Q, Kahn BB. Decreased clearance of serum retinol-binding protein and elevated levels of transthyretin in insulin-resistant ob/ob mice. Am J Physiol Endocrinol Metab 294: E785–E793, 2008. First published February 19, 2008; doi:10.1152/ajpendo.00521.2007.—Serum retinol-binding protein (RBP4) is secreted by liver and adipocytes and is implicated in systemic insulin resistance in rodents and humans. RBP4 normally binds to the larger transthyretin (TTR) homotetramer, forming a protein complex that reduces renal clearance of RBP4. To determine whether alterations in RBP4-TTR binding contribute to elevated plasma RBP4 levels in insulin-resistant states, we investigated RBP4-TTR interactions in leptin-deficient ob/ob mice and high-fat-fed obese mice (HFD). Gel filtration chromatography of plasma showed that 88–94% of RBP4 is contained within the RBP4-TTR complex in ob/ob and lean mice. Coimmunoprecipitation with an RBP4 antibody brought down stoichiometrically equal amounts of TTR and RBP4, indicating that TTR was not more saturated with RBP4 in ob/ob mice than in controls. However, plasma TTR levels were elevated approximately fourfold in ob/ob mice vs. controls. RBP4 injected intravenously in lean mice cleared rapidly, whereas the t1/2 for disappearance was approximately twofold longer in ob/ob plasma. Urinary fractional excretion of RBP4 was reduced in ob/ob mice, consistent with increased retention. In HFD mice, plasma TTR levels and clearance of injected RBP4 were similar to chow-fed controls. Hepatic TTR mRNA levels were elevated approximately twofold in ob/ob but not in HFD mice. Since elevated circulating RBP4 causes insulin resistance and glucose intolerance in mice, these findings suggest that increased TTR or alterations in RBP4-TTR binding may contribute to insulin resistance by stabilizing RBP4 at higher steady-state concentrations in circulation. Lowering TTR levels or interfering with RBP4-TTR binding may enhance insulin sensitivity in obesity and type 2 diabetes.

RESISTANCE TO INSULIN ACTION is a major risk factor for type 2 diabetes, cardiovascular disease, and early mortality (16, 39). Multiple factors secreted by adipocytes contribute to regulation of systemic insulin sensitivity, fuel metabolism, energy balance, cardiovascular function, and immune function (27, 41). Serum retinol-binding protein (RBP4) is secreted from liver and adipocytes. Previously, its only known function was to deliver retinol (vitamin A) to tissues (46). We recently discovered that RBP4 is elevated in serum in insulin-resistant rodents and humans (23, 59). Serum levels correlate highly with the magnitude of insulin resistance and with many other features of the “metabolic syndrome” (2, 9, 20, 23, 28, 32, 49, 50, 56, 59), a constellation of insulin resistance and cardiovascular risk factors. Experimentally elevating serum RBP4 levels in mice causes insulin resistance, whereas lowering serum RBP4 in normal mice or in mice on a high-fat diet (HFD) enhances insulin sensitivity (59). A few studies have not found a correlation between insulin resistance and serum RBP4 (6, 7, 45, 55, 57), which may be due to methodological problems (22). Recent human genetic studies link single nucleotide polymorphisms in the RBP4 gene to altered insulin secretion and insulin sensitivity (15) and type 2 diabetes (36), raising the possibility that RBP4 might be involved in the pathogenesis of diabetes in some humans. In this study, we aimed to determine whether altered clearance of RBP4 could contribute to its elevation in serum in insulin-resistant states.

Although the major site of RBP4 synthesis and secretion is the hepatocyte, other organs and tissues express RBP4, including adipose tissue (46). RBP4 mRNA and protein are upregulated in adipose tissue in some insulin-resistant states (30, 50, 59). RBP4 is a compact, globular, 21-kDa protein that is filtered freely through the renal glomerular membrane. However, RBP4 normally binds to the larger (56 kDa) transthyretin (TTR) homotetramer to form a protein complex that resists glomerular filtration and reduces renal clearance of RBP4. TTR is also the major thyroid-binding protein in mice. Interestingly, TTR-deficient mice [TTR-knockout (KO)] have normal thyroid function due to enhanced production of triiodothyronine in tissues (37) but have markedly reduced plasma retinol and RBP4 levels (5% of wild-type levels) (17). Moreover, compounds that interfere with RBP4 binding to TTR, such as certain synthetic retinoids, profoundly reduce serum RBP4 levels (3, 18). Thus, formation of an RBP4-TTR complex in serum is critical for maintaining RBP4 levels. Conditions that increase RBP4-TTR binding affinity in serum could be key determinants of serum RBP4 levels in vivo. Interestingly, circulating TTR levels are elevated in some obese, insulin-resistant people in conjunction with increased serum RBP4 (30).

In vitro studies in the presence of saturating concentrations of RBP4 have suggested more than one binding site for RBP4 on TTR (35). However, under normal conditions in vivo, RBP4 and TTR are thought to exist as a 1:1 molar complex due to the limiting concentration of RBP4 compared with TTR (46). Studies (21) have reported a three- to fivefold excess of TTR over RBP4 in human and rodent serum. Therefore, one potential mechanism contributing to elevated serum RBP4 levels in insulin-resistant states could be altered stoichiometry of RBP4-TTR binding. For many years, it was thought that circulating levels of retinol-RBP4 remained very constant, changing only in re-
spontaneous extremes in nutritional intake of vitamin A, protein, calories, and zinc or to hormonal factors, stress, or some disease states (46). Although some mechanisms responsible for maintaining and regulating RBP4 levels in the circulation have been characterized, the processes involved in maintaining abnormally elevated RBP4 levels in insulin-resistant states have not been investigated, partly because chronic elevation of RBP4 has only recently been described (23, 59). Here we investigate whether alterations in RBP4-TTR binding could contribute to the elevated serum RBP4 levels that are characteristic of obesity, type 2 diabetes, and the “metabolic syndrome.”

METHODS

Mice and diets. Female ob/ob mice and lean littermate controls (either +/+ or ob/+ ) were obtained from Jackson Laboratories, and female FVB mice were obtained from Charles River Laboratories. Mice with a targeted disruption of the RBP4 gene (RBP4-KO) were generously provided by Drs. Max Gottesman and William Blaner. (Columbia University, New York, NY). RBP4-KO mice were of mixed C57BL/6J × 129/Sv background. All mice were fed Formula chow diet 5008 (4.5% of calories from fat). After an acclimation period of 1 wk, female FVB mice were randomly assigned into chow or HFD groups. HFD mice were fed a diet high in fat [55% of calories derived from corn oil (18%) and lard (37%); Harlan Teklad 93075] for 16 wk. Mice were housed four per cage in a temperature-controlled room and were maintained on a 14:10-h light-dark cycle. Mice had ad libitum access to both food and water.

Purification and analysis of recombinant RBP. cDNA encoding human ob/ob mice as expected, indicating insulin resistance and impaired glucose homeostasis (insulin: lean 225 mg/dl, +/+; P < 0.01). We investigated RBP4-TTR interactions in two obese, insulin-resistant models: leptin-deficient ob/ob mice, because they exhibit the highest levels of RBP4 among insulin-resistant models: leptin-deficient ob/ob mice, because they exhibit the highest levels of RBP4 among insulin-resistant states have been studied (59), and mice with obesity due to HFD feeding, because of the relevance to dietary obesity in humans. ob/ob mice (females, age 6 wk) exhibit approximately twofold increased body weight (Fig. 1A, left) and approximately fourfold increased serum RBP4 levels compared with their lean littermate controls (Fig. 1A, right).

RESULTS

We investigated RBP4-TTR interactions in two obese, insulin-resistant models: leptin-deficient ob/ob mice, because they exhibit the highest levels of RBP4 among insulin-resistant states have been studied (59), and mice with obesity due to HFD feeding, because of the relevance to dietary obesity in humans. ob/ob mice (females, age 6 wk) exhibit approximately twofold increased body weight (Fig. 1A, left) and approximately fourfold increased serum RBP4 levels compared with their lean littermate controls (Fig. 1A, right). Plasma insulin and glucose measured in the ad libitum-fed state were elevated in ob/ob mice as expected, indicating insulin resistance and impaired glucose homeostasis (insulin: lean 1.6 ± 0.3 ng/ml, ob/ob 144 ± 18 ng/ml, P < 0.01; glucose: lean 225 ± 12 mg/dl, ob/ob 334 ± 36 mg/dl, P < 0.01). We used size exclusion (gel filtration) chromatography to analyze...
The majority of immunoreactive RBP4 and TTR eluted together as a peak at the same retention volume as the purified RBP4-TTR complex calibrator (Fig. 1, A and C). A small amount of immunoreactive RBP4 (6% of total RBP4 in ob/ob, 12% in lean) eluted after this major peak in two subsequent fractions (fractions 16.2–18 and 18–19.8 ml; Fig. 1C). These fractions represent non-TTR-bound RBP4, since there was no associated TTR immunoreactivity, and could represent unknown forms of low-molecular-weight RBP4 aggregates. Therefore, despite an approximately fourfold elevation of plasma RBP4 in ob/ob mice, very little “free” RBP4 is detected in plasma, and the majority appears to be contained in a complex consistent in molecular size with the RBP4-TTR complex.

A very small fraction of TTR has been detected as a component of plasma chylomicrons in the fasted condition or after lipid intake (48). In the present study, immunoreactive RBP4 or TTR did not coelute with chylomicrons in the plasma of ob/ob or lean control mice; plasma was obtained in the fed state, when chylomicrons would be relatively high (retention volume 7.2–9 ml; Fig. 1, A and C). TTR plays a critical role in stabilizing RBP4 in circulation (17), and since the majority of RBP4 is associated with TTR in ob/ob mice, we sought to determine 1) whether the binding affinity and/or capacity of TTR for RBP4 is altered in ob/ob or lean control mice; plasma was obtained in the fed state, when chylomicrons would be relatively high (retention volume 7.2–9 ml; Fig. 1, A and C).

We previously reported that intraperitoneal (ip) injection of purified recombinant RBP4 causes elevation of plasma RBP4 to levels that are comparable with those observed in ob/ob mice (59) and in some insulin-resistant human subjects (23). However, the levels decline relatively rapidly after peak levels are achieved. Since purified recombinant RBP4 exhibits normal binding to TTR in vitro (59), we hypothesized that binding capacity of TTR for RBP4 may be exceeded in the setting of an acute elevation of circulating RBP4. To test this, we analyzed the RBP4-TTR complex following ip injection of purified RBP4 in normal FVB mice. Plasma RBP4 concentrations reached a peak at ~2 h after ip injection (Fig. 2A) and declined relatively rapidly to a normal baseline level within 24 h. Gel
filtration chromatography of plasma obtained at the peak concentration of RBP4 (2 h after injection) revealed that 60% of RBP4 coeluted with TTR at the same retention volume (fraction 14.4–16.2 ml; Fig. 2B) observed for the purified RBP4-TTR complex and for the endogenous RBP4-TTR complex in ob/ob and lean mouse plasma (Fig. 1C). The remaining 40% of injected RBP4 eluted at higher retention volumes, consistent with the presence of “free” RBP4 protein or unknown forms of RBP4 aggregate, as seen in Fig. 1C. Furthermore, RBP4-TTR stoichiometry did not differ between RBP4-injected mice and noninjected control mice at the time of peak RBP4 concentration (data not shown).

We further characterized RBP4-TTR binding by Western blotting smaller volume chromatographic fractions in the region of interest, resulting in a higher resolution analysis (Fig. 3). To test the sensitivity of the system to changes in plasma RBP4 concentrations, we first performed the analysis on plasma from lean chow-fed control mice and ob/ob mice (not shown). HRP4 clearance was measured 15 min after intravenous injection were ~25% greater in lean mice than in ob/ob mice (Fig. 4B), which may reflect greater dilution of the bolus in the expanded blood volume of ob/ob mice (61). Injected hRBP4 disappeared rapidly from plasma during the first hour after injection in both lean and ob/ob mice (Fig. 4C). We used a one-compartment kinetic model to determine the rate of disappearance of hRBP4 starting at 1 h postinjection, since plasma levels were matched for both models at that time point. The t1/2 for disappearance of RBP4 from ob/ob mouse serum was more than twofold greater than in lean mice (ob/ob 111.2 ± 9.9 vs. lean control 53.9 ± 5.6 min, P < 0.001; Fig. 4C). Therefore, ob/ob mice exhibited decreased clearance of RBP4 from plasma, consistent with the observation of enhanced RBP4-binding capacity of TTR in ob/ob plasma. To determine whether clearance of circulating RBP4 is altered in ob/ob mice, we injected purified hRBP4 intravenously and monitored its disappearance from serum. We used hRBP4 for these studies because it can be distinguished from endogenous mouse RBP4 due to its slower mobility on SDS-PAGE, and in separate studies we found that its clearance is similar to that of injected mouse RBP4 (not shown). Moreover, hRBP4-transgenic mice exhibit an approximately threefold elevation of RBP4 in plasma, indicating that endogenous mouse TTR can interact with and stabilize hRBP4 (38). We tested the ability of injected hRBP4 to interact with endogenous mouse TTR in plasma of normal mice by performing gel filtration analysis of plasma. After injection of purified hRBP4, we found that all of the hRBP4 coeluted with endogenous mouse RBP4 and TTR in the expected fraction for the RBP4-TTR complex (Fig. 4A).

In both lean and ob/ob mice, injection of hRBP4 produced elevated levels of plasma RBP4. RBP4 concentrations measured 15 min after intravenous injection were ~25% greater in lean mice than in ob/ob mice (Fig. 4B), which may reflect greater dilution of the bolus in the expanded blood volume of ob/ob mice (61). Injected hRBP4 disappeared rapidly from plasma during the first hour after injection in both lean and ob/ob mice (Fig. 4C). We used a one-compartment kinetic model to determine the rate of disappearance of hRBP4 starting at 1 h postinjection, since plasma levels were matched for both models at that time point. The t1/2 for disappearance of RBP4 from ob/ob mouse serum was more than twofold greater than in lean mice (ob/ob 111.2 ± 9.9 vs. lean control 53.9 ± 5.6 min, P < 0.001; Fig. 4C). Therefore, ob/ob mice exhibited decreased clearance of RBP4 from plasma, consistent with the observation of enhanced RBP4-binding capacity of TTR in ob/ob plasma. Further supporting this observation, we found that ob/ob mice exhibited ~70% reduced fractional excretion of RBP4 compared with lean littersmates [0.040 ± 0.010 (lean) vs. 0.012 ± 0.005 (ob/ob), P < 0.05], calculated on the basis of steady-state RBP4 and creatinine concentrations in plasma and urine. The reduced RBP4 fractional excretion was not due to impaired renal function since creatinine clearance was similar in both groups.

To determine whether enhanced RBP4-binding capacity of TTR and decreased RBP4 clearance are features of other insulin-resistant states, we studied FVB mice with obesity and insulin resistance due to HFD feeding (55% fat calories). Baseline RBP4 concentrations in lean chow-fed FVB control mice were similar to those in lean control littersmates of ob/ob mice (not shown). HFD mice are less obese and exhibit less severe insulin resistance and glucose intolerance than ob/ob mice. Sixteen weeks of HFD feeding resulted in 34% increased body weight (Fig. 5A), threefold elevated plasma insulin (CHOW 1.0 ± 0.1 ng/ml, HFD 3.3 ± 0.9 ng/ml, P < 0.003), modestly elevated plasma glucose (CHOW 128.9 ± 6.0 mg/dl, HFD 165 ± 8.7 mg/dl, P < 0.003) in the ad libitum-fed state, and a twofold elevation of endogenous plasma RBP4 levels (Fig. 5B) relative to lean chow-fed control mice. In contrast to
ob/ob mice, plasma TTR levels were not elevated in HFD-fed mice compared with lean chow-fed control mice (Fig. 5C). Similar results were obtained in HFD-induced obese mice on the C57BL/6J X 129/Sv mixed background (not shown).

Thus, elevated plasma TTR concentrations do not play a role in stabilizing the twofold elevated levels of plasma RBP4 in mice that are insulin-resistant due to HFD. However, this observation does not rule out the possibility that RBP4-TTR binding affinity could be increased in mice fed HFD, causing reduced dissociation of the RBP4-TTR complex and decreased clearance of free RBP4. To test this, we measured clearance of injected hRBP4 in HFD mice using the same method employed for ob/ob mice. At 15 min after intravenous injection, hRBP4 reached the same peak concentrations in plasma of HFD- and chow-fed mice (Fig. 5D), and the t_{1/2} of clearance of hRBP4 was identical for both groups (56 ± 6 min for CHOW vs. 51 ± 7 min for HFD; Fig. 5, D and E) and similar to the t_{1/2} observed in lean mice (Fig. 4C). Furthermore, RBP4-TTR stoichiometry did not differ between mice fed chow and mice fed HFD (Fig. 5F).

To determine whether differences in plasma TTR levels in these models reflect altered expression in liver, which could result in increased secretion, we measured TTR mRNA in liver, the primary tissue source of circulating TTR. TTR mRNA was increased in liver of ob/ob mice but not in mice on HFD relative to their respective lean controls (Fig. 5G), consistent with the elevated circulating TTR levels in ob/ob but not in HFD mice. In contrast, both ob/ob and HFD-fed mice exhibit reduced RBP4 mRNA in liver relative to lean controls (data not shown). RBP4 mRNA per gram of adipose tissue is not increased in adipose tissue of ob/ob or HFD-fed mice relative to lean controls, unlike in obese humans (30, 50, 59). However, in both mouse models, RBP4 mRNA expressed per fat pad is increased due to the expanded fat mass (not shown).

Therefore, increased fat mass in these models may contribute, at least in part, to elevated serum RBP4 concentrations.

Together these data indicate that there are multiple mechanisms for elevation of plasma RBP4 in insulin-resistant states. Increased levels of circulating TTR may contribute to increased plasma RBP4-binding capacity and altered RBP4 clearance in some but not all states of insulin resistance. Since elevation of circulating RBP4 causes insulin resistance and glucose intolerance in mice, these findings further suggest that increased TTR or alterations in RBP4-TTR binding may contribute to the development or worsening of insulin resistance by stabilizing RBP4 at higher steady-state concentrations in circulation.

DISCUSSION

RBP4 is elevated in many studies of insulin-resistant mice and human subjects (2, 9, 20, 23, 28, 32, 49, 50, 56, 59). Lack of elevation in a few studies may be due to methodological problems in measuring serum RBP4 levels (22). Although RBP4 is expressed primarily in liver under normal conditions, adipose tissue may be an important secondary source of RBP4 in insulin-resistant states (53, 59). RBP4 concentrations may be elevated up to fivefold above the normal range in some insulin-resistant human subjects and from 4- to >10-fold elevated in ob/ob mice (Ref. 59 and the present study). Extreme elevations in circulating RBP4 may reflect altered production and/or altered clearance of RBP4 from circulation. Here we report that impaired clearance of RBP4 from circulation may contribute to the very high concentrations of RBP4 observed in ob/ob mice but not to the more modest approximately twofold elevation observed in mice that are obese from HFD. In addition, we found increased RBP4-binding capacity in plasma that appears to be secondary to a fourfold increase in TTR concentrations as a result of increased fat mass in these models.
observed in \( \text{ob/ob} \) mice but not in mice fed HFD. Since TTR stabilizes RBP4 in serum (17), elevated TTR concentrations may play a role in maintaining the very high RBP4 concentrations observed in \( \text{ob/ob} \) mice. The important role of TTR in determining circulating RBP4 levels is also evident in prior observations that TTR-KO mice have extremely low circulating RBP4 levels (\(<5\%\) of those observed in wild-type mice) due to more rapid renal clearance of RBP4 from circulation (54). Since serum TTR levels are elevated in some insulin-resistant humans (30), these findings may reveal at least one mechanism for the elevation of RBP4 in some obese and type 2 diabetic humans.

The potential importance of high TTR levels in insulin-resistant states is further highlighted by the fact that TTR and RBP4 were elevated in serum in human subjects with lipid profiles that were associated with increased cardiovascular risk (62). The authors of that study concluded that TTR, as well as RBP4, is a marker of dyslipidemia, “overnutrition,” and possibly the metabolic syndrome in humans. Whether TTR could have metabolic effects that contribute to insulin resistance independent of RBP4 is not known, although studies (34, 43) suggest TTR exerts effects on triacylglycerol synthesis and glucose transport through acylation-stimulating protein. TTR has also been shown (40) to affect insulin secretion.

Our findings suggest that delayed RBP4 clearance in some obese models such as \( \text{ob/ob} \) mice may be due, at least in part, to elevated TTR levels. However, the elevated serum RBP4 levels in HFD mice are not associated with elevated serum TTR. Since TTR circulates in a three- to fivefold molar excess over RBP4, only ~20% of serum TTR is bound to RBP4 normally. Thus, the RBP4 elevation in this model probably reflects occupancy of an increased number of TTR molecules by RBP4. The mechanism by which TTR molecules act to retain an increased number of RBP4 molecules could involve structural modifications of TTR that affect TTR-binding affinity for RBP4 and thereby influence RBP4 clearance in insulin-resistant models. The only known modulators of RBP4-TTR affinity are synthetic retinoids that cause a conformational...
creatinine clearance was normal in...affected with early stages of diabetic nephropathy is not likely to...urinary RBP4 excretion. Therefore, renal dysfunction associated (1, 19, 25, 26, 44, 60) with increased insulin-resistant states, including type 2 diabetes in humans, is generally associated with increased chronic renal dysfunction without anuria/oliguria in insulin-resistant states, including type 2 diabetes in humans, is generally associated (1, 19, 25, 26, 44, 60) with increased...Since ob/ob mice and HFD-fed mice were different strains in this study, it remains possible that differences in genetic background might explain some differences in RBP4 clearance and serum TTR concentrations observed in the two models. However, we found that the same HFD feeding protocol caused a similar magnitude of RBP4 elevation with unchanged TTR concentrations in mixed C57BL/6 × 129/Sv strain mice (not shown). Further work is necessary to determine whether certain strains may be more or less susceptible to impaired clearance of RBP4 in insulin-resistant states.

Since ob/ob mice exhibit multiple abnormalities related to their leptin-deficient, insulin-resistant, and severely obese state (5, 8, 11, 29, 31), the question arises whether the decreased RBP4 clearance could reflect generally diminished renal function compared with the lean controls. This is highly unlikely since chronic renal dysfunction without anuria/oliguria in insulin-resistant states, including type 2 diabetes in humans, is generally associated (1, 19, 25, 26, 44, 60) with increased urinogenic RBP4 excretion. Therefore, renal dysfunction associated with early stages of diabetic nephropathy is not likely to result in impaired clearance of RBP4. In fact, we found that creatinine clearance was normal in ob/ob mice, but the fractional urinary excretion of RBP4 was markedly reduced. Thus, the retention of RBP4 in plasma does not result from advanced renal dysfunction and is likely to be due, at least in part, to elevated TTR.

It is not yet known whether the increased hepatic expression of TTR and elevated serum TTR concentrations observed in ob/ob mice result primarily from leptin deficiency or secondarily from the state of severe obesity and insulin resistance in this model. However, we found that short-term leptin therapy (over the course of 24 h) does not affect serum RBP4 or TTR levels in ob/ob mice compared with saline injected controls (data not shown). We did not examine the effect of longer-term leptin replacement therapy in ob/ob mice due to the rapid effects on food intake and body weight, which would confound interpretation of the results (24). Studies of transcriptional control of TTR expression (12–14, 58) have identified DNA binding sites for several liver-enriched transcription factors that could be involved in the induction of TTR expression in ob/ob mice.

In addition to providing new insight into potential mechanisms for elevated RBP4 in the setting of some insulin-resistant states, our findings further emphasize the utility of targeting the RBP4-TTR complex as a pharmacological strategy for treating insulin resistance and type 2 diabetes. Treatment of obese HFD-fed mice with fenretinide, a drug that lowers RBP4 by reducing its affinity for TTR, thereby increasing its renal excretion, improves insulin sensitivity and glucose intolerance (59). Even in the setting of elevated TTR concentrations, fenretinide or other drugs that reduce RBP4-TTR binding affinity would be predicted to lower serum RBP4 levels and improve insulin-glucose homeostasis. Therefore, insulin-resistant human subjects with or without elevated TTR levels may benefit from lowering serum TTR levels or from targeted pharmacological disruption of the RBP4-TTR complex.

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