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Role of human pregnane X receptor in high fat diet-induced obesity in pre-menopausal female mice

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Abstract

Obesity is a complex metabolic disorder that is more prevalent among women. Until now, the only relevant rodent models of diet-induced obesity were via the use of ovariectomized (“postmenopausal”) females. However, recent reports suggest that the xenobiotic nuclear receptor pregnane X receptor (PXR) may contribute to obesity. Therefore, we compared the roles of mouse and human PXR in diet-induced obesity between wild type (WT) and PXR-humanized (hPXR) transgenic female mice fed either control or high-fat diets (HFD) for 16 weeks. HFD-fed hPXR mice gained weight more rapidly than controls, exhibited hyperinsulinemia, and impaired glucose tolerance. Fundamental differences were observed between control-fed hPXR and WT females: hPXR mice possessed reduced estrogen receptor α (ER α) but enhanced uncoupling protein 1 (UCP1) protein expression in white adipose tissue (WAT); increased protein expression of the hepatic cytochrome P450 3A11 (CYP3A11) and key gluconeogenic enzymes phosphoenolpyruvate carboxykinase and glucose 6-phosphatase, and increased total cholesterol. Interestingly, HFD ingestion induced both UCP1 and glucokinase protein expression in WT mice, but inhibited these enzymes in hPXR females. Unlike WT mice, CYP3A11 protein, serum 17 β -estradiol levels, and WAT ER α expression were unaffected by HFD in hPXR females. Together, these studies indicate that the hPXR gene promotes obesity and metabolic syndrome by dysregulating lipid and glucose homeostasis while inhibiting UCP1 expression. Furthermore, our studies indicate that the human PXR suppresses the protective role of estrogen in metabolic disorders. Finally, these data identify PXR-humanized mice as a promising *in vivo* research model for studying obesity and diabetes in women.

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Keywords

obesity; high-fat diet; pregnane X receptor; nuclear receptors; type 2 diabetes; females

1. Introduction

The prevalence of obesity has increased worldwide at an alarming rate and is now recognized as a serious global health problem [1]. In the US, obesity affects every age-group of the population, with 1 in 3 adults considered to be clinically obese [2]. In the context of this global obesity pandemic, a clear gender disparity exists, with women at greater risk of developing obesity than men [2]. Further, obesity is also a disease of racial disparity, in which African-American women are more frequently afflicted than Caucasian women [3]. The biological mechanisms that contribute to these gender and racial disparities remain known.

Similar to humans, several studies have reported differential susceptibility to obesity upon high-fat diet (HFD) ingestion by some strains of rats and mice [4,5,6,7]. Reports indicate that C57BL/6 (B6) mice will develop severe obesity, hyperglycemia, and insulin resistance if weaned unto a HFD compared to other strains, such as C3H/He, 129/Sv, and A/J mice, which are resistant to obesity and diabetes [7,8,9,10,11,12]. Interestingly, Similar to the B6 mice, AKR/J mice are also sensitive to the development of obesity by a HFD, but are less hyperglycemic [7]. Several factors influence the development of obesity including genetics, sedentary lifestyles, and dietary fat amount and type [13,14,15]. Interestingly, the specific genes that determine sensitivity to dietary obesity remain unknown [13,14].

With respect to gender differences in obesity, both clinical data and animal studies indicate that sex hormones (estrogens in females and androgens in males) are the chief drivers of sexual dimorphisms; gonadal steroids contribute to the regulation of food intake, body weight, and lipid metabolism [16,17]. In females, the loss of estrogen production, either following menopause in humans or ovariectomy in rodents, frequently leads to obesity, suggesting that estrogens appear to play a role in lipid homeostasis to reduce overall body adiposity [18,19].

Consumption of a high caloric diet, rather than genetic makeup, is the most common cause of obesity in humans [20]. Therefore, a clinically relevant animal model of HFD-induced obesity would provide a valuable tool for investigating the molecular basis of obesity in women. Unfortunately, unlike humans, female rodents are more resistant to HFD-induced obesity than their male counterparts, and among female rodents, only ovariectomized animals are capable of significant HFD-induced weight gain [21]. Therefore, female mice are less prone to develop diabetes than humans [22]. Thus, data from both human and animal studies suggest that estrogen may not be the only factor that influences sexual dimorphism in obesity.

Numerous studies have utilized ovariectomy with or without repletion of estrogen and/or compared male and female mice provided a HFD as models to explore the contribution of estrogen to the sexual dimorphisms in obesity [18,19]. Obesity is the product of a chronic

positive energy balance, and the health risks associated with obesity vary depending on the amount and location of adipose tissue [23,24,25]. While the ovariectomized rodent model shares similar characteristics with humans, this model requires a high level of surgical skill and does not completely match the physiology of postmenopausal women, most of whom retain their ovaries. For example, both ovariectomy in female rodents and menopause result in weight gain and increased adipose tissue mass, however, ovariectomy preferentially increases subcutaneous fat whereas menopause increases the metabolically more deleterious intraabdominal visceral fat [24,25,26,27]. Furthermore, ovariectomy leads to an immediate cessation of estrogen secretion and gain in lean body mass, whereas menopause occurs after a gradual cessation of estrogen secretion resulting in loss of lean body mass [26,27]. These dissimilar effects of ovariectomy and menopause suggest that the metabolic consequences of ovariectomy differ from that caused by menopause-induced obesity and that ovariectomy may not fully represent a rodent model for female obesity.

Nevertheless, estrogens and estrogen receptors (ERs) have been the primary focus of studies attempting to unravel the molecular basis of sexual dimorphisms in obesity. Estrogen activity occurs via the ER, a ligand-dependent transcription factor that consists of distinct modular domains, each with unique biological functions [28,29]. Importantly, ER-mediated transcriptional activity modulates other nuclear receptors, including the peroxisome proliferator-activated receptor α (PPAR α), the constitutive androstane receptor (CAR), and the pregnane X receptor [PXR; NR1I2, its human homologue, steroid and xenobiotic receptor (SXR)] [30,31,32]. While the role of nuclear receptors in obesity is now well established, information is lacking about the contribution of nuclear receptors to the gender disparity of obesity incidence in humans and the functional differences between mouse and human nuclear receptors in diet-induced obesity.

PXR expressed primarily in the liver, intestine and kidney is a xenobiotic sensing nuclear receptor, involved in detoxification of drugs and other foreign chemicals [33]. While previous studies have established functional links between glucose homeostasis and PXR, the *in vivo* significance of PXR function in obesity and metabolic syndrome has not been fully explored [34,35,36]. The sequences of human and mouse PXRs share nearly 77% amino acid identity across the C-terminal ligand binding domain (LBD), 96% amino acid identity in the N-terminal DNA-binding domain, and the two PXRs display similar tissue-specific expression patterns [33]. However, there are major differences in responses to ligand activation by human and mouse PXR that are likely due to differences in LBD sequence [37]. As a result, some chemicals that activate human PXR usually have little effect on the mouse PXR and vice versa [38,39]. For example, rifampicin, phytoestrogens, and corticosterone potently activate human PXR, but not mouse PXR. In contrast, pregnenolone-16 α -carbonitrile (PCN) and dexamethasone strongly activate mouse, but not human PXR [38].

To overcome the species differences in ligand specificity, PXR-humanized (hPXR) transgenic mice have provided a relevant *in vivo* model of the human xenobiotic response [40]. The hPXR mice were generated by bacterial artificial chromosome (BAC) transgenesis, in which the transgene contains the complete human PXR gene and the 5'- and 3'-flanking sequences and then bred with PXR-KO mice to produce hPXR mice carrying a

C57BL/6 genetic background [40]. hPXR mice selectively express PXR in the liver and intestine in parallel with cytochrome P450 3A4 (CYP3A4), used to detoxify a wide range of substances [40]. Treatment with PXR ligands revealed a clear species difference between WT and hPXR mice in their response to xenobiotics, suggesting that this BAC transgenic hPXR mouse model is useful for investigating human PXR function *in vivo* [40]. Indeed, recent reports have demonstrated the utility of this whole body hPXR mouse model in exploring the xenobiotic and endobiotic role of PXR [40,41,42,43,44,45]. Our group and others have shown that the male mouse PXR (mPXR) promotes obesity, however, whether PXR regulates obesity with sexual dimorphism remains unknown [45,46]. Moreover, the effect of the human PXR gene on ER- and diet-induced obesity in a mouse model requires investigation. At the same time, gender-based obesity research has lacked a valid animal model that fully recapitulates the human disease.

To resolve discrepancies in the gender-specific molecular pathology of obesity between rodents and humans, female WT and humanized PXR transgenic mice were investigated with HFD treatment. Our results indicate that the human PXR gene when expressed transgenically in female mice produces an animal model that becomes rapidly obese, glucose intolerant, diabetic, and hyperinsulinemic when exposed to a HFD. These results suggest that human PXR promotes obesity in female mice and that PXR-humanized mice will serve as a valuable *in vivo* model for obesity research in humans.

2. Materials and Methods

2.1. Animals

Breeding-pairs of adult male and female C57BL/6 (WT) mice were purchased from Jackson Laboratories (Bar Harbor, ME). All mice were housed in polycarbonate cages on racks directly vented via the facility's exhaust system at 22°C with a 12/12-h light/dark cycle at the Animal Resources Complex at North Carolina Central University. Breeding pairs of hPXR mice on a C57BL/6 background were transferred from the colony housed at the National Cancer Institute (NIH, Bethesda, Maryland) [40]. Age-matched female WT and hPXR mice, were each randomly separated at weaning (~3 weeks old) into two groups ($n = 7-11$ for each group) and fed with either a control diet (12% of calories as fat; Research Diet catalogue #: D03032702); or a high-fat diet (HFD, 45% of calories from fat; Research Diets catalogue #: D03032705) for 16 weeks. The gross energy density in the control diet and the HFD were calculated to be 3.8 and 4.7 Kcal/g, respectively. Food and water were provided *ad libitum* throughout the entire feeding period. However, we observed that both the control- and HFD-fed mice were most active and fed more at night. Throughout the experimental period, the mice were housed in groups of 3-5 mice/cage. All diets were prepared by Research Diets Inc. (New Brunswick, NJ) and the detailed diet composition was previously described except that HFD in this study provided 45% of calories from lard and soybean oil, instead of 43% [47]. Furthermore, the HFD and the control chow diets were matched in the type of fat and other nutrients. The food consumption and the change in body weight were monitored weekly. All procedures conducted in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals were approved by the North Carolina Central University Institutional Animal Care and Use Committee. After 16 weeks on the diet, mice

were anesthetized with isoflurane and sacrificed in the morning (09:00–11:00 hours) without starvation. Mice were not starved overnight before sacrifice because previous reports indicate that fasting induces PXR expression in the liver [48,49]. Furthermore, a detrimental role of PXR activation by both the human and rodent agonists on glucose tolerance has been reported [50]. After sacrifice, sections of liver, brown adipose tissue (BAT, removed from the back), and WAT were rapidly dissected, weighed, snap-frozen in liquid nitrogen and kept at -80°C . The WAT comprised parametrial, perirenal, and mesenteric fat. Blood samples collected by cardiac puncture were centrifuged at 3000 rpm for 15 min to collect serum to determine liver enzymes, triglycerides and cholesterol, glucose, 17β -estradiol, leptin, and insulin concentrations.

2.2. Serum alanine aminotransferase (ALT), cholesterol, triglyceride, glucose, insulin, leptin, and 17β -estradiol measurements

Blood was collected by cardiac puncture and serum processed and stored at -80°C . Serum ALT activity and glucose levels were determined with the ALT/AST (parts # 12-788) and Lipid profile-GLU (parts # 10-991) Test Cassettes, respectively, using the Cholestech LDX analyzer (Cholestech Corporation, Hayward, CA) [51]. Serum leptin (catalog # 90030) and insulin (catalog # 90080) levels were determined using enzyme-linked immunosorbent assay (ELISA) kits according to protocols provided by the kit manufacturers (Crystal Chem Inc., Dowers Grove, IL). Serum triglyceride and cholesterol levels were quantified using a Triglyceride (catalog #s, 461-08992, 461-09092, and 461-01601) and Cholesterol (catalog # 439-17501) test kits (Wako pure Chemical Industries, Richmond, VA). Serum levels of 17β -estradiol were determined using an ELISA kit (catalog # 582251, Cayman Chemical, Ann Arbor, MI).

2.3. Hepatic triglyceride, cholesterol, and nonesterified free fatty acid (NEFA) levels

Total liver lipids were extracted from 100 mg of liver homogenate using methanol and chloroform as previously described [52]. Hepatic triglyceride, cholesterol, and NEFA levels were quantified using a Triglyceride, Cholesterol, and the HR series NEFA-HR (2) (Catalog #s 999-34691, 995-34791, 991-34891, 993-35191, and 276-76491) test kits (Wako pure Chemical Industries, Richmond, VA).

2.4. Glucose tolerance test (GTT)

GTT were performed on WT and hPXR mice ($n = 6/\text{group}$) on the control diet or HFD for 15 weeks using females. Glucose tolerance tests were conducted in 12-15 hours fasting mice, before a single dose of D-glucose (10% solution in water, 10 mL/kg) was intraperitoneally (i.p.) injected. Glucose levels were measured from blood collected from the tail vein immediately before and 15, 30, 60, 90, and 120 min after the injections using Contour TS blood glucose meter (Bayer HealthCare LLC, Mishawaka, IN), an instrument that reads blood glucose levels.

2.5. Preparation of liver and white adipose tissue (WAT) extracts for Western blot analyses

Frozen livers, were homogenized at 4°C and then subjected to Western blot analysis as described previously [53]. Frozen WAT (500 mg of parametrial fat) were homogenized at

4°C, followed by centrifugation at 14,000 rpm for 20 min as we described previously [45]. The supernatant was collected as the cytosolic fraction. The liver homogenate or cytosolic fraction (30–40 µg/lane), were mixed in Laemmli loading buffer containing β-mercaptoethanol, boiled for 5 min, and were separated by 10 or 15% SDS-PAGE gels, and transferred to polyvinylidene difluoride (PVDF) membrane. The membranes were probed overnight with rabbit polyclonal primary antibodies from Abcam Inc. (Cambridge, MA) at a concentration of 1:1000 for anti-CYP2E1 (ab28146), anti-glucokinase (ab37796), anti-glucose 6-phosphatase (G6Pase, ab83690), anti-UCP1 (ab10983) or anti-PEPCK1 (ab70358). Primary antibodies from Santa Cruz Biotechnology, Inc., (Santa Cruz, CA) were used at a concentration of 1:200 for rabbit polyclonal anti-ERα (Sc-542), rabbit polyclonal anti-ERβ (Sc-8974), or goat polyclonal anti-CYP3A (Sc-30621). Blots were then incubated with the appropriate peroxidase-conjugated anti-rabbit IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) secondary antibodies diluted in TBST plus 1% milk for 60 min at room temperature. Following probing, the blots were stripped and reprobed with anti-α-tubulin antibody (Cell Signaling Technology, Danvers, MA) at a concentration of 1:1000. Proteins were viewed using enhanced chemiluminescence. The intensities of the bands were quantified using ImageJ software (U. S. National Institutes of Health, Bethesda, MD). Protein contents in the liver homogenate and cytosolic fractions prepared from the WAT were determined by the BCA™ protein assay kit (Thermo Scientific, Rockford, IL).

2.6. Statistical Analysis

Data are presented as means ± SEM (n = 7–11). Statistical analysis was performed using one-way ANOVA followed by Bonferroni post-hoc test. A *P* value of < 0.05 was considered statistically significant. The analyses were performed with the statistical software IBM SPSS Statistics 20 (Armonk, NY).

3. Results

3.1 The hPXR gene predisposes female mice to high fat diet-induced obesity

We characterized the role of the human PXR gene in a widely studied HFD-induced model of obesity using female WT and hPXR mice. While the effect of HFD on weight gain in WT mice was not apparent until after 6 weeks (Fig. 1a), body weight differences between hPXR mice fed control diet and those fed a HFD were statistically significant as early as 2 weeks from the start of their respective diets (Fig. 1a). Within this same timeframe (2 weeks), significant body weight differences were also observed between the hPXR and WT genotypes receiving a HFD; these genotype differences in response to HFD persisted until the end of the study at 16 weeks (Fig. 1a). By the end of the study period, HFD-fed hPXR mice had gained an average of 30.3 g, while HFD-fed WT mice gained only 20.3 g (Fig. 1b). This difference in weight gain between the two genotypes did not occur among animals fed control chow (Fig. 1a and b).

Consonant with trends in weight gain, animals of both genotypes ate more on a HFD than on a normal diet (Fig. 1c). Also consistent with weight gain patterns, there was no difference in daily food intake between genotypes on the control diet. Curiously, however, even though

HFD-fed hPXR animals gained more weight than their WT counterparts, this weight gain came without an increase in food intake by hPXR mice (Fig. 1c).

HFD ingestion increased parametrial and perirenal fat pads to the same extent in both WT and hPXR mice (Fig 2a and b). In contrast, the increase in mesenteric fat pads by HFD ingestion was higher in hPXR mice (410%) than in WT mice (230%, Fig. 2c). Furthermore, HFD ingestion increased the weight of the BAT to a greater extent in hPXR mice (312%) than in WT mice (224%, Fig. 2d). HFD ingestion also increased liver weight only in hPXR (180%) mice (Fig 2e). Taken together, these data indicate that the human PXR gene rapidly predisposes female mice to HFD-induced obesity and imply that PXR-dependent weight gain occurs in mesenteric fat, BAT, and liver but does not result from increased food intake.

3.2 Basal uncoupling protein 1 (UCP1) protein expression is elevated, but suppressed by HFD in WAT of hPXR mice

To attempt to define the cause of severe obesity in hPXR mice upon HFD ingestion, we examined the expression of the BAT specific-protein, UCP1. Previous reports indicate that induction of UCP1 in WAT is effective at reducing adiposity, insulin resistance, and obesity [54,55,56]. Western blot analysis indicated that the UCP1 protein was barely detectable in WAT of WT mice fed the control diet (Fig 3a). However, unexpectedly, a strong up-regulation (5.4-fold) of the UCP1 protein in WAT of control chow-fed hPXR mice compared to WT mice fed the same diet was observed (Fig 3a). Most interestingly, while UCP1 protein was induced in WT mice (2.5-fold), the UCP1 protein was almost completely inhibited in hPXR mice, when both WT and hPXR mice were placed on a HFD (Fig. 3a). We also explored the association between mouse vs. human PXR and WAT ER α levels using Western blot analysis. Among control diet-fed animals, WAT ER α expression was markedly decreased (15% of WT mice on control diet) in the hPXR mice (Fig. 3b). Further, HFD feeding significantly decreased ER α protein levels in WT mice (24% of chow-fed animals) (Fig. 3b). Somewhat surprisingly, HFD ER α protein levels in hPXR mice were unresponsive to HFD feeding (Fig. 3b). We also examined ER β protein levels in WAT. Unexpectedly, ER β protein levels did not differ significantly among control-fed or HFD-fed mice of either genotype (Fig. 3c). Although hPXR animals may have had slightly reduced ER β expression levels, these differences were not statistically significant.

3.3 The hPXR mice exhibit hyperinsulinemia under basal conditions

Levels of serum 17 β -estradiol tended to be lower in control chow-fed hPXR mice (68%), compared with WT controls; but the difference failed to reach statistical significance (Fig. 4a). However, the levels of serum 17 β -estradiol were significantly reduced only in HFD-fed WT mice (30% of control-fed WT mice) (Fig. 4a). While serum insulin levels remained unchanged in HFD-fed WT mice, the levels were increased 4.7-fold in hPXR mice fed a HFD (Fig. 4b). Serum insulin levels in control-fed hPXR mice were elevated (2.0-fold) (Fig. 4b). Furthermore, HFD feeding elevated fasting blood glucose levels in both WT and hPXR mice (data not shown). Serum levels of alanine aminotransferase (ALT, a marker of liver injury) increased after HFD ingestion by 1.5- and 1.6-fold in WT and hPXR mice, respectively (Fig. 4c). Basal serum cholesterol levels were elevated (21%) in hPXR mice fed the control chow diet, compared to control chow-fed WT mice (Fig. 4d). As expected, HFD

feeding increased serum cholesterol levels in both mice lines where the increase in serum cholesterol levels by HFD in hPXR mice (44%) was significantly higher than that in HFD-fed WT mice (32%) (Fig. 4d). In contrast, HFD increased serum triglycerides by 36% in hPXR mice alone (Fig. 4e). HFD-feeding increased leptin levels in WT (4.0-fold) and hPXR (3.0-fold) mice above the levels found in control-fed mice (Fig. 4f). We also noted that basal (control diet) serum leptin levels were higher in hPXR mice (1.5-fold) than in WT mice, but these differences were not statistically significant (Fig. 4f).

3.4 The basal hepatic protein levels of CYP3A11 are up-regulated while ER α are suppressed in hPXR mice

In this study, HFD ingestion decreased serum 17 β -estradiol levels in WT mice (Fig. 4a). Among the cytochrome P450 enzyme system, the current study focused only on CYP3A11 and CYP2E1 based on previous reports indicating that CYP3A, a PXR target gene, can metabolize steroid hormones including estrogen, while CYP2E1 participates in oxidation of a variety of xenobiotic compounds [57,58]. Furthermore, the biological functions of estrogen are mediated by binding to its receptors including ER α [59]. Western blot analyses were used to examine hepatic CYP3A11, CYP2E1 and ER α expression. Basal hepatic CYP3A11 protein levels were increased in hPXR (1.8-fold) mice (Fig. 5a). HFD ingestion did not increase CYP3A11 protein levels in hPXR mice, however, it increased the levels in WT (1.5-fold) mice (Fig 5a). CYP2E1 expression in hPXR animals on either diet was not different compared to that in control-fed WT mice. HFD feeding increased CYP2E1 protein levels in WT (2.6-fold) mice and the increase was significantly higher than in HFD-fed hPXR mice (Fig. 5b). The basal hepatic ER α protein levels were decreased by 57% in hPXR mice, compared to control chow-fed WT mice (Fig. 5c). HFD did not have any significant effect on hepatic ER α expression in both genotypes (Fig. 5c).

3.5 The human PXR gene disrupts hepatic cholesterol homeostasis

Previous reports have shown that elevated levels of NEFA in the liver is a stimulus for gluconeogenesis [60]. It is interesting to note that both hepatic cholesterol and NEFA levels were elevated in hPXR mice on either diet (Fig. 6a and c), in contrast to the increase in liver weight seen only in HFD-fed hPXR females (Fig. 2e). Both genotypes had increases in hepatic triglyceride levels on a HFD, which were more pronounced in hPXR mice (Fig. 6b).

3.6 Glucose tolerance is impaired in both HFD-fed WT and hPXR mice

To establish whether glucose clearance varies between females that possess mouse vs. human PXR, we performed intraperitoneal glucose tolerance tests (IPGTT) on the control- and HFD-fed PXR genotypes. Serum glucose levels peaked at 15 minutes after glucose challenge in both WT and hPXR mice fed the control diet (Fig. 7a). WT mice fed a HFD cleared systemic glucose spikes more efficiently and more rapidly than their hPXR counterparts (Fig. 7a and b) did. Glucose elimination was severely impaired in HFD-fed hPXR mice and the total systemic glucose [represented by the area under the curve (AUC)] significantly increased by 121%, as the result of excess dietary lipids, indicative of glucose intolerance (Fig. 7a and b). It was also noted that the AUC in HFD-fed WT animals significantly increased by 67% compared to their control-fed counterparts (Fig. 7a and b).

3.7 Gluconeogenic enzymes PEPCK1 and G6Pase protein are constitutively activated in hPXR mice

Western blot analyses were employed to establish whether the hyperglycemia and severely impaired glucose tolerance observed in HFD-fed hPXR mice resulted from altered expression of liver gluconeogenic enzymes such as PEPCK1 and G6Pase and glucokinase involved in glycolysis [61]. Hepatic PEPCK1 protein levels in hPXR mice fed the control diet were increased 5.0 fold compared with WT mice on the same diet (Fig. 8a). However, HFD feeding did not, further increase the high basal PEPCK1 protein levels in hPXR mice, but did enhance PEPCK1 protein expression in WT mice (3.1-fold), but significantly lower than the level found in HFD-fed hPXR mice (Fig. 8a). The basal protein levels of G6Pase, were increased in control chow-fed hPXR (2.9-fold) mice (Fig. 8b). HFD suppressed G6Pase protein levels in WT mice by 47%, but not in HFD-fed hPXR mice and G6Pase levels remained significantly higher than in HFD-fed WT mice (Fig. 8b). HFD ingestion increased the glucokinase protein in WT (2.1-fold) mice, however, it inhibited the glucokinase protein by 64% in hPXR mice (Fig. 8c).

Discussion

The major finding of this study is that the human, but not mouse, PXR gene significantly potentiated HFD-induced obesity, glucose intolerance, and insulin resistance. Our data also indicate that the increased sensitivity of female hPXR mice to HFD-induced obesity was not the result of increased food intake relative to their HFD-fed WT counterparts, but must be due to the function of the PXR gene alone and in combination with exposure to HFD. Specifically, while both HFD-fed WT and hPXR mice were hyperphagic in comparison with control chow-fed mice, there was no difference in food intake between the two HFD-fed genotypes. An obesity metabolic phenotype occurs in HFD-fed hPXR mice, which was associated with decreased basal protein levels of WAT ER α , but increased expression of the hepatic gluconeogenic enzymes PEPCK1 and G6Pase, compared to levels of these enzymes in WT mice fed the control diet. Interestingly, two of these phenomena, reduced WAT ER α protein expression and enhanced hepatic PEPCK1 protein expression were also present in the HFD-induced obesity and a type 2 diabetic profile in our female WT mice. Unexpectedly, it was observed that hPXR mice possess increased basal expression of the UCP1 in WAT compared to WT mice. The most important difference between these two genetic strains, after HFD ingestion, is inhibition of WAT UCP1 and hepatic glucokinase protein in hPXR mice leading to robust obesity and severe glucose intolerance, in contrast to induction of both UCP1 and glucokinase protein in WT mice, probably leading to increased energy expenditure and some protection against diet-induced obesity in female rodents.

The increased health risks associated with obesity vary depending on the amount and location of adipose tissue [23,24,25]. Adipose tissue exists in two forms, WAT and BAT. WAT stores excess energy in the form of triglycerides and releases molecular signals such as leptin to inform the body of its overall energy state, while the main function of BAT is to regulate thermogenesis, producing heat instead of ATP [62,63,64]. WAT distributed in the abdominal viscera is associated with a greater risk of metabolic disorders [23,24,25]. Thus, the size of the different intra-abdominal WAT depots and BAT in both control chow-fed and

HFD-fed WT and hPXR mice was investigated in this study. As expected, HFD promoted an increase in weight of fat pads in both WT and hPXR mice, resulting in a significant increase in body weight compared with mice fed the control diet. However, compared with WT mice fed a HFD, fat accumulation in WAT, especially mesenteric WAT and BAT, were significantly higher in HFD-fed hPXR mice, and not an excess buildup in parametrial and perirenal fat. Interestingly, the selective deposition of fat in the mesentery in the hPXR mice models a common form of human obesity, since majority of the increased fat mass in diabetic, obese humans is found in the mesentery [24,65]. Liver weight was also higher in HFD-hPXR mice compared with WT mice fed the same diet. Obesity is also prevalent in children, with more than 17 percent of United States children and adolescents classified as obese [2]. Obesity among children is a significant risk factor for type 2 diabetes and insulin resistance [66]. The importance of developmental origins of adult diseases as a contributor to the epidemic of obesity has been recognized [67]. Interestingly, reports indicate that offspring exposed to maternal obesity were at increased risk of developing components of metabolic syndrome (obesity, dyslipidemia, insulin resistance and hypertension) supporting the concept of fetal programming [67,68]. Thus, it will be of interest to examine the development of metabolic syndrome in the offspring of hPXR dams that were fed a HFD during pregnancy compared with dams that received control chow diets. Overall, our data indicate that increased fat mass together with enlarged organs may contribute to the development of increased body weight in hPXR mice.

BAT is a major site involved in energy dissipation and the protein responsible for BAT thermogenesis is UCP1 [64]. UCP1 expression does not normally occur in muscle or white fat, however, previous reports indicate that ectopic UCP1 expression in muscle or WAT causes an increase in energy expenditure and prevents obesity [54,55,56]. Previous reports have shown that overexpression of UCP1 reduces weight gain in obesity prone-mice [56,69,70]. Moreover, the ability of the A/J mice to resist diet-induced obesity compared to the obesity-prone C57BL/6J mice is associated with a strain-specific increase in UCP1 expression in adipose tissue [70]. Unexpectedly, we observed that UCP1 protein was strongly induced in parametrial WAT of control chow-fed hPXR mice, but barely detectable in WT mice. Interestingly, while HFD ingestion induced UCP1 protein expression in WT mice, in contrast, it completely obliterates UCP1 protein expression in hPXR mice. Thus, the failure to upregulate or maintain WAT UCP1 expression to promote thermogenesis and increase in energy dissipation, may contribute to lipid accumulation and obesity in HFD-fed hPXR mice. Conversely, induction of WAT UCP1 protein expression in WT mice will increase thermogenesis capacity in WAT, promote energy dissipation and suppress obesity in WT mice fed the HFD diet as seen in the current study. Although, the reason for the unexpected UCP1 expression in WAT in hPXR mice requires further investigation, it is well established that leptin regulates UCP1 gene [71]. Furthermore, the PPAR γ agonist pioglitazone, essential fatty acids and retinoids have been reported to activate UCP1 gene expression [72,73,74,75]. We have recently shown that the basal serum leptin levels are higher in both male PXR-KO and hPXR mice than in WT mice [45]. The current study also observed a slightly higher, though not significantly, basal serum leptin levels in the female hPXR mice than in WT mice. Hence, it is plausible to suggest that the hPXR gene may

encode for brown fat cell properties in white fat cells by indirectly increasing leptin secretion.

Evidence indicates functional interactions between sex steroids and insulin in several tissues [76]. Previous reports have shown that the deficiency of estrogens or estrogen receptors results in visceral fat accumulation, impairment of insulin sensitivity and lipid metabolism, hypercholesterolemia, and cardiovascular disease risk [77,78]. Furthermore, estrogen withdrawal causes obesity and increased appetite in rodents [79]. In this study, both hPXR mice and WT mice fed a HFD were hyperphagic and obese, compared to their respective chow-fed littermates. The hPXR mice on the control chow diet also exhibited hypercholesterolemia, an important risk factor associated with estrogen deficiency [77,80,81]. In mice, the estrous cycle, an indicator of reproductive function, is 4-5 days with four stages, (proestrus, estrus, diestrus and metaestrus) and is associated with significant variation in the serum estrogen concentrations [82,83]. Interestingly, disruption of the estrous cycle occurs in female obese rodents [84,85]. Reports indicate that total energy intake of female rodents decrease when 17 β -estradiol levels are high (in estrus) and increases when estradiol levels are low (in diestrus) [86,87]. In this study, serum 17 β -estradiol levels were also measured, however, the mice were not sacrificed based on the stage of the estrous cycle. While 17 β -estradiol concentrations in hPXR mice fed control diet tended to be lower, significantly reduced levels of 17 β -estradiol were observed only in HFD-fed WT mice. Thus, although our mice may be at different stages of estrus at sacrifice, it is possible to suggest that the phenotype observed in both female WT and hPXR mice fed a HFD may be due to alterations in the estrogen/ER signaling.

Two major ERs, alpha and beta (ER α and ER β), mediate the physiological signaling of estrogens (17 β -estradiol) [59]. We therefore examined ER α and/or ER β expression in WAT and liver of both hPXR and WT mice. Unexpectedly, the basal ER α expression in WAT and liver were markedly lower in hPXR mice fed the control chow diet compared with WT mice fed this diet. ER α -knockout mice and aromatase-knockout mice, which are unable to synthesize estrogen, both exhibit adipocyte hyperplasia and obesity [77,88]. Interestingly, like the ER α -knockout mice, hPXR mice on a control chow diet possessed elevated fasting blood glucose (data not shown), serum insulin and hepatic cholesterol levels [77,80]. In contrast, ER β -knockout mice have normal fasting blood glucose, glucose tolerance, and cholesterol levels, suggesting that ER α , but not ER β , has a protective role in metabolic disorders [89]. Intriguingly, hPXR animals expressed only low WAT ER α levels but had normal ER β levels. These findings strongly suggest that the increase in WAT accumulation and obesity in HFD-fed hPXR females might result from decreased WAT ER α expression. Along these same lines, the moderate obesity observed in HFD-fed WT mice also corresponded with decreased WAT ER α protein levels, suggesting that ER α signaling is a major factor involved in HFD-induced obesity and diabetes in female mice of both genotypes. Together, an intriguing finding here is that resistance to diet-induced obesity in female non-ovariectomized rodents may not be solely due to the ER α , but instead may be due to induction of WAT UCP1 protein by HFD.

Our results resemble the metabolic status of humans who lack ER α and are insulin resistant [90]. In premenopausal females, ovaries are the principal site for the biosynthesis of

estrogen and the major contributors to systemic estrogen levels [91]. However, extragonadal tissues, including adipose tissue, become important as the organism ages for the maintenance of adequate local estrogen levels [91]. Since hPXR female mice are fertile (and therefore possess functional ovaries), it is likely that the suppressive effects of hPXR on estrogen/ER signaling observed in these mice involves only peripheral tissues capable of estrogen biosynthesis such as WAT.

PXR mediates the genomic effects of several steroid hormones including estrogen, progesterone, pregnenolone, and glucocorticoids in the mouse, rat, and human [92,93]. Besides liver and intestine, PXR expression occurs in reproductive organs including ovary, uterus, and placenta, and PXR mRNA levels are significantly increased during pregnancy in these organs [58,94]. Moreover, PXR ligands such as endogenous steroids including estrogen, progesterone, and pregnenolone activate PXR-mediated transcriptional activity. Once activated, PXR binds to specific DNA sequences as a heterodimer with the retinoid X receptor (RXR) to promote xenobiotic metabolism and elimination [37,93,94]. In contrast, ligand-bound ER α either binds to DNA directly as a homodimer or interacts indirectly with DNA by tethering to other transcriptional factors in estrogen-responsive target genes [95]. Interestingly, ligand binding induces conformational changes in both hPXR and ER α , leading to the recruitment of co-activators or co-repressors that ultimately determine biological activity [96]. Previous reports indicate that xenobiotic nuclear receptors such as hPXR share a similar pool of co-activators with the ERs, providing a platform for inhibitory “cross-talk” between nuclear receptors [97,98]. In this respect, recent studies have demonstrated that ER-mediated transcriptional activity influences other nuclear receptors [30,31,32]. In this study hepatic protein expression of CYP3A, a PXR target gene was elevated in control chow-fed hPXR mice, suggesting that PXR is activated in these mice [58]. Whether the decreased WAT and hepatic ER α expression observed in hPXR mice is due to suppression of or competition with ER α by overexpressed and activated hPXR or the involvement of a distinct nuclear receptor co-activator/co-repressor of an hPXR-ER α interaction remains to be determined. In this study, the basal hepatic ER α protein levels were decreased in the livers of hPXR mice, and since adipose tissue does not express hPXR, it is possible that the decreased WAT ER α is secondary to the liver effect. Interestingly, CAR, a sister xenobiotic hormone receptor of PXR which is not expressed in adipose tissue, is also able to transduce its metabolic effects in both WAT and BAT [99]. Thus, both hPXR-induced and HFD-induced suppression of ER α protein expression in WAT and liver cause obesity and glucose intolerance in both hPXR and WT mice fed a HFD.

Excessive glucose production by the liver contributes significantly to diabetic hyperglycemia. Mounting evidence indicates that PXR is capable of down-regulating the expression of gluconeogenic genes [34,35,36]. However, the present study demonstrates that hPXR impairs glucose tolerance in the presence of high serum lipids, raising doubts about the anti-diabetogenic actions of PXR [35,36,99]. The current data indicate that induction of the key hepatic gluconeogenic enzymes, PEPCK1 and G6Pase, in hPXR mice is independent of their dietary regimen. Furthermore, HFD-fed hPXR mice became more glucose intolerant than HFD-fed WT mice. HFD-fed hPXR mice also exhibited decreased insulin sensitivity. Supportive of these findings is a recent report indicating a detrimental role of PXR activation by both the human and rodent agonists on postprandial glucose

tolerance [50]. In the liver, glucokinase determines the rate of glucose utilization and glycogen synthesis by catalyzing the phosphorylation of glucose to glucose-6-phosphate [100]. Moreover, glucokinase-deficient mice die with severe hyperglycemia [101]. In this study, HFD increased glucokinase protein expression while inhibiting that of G6Pase in WT mice. In contrast, HFD-induced increases in glucokinase protein levels were inhibited while maintaining the elevated G6Pase protein levels in hPXR mice. We speculate that the hyperglycemia and impaired glucose tolerance seen in HFD-fed hPXR mice were due to reduced levels of glucokinase and lost capacity to use or store glucose in the livers of these mice. Together, these data suggest that much of the insulin resistance, hyperglycemia, and impaired glucose tolerance in HFD-fed hPXR mice can be accounted for by 1) overexpression of PEPCK1, 2) marked induction of G6Pase protein, 3) downregulation of glucokinase, and 4) defective inhibition of PEPCK1 expression by insulin or hPXR.

The current study reveals that female hPXR mice fed a HFD constitute a suitable *in vivo* rodent model to elucidate the molecular mechanisms linking HFD and impaired insulin signaling in women. Our collective observations suggest that the hPXR gene directly or indirectly promotes weight gain and aggravates diet-induced obesity associated with type 2 diabetes in females. Importantly, these findings are in agreement with a recent report by our group and others indicating that ablation of the PXR gene in male mice protects against obesity [45,46]. Considered together, both our previous and current reports indicate that the hPXR genotype is associated with the development of HFD-induced hyperglycemia in both genders [45].

In summary, this report demonstrates that female hPXR mice have markedly reduced basal WAT and hepatic ER α levels but elevated WAT UCP1, hepatic cholesterol, CYP3A11, PEPCK1, and G6Pase protein levels. When subjected to a HFD, hPXR mice display exacerbated diet-induced obesity, hyperinsulinemia, and severely impaired glucose tolerance compared to WT mice subjected to similar diet regimen. Together, these data provide the first evidence that the hPXR genetically engineered in female mice positively regulates obesity, insulin signaling, and glucose metabolism. The impairment of ER α expression in WAT by hPXR and HFD-induced inhibition of both UCP1 in WAT and hepatic glucokinase induction resulting in obesity makes a significant contribution to our understanding of the discrepancy between diet-induced obesity associated metabolic disease in female rodents and women.

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Abbreviations

HFD	high-fat diet
PXR	pregnane X receptor
mPXR	mouse PXR
hPXR	PXR-humanized or human PXR
WT	wild type
WAT	white adipose tissue
BAT	brown adipose tissue
ALT	alanine transaminase
PPARs	peroxisome proliferator-activated receptors
LBD	ligand binding domain
H&E	hematoxylin and eosin
GTT	Glucose tolerance test
IPGTT	intraperitoneal glucose tolerance test
CYP2E1	cytochrome P450 2E1
CYP3A11	cytochrome P450 3A11
AUC	area under the curve
PEPCK1	phosphoenolpyruvate carboxykinase 1
PCN	pregnenolone 16 α -carbonitrile
UCP1	uncoupling protein 1
ERα	estrogen receptor α
ERβ	estrogen receptor β
CAR	constitutive androstane receptor
NEFA	nonesterified free fatty acid
G6Pase	glucose 6-phosphatase

Fig. 1.

Body weight, weight gain, and average food intake of mice during a 16 week control diet or a high-fat diet (HFD) regimen. Female wild type (WT) and hPXR mice were fed a control or a HFD for 16 weeks. Body weight was recorded weekly. The gross energy density in the control diet and the HFD were calculated to be 3.8 and 4.7 Kcal/g, respectively. Time-dependent weight gain in mice maintained on control or a HFD (a); total weight gain (b); and average food intake (c). Data represent mean $\pm$ SEM ($n = 7-11$). $\#P < 0.05$ between mice fed control and HFD. $^{\dagger}P < 0.05$ between WT mice fed a HFD and hPXR mice fed a HFD.

Fig. 2.

Adipose tissue and liver weights in female wild type (WT) and PXR-humanized (hPXR) transgenic mice fed control or a high-fat diet (HFD) for 16 weeks. After 16 weeks on the diet, mice were sacrificed without starvation and sections of white adipose tissue (WAT) comprising [parametrial fat (a), perirenal fat (b), and mesenteric fat (c)], brown adipose tissue (BAT, removed from the back) (d), and liver (e) were rapidly dissected and weighed. Data represent mean $\pm$ SEM (n = 7-11). # P < 0.05 between mice fed control and HFD. † P < 0.05 between WT mice fed a HFD and hPXR mice fed a HFD.

Fig. 3.

Immunoblot analysis of white adipose tissue (WAT) uncoupling protein 1 (UCP1), estrogen receptor α (ER α), and (ER β) in female wild type (WT) and PXR-humanized (hPXR) transgenic mice fed control diet or a HFD for 16 weeks. Cytosolic fractions prepared from frozen WAT (500 mg of parametrial fat) (30 μ g/lane) of mice fed a control diet or a HFD were electrophoresed on 10 or 15% SDS-polyacrylamide, transferred to nitrocellulose membranes, and incubated with anti-UCP1 (a), anti-ER α (b), and anti-ER β (c) antibody as indicated in representative blots. Quantitative analysis of UCP1, ER α , and ER β levels plotted after normalizing with α -tubulin in WT and hPXR mice fed control diet ($\square$) or a HFD ($\blacksquare$) for 16 weeks. The intensities of the bands were quantified using ImageJ software (U. S. National Institutes of Health, Bethesda, MD, USA). Data represent mean $\pm$ SEM of 2 independent experiments from 3-4 mice/group. $^*P < 0.05$ between WT mice fed control diet and hPXR mice fed the control diet. $^{\#}P < 0.05$ between mice fed control and HFD.

Fig. 4.

Serum 17 β -estradiol, insulin, alanine aminotransferase (ALT), cholesterol, and triglyceride, and leptin levels in mice fed control diet or high-fat diet (HFD). Female wild type (WT) and PXR-humanized (hPXR) transgenic mice were fed control diet (□) or a HFD (■) for 16 weeks. 17 β -estradiol (a), insulin (b), ALT (c), cholesterol (d), triglyceride (e), and leptin (f) levels in serum were determined as described in Materials and Methods. Data represents means $\pm$ SEM (n = 5-6). * P < 0.05 between WT mice fed control diet and hPXR mice fed the control diet. # P < 0.05 between mice fed control and HFD. † P < 0.05 between WT mice fed a HFD and hPXR mice fed a HFD.

Fig. 5. Immunoblot analysis of hepatic cytochrome P450 (CYP) 3A11, CYP2E1, and estrogen receptor α (ER α) in female wild type (WT) and PXR-humanized (hPXR) transgenic mice fed control diet or a HFD for 16 weeks. Liver homogenate (40 μ g/lane) prepared from mice fed control diet or a HFD were electrophoresed on 10% SDS-polyacrylamide, transferred to nitrocellulose membranes, and incubated with anti-CYP3A11 (a), anti-CYP2E1 (b) or anti-ER α (c), as indicated in representative blots. Quantitative analysis of CYP3A11, CYP2E1, and ER α protein levels plotted after normalizing with α -tubulin in WT and hPXR mice fed control diet (□) or a HFD (■) for 16 weeks. The intensities of the bands were quantified using ImageJ software (U. S. National Institutes of Health, Bethesda, MD, USA). Data represent mean $\pm$ SEM of 2 independent experiments from 3-4 mice/group. $^*P < 0.05$ between WT mice fed control diet and hPXR mice fed the control diet. $^{\#}P < 0.05$ between

mice fed control and HFD, $^{\dagger}P < 0.05$ between WT mice fed a HFD and hPXR mice fed a HFD.

Fig. 6.

Hepatic cholesterol, triglyceride and nonesterified fatty acid (NEFA) levels in female wild type (WT) and PXR-humanized (hPXR) transgenic mice fed control or a high-fat diet (HFD) for 16 weeks. Hepatic cholesterol levels (a), hepatic triglyceride levels (b), and hepatic NEFA levels (c), from different treatment groups were performed as described in Materials and Methods. Data represents means $\pm$ SEM ($n = 5-6$). * $P < 0.05$ between WT mice fed control diet and hPXR mice fed the control diet. # $P < 0.05$ between mice fed control and HFD. † $P < 0.05$ between WT mice fed a HFD and hPXR mice fed a HFD.

Fig. 7. Blood glucose levels during glucose tolerance test (GTT) in mice fed control or HFD. Female wild type (WT) and PXR-humanized (hPXR) transgenic mice were fed control diet or a HFD for 16 weeks. GTT was performed after mice (WT and hPXR) had been on the control diet or HFD for 15 weeks ($n = 6/\text{group}$). Intraperitoneal glucose tolerance test (IPGTT) was conducted in overnight feed-deprived conditions (12-15 hours of starvation) and mice were intraperitoneally (i.p.) injected a single dose of D-glucose (10% solution in water, 10 mL/kg). Glucose levels were measured from blood collected from the tail vein immediately before and after 15, 30, 60, 90, and 120 minutes using Contour TS blood glucose meter (Bayer HealthCare LLC, Mishawaka, IN), an instrument that reads blood glucose levels (a). Area under the curve (AUC) for the GTT were calculated using Sigma Plot 12.0 (Systat Software Inc, San Jose, CA), to estimate glucose tolerance in corresponding mice (b). [#] $P < 0.05$ between mice fed control and HFD. [†] $P < 0.05$ between WT mice fed a HFD and hPXR mice fed a HFD.

Fig. 8.

Immunoblot analysis of hepatic PEPCK1, glucose 6-phosphatase (G6Pase), and glucokinase levels in female wild type (WT) and PXR-humanized (hPXR) transgenic mice fed control diet (□) or a HFD (■) for 16 weeks. Liver homogenate (40 µg/lane) from mice fed control diet or a HFD were electrophoresed on 10% SDS-polyacrylamide, transferred to nitrocellulose membranes, and incubated with anti-PEPCK1, anti-G6Pase, or anti-glucokinase antibody as indicated in representative blots (a, b and c). Quantitative analysis of PEPCK1 protein after normalizing with α-tubulin (a), G6Pase protein after normalizing with α-tubulin (b) and glucokinase protein after normalizing with α-tubulin (c) in WT and hPXR mice fed control diet or a HFD for 16 weeks. The intensities of the bands were quantified using ImageJ software (U. S. National Institutes of Health, Bethesda, MD). Data represent mean ± SEM of 2 independent experiments from 3-4 mice/group. **P* < 0.05 between WT mice fed control diet and hPXR mice fed the control diet. #*P* < 0.05 between

mice fed control and HFD, $^{\dagger}P < 0.05$ between WT mice fed a HFD and hPXR mice fed a HFD.