DECREASED CLEARANCE OF SERUM RETINOL BINDING PROTEIN AND ELEVATED LEVELS OF TRANSTHYRETIN IN INSULIN-RESISTANT OB/ OB MICE

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Running Title: plasma RBP4 clearance in insulin-resistant states.

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. Co-immunoprecipitation 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 ~4-fold elevated in ob/ob mice versus controls. RBP4 injected i.v. in lean mice cleared rapidly, whereas the $t_{1/2}$ for disappearance was >2-fold 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 ~2-fold 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). 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 while lowering it in normal mice or mice on a high fat diet 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 up-regulated in adipose tissue in some insulin resistant states (30, 50, 59). RBP4 is a compact, globular 21 KDa protein that is freely filtered 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 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 wildtype levels) (17). Moreover, compounds which 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 sera 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 to TTR (46). Studies have reported a 3-5-fold excess of TTR over RBP4 in human and rodent serum (21). 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 remain very constant, changing only in response to extremes in nutritional intake of vitamin A, protein, calories and zinc, or to hormonal factors, stress, or some disease states. Although some mechanisms responsible for maintaining and regulating retinol-RBP4 levels in the circulation have been characterized, the processes involved in maintaining abnormally elevated retinol-RBP4 levels in insulin resistant states have not been investigated, partly because chronic elevation of RBP4 has only recently been described. 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 Dr. Max Gottesman and Dr. William Blaner, (Columbia University, NY). RBP4 KO mice were mixed C57BL/6J x 129/Sv background. All mice were fed Formulab chow diet 5008 (4.5% calories from fat). After an acclimation period of 1 week, female FVB mice were randomly assigned into chow or high fat (HFD) groups. HFD mice were fed a diet high in fat (55% calories derived from corn oil (18%) and lard (37%); Harlan Teklad 93075) for 16 weeks. Mice were housed 4 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 RBP4 (hRBP4) or mouse RBP4 (mRBP4) lacking the N-terminal 17 amino acids signal sequence was expressed in E.coli and purified as previously described (59). Briefly, an IPTG-inducible expression system yielded bacterial inclusion bodies containing >50% pure recombinant hRBP4. The inclusion bodies were solubilized in 5 M guanidine-hydrochloride denaturing buffer, RBP4 was refolded in the presence of retinol and then subjected to anion exchange chromatography.

Purified hRBP4 bound retinol efficiently, based on approximately equal UV absorbance ratios at wavelengths of 280 nM (detecting purified hRBP4 protein) and 330 nm (detecting retinol). The quality of refolding of purified recombinant hRBP4 was further assessed by measuring its interaction with a column matrix composed of 1 mg of human transthyretin (Sigma) crosslinked to 1 ml of NHS-sepharose (GE Healthcare). As expected, >90% of the purified hRBP4 was retained on the transthyretin affinity column under physiological salt and pH conditions, and the retained RBP4 was quantitatively eluted under high pH/low salt conditions. Endotoxin was measured by Limulus amoebocyte assay (Cambrex/Biowhittaker) to be less than 0.01 endotoxin units per microlitre for both the RBP4 and vehicle control solutions after endotoxin removal, which is less than the ambient endotoxin levels of reverse-osmosis double-deionized
water (Millipore). Purified RBP4 protein was dialyzed in a buffer containing 10 mM Hepes, 100 mM NaCl, stored frozen at stock concentrations of 7-8 mg/ml and protected from exposure to light.

_Gel Filtration Chromatography for Separation of RBP4-TTR Complexes_—Plasma, plasma (0.1 ml) or recombinant protein was loaded onto a Superdex™ 200, Tricorn 10/300 GL column connected to an ÄKTÅ™ purifier 10 system (GE Healthcare). Protein complexes were separated by passing phosphate-buffered saline at 0.5 ml/min for 90 min at room temperature. Eluting proteins were detected by absorbance at 280 nm and collected in 1.8 ml fractions for analysis by SDS-PAGE. Resolution of separation was increased by collecting smaller (0.3 ml) fractions for some analyses.

**Western blotting for plasma RBP4 and TTR**—Plasma was diluted 30 times in 1x SDS-PAGE sample buffer, proteins were separated by 15% SDS-PAGE and transferred to nitrocellulose. Mouse and human RBP4 proteins were detected using an anti-human RBP4 polyclonal antibody (#A0040, DAKO, Germany). This antibody also recognizes mouse RBP4 but with ~3 times lower affinity (data not shown). TTR was detected with an anti-rat TTR polyclonal antibody generously provided by Dr. William Blaner, (Columbia University, NY). Quantification was performed with GeneGnome chemiluminescence imaging system and GeneTools software (Syngene, MD). One microliter plasma was used to detect RBP4 and only 0.3 µl plasma for the more abundant TTR. Gel filtration fractions (8 µl of each fraction) were analysed similarly.

_Immunoprecipitation of plasma RBP4-TTR complex_—mouse plasma was subjected to immunoprecipitation using anti-human RBP4 followed by SDS-PAGE. Western blot for RBP4 and TTR was then performed and levels of these proteins were quantified as described above. Plasma from RBP4 KO mice was used to control for non-specific background.

**RBP4 pharmacokinetics**—Mice were injected with recombinant human RBP4 (100 µg) via tail vein and 20 µl blood was collected at intervals (15 min, 1, 2, 4 and 24 h). Mice had _ad libitum_ access to both food and water. Plasma (0.3 µl) was subjected to SDS-PAGE and western blotting. RBP4 concentrations were quantified as described above and 2-parameter exponential curves plotted with 1 h to 24 h time points for
each mouse using SigmaPlot (Systat Software, CA). The 2-parameter exponential equation $C_t = a \cdot \exp(-b \cdot x)$ was used to calculate $t_{1/2}$, where $C_t$ is the plasma concentration at time $x$.

**Fractional excretion of RBP4** — The entire volume of urine spontaneously produced over 6 or 8 hr intervals from individual mice was collected at room temperature. Plasma was collected during the urine collection period. Urine RBP4 was measured using an ELISA for mouse or rat RBP4 (Adipogen, Korea). Creatinine was measured in urine and plasma using a colorimetric assay (Bio Assay Systems) according to the manufacturer's instructions. Creatinine clearance was calculated as follows: (urine creatinine divided by plasma creatinine) x (urine volume per minute). Fractional excretion of RBP4 was calculated as follows: (urine RBP4 x plasma creatinine) divided by (plasma RBP4 x urine creatinine).

**mRNA preparation and quantitative PCR** — Total RNA was extracted from liver of fed mice using Trizol reagent (Molecular Research Center, OH). Mouse TTR mRNA was quantified using the Taqman gene expression assay Mm00443267_m1 (Applied Biosystems, CA). 18S mRNA was also measured and used to normalize TTR mRNA values.

**Measurement of serum insulin and glucose levels** — Serum insulin levels were determined by enzyme-linked immunosorbent assay (Crystal Chem Inc., IL). Serum glucose levels were determined by glucose oxidase assay.

**Statistical Analyses**—All data are expressed as means ± S.E.M. Significance is set at $p < 0.05$. 
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 mouse models we have studied (59), and mice with obesity due to high fat diet (HFD) feeding, because of the relevance to dietary obesity in humans. ob/ob mice (females, age 6 weeks) exhibit ~2-fold increased body weight (Fig. 1A, left panel) and ~4-fold increased serum RBP4 levels compared with their lean littermate controls (Fig. 1A, right panel). 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 1.6 ± 0.3 ng/ml lean and 144 ± 18 ng/ml ob/ob, p<0.01, glucose 225 ± 12 mg/dL lean and 334 ± 36 mg/dL ob/ob, p< 0.01). We used size-exclusion (gel-filtration) chromatography to analyze the RBP4-TTR complex. The gel-filtration column was calibrated by determining individual retention volumes for plasma chylomicrons, purified RBP4-TTR (pre-complexed), and purified RBP4 alone (Fig. 1B). Bovine serum albumin (66 kDa) was tested as a separate calibrator and found to elute just after the RBP4:TTR complex (77 kDa) consistent with its relative molecular weight (not shown).

Lean and ob/ob plasma samples were subjected to gel filtration chromatography and column fractions were analyzed by western blotting (Fig 1C). The majority of immunoreactive RBP4 and TTR eluted together as a peak at the same retention volume as the purified RBP4-TTR complex calibrator (Figs. 1B and 1C). 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 (Fig. 1C, fractions 16.2-18 ml and 18-19.8 ml). 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 ~4-fold 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 co-elute
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 (Fig. 1B and 1C, retention volume 7.2-9 ml).

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 (i) whether the binding affinity and/or capacity of TTR for RBP4 is altered in ob/ob mouse plasma and (ii) whether clearance of circulating RBP4 is reduced in ob/ob mice. Western blotting of ob/ob plasma revealed that TTR concentrations are elevated ~4-fold in plasma of ob/ob vs. lean mice (Fig. 1D). To determine whether TTR, which is normally present in molar excess to RBP4, binds more than one molecule of RBP4 in plasma of ob/ob mice, we analyzed the relative stoichiometries of RBP4 and TTR in RBP4 immunoprecipitates by western blotting. All of the RBP4 present in plasma was immunoprecipitated as determined by western blotting of the plasma supernatant after immunoprecipitation (not shown). The stoichiometry of RBP4 and TTR co-immunoprecipitation was the same in ob/ob and lean mice (Fig. 1E) suggesting no change in the stoichiometry of RBP4 and TTR binding in ob/ob mice.

We previously reported that intraperitoneal (IP) injection of purified recombinant RBP4 causes elevation of plasma RBP4 to levels that are comparable to 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 hrs after IP injection (Fig 2A) and declined relatively rapidly to a normal baseline level within 24 hrs. Gel filtration chromatography of plasma obtained at the peak concentration of RBP4 (2 hrs after injection) revealed that 60% of RBP4 co-eluted 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
previously in Fig 1C. Furthermore, RBP4:TTR stoichiometry did not differ between RBP4 injected mice and non-injected 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 RBP4-injected and RBP4 KO mice. Increased RBP4-TTR binding in serum of RBP4-injected mice and loss of RBP4-TTR binding in RBP4 KO mice could be detected as a shift in TTR size distribution to lower and higher retention volumes respectively (Fig 3A upper panel quantitation; lower panel representative gels). RBP4 in plasma of ob/ob mice displayed a size distribution very similar to that observed in lean control mice (Fig. 3B graph and 1st and 3rd gels) and in normal wild type control mice (Fig. 3A) suggesting no change in the stoichiometry of RBP4-TTR binding in ob/ob plasma. However, the distribution of TTR in plasma of ob/ob mice was shifted slightly toward lower retention volumes compared to lean mice, suggesting that a 4-fold increase in TTR in ob/ob mice may lead to an increase in the binding of TTR to other small molecular weight circulating factors. Together these findings indicate that the total circulating pool of TTR in ob/ob plasma provides an increased binding capacity for RBP4.

To determine whether clearance of circulating RBP4 is altered in ob/ob mice, we injected purified human recombinant RBP4 (hRBP4) intravenously (IV) 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 a ~3-fold 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 co-eluted 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 minutes after i.v. 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 hour post-injection, since plasma levels were matched for both models at that time point. The t½ for disappearance of RBP4 from ob/ob mouse serum was more than 2-fold greater than in lean mice (111.2 ±9.9 min vs. 53.9±5.6 min lean control, p<0.001, Fig. 4C). Therefore, ob/ob mice exhibit 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 as compared to lean littermates (0.040 ± 0.010 lean vs. 0.012 ± 0.001 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 high fat diet feeding (HFD, 55% fat calories). Baseline RBP4 concentrations in lean chow-fed FVB control mice were similar to those in lean control littermates 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 high fat diet feeding resulted in 34% increased body weight (Fig. 5A), 3-fold elevated plasma insulin (1.0 ± 0.1 ng/ml CHOW and 3.3 ± 0.9 ng/ml HFD, p<0.003), modestly elevated plasma glucose (128.9 ± 6.0 mg/dL CHOW and 165 ± 8.7 mg/dL HFD, p< 0.003) in the ad libitum fed state and a 2-fold 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 as compared to 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 2-fold 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 fifteen minutes after i.v. injection, hRBP4 reached the same peak concentrations in plasma of HFD and chow-fed mice (Fig 5D), and the t½ of clearance of hRBP4 was identical for both groups (56 ±6 min for chow vs. 51±7 min for HFD, Fig 5D and 5E) and similar to the t½ 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). While 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 5-fold above the normal range in some insulin resistant human subjects and from 4- to >10-fold elevated in ob/ob mice [(59) and this publication]. 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 ~2-fold 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 4-fold increase in transthyretin concentrations observed in 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 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 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 suggest TTR exerts effects on triacylglycerol synthesis and glucose transport through acylation stimulating protein (34, 43). TTR has also been shown to affect insulin secretion (40).

Our findings suggest that delayed RBP4 clearance in some obese models such as 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 3-5 fold 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 change upon binding RBP4 and reduce the affinity of RBP4 for TTR (3, 18), and changes in the primary sequence of TTR due to genetic mutation. More than 80 variants of TTR have been identified, many due to their role in forming or preventing amyloidogenic intermediates and amyloid fibrils in familial amyloidosis (10, 42). One of these variants (Ile84Ser mutation) has a dramatically reduced affinity for RBP4 (4) while an individual with the compound mutation Arg104His/Val30Met was found to have high serum TTR and RBP4 levels (51). No information regarding insulin-glucose homeostasis was reported in either case. In addition to genetic variability, several post-translationally modified forms of TTR have been identified in serum of healthy humans and in subjects with familial amyloidosis (33, 47, 52, 63). Future studies are indicated to assess whether genetic or post-translational variants of TTR contribute to elevation of RBP4 and insulin resistance in human subjects.
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 x 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 to 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 with increased urinary RBP4 excretion (1, 19, 25, 26, 44, 60). 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 24 hrs) does not affect serum RBP4 or TTR levels in ob/ob mice compared to 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 have identified DNA binding sites for several liver-enriched transcription factors (12-14, 58) which 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 and 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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Footnotes:

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Abbreviations: RBP4, retinol binding protein 4; TTR, transthyretin; HFD, high-fat diet;
FIGURE LEGENDS

FIGURE 1.

**RBP4 protein is elevated in plasma of ob/ob mice and co-fractionates with an equimolar amount of TTR at a column volume consistent with the size of the RBP4-TTR complex.**  
**A.** Body weight and plasma RBP4 levels determined by western blotting in lean and ob/ob mice in the fed state at 6 weeks of age. Data are means ± SEM of 4 mice per genotype, *p<0.001.** B.** Elution profile of chylomicrons in mouse plasma, purified and precomplexed mouse RBP4-TTR, and purified recombinant mouse RBP4 alone. Purified proteins were detected in column eluents by monitoring absorbance at 280 nm. Plasma chylomicrons were detected by monitoring retinyl ester absorbance at 330 nm.  
**C.** Western blotting of RBP4 and TTR in plasma pooled from 2 lean and 2 ob/ob mice separated by gel filtration chromatography (column eluent fractions 7.2 to 21.6 ml). These data are representative of 3 experiments on a total of 6 mice per genotype.  
**D.** TTR levels in plasma from lean and ob/ob mice (determined by western blotting). The same mice were used as in panel A. Data are means ± SEM of 4 mice per genotype. *p<0.001.** E.** Relative levels of RBP4 and TTR present in the RBP4-TTR complex co-immunoprecipitated from plasma of ob/ob (N=5) or lean littermates (N=3) using anti-RBP4 antibody and detected by western blotting with anti-RBP4 antibody or anti-TTR antibody. *p<0.03 versus lean.

FIGURE 2.

**Injected RBP4 binds to endogenous TTR in the circulation, but is cleared within 24 hours.**  
**A.** normal FVB mice were injected with 400 μg recombinant mouse RBP4 ip in the ad lib fed state and sacrificed after 0, 1, 2, 6 and 24 hours. Mice were euthanized, cardiac bleeds were performed and plasma RBP4 was measured by western blotting, n=5 at each time point. *p<0.001 versus levels at times 0 and 24 hours.** B.** plasma from mice sacrificed 2 hours after injection with mouse RBP4 (mRBP4 injected) and
from control littermates that were not injected (No injection control) was subjected to gel filtration chromatography. Fractions 7.2 to 21.6 ml were subjected to SDS-PAGE and western blotting for RBP4 and TTR. These data are representative of 2 experiments on a total of 8 mice per genotype.

FIGURE 3.

**Analysis of size distribution of the RBP4:TTR complex by high resolution gel filtration chromatography.** A. Top. Effects of saturating RBP4 concentrations or absence of RBP4 on the size-distribution (eluent fractions 14.1 to 15.9 ml) of TTR in plasma of normal control mice (○), plasma from normal mice in which RBP4 binding sites on TTR has been saturated by injection of purified RBP4 (▲), plasma of RBP4 knockout (KO) mice in which no RBP4 is bound to TTR (●). Bottom panels. Western blotting of column fractions (14.1 to 15.9 ml) from mouse plasma with antibodies to RBP4 and/or TTR, corresponding to the size-distribution graph above. WT=wildtype. Data are representative of 3 mice per genotype. B. Top. Size distribution of RBP4 in plasma of ob/ob mice (○) or lean littermate control mice (●). The graph shows quantification of bands from western blotting fractions 14.1 to 15.6 which contain the RBP4-TTR complex (Fig 1C); Bottom panels. Representative western blots. The volume of ob/ob plasma fractionated was adjusted to approximate the quantity of RBP4 present in lean control plasma. Similar results were obtained in 3 mice of each genotype.

FIGURE 4

**Altered pharmacokinetics of plasma RBP4 in ob/ob mice.** A. Plasma from a normal FVB mouse, 3 hours after injection with recombinant human RBP4 (hRBP4) and a control mouse which was not injected was subjected to gel filtration chromatography. Fractions 7.2 to 21.6 ml (low resolution separation) were subjected to SDS-PAGE and western blotting for RBP4 and TTR. These data are representative of 3 experiments. B, lean and ob/ob mice were injected with hRBP4 (100 µg) via tail vein, then hRBP4 levels
in plasma were measured at 15 min, 1, 2, 4 and 24 h. C, Two-parameter exponential curve fits of hRBP4 levels in plasma of individual mice at 1, 2, 4 and 24 h were used to calculate t_{1/2} of disappearance from plasma after intravenous injection using a one-compartment model. For panels B, and C, values are means ± S.E.M. *p ≤ 0.02 versus lean, n=16 per group.

FIGURE 5.

Unaltered pharmacokinetics of plasma RBP4 in high-fat diet induced obese mice with elevated plasma RBP4 levels. A, body weight B, plasma RBP4 and C, plasma TTR as determined by western blotting in high-fat diet induced obese (HFD) and chow fed (CHOW) mice after 16 weeks of diet. D, CHOW and HFD mice were injected with recombinant human RBP4 (100 µg) via tail vein, then hRBP4 levels in plasma were measured at 15 min, 1, 2, 4 and 24 h. E, Two-parameter exponential curve fits of hRBP4 levels in plasma of individual mice at 1, 2, 4 and 24 h were used to calculate t_{1/2} of disappearance from plasma after intravenous injection. F, Relative levels of RBP4 and TTR present in the RBP4-TTR complex co-immunoprecipitated from plasma of CHOW or HFD mice using anti-RBP4 antibody and detected by western blotting with anti-RBP4 antibody or anti-TTR antibody. G, RNA was extracted from liver of lean, ob/ob (left panel) CHOW and HFD mice (right panel) and TTR and 18S mRNA were measured by RT-PCR. TTR mRNA is expressed relative to 18S mRNA. All values are means ± S.E.M. For panels A, B and F, *p <0.001 versus CHOW, n=7 CHOW and n=8-9 HFD. For panel G. *p<0.05 versus lean, n=6-8 in each group.
Figure 1

A

- **Body weight**: Bar graph showing body weight in grams for lean and ob/ob mice.
- **Plasma RBP4**: Bar graph showing plasma RBP4 levels in fold over lean for lean and ob/ob mice.

B

- **Plasma chylomicrons**: Absorbance graph showing peaks at >700 kDa.
- **RBP4-TTR**: Absorbance graph showing peaks at ~77 kDa and 21 kDa.

C

- **Retention volume (ml)**: Graph showing retention volume in milliliters with peaks at 7.2, 9.0, 10.8, 12.6, 14.4, 16.2, 18, 19.8, and 21.6.
- **Western blots**: Blots for RBP4 and TTR in lean and ob/ob mice.
Figure 1 cont

**D**

**E**

TTR co-immunoprecipitated with RBP4 antibody

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Figure 2

A

plasma RBP4

(fold over 0 h time)

Time after i.p. injection (h)

B

Retention volume (ml)

No injection control

mRBP4 injected

No injection control

mRBP4 injected

RBP4

TTR
Figure 4

A

Retention volume (ml)

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no injection control

hRBP4 injected

B

RBP4 disappearance

C

t$_{1/2}$ injected RBP4 in plasma

---

**Lea**n

**ob/ob**
Figure 5

A  body weight

B  plasma RBP4

C  plasma TTR

D  RBP4 disappearance

E  t1/2 injected RBP4 in plasma

F  TTR co-immunoprecipitated with RBP4 antibody

G  liver TTR mRNA levels