Identification and Characterization of a Non-retinoid Ligand for Retinol-binding Protein 4 Which Lowers Serum Retinol-binding Protein 4 Levels in Vivo*

Alykhan Motani†, Zhulun Wang‡, Marion Conn†, Karen Siegler†, Ying Zhang†, Qingxiang Liu‡, Sheree Johnstone§, Haoda Xu¶, Steve Thibault‡, Yingcai Wang‡, Pingchen Fan‡, Richard Connors§, Hoa Le¶, Guifen Xu†, Nigel Walker†, Bei Shan†, and Peter Coward‡

From the Departments of †Metabolic Disorders, ‡Molecular Structure, §Chemistry, and ¶Pharmacokinetics and Drug Metabolism, Amgen, Inc., South San Francisco, California 94080

Retinol-binding protein 4 (RBP4) transports retinol (vitamin A) from the liver to extrahepatic tissues, and RBP4 lowering is reported to improve insulin sensitivity in mice. We have identified A1120, a high affinity (Kᵢ = 8.3 nM) non-retinoid ligand for RBP4, which disrupts the interaction between RBP4 and its binding partner transthyretin. Analysis of the RBP4-A1120 co-crystal structure reveals that A1120 induces critical conformational changes at the RBP4-transthyretin interface. Administration of A1120 to mice lowers serum RBP4 and retinol levels but, unexpectedly, does not improve insulin sensitivity. In addition, we show that Rbp4−/− mice display normal insulin sensitivity and are not protected from high fat diet-induced insulin resistance. We conclude that lowering RBP4 levels does not improve insulin sensitivity in mice. Therefore, RBP4 lowering may not be an effective strategy for treating diabetes.

RBP4 is a serum protein that transports retinol (vitamin A) from the liver to extrahepatic tissues (1). The majority of RBP4 is expressed in the liver, with ~15–20% expressed in adipose tissue (2). In the serum, RBP4 is present as a complex with transthyretin (TTR), which effectively increases the molecular weight of RBP4 and protects it from glomerular filtration. Thus disruption of the RBP4-TTR complex in vivo by administration of the synthetic retinoid fenretinide (N-(4-hydroxyphenyl)retinamide) results in a rapid reduction in serum RBP4 levels (3, 4).

Although the major physiological ligand for RBP4 appears to be retinol, RBP4 can bind to other endogenous and synthetic retinoids. For example, using biochemical assays, RBP4 has been shown to bind to retinol, all-trans- and 13-cis-retinoic acid, retinyl acetate, N-(ethyl)retinamide, and fenretinide (4–6). In addition, x-ray diffraction analysis of a variety of RBP4-retinoid co-crystal structures has demonstrated that these retinoids bind to the same site as retinol, with the cyclohexene ring buried within the internal cavity and the polar head group pointing toward the exterior of the protein (7–9). The loop regions of RBP4 surrounding the entrance of the binding cavity form the binding site for TTR, with the binding of retinol (in particular the presence of the hydroxyl group) increasing the affinity of RBP4 for TTR by a factor of ~4 (10). The binding of fenretinide, however, has the opposite effect. Through a combination of steric hindrance (from the bulky phenylamide head group) and changes in the position of the loop regions of RBP4 located at the TTR binding interface, fenretinide completely disrupts the binding of RBP4 to TTR (4, 9).

Recent reports have suggested that, in addition to its role in vitamin A transport, RBP4 may also be involved in the development of insulin resistance. For example, Yang et al. reported that mice overexpressing an RBP4 transgene and mice injected with recombinant RBP4 protein become insulin-resistant, and that reduction of RBP4 levels in mice either by gene ablation or treatment with fenretinide improves insulin sensitivity (11). In humans, Graham et al. showed that elevated serum RBP4 levels are correlated with the magnitude of insulin resistance in subjects with obesity, impaired glucose tolerance, and type 2 diabetes (12). This has been confirmed by some studies (13–16) but not by others (17–20), leaving in question the relevance of RBP4 as a marker or therapeutic target for insulin resistance in humans.

To further understand the involvement of RBP4 in insulin resistance, we conducted a high throughput screen to identify non-retinoid ligands for RBP4. We report here the identification and characterization of A1120, a non-retinoid small molecule that binds with high affinity to RBP4 and displaces TTR from an RBP4-TTR complex. The co-crystal structure of A1120 with RBP4 shows that A1120 binds to the same site as retinol and induces changes in the orientation (compared with the retinol bound form) of loops at the RBP4-TTR interaction interface. When administered to mice, A1120 lowers RBP4 and retinol levels in a dose-dependent manner, to a similar extent as seen with fenretinide. However, unlike fenretinide, A1120 does not have beneficial effects on insulin resistance. In addition, our experiments show that Rbp4−/− mice are not resistant to high fat diet-induced insulin resistance and are indistinguishable from their wild-type litter-
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mates with respect to insulin sensitivity. These results suggest that the insulin-sensitizing effects of fenretinide are not mediated by RBP4 and indicate that lowering RBP4 levels does not significantly improve the diabetic state in mice.

EXPERIMENTAL PROCEDURES

Synthesis of A1120—A solution of methyl 2-isocyanatobenzoate (10.00 g, 56.4 mmol) in tetrahydrofuran (30 ml) was slowly added to a solution of 4-(2-(trifluoromethyl)phenyl)piperidine hydrochloride (14.3 g, 53.8 mmol, Sigma) and triethylamine 99% (8.99 ml, 64.5 mmol) in tetrahydrofuran (120 ml) at 0 °C. The mixture was removed from the cooling bath and stirred at room temperature for 15 min, at which time LC/MS analysis indicated that the reaction was complete. EtOH (75 ml) and aqueous LiOH (2N, 150 ml) were then added, and the resulting mixture was extracted with EtOAc (2 × 600 ml). The EtOAc extract was dried over MgSO\(_4\), and concentrated to an off-white solid. Recrystallization from EtOAc yielded 14.0 g (66%) of 2-(4-(2-(trifluoromethyl)phenyl)piperidin-1-carboxamido) benzoic acid as a white solid, which was homogeneous by analytical high-performance liquid chromatography (>99%): mp: 156.3–157.1 °C; MS electrospray ionization (pos.) m/e 393.0 (M+H)\(^+\) (calcd. for C\(_{20}\)H\(_{22}\)F\(_3\)N\(_2\)O\(_4\), 393.1); \(^1\)H NMR (400 MHz, DMSO-\(d_6\)): \(\delta\) ppm 13.55 (1H, br.s.), 10.97 (1 H, s), 8.43 (1H, \(d, J = 7.8\) Hz), 7.96 (1H, dd, \(J = 7.8, 2.0\) Hz), 7.51–7.71 (4H, m), 7.41 (1 H, t, \(J = 7.6\) Hz), 7.01 (3 H, dd, \(J = 15.3, 1.2\) Hz), 4.17–4.28 (2 H, m), 2.96–3.14 (3H, m), 1.69–1.83 (4H, m); \(^{13}\)C NMR (101 MHz, DMSO-\(d_6\)): \(\delta\) ppm, 170.43, 153.55, 144.23, 143.30, 134.05, 132.80, 130.96, 128.66, 126.67, 120.49, 118.89, 114.57, 44.09, 38.10, and 32.75.

Biochemical Assays—The RBP4 scintillation proximity and FRET assays were performed as described previously (21). CRBP1 pENTR 221 CDNA was purchased from Invitrogen and subcloned into pET300/NT-DEST (Invitrogen). Soluble recombinant His-tagged protein was made in Escherichia coli, purified on nickel-nitrilotriacetic acid resin (Qiagen), and biotinylated as described for RBP4 (21). The CRBP1 scintillation proximity assay was performed as described for RBP4.

Protein Preparation and Crystallization—The protein preparation was carried out as described (22). In brief, the truncated form of human RBP4 (residues 19–201) was cloned into a pET21a vector (Novagen) by PCR. The recombinant protein with a N-terminal His\(_6\) tag was expressed in E. coli BL21(DE3) cells (Novagen), grown in 2\(\times\)YT medium (Sigma) at 30 °C and induced by 1 mM isopropyl 1-thio-\(\beta\)-d-galactopyranoside for 4 h before harvesting. Inclusion bodies containing recombinant RBP4 were isolated by centrifugation and washed once with 1% Triton and then twice with cold water. The protein was solubilized in 7 mM guanidine HCl, 10 mM dithiothreitol, 10 mM EDTA, and 50 mM Tris, pH 8.5, and then diluted to 1 mg/ml with 5 mM guanidine HCl in 50 mM Tris, pH 8.5. Refolding of RBP4 was carried out by rapid, 10-fold dilution in refolding buffer (0.5 mM guanidine HCl, 4 mM GSH, and 0.4 mM oxidized glutathione, and 50 mM Tris, pH 7.9), followed by a gel filtration over Superdex 75 (Amersham Biosciences). The protein was further purified by anion exchange over Mono Q (Amersham Biosciences), followed by cation exchange over Mono S (Amersham Biosciences). The Mono S eluant was then applied to a Superdex 75 column and eluted in a buffer of 200 mM NaCl, 20 mM Tris-HCl, pH 7.4, and 1 mM dithiothreitol. The protein was then concentrated to 15 mg/ml for crystallization. The RBP4 co-crystals with A1120 were grown at 16 °C in a sitting drop with 1.3 \(\mu\)l of the protein solution and 1.3 \(\mu\)l of the well solution containing 0.4 M magnesium formate, 0.1 M guanidine HCl, 0.1 M Bis-Tris, pH 6.5. The crystals were transferred into the mother liquor with additional 10% glycerol and flash frozen in liquid nitrogen.

Data Collection, Structure Determination, and Refinement—The x-ray diffraction data set for the A1120-RBP4 co-crystal was collected on a synchrotron radiation beamline (5.0.2) at the Advanced Light Source in Berkeley, CA, to a resolution of 2.9 Å. The data were integrated using MOSFLM (23) and scaled in CCP4 (24). The structure was solved by molecular replacement with Phaser (25) using a previously published retinol binding protein structure (PDB code: 1RBP) as a search model. Several rounds of model building were done using Quanta (Accelrys). The program REFMAC (26) in CCP4 was used for structural refinement. The atomic coordinates have been deposited in the Research Collaboratory for Structural Bioinformatics Protein Data Bank under accession code 3FMZ.

Serum RBP4 and Retinol Measurements—Serum RBP4 was measured using the Dual Mouse/Rat RBP4 ELISA kit from AdipoGen (Seoul, South Korea) according to the manufacturer’s instructions. To measure serum retinol, samples were diluted with phosphate-buffered saline, and aliquots (50 \(\mu\)l) were extracted with 3 volumes of acetonitrile containing internal standard. After vortexing and centrifugation, the supernatant was collected and subjected to reversed-phase liquid chromatography with tandem mass spectrometry with a Cohesive LX2 system (ThermoFisher Scientific, Waltham, MA) connected inline with a Sciex API 4000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA). The LX2 system consisted of Agilent 1200 pumps and a CTC autosampler. The LC column was a Shiseido Capcell PAK UG C18 (50 × 2.0 mm, 3 \(\mu\)m, Phenomenex, Torrance, CA) maintained at 40 °C. Mobile phases were: A, 0.1% formic acid in water; and B, 0.1% formic acid in acetonitrile. The LC gradient started with 5% B for 1 min, and then ramped to 60% B in 1 min followed by a linear increase to 95% B over 2 min, and held at 95% B for 1 min. The flow rate was 0.6 ml/min. Samples were maintained at 10 °C in the autosampler. MS detection was carried out in positive ionization mode with multiple reaction monitoring. Source parameters were as follows: ion spray voltage, 5500 V; curtain gas, 20; collision gas, 6; ion source for gases 1 and 2, 50; and temperature, 550 °C. The multiple reaction monitoring transitions used for quantification were m/z 269.3 → 92.9 (retinol) and 435.2 → 264.3 (calibration standard). For retinol, [M+H+18]\(^+\) was selected by Q1 monitoring due to its in-source loss of water. Calibration standards were prepared separately by spiking the standard into phosphate-buffered saline, followed by sample extraction as described above. The concentrations of quality control standards were 10, 100, and 1000 \(\mu\)g/liter. Calibration curves were obtained by regression of peak area ratios (analyte to internal standard) versus the theoretical concentrations of the plasma calibration standards.
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**In Vivo Experiments**—Male B6D2F1 mice (4–5 weeks of age, Harlan, IN) were maintained on irradiated normal chow (Teklad, Harlan, IN) and supplied with drinking water purified by reverse osmosis. Animals were housed in a room adjusted to a 12-h light/12-h dark cycle beginning at 0600 and 1800, respectively. Animal holding room temperature and humidity were in conformance with the “Guide for the Care and Use of Laboratory Animals” (National Research Council, National Academy Press, Washington, D. C., 1996), and studies were performed in accordance with Amgen Inc. “Research and Development Laboratory Animal Care and Use Policy” and under protocols approved by the Institutional Animal Care and Use Committee.

For acute studies, non-fasted, chow fed mice (8–10 weeks of age, Harlan, IN) were maintained on irradiated normal chow (Teklad 2198) or high fat diet (BioservS1850, 60kCal% fat, Frenchtown, NJ) and supplied with drinking water purified by reverse osmosis. Animals were housed in a room adjusted to a 12-h light/12-h dark cycle beginning at 0600 and 1800, respectively. Animal holding room temperature and humidity were in conformance with the “Guide for the Care and Use of Laboratory Animals” (National Research Council, National Academy Press, Washington, D. C., 1996), and studies were performed in accordance with Amgen Inc. “Research and Development Laboratory Animal Care and Use Policy” and under protocols approved by the Institutional Animal Care and Use Committee.

For acute studies, non-fasted, chow fed mice (8–10 weeks of age) were administered test articles prepared in 1% Tween 80, 1% methylcellulose in autoclaved milliQ water at the indicated doses in a volume of 10 ml/kg. For non-terminal sampling, blood was collected from conscious animals via a tail nick. For terminal samples, mice were decapitated without anesthesia, and whole blood was collected for isolation of serum by centrifugation. For oral glucose tolerance and insulin suppression tests, mice were fasted for 4 h from the start of the light cycle (6 a.m.). Mice were then challenged with glucose (2 g/10 ml per os) or insulin (1 U/10 ml/kg, intraperitoneally), and blood was sampled via tail nick at the indicated time points. Blood glucose levels were determined directly in whole blood by glucometer (Accu-Chek, Roche Applied Science) or in serum by colorimetric assay (Glucose Trinder, Sigma). Insulin was determined in serum by ELISA (Ultrasensitive Mouse Insulin, ALPCO Diagnostics).

For chronic treatment studies, test articles were prepared as food admixture based on pre-determined average daily food intake. Mice fed a high fat diet for 6 weeks (diet-induced obese mice) were randomized into treatment groups based on fed state body weight, glucose, and insulin levels. Age-matched mice on chow diet were used as controls. Body weight and food intake were measured weekly.

Male Rbp4−/− and Rbp4+/+ (wild-type control) mice were derived from mating of heterozygous (Rbp4+/−) mice licensed from Columbia University. Animals were genotyped by Southern blot as described (27), and these results were confirmed by Western blot (using in-house generated rabbit polyclonal sera) and/or ELISA. For phenotypic analyses, all studies were conducted using age-matched, wild-type controls. Mice were maintained on chow (Teklad 2198) or challenged with high fat diet (BioservS1850).

**Statistical Analyses**—Data are expressed as mean ± S.E., for the indicated number of animals per treatment group. Statistical comparisons were performed by one- or two-way repeated measures ANOVA, followed by post-tests correcting for multiple comparisons (Dunnett or Bonferroni, respectively) performed in GraphPad Prism 5.0.

**RESULTS AND DISCUSSION**

**Identification of a Novel Non-retinoid RBP4 Ligand**—Using a scintillation proximity assay (21), we screened the Amgen chemical library for compounds that displaced radiolabeled retinol from human RBP4. Several leads were identified, and subsequent medicinal chemistry efforts yielded A1120 (Fig. 1A). The IC50 of A1120 was 8.3 nM (Fig. 1B), 66 nM on mouse RBP4 (Fig. 1C), and 3.4 M, as shown on the right side. The IC50 of A1120 was 90 nM on human RBP4 (Fig. 1D), and 30 μM for hCRBP1 (n = 11 independent determinations), 66 nM for mRBP4 (n = 4), and >30 μM for hCRBP1 (n = 4). E, concentration-response curves in the FRET assay. Retinol was dosed in the presence of RBP4, TTR, and increasing concentrations of A1120 (0–1 μM).

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**FIGURE 1. In vitro characterization of A1120.** A, chemical structure of A1120. B–D, representative radioligand binding isotherms for human RBP4 (B), mouse RBP4 (C), and human CRBP1 (D). In each case, [3H]retinol was used as the radioligand. IC50 values were 90 nM for hRBP4 (n = 11 independent determinations), 66 nM for mRBP4 (n = 4), and >30 μM for hCRBP1 (n = 4). E, concentration-response curves in the FRET assay. Retinol was dosed in the presence of RBP4, TTR, and increasing concentrations of A1120 (0–1 μM, as shown on the right side). The IC50 of A1120 was 8.3 nM (n = 3).
between CRBP1 and RBP4 (28). Fig. 1D shows that A1120 does not bind to CRBP1. In addition, A1120 was selective against a panel of G-protein-coupled receptor, kinase, and enzyme targets (data not shown). Thus A1120 appears to bind specifically to RBP4.

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We have previously described a fluorescence resonance energy transfer (FRET) assay that measures the interaction between RBP4 and TTR (21). Because retinol increases the basal interaction between RBP4 and TTR, it increases the FRET signal in a dose-dependent manner. Conversely, because fenretinide disrupts the interaction between RBP4 and TTR, it decreases the FRET signal in a dose-dependent manner. In addition, because fenretinide and retinol both bind to the same site on RBP4, fenretinide will also right-shift a retinol dose-response curve. As shown in Fig. 1E, A1120 behaves qualitatively similar to fenretinide in the FRET assay; it lowers the basal response and right-shifts the retinol dose-response curve, indicating that A1120 also disrupts the interaction between RBP4 and TTR. Using the FRET assay, we determined the affinity of A1120 for RBP4 to be 8.3 nM (Fig. 1E). Thus A1120 binds to RBP4 with higher affinity than either retinol or fenretinide ($K_i = 125$ nM for each (21)).

The overall fold of RBP4 in the A1120 co-crystal is the same as reported previously for retinol-bound RBP4 (7, 29, 30). It consists of a core $\beta$-barrel of eight up-and-down $\beta$ strands, an N-terminal coil, and a C-terminal $\alpha$-helix followed by a coil region (Fig. 2A). The co-crystal structure showed well resolved electron density for A1120 in the central cavity of the $\beta$-barrel (the same site occupied by retinol), with the trifluoromethylphenyl ring binding deep in the pocket and the benzoic acid moiety sitting at the opening (Fig. 2B). The carbonyl of the carboxamido accepts a hydrogen bond from the backbone amide of Leu-37. The carboxylic moiety of the benzoic acid forms a salt bridge with Arg-121 as well as a hydrogen bond with the hydroxyl group of Tyr-90. The rest of the molecule makes numerous van der Waals contacts with the protein.

Although the $\beta$-barrel core structure of RBP4 shows very little change upon A1120 binding, conformational changes are observed in the loop regions surrounding the opening of the binding cavity, including loop $\beta3$–$\beta4$ (residues from 63 to 68) and loop $\beta5$–$\beta6$ (residues from 92 to 99) (Fig. 2C). These loop regions form part of the TTR interaction interface (10, 31), and

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**FIGURE 2. Co-crystal structure of human RBP4 with A1120.** A, overall view of A1120-bound RBP4 structure. The RBP4 protein is shown in schematic representation with a spectrum color from blue (N terminus) to red (C terminus). A1120 is shown in stick representation with magenta for carbon atoms, red for oxygen atoms, blue for nitrogen atoms, and pale cyan for fluorine atoms. The $2F_o - F_i$ electron density map, contoured at 1$\sigma$, for A1120 is shown in gray mesh. B, protein-ligand interactions for A1120. The protein is shown in both stick (spectrum color) and molecular surface (wheat color) representations. The hydrogen bonds between RBP4 and A1120 are shown as black dashed lines. C, superposition of the A1120-RBP4 co-crystal structure and retinol-RBP4 co-crystal structure (PDB code: 1RBP). The carbon atoms are colored in gray for the retinol-bound structure. D, superposition of the A1120-RBP4 co-crystal structure on the RBP4-TTR complex structure (PDB code: 1QAB). The TTR tetramer is shown in molecular surface representation. The RBP4 proteins are shown in schematic representation with the RBP4 protein shown in gray in the TTR complex and in spectrum color in the A1120-bound form.
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![Graphs showing the effects of A1120 on serum RBP4 and retinol levels.](image)

A1120 Lowers Serum RBP4 and Retinol Levels—Because in vivo administration of fenretinide results in decreased serum RBP4 and retinol levels (3, 32), we performed experiments to determine if A1120 would show the same effect. For all our in vivo experiments, we used B6D2F1 mice, a hybrid F1 strain between C57Bl/6 and DBA/2. These animals are well suited for studying diabetes, because they quickly develop insulin resistance on a high fat diet. Fig. 3 (A and B) shows that administration of an oral dose of 30 mg/kg A1120 to B6D2F1 mice resulted in a decrease in serum RBP4 and retinol levels, with the peak reduction in both occurring 12 h after administration. Administration of 30 mg/kg fenretinide resulted in reductions in RBP4 and retinol that followed a similar time course, although the magnitude of the effect was not as great as for A1120. Fig. 3C shows that the RBP4 lowering by A1120 is dose-dependent, with the reductions seen at 5 and 30 mg/kg similar to that seen with 30 mg/kg fenretinide (all measurements taken 4 h after compound administration). Overall, the data in Fig. 3 show that A1120 is at least as potent and efficacious as fenretinide in reducing serum levels of RBP4 and retinol.

Evaluation of the Anti-diabetic Activity of A1120—Yang et al. showed that fenretinide improves insulin sensitivity in diet-induced obese mice (11), and so we used a similar experimental design to evaluate the anti-diabetic efficacy of A1120. B6D2F1 mice fed a high fat diet for 6 weeks were administered fenretinide, A1120, the PPARγ agonist rosiglitazone, or vehicle ad libitum as a diet admixture. A Chow-fed group was included as a control. After 4–6 weeks of compound administration, serum was collected for RBP4, retinol, and baseline glucose measurements, and the animals were subjected to oral glucose tolerance and insulin suppression tests.

As expected, animals receiving fenretinide and A1120 showed significant RBP4 and retinol lowering (Fig. 4, A and B). Rosiglitazone treatment resulted in slightly decreased RBP4 levels, a finding consistent with published data in adipose-Glut4−/− mice (11). However, the effect was minor, and similar results were not seen consistently in additional studies in diet-induced and genetic rodent models of diabetes (Fig. 5A and data not shown). Chow-fed animals had slightly lower RBP4 levels than the high fat fed animals, a finding consistent with data in the literature (11).

Fig. 4C shows that, after 4 weeks, fenretinide-treated animals had lower basal glucose levels compared with vehicle-treated animals. This finding is consistent with published data (11). However, despite even lower plasma RBP4 levels in the A1120-treated group (Fig. 4A), basal glucose levels were not significantly different from vehicle. Rosiglitazone-treated animals showed a very pronounced lowering of basal glucose levels, to a level similar to that seen in chow-fed animals. An insulin suppression test was also conducted after 4 weeks of compound treatment, measuring glucose excursions in response to an intraperitoneal injection of insulin (Fig. 4D). Compared with vehicle-treated animals, fenretinide-treated animals only showed an improvement in glucose levels at the first time point, and A1120-treated animals did not show an improvement at any time point. However, animals in both the rosiglitazone and
Chow-fed groups had significantly decreased glucose levels at all time points measured. An oral glucose tolerance test was conducted after 6 weeks of compound administration, measuring both glucose and insulin levels in animals in response to oral administration of glucose (Fig. 4, E and F). Effects of compound administration on baseline glucose were no longer evident in any of the treatment groups. Compared with the vehicle group, animals treated with fenretinide or A1120 did not exhibit any improvement in glucose levels following the oral glucose challenge. Rosiglitazone-treated animals showed statistically significant improvement in glucose levels at all time points measured, reaching the level seen in chow-fed animals. Insulin levels determined during the oral glucose tolerance test were significantly lower at all time points in fenretinide-treated animals compared with vehicle-treated controls (Fig. 4F). However, A1120-treated animals did not show any lowering of insulin (Fig. 4F), despite showing greater RBP4 lowering than fenretinide (Fig. 4A). Animals treated with rosiglitazone had insulin levels comparable to chow-fed animals.

The data in Fig. 4 demonstrate that, even though fenretinide and A1120 both lower RBP4 levels, only fenretinide is able to improve insulin sensitivity (as shown by improved insulin levels during the oral glucose tolerance test). Because the magnitude and duration of RBP4 lowering is similar for both compounds (Fig. 3), it is unlikely that differences in these parameters can explain the result. Instead, it seems more likely that the insulin-sensitizing effects of fenretinide are not mediated through RBP4. A possible alternative mechanism may involve modulation of nuclear retinoid receptors, which have been shown to respond to fenretinide (33).

Phenotype of Rpb4−/− Mice—To further investigate the role of RPB4 in insulin resistance, we characterized Rpb4−/− animals, which had been previously reported to have lower plasma glucose levels than their wild-type littermates following an intraperitoneal insulin injection (11). Fig. 5A shows that glucose levels in Rpb4−/− mice on a chow diet were the same as those in wild-type littermate controls following an insulin injection. This result is consistent with the data in Fig. 4D, where neither fenretinide nor A1120 treatment improved glucose levels in response to insulin. However, the result is not consistent with the previously published results (11). Although the Rpb4−/− line used in both studies is the same (27), the line is not inbred, and thus genetic variability may explain the different results. To minimize potential effects of genetic variability, we phenotyped a large number of animals (n = 28 in the wild-type group and n = 15 in the Rpb4−/− group).

We next performed an experiment to determine if the Rpb4−/− mice were protected from high fat diet-induced insulin resistance. Consistent with the data from the chow-fed animals, there was no difference in glucose disposal following an insulin challenge between the wild-type and
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*Rpb4/H11002* animals even after 9 weeks of high fat feeding (Fig. 5B). Furthermore, there was no difference in the magnitude of the response between the wild-type and *Rpb4/H11002* mice. Combined with the data shown in Fig. 4, where rosiglitazone only moderately reduced RBP4 levels but showed dramatic improvements in insulin sensitivity and A1120 dramatically reduced RBP4 levels but showed no improvement in insulin sensitivity, this indicates that the anti-diabetic effects of rosiglitazone are unlikely to involve RBP4.

In summary, we have identified a non-retinoid RBP4 ligand that lowers RBP4 and retinol levels in vivo, but does not improve insulin resistance. In addition, we characterized *Rpb4/H11002* mice and demonstrated that they are indistinguishable from their wild-type littermates with respect to insulin sensitivity when fed a chow or high fat diet, or when treated with the PPARγ agonist and insulin sensitizer rosiglitazone. These data indicate that RBP4 lowering does not significantly influence the diabetic state in mice. The relevance of these findings to humans is not clear, but there is considerable debate in the literature over the involvement of RBP4 in insulin resistance, with multiple studies reporting conflicting results (12–20). Recently, in a comparison of normal and type 2 diabetic patients, it was found that kidney function but not diabetes determined serum RBP4 levels (34). Clearly, additional studies will be needed to definitively establish any potential link between RBP4 and insulin resistance in humans.

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