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ORIGINAL ARTICLE

Sexual dimorphism in developmental and diet-dependent circulating retinol binding protein 4

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Summary

Objective

Retinol binding protein 4 (RBP4) transports vitamin A (Retinol) in the blood and contributes mechanistically to the linkage between obesity, insulin resistance and associated comorbidities including type 2 diabetes mellitus, coronary artery and neoplastic diseases. Circulating RBP4 levels have variably been associated with body mass and gender differences. Many of these differences have been demonstrated after limited dietary interventions, and/or at single unique time points. This study investigated the impact of sex and age as biologic variables as well as high versus low fat diets on development of obesity, RBP4 levels and insulin resistance in C57BL/6J mice.

Methods

Male and female C57BL/6J mice were fed for 400 days with either low or high fat diets. Female mice were also evaluated on same diets after ovariectomy or sham ovariectomy. Mice were monitored for changes in weight, circulating levels RBP4, glucose and insulin at 100-day intervals and also by 2-hour glucose tolerance tests.

Results

All mice on low or high fat diets gained weight. Mice on high fat diets showed significantly greater weight gain than those on low fat. Male mice showed significantly greater weight gain compared with females on corresponding diet. Male mice compared with females already showed significantly higher RBP4 levels even before starting diets. Sex differences were maintained for more than 1 year. Gender differences in RBP4 were associated with significant differences in development of glucose intolerance and insulin resistance.

Conclusions

Male compared with female C57BL/6J mice show significant gender differences in circulating RBP4 levels from 6 weeks of age, extending more than 1 year. Gender differences in RBP4 may be mechanistically associated with protection against glucose intolerance and insulin resistance. Targeting RBP4 pathways could be useful to disrupt gender differences in insulin resistance and disparities in comorbidities.

Keywords: Insulin resistance, obesity, retinol binding protein 4.

Introduction

Retinol binding protein 4 (RBP4) is a 21-kDa plasma protein that binds and transports Vitamin A (Retinol) in the blood (1,2). It is synthesized in multiple organs, including liver and adipose tissue where it is designated one of many adipokines (3). Circulating levels of RBP4 increase with obesity where they contribute to promotion of several obesity-associated comorbidities, including cardiovascular (4,5), metabolic (6) and neoplastic disorders (7). In at least one study, RBP4 has been shown to be associated with increased liver, but not total body, visceral or

subcutaneous fat (8). In another study, plasma RBP4 was not increased in mice engineered to overexpress RBP4 in adipocytes (9), and in another study, liver RBP4 knockout mice indicate that most circulating RBP4 in obesity is primarily secreted by liver (10). Moreover, variations in circulating RBP4 levels suggest pleiotropic determinants which may contribute to obesity-associated comorbidities.

Maintenance of normal weight should provide the most effective control of obesity and associated increase of RBP4 and its impact on obesity associated comorbidities. However, the current status of the obesity pandemic with 650 million obese adults and 110 million obese children on a world-wide basis (11) indicates not only the magnitude, but also the refractoriness of this problem and the need for better understanding of this metabolic disorder, its physiologic determinants and potentially innovative strategies for control of its consequences.

Mechanistically, RBP4 reduces expression of the insulin-response glucose transporter, GLUT 4, in adipose tissue and skeletal muscle leading to insulin resistance and impaired glucose tolerance (6). High circulating RBP4 levels are associated with increased risk of coronary heart disease (4). RBP4 has been shown to increase expression of toll-like receptors and myeloid-dependent primary response gene 88 and associated inflammatory response and cardiomyocyte hypertrophy contributing to cardiac hypertrophy and ischemic heart disease (12). RBP4 contributes to obesity promoted cancers at multiple levels along the neoplastic continuum (7). Thus, elevated RBP4 has been shown to be associated with an increased incidence of colorectal adenoma (CRA) (13). Moreover, RBP4 stimulates growth of established colorectal cancer (CRC) by binding and activating the membrane receptor STRA6 triggering downstream activation of the pro-oncogenic JAK2/STAT3/5 pathway (14). Thus, RBP4 serves to promote cancer development and progression by a multi-level cascade including direct activation of the JAK2 pathway and indirectly by stimulating insulin resistance and elevated insulin levels (3,14). Accordingly, since RBP4 contributes to the linkage between obesity and several of its comorbidities, including type 2 diabetes mellitus, coronary artery disease (CAD), and cancer, it is important to determine factors affecting circulating levels including the importance of gender and age.

In this regard, some studies suggest sex differences in circulating RBP4 levels (15,16), others do not (17), even though they show differences in adipose tissue RBP4 mRNA expression studies showing female greater than male (17). To evaluate these differences, the effects of sex and age, as biologic variables, were compared on the consequences of low and high fat diets on obesity development, circulating RBP4 levels and insulin resistance over an extended time course in male and female C57BL/6J mice. The contribution of ovarian function to the observed differences was further evaluated by comparing effects in control females to those in ovariectomized (OVX) and sham ovariectomized (SHAM) mice.

Materials and methods

C57BL/6J mice were originally purchased from Jackson Laboratories (Bar Harbour, ME) and used to develop breeding colonies at Case Western Reserve University, which supplied all animals used in these studies. Mice were housed in polysulfonate micro isolator cages, maintained at 25° C on a 12-hour reverse light/dark cycle, fed an autoclaved standard diet, Prolab Isopro RMH 3000, C (P3000) from Lab Diet (Brentwood, MO) and autoclaved water *ad libitum*. Colonies were propagated by placing single males with a pair of females until females conceived and delivered litters. Breeding pairs were maintained together for approximately 1 year or until reproductive activity decreased at which time breeders were replaced with younger mice derived from the colony or purchased from Jackson Laboratories. At 3 weeks of age, mice were weaned and provided with standard chow diet, P3000 until assignment to experimental diets and/or procedures.

At 6 weeks of age, blood was sampled, and mice were distributed to four conditions, two experimental diet groups each, for a total of eight groups, 15 mice each as follows: control males fed high fat low sucrose (HF LS), control males fed low fat low sucrose (LF LS), control females fed HF LS; control females fed LF LS; OVX females fed HF LS; OVX females fed LF LS; Sham OVX females fed HF LS; Sham OVX females fed LF LS.

Mice were maintained on experimental diets, incubated two to five mice per cage, inspected by trained animal care technicians every other day for general health status, weight measured and recorded at weekly intervals. At 100-day intervals, mice were weighed, fasted overnight, sedated with Isoflurane and phlebotomized from retro orbital sinus. Whole blood was assayed immediately for glucose levels. Plasma was separated by centrifugation of EDTA collected blood and stored at −80°C.

At termination of experiment, mice were euthanized by physical disruption of brain activity using cervical dislocation. All procedures were in compliance and approved by CWRU IACUC.

Experimental diets

Experimental diets, as previously described (18), were obtained from Research Diets Inc. (New Brunswick NJ). High

fat diet (HFD) (D12330:Kcal% – 17% protein, 58% saturated fat, 26% carbohydrate [0 Kcal sucrose, 700 Kcal corn starch]); low fat diet (LFD) (D12328: Kcal% – 17% protein, 11% saturated fat, 73% carbohydrate [0 Kcal sucrose, 3,340 Kcal corn starch]). Hydrogenated coconut oil was used for fat in all diets. Diets were matched for micronutrients and caloric density including Vitamin A acetate which is present in both diets at 4,000 IU/5,565 calories. These diets were formulated with AIN-76 A Vitamin mix and provide the same level of Vitamin A as the AIN-93 A series. The composition of these diets has remained unchanged since their inception (18) (and Research Diets Inc. Product Information and Personal Communication).

Surgical procedures

Bilateral ovariectomies were performed in randomly selected females at 6 weeks of age. Mice were weighed, fasted overnight, anaesthetised with isoflurane and phlebotomized by capillary tube from retro orbital sinus. Fur was removed from surgical sites which were then cleaned with betadine. Bilateral incisions were made parallel to spine along posterior flanks just above knees. Ovaries and surrounding tissues were identified, externalized, then excised, remaining tissues reinserted through muscle following which skin was closed with auto clips which were subsequently removed 10 days following surgery. Mice were kept warm on a heating pad until motility recovered following which they were returned to home cages. Post-surgical mice were provided with carprofen, 0.5 mg dL^{-1} for 3 days post-surgery. Sham OVX mice were treated in exactly same manner including closure of flank incisions with autoclips then managed as above.

Glucose tolerance curves

Mice were fasted overnight and subsequently injected intraperitoneally with glucose (2 g glucose/kg body weight). Blood was collected from the tail vein and glucose levels measured at 0, 15, 30, 60 and 120 minutes using an Ultra Touch Glucose Meter®.

Analytics

Retinol binding protein 4 (RBP4) levels were measured in plasma samples by enzyme-linked immunosorbent assays using mouse RBP4 Quantikine MRBP40 from R&D Systems (Minneapolis, MN) according to manufacturer's instructions. All samples were analysed in duplicate with results reported as mean values.

Insulin was quantified using MAGPIX technology (Millipore; Bellerica, MA) based on the Luminex (Luminex Corp; Austin TX) soluble phase format. Quantification was done using a commercial kit (Millipore; Bellerica, MA) according to manufacturer's instructions. Samples were analysed in duplicate with results reported as mean values.

Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as previously described (19–21). HOMA-IR for male mice on normal chow diet at beginning of experiment, 42 days of age, was 2.48 ± 1.06. Accordingly, 3.5 was set as cutoff for normal. Insulin resistance is designated as Moderate for HOMA-IR $\geq$ 3.9–14.9 and Severe for HOMA IR $\geq$ 15.

Statistics

Studies were performed on multiple animals; values are presented as means with standard deviation when otherwise not specified. Differences in RBP4, glucose, insulin and HOMA-IR between two groups of mice were assessed via a standard t-test. In order to see which variables were most predictive of circulating RBP4 levels, we then performed multivariate statistical analysis, treating each sample as an independent measurement. For the multivariate analyses, a linear regression with RBP4 as the outcome was done on samples obtained at 1, 100 and 400 days including age, gender, having ovaries and weight as covariates. Backward stepwise regression model was incorporated to identify the best fitting model. *P*-values <0.05 were considered statistically significant. All *p*-values presented here are two sided.

Results

Figure 1 shows the relation of RBP4 to different parameters of adiposity: (A) weight across life span, (B) weight at 400 days on diet and (C) BMI. This data shows that RBP4 is dependent on weight (A) R^2 = 0.30 and (B) R^2 = 0.47, or BMI (C) R^2 = 0.47, with moderate variability. To determine the contributions of other factors affecting weight dependence on circulating RBP4, we performed linear regression to evaluate the impact of diet, age, gender and presence of ovaries. The best fitting multivariate progression model included weight ($p < 0.0001$), diet ($p = 0.0003$) and having ovaries ($p = 0.021$), suggesting all are important, although not independent, predictors of RBP4.

Figure 2 shows the age-dependent effects of LFD and HFD in the different mouse groups. As shown in 2A, at 6 weeks of age (Diet Day 0), when mice were distributed to different diets or subject to surgical procedures, male mice already weighed more than female mice. (20.75 [± 0.95] g vs. 17.52 [± 1.26] g, $p = 0.00041$). All mice within each gender subsequently gained weight over the experimental time course. Each HFD fed group

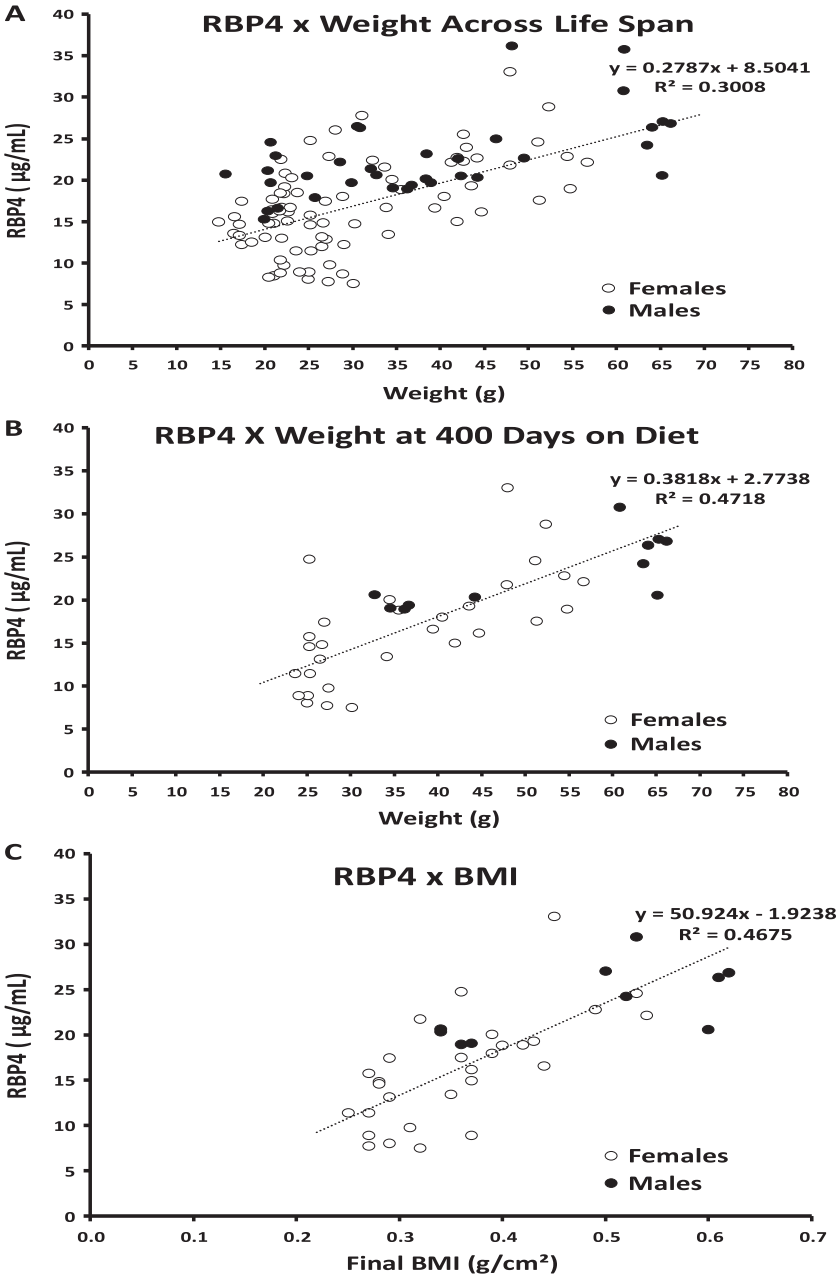

Figure 1 Association RBP4 plasma levels with body mass. A, Weight and RBP4 at multiple time points across life span 42 to 500 days on diet; B, weight and RBP4 at single time point of 400 days on diet; C, BMI and RBP4 at 400 day time point. Each point represents mean value for duplicate determinations on a single mouse, (0) females, (•) males.

showed greater weight gain than the corresponding LFD fed group. Although, HFD fed male and female mice both gained weight, female mice acquired less body mass than males, indicating a persistent gender difference. By 400 days, OVX female mice fed HFD showed greater, but not significantly different weight gain compared with normal females fed HFD, (45.78 (± 7.31) g vs. 37.96 (± 11.94) g $p = 0.76$) and did not reach the same weight as HFD fed males (59.41 (± 1.31) g $p = 0.00016$) indicating that the sex difference between male and female mice is not strongly under ovarian control.

Figure 2B shows the effects of diet, development and gender on RBP4, over 400-day period. At 6 weeks of age, before any dietary difference or surgical manipulation, male mice already showed higher RBP4 levels than females (19.7 (± 3.22) $\mu g\ mL^{-1}$ vs. 14.2 (± 1.58) $\mu g\ mL^{-1}$, $p = 0.0004$). Although male mice fed LFD gained weight over the 400 day experimental course, they maintained

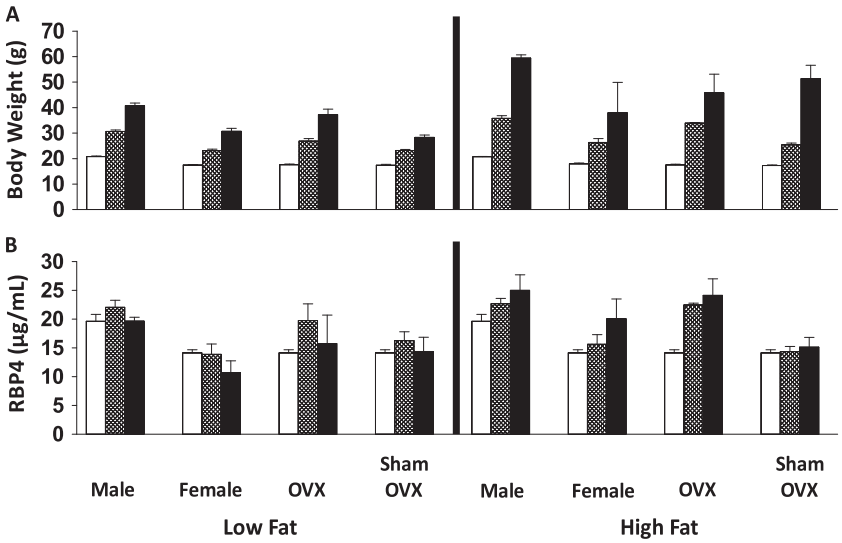

Figure 2 Serial measurement change in body weight (A) and plasma RBP4 (B) over 400 day feeding low fat diet (left of midline) or high fat diet (right of midline) at start of diet, day 0 □, 100 days on diet ▒, 400 days on diet ■. Each box represents mean (± SD) of three or more mice.

a relatively constant RBP4 level (0 to 100 days $p = 0.18$, 0 to 400 days $p = 0.46$). Female mice on the same LFD showed a lower RBP4 level over the same time (0 days 14.13 ± 1.68 μg mL^{-1} to 400 days 10.71 ± 2.05 μg mL^{-1}, $p = 0.0053$). Nonetheless, for mice fed LFD, the higher level RBP4 in males compared with females, persisted for the duration of the experiment. Differences in RBP4 among OVX (15.73. ± 4.98) μg mL^{-1} and normal female (10.71 ± 2.05) μg mL^{-1} mice, fed LFD, at 400 days, did not reach statistical significance ($p = 0.070$). However, RBP4 levels in OVX LF fed mice trended towards LFD fed males (19.66 ± 0.76 μg mL^{-1}, $p = 0.12$ for difference in males from OVX mice).

Both male and female mice fed HFD showed a continuous increase in RBP4 (male 400 day vs. 0 day, $p = 0.0032$; female 400 day vs. 0 day $p = 0.0030$). Moreover, after 400 days on HFD, male mice relative to female mice still show elevated RBP4 ($p = 0.0037$). Although OVX compared with normal females fed HFD appear to have higher RBP4, these values did not reach statistical significance ($p = 0.17$). These results show that both diet and sex make an independent effect in determining RBP4 levels.

These data show that male mice on HFD have increased RBP4 relative to those on LFD (400 days, 25.00 (± 2.72) μg mL^{-1} vs. 19.66 (± 0.68) μg mL^{-1} $p = 0.00032$). Likewise, female mice on HFD show similar results (400 days, 20.07 A (± 3.45) μg mL^{-1} vs. 10.71 (± 2.05 μg mL^{-1}) $p = 0.0030$. However, female mice maintain lower RBP4 levels than male mice on same diet $p = 0.0010$). Differences in RBP4 levels in OVX mice compared with normal females fed LFD or HFD increased towards male values. Thus, normal female mice show a difference in RBP4 at beginning of experiment, and on a LFD, this gender difference is maintained for at least 400 days of experiment during which time mice undergo normal growth, development, maturation and weight gain. Likewise, female mice on a HFD show increased RBP4 but not as much as HFD fed males. OVX females fed HFD showed a further increase in RBP4 indicating that the restriction in HFD induced RBP4 increase is partially under ovarian control.

To determine whether there are sex differences in consequences of diet, obesity and regulation of RBP4 and its association with insulin resistance, FBG, plasma insulin and HOMA IR were determined at multiple time points among the eight mouse groups. Figures 3A and B show that before starting experimental diets at 6 weeks, male mice compared with females showed similar FBG (127.00 ± 30.63 mg dL^{-1} vs. 117.3. ± 31.17 mg dL^{-1}, $p = 0.48$) and insulin (54.9 ± 29.49 p mol L^{-1} vs. 36.3, ± 7.86 p mol L^{-1} $p = 0.47$). Mean calculated HOMA IR at that time for males was 2.48 (± 1.10), while for females was 1.32 (± 0.57) ($p = 0.28$-). Over the course of this experiment, both normal male and female LFD fed mice showed no increase in HOMA IR compared with baseline values ($p > 0.05$). However, by day 300, OVX, but not control female mice, showed increase in HOMA IR (11.16 vs. 0.92, $p < 0.0001$) indicating development of moderate insulin resistance in the OVX animals. Interestingly, RBP4 levels did not differ significantly between OVX and control mice fed LFD at this time. In contrast to the effects of LFD in male mice, those fed HFDs developed moderate insulin resistance at day 100 (HOMA

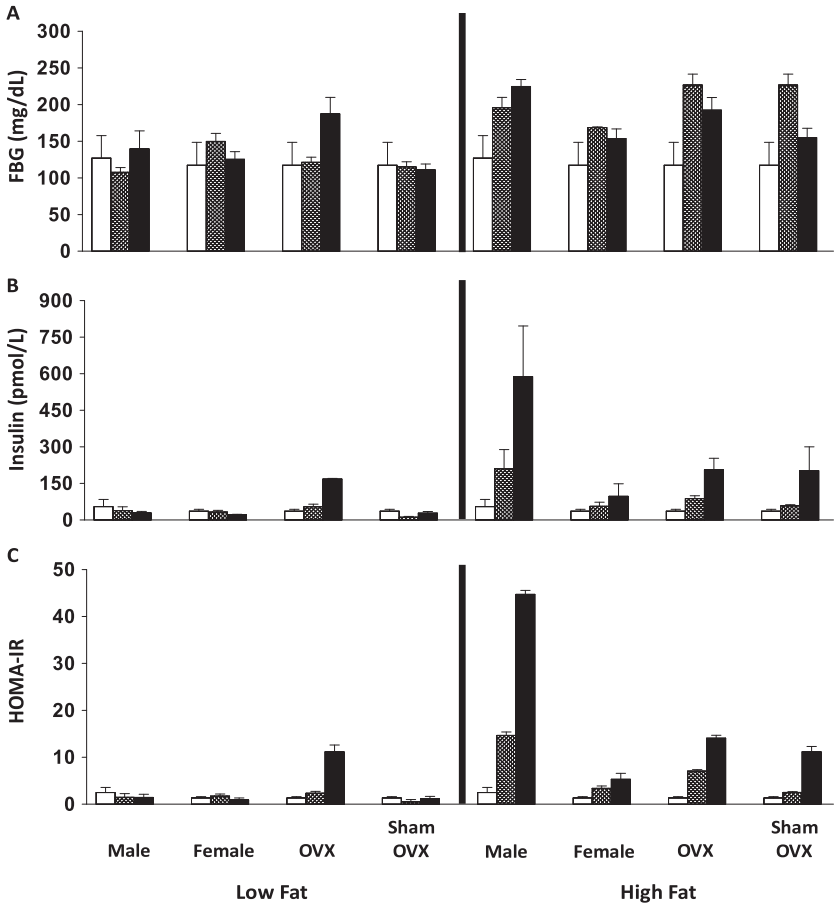

Figure 3 Serial measurement change in fasting blood glucose (A), plasma insulin (B), and HOMA-IR (C) over 300-day feeding low fat diet (left of midline) or high fat diet (right of midline at start of diet, day 0 □, 100 days on diet ▒, 300 days ■. Each box represents mean (± SD) of three or more mice.

IR 14.66 vs. 1.47, $p < 0.0001$) and severe insulin resistance at day 300 (HOMA IR 44.71 vs. 1.44, $p < 0.0001$). Control female mice fed HFD developed moderate insulin resistance by day 100 (HOMA IR 3.38 vs. 1.73, $p = 0.087$) with a continued increase by day 300 (HOMA IR 5.32 vs. 0.92, $p = 0.0007$). At 300 days, insulin resistance in HFD fed mice was more severe in males than females (HOMA IR 44.71 vs. 5.31, $p < 0.0001$); HFD fed OVX mice showed greater IR than control females (HOMA IR 11.14 vs. 5.32, $p = 0.0010$) but did not reach the levels of HFD fed males.

To determine the impact of HFD associated elevation of RBP4 on insulin resistance in the eight experimental mouse groups, 2-hour glucose tolerance curves were performed on three mice from each group. Figure 4A shows that male and female mice fed LFD showed peak serum glucose levels at 15 to 30 minutes following which they decreased towards normal levels by 2 hours. On HFD, both male and female mice each continued with elevated glucose beyond 15 minutes, such that females achieved peak levels at 30 minutes before beginning to decrease towards normal by 120 minutes. In contrast, HFD fed males maintained markedly elevated glucose levels between 30 and 60 minutes which remained elevated at 450 mg dL^{-1} even after 2 hours. GTT results are compared as areas under curve (AUC) in Figure 4B. Compared with LFD fed females, LFD fed males showed slightly higher AUCs, but this difference was not statistically significant ($p = 0.18$). OVX LFD fed females compared with LFD fed female controls showed slightly elevated AUC's, but again not statistically different ($p = 0.40$). In all cases, both male and female mice fed HFD showed greater AUCs than corresponding LFD fed animals reaching significant levels of difference in males ($p = 0.034$), females ($p = 0.048$) but not OVX females ($p = 0.044$). HFD fed males showed the highest AUC which was significantly greater than females and OVX females. Thus, at 300 days, HFD fed males showed much greater consequences of insulin resistance.

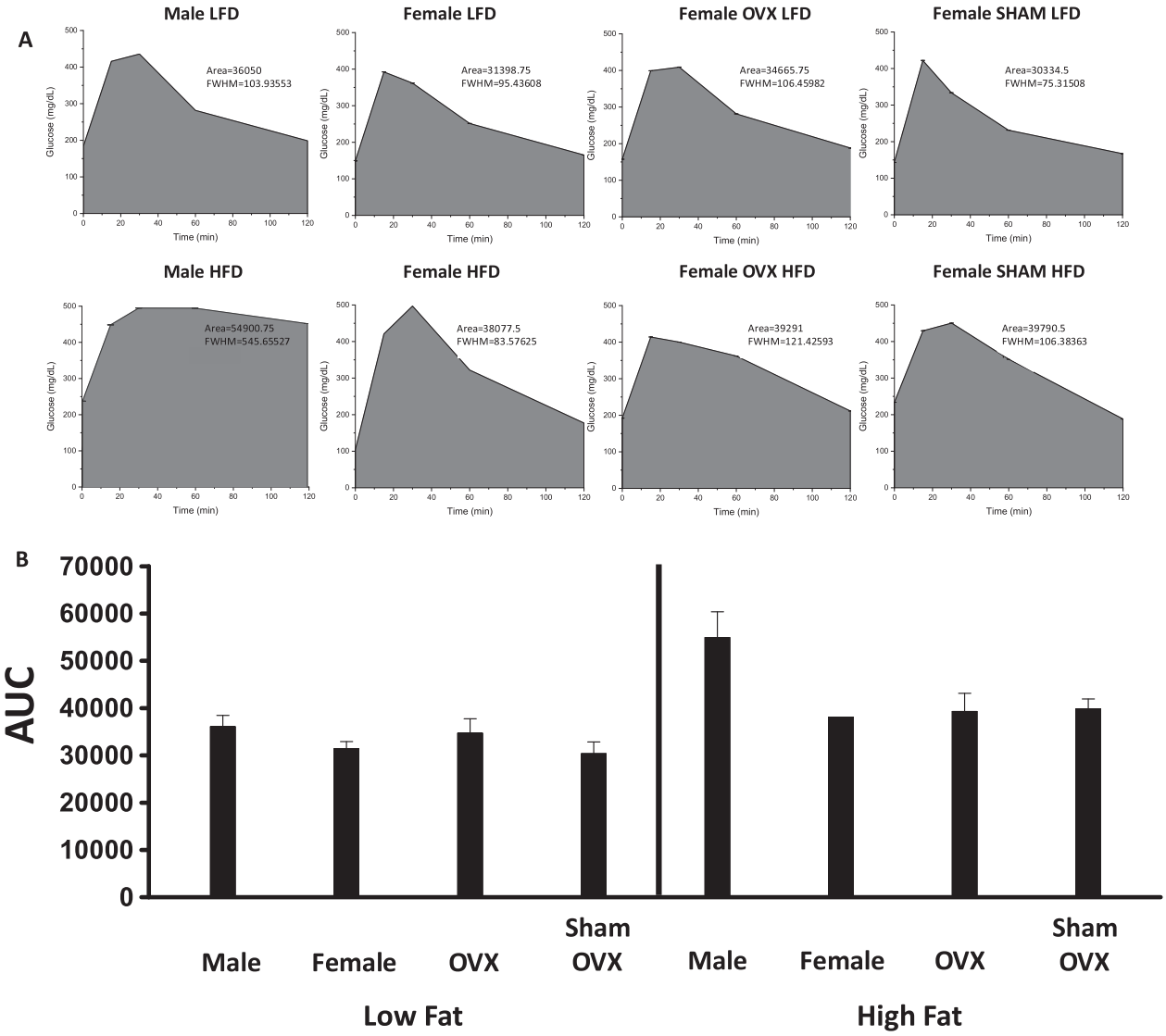

Figure 4 Two-hour glucose tolerance curves in eight mouse groups at 300 days on low fat or high fat diets. Each curve is mean value for three mice except high fat fed females which was limited to one mouse. (A) Top row, low fat fed mice; (B) bottom row, high fat fed mice. (B) integrated areas under curve mean (± SD) arbitrary units for each group.

Discussion

This study was designed to evaluate the gender-dependent effects of high compared with low fat diets on weight gain, RBP4 levels and the RBP4-dependent consequence of insulin resistance. The results show that males compared with females have greater circulating RBP4 from early age extending over time periods of at least 400 days and although both male and female mice fed HFD show progressive increase in RBP4, the male greater than female difference was maintained. The greater increase in RBP4 in HFD fed males compared with females was associated with development of severe insulin resistance in males but not females. The effect in males was partially recapitulated in OVX females, thereby demonstrating an important regulatory effect of ovarian function. The observation that RBP4 levels in OVX mice do not fully reach the levels in males may be due to further stimulation of RBP4 levels by testosterone (16). In summary, RBP4 is greater in males than females from early time points, i.e. 6 weeks of age, and maintains this difference on both low and high fat diets, in the absence or presence of obesity, over extended periods of at least 400 days. In addition, RBP4 associated consequence, such as insulin resistance, shows a similar sex difference.

These findings suggest that RBP4 may contribute to the higher incidence of type 2 diabetes mellitus and CAD in males vs. pre-menopausal females (22–25) . The

results are suggestive also that gender difference in RBP4 may contribute to the observation of lower incidence of CRA and CRC in females compared with males (26). Accordingly, increase in RBP4 following OVX and resultant increase in insulin resistance may be a model for increased RBP4 in the post-menopausal state and be associated with increased CAD, CRA and CRC in post-menopausal women. These results indicate that female mice compared with males are protected against HFD induced weight gain, insulin resistance, RBP4 elevation and glucose intolerance, and these effects are partially dependent on ovarian hormones. Since genetic interference with RBP4 expression improves insulin resistance (6), these findings suggest that targeting RBP4 levels or activity could decrease some gender disparities in insulin resistance and obesity comorbidities. Thus, pharmacologic strategies targeted at interference with RBP4 synthesis and/or activity should be evaluated to reduce some of the comorbid effects of obesity. These results further suggest the importance of monitoring RBP4 levels as part of weight loss and/or exercise interventions.

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Disclosure

The authors declared no conflict of interest.

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