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# **Effects of Adipocyte Aryl Hydrocarbon Receptor Deficiency on PCB-Induced Disruption of Glucose Homeostasis in Lean and Obese Mice**

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**Running title:** Adipocyte AhRs mediate PCB-induced diabetes

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## Abstract

**Background:** Coplanar polychlorinated biphenyls (PCBs) promote adipocyte inflammation and impair glucose homeostasis in lean mice. When lipophilic PCBs were administered to obese mice the diabetes promoting effects were observed only during weight loss. The molecular mechanisms linking PCB exposures to impaired glucose metabolism are unclear.

**Objectives:** This study tested the hypothesis that coplanar PCBs act at adipocyte aryl hydrocarbon receptors (AhRs) to promote adipose inflammation and impair glucose homeostasis in lean and in obese mice during weight loss.

**Methods and Results:** PCB-77 administration impaired glucose and insulin tolerance in LF-fed control (*AhR<sup>fl/fl</sup>*), but not in adipocyte AhR deficient mice (*AhR<sup>AdQ</sup>*). Unexpectedly, *AhR<sup>AdQ</sup>* mice exhibited increased fat mass when fed standard, low fat (LF) or high fat (HF) diet. When fed a HF diet both genotypes became obese, but *AhR<sup>AdQ</sup>* mice administered vehicle (VEH) exhibited increased body weight, adipose mass, adipose inflammation, and impaired glucose tolerance compared to *AhR<sup>fl/fl</sup>* controls. Impairment of glucose homeostasis in response to PCB-77 was not observed in obese mice of either genotype. However, upon weight loss, *AhR<sup>fl/fl</sup>* mice administered PCB-77 exhibited increased adipose tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) mRNA abundance and impaired glucose homeostasis compared to VEH. In contrast, PCB-77 had no effect on TNF- $\alpha$  or glucose homeostasis in *AhR<sup>AdQ</sup>* mice exhibiting weight loss.

**Conclusions:** These results demonstrate that adipocyte AhR mediates PCB-induced adipose inflammation and impairment of glucose homeostasis in mice. Moreover, deficiency of AhR in adipocytes augmented the development of obesity, indicating that endogenous ligand(s) for AhR regulate adipose homeostasis.

## Introduction

The aryl-hydrocarbon receptor (AhR) has established roles in toxicology and phase I drug metabolism (Tijet et al. 2006). Moreover, since mice with whole body AhR deficiency exhibit organ abnormalities (Abbott et al. 1999; Fernandez-Salguero et al. 1997; Harstad et al. 2006; Lahvis et al. 2005; Vasquez et al. 2003), endogenous ligand activation of this receptor has been implicated in the control of proliferation and/or differentiation of various cell types. Several studies have demonstrated marked sequestration of xenobiotic ligands of AhR, including lipophilic coplanar polychlorinated biphenyls (PCBs) (Brown and Lawton 1984; Fukano and Doguchi 1977; McFarland and Clarke 1989), in adipose tissue. Rather than serve as an inert storage reservoir for PCBs (Bourez et al. 2012; Bourez et al. 2013), adipocyte AhR activation by coplanar PCB AhR ligands promoted adipose inflammation (Arsenescu et al. 2008; Kim et al. 2012). In addition, administration of coplanar PCBs to lean mice impaired glucose and insulin tolerance, and these effects were abolished by an AhR antagonist (Baker et al. 2013b). Impaired glucose homeostasis in mice exposed to coplanar PCBs was associated with an adipose-specific increase in expression of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), a cytokine linked to impairment of insulin-stimulated glucose uptake. These results suggest that in addition to serving as a storage reservoir, adipocytes respond to PCBs to promote inflammation and negatively influence glucose homeostasis.

Due to bio-accumulation in adipose lipids, the total body burden of PCBs is increased in obese rodents and humans (Kim et al. 2011; Myre and Imbeault 2014; Pelletier et al. 2003).

Interestingly, in contrast to lean mice where administration of PCB-77 impaired glucose and insulin tolerance (Baker et al. 2013b), when PCB-77 was administered to mice made obese from

consumption of a high fat (HF) diet there was no effect on glucose or insulin tolerance. In contrast, during periods of weight loss, PCB concentrations in adipose tissue decrease and serum concentrations of PCBs increase (Chevrier et al. 2000; Irigaray et al. 2006). Moreover, when obese mice administered PCB-77 were subjected to weight loss, they exhibited impairments in glucose and insulin tolerance that blunted the beneficial effects of weight loss (Baker et al. 2013b). One possible explanation for this observation is that hydrophobic PCBs are released upon lipolysis within adipocytes during weight loss to act systemically, or alternately interact with the cytosolic AhR within adipocytes.

In this study, we hypothesized that coplanar PCBs promote insulin resistance and impair glucose homeostasis through adipocyte-specific AhR activation. To test this hypothesis, we generated mice with adipocyte AhR deficiency. Since coplanar PCBs impair glucose homeostasis in lean mice, we defined the role of adipocyte AhR on PCB-induced impairment of glucose and insulin tolerance in mice fed a low fat (LF) diet. Moreover, since the bio-accumulation of lipophilic PCBs is altered with obesity and upon weight loss, we examined the role of adipocyte AhR during the development of HF diet-induced obesity and during weight loss in obese mice previously exposed to PCB-77.

## **Materials and Methods**

### **Chemicals**

3,3',4,4'-tetrachlorobiphenyl (PCB-77) was purchased from AccuStandard Inc. (New Haven, CT).

## **Animal treatments and sample collection**

All experiments met the approval of the Animal Care and Use Committee of the University of Kentucky. Animals used in this study were treated humanely and with regard for the alleviation of suffering. Mice were maintained in Individually Ventilated Cages (Tecniplast Sealsafe Plus Mouse Green Line) with an automated water system (ad libitum; Endstrom), with aspen wood chip bedding (Harlan Teklad Sani-Chips) and cotton nesting squares (Neslet from Ancare) containing a small amount of shredded paper (Enviro-Dri; Shepherd Specialty Papers) for enrichment. The light/dark cycle was 14/10 hours, respectively, with a temperature of 70°C (plus or minus 2°C) and humidity ranging from 30-70%. AhR-floxed ( $AhR^{fl/fl}$ ) mice with loxP sites flanking exon 2 were a generous gift of Dr. Mary Walker, University of New Mexico (Agbor et al. 2011). Female  $AhR^{fl/fl}$  mice were bred to hemizygous transgenic male Cre mice under control of an adiponectin/promoter/enhancer (B6;FVB-Tg(Adipoq-cre)1Evdr/J; The Jackson Laboratory). Male  $AhR^{fl/fl}$  littermate controls and  $AhR^{AdQ}$  mice were used in all studies. There were no overt differences in health or appearance between genotypes at the start of the study. Mice of each genotype were randomly assigned to study groups for each specific study. A total of 86 mice were used in these studies, of which  $n = 37$  were  $Ahr^{fl/fl}$ , and  $n = 49$  were  $Ahr^{AdQ}$ . Of the total 86 mice,  $n = 27$  were administered vehicle (VEH, tocopherol-stripped safflower oil), and  $n = 28$  mice were administered PCB-77.

In studies confirming the efficiency and specificity of adipocyte AhR deletion, male mice of each genotype ( $n = 5$ /genotype) were fed standard mouse diet (Harlan Teklad 2918 Global Rodent Diet; irradiated) ad libitum from weaning to 2 months of age.

In studies examining lean mice, male mice (2 months of age) of each genotype were administered VEH or PCB-77 (50 mg/kg; by oral gavage at approximately 10am given as two separate doses over 2 weeks;  $n = 3-8$  mice/group (Baker et al. 2013b) and fed a LF diet (10% kcal as fat, D12450B; Research Diets) ad libitum. Mice in each group were examined 48 hours after the last administered dose (week 2).

For studies on obese mice, mice (2 months of age) were fed a HF diet (60% kcal as fat, D12492; Research Diets) ad libitum for 12 weeks to promote the development of obesity. Mice of each genotype were administered VEH or PCB-77 (50 mg/kg; by oral gavage at approximately 10am given as 4 doses in weeks 1, 2, 9, and 10;  $n = 6-8$  mice/group). For weight loss studies, after 12 weeks of HF feeding, mice of each genotype and treatment group were placed on LF diet for 4 weeks to induce weight loss.

For all studies, body weights were quantified weekly at approximately 9am. At study end point (approximately 6am), mice were transferred to the investigator's laboratory using an stainless steel cart, and then anesthetized [ketamine/xylazine, 10/100 mg/kg, by intraperitoneal (ip) injection beginning at approximately 11am] for exsanguination and tissue harvest (liver, subcutaneous adipose (SubQ), retroperitoneal adipose (RPF), epididymal adipose (EF), interscapular brown adipose tissue (BAT), kidney, brain, heart)).

### **Measurement of body composition**

Body composition of mice was determined by nuclear magnetic resonance spectroscopy [EchoMRI (magnetic resonance imaging)] at baseline (before diet administration) and at study endpoint. Briefly, conscious mice were placed in clear, cylindrical plastic tube (sized by animal weight) at approximately 10am. The tubes have holes for breathing and are maintained in a

horizontal plane during the procedure. Three sequential scans (taking approximately 2 minutes each) are conducted for each animal. The scanner applies an external magnetic field at a level of 5-gauss beyond the surface of the system in a 9 inch radius from the edge of the animal holder. An equal field is present on the opposite side of the system. Following completion of the scan the tubes are cleaned with soap and water using a long bottle brush. For this measurement, mice were transported using a stainless steel cart to a procedure room within the Division of Laboratory Animal Resources (DLAR), and then returned to the room where they were housed using the same cart.

### **Glucose (GTT) and insulin (ITT) tolerance tests**

Mice were fasted for 4 or 6 hours for ITT or GTT, respectively, and blood glucose concentrations were measured by tail vein using a hand held glucometer (Freedom Freestyle Lite, Abbott Laboratories). For GTT, mice were transferred to a procedure room within the DLAR, injected i.p. with D-glucose (Sigma, 20% in saline, 10  $\mu\text{L/g}$  of body weight at approximately 1pm) and blood glucose concentrations were quantified at 0, 15, 30, 60, 90, and 120 minutes. For ITT, mice were transferred to a procedure room within DLAR, injected i.p. with human insulin (Novolin, 0.0125  $\mu\text{M}$  in saline/g of body weight at approximately 2pm), and blood glucose concentrations were quantified at 0, 30, 60, 90, and 120 minutes. Total area under the curve (AUC; arbitrary units) was calculated as previously described (Baker et al. 2013b).

### **Quantification of PCB-77 and hydroxylated metabolites in tissues**

Tissue samples (EF, liver) were weighed and homogenized in  $\text{dH}_2\text{O}$ . Cold acetonitrile and 50  $\mu\text{L}$  of internal standard (10  $\mu\text{M}$   $^{13}\text{C}$  labeled d6-PCB-77) were added to homogenates which were

vortexed, sonicated, and centrifuged at 15,000 rpm. Supernatants were transferred to glass vials and pellets re-extracted with cold acetonitrile and the process repeated twice. Pooled supernatants were dried under nitrogen and reconstituted in 100  $\mu$ L of 99/1 methanol/dH<sub>2</sub>O containing 0.5% formic acid and 0.1% ammonium formate. PCB-77 and hydroxy-PCB-77 were measured using LC-MS/MS as described previously (Baker et al. 2013b).

### **Extraction of RNA and quantification of mRNA abundance using real-time polymerase chain reaction**

Total RNA was extracted from tissues (liver, adipose, soleus muscle) using the SV Total RNA Isolation System kit (Promega Corporation), per the manufacturer's instructions. RNA concentrations were determined using NanoDrop 2000 spectrophotometer (Thermo Scientific). cDNA was synthesized from 0.4  $\mu$ g total RNA with qScript cDNA SuperMix (Quanta Biosciences) in the following reaction: 25°C for 5 minutes, 42°C for 30 minutes, and 85°C for 5 minutes. The cDNA was diluted to 0.4 ng/ $\mu$ L and amplified with an iCycler (Bio-Rad) and the Perfecta SYBR Green Fastmix for iQ (Quanta Biosciences). Using the difference from GAPDH rRNA (reference gene) and the comparative Ct method, the relative quantification of gene expression in each sample was calculated. Primers (Eurofins MWG Operon) were designed using the primer design program available from PubMed.gov (sequences presented in Table 1). The PCR reaction was as follows: 94°C for 5 minutes, 40 cycles at 94°C for 15 seconds, 58°C or 64°C (based on tested primer efficiency) for 40 seconds, 72°C for 10 minutes, and 100 cycles from 95°C to 45.5°C for 10 seconds. Primer sequences were as follows: *AhR*, forward 5'-GACCAAACACAAGCTAGACTTCACACC, reverse 5'-CAAGAAGCCGGAAACTGTCATGC; *CYP1A1*, forward 5'-AGTCAATCTGAGCAATGAGTTTGG-3', reverse 5'-GGCATCCAGGGAAGAGTTAGG-3';

*GAPDH*, forward 5'-GCCAAAAGGGTCATCATCTC-3', reverse 5'-GGCCATCCACAGTCTTCT-3'; *TNF- $\alpha$* , forward 5'-CCCACTCTGACCCCTTACTC-3', reverse 5'-TCACTGTCCCAGCATCTTGT-3; F4/80, Forward 5'-CTTTGGCTATGGGCTTCCAGTC-3', Reverse 5'-GCAAGGAGGACAGAGTTTATCGTG-3''.

### **Quantification of adipocyte size and cell number**

Sections of formalin (10% wt/vol) fixed pieces of subcutaneous adipose tissue were stained with hematoxylin and eosin. Images of slides were taken at 10 $\times$  magnification. Via use of the “detect edges” setting, image threshold, and object count features of NIS Elements software (Nikon Instruments, Inc.), the area of each adipocyte and the number of adipocytes within a 700  $\times$  700  $\mu$ m measurement frame were quantified. Adipocyte size was calculated on three measurement frames within each section of adipose tissue (n = 3 sections/mouse) from mice in each group (n = 3 mice/group).

### **Differentiation of adipocytes from the stromal vascular fraction (SVF) of subcutaneous adipose tissue**

Subcutaneous adipose tissue was taken from the inguinal region, minced, and incubated in basal medium (Zenbio) supplemented with collagenase (1 mg/ml) and penicillin/streptomycin mixture (5%) for 1 h with shaking at 37°C as previously described (Rodbell 1964). Two days after SVF cells had reached 100% confluence, media was changed to differentiation medium (OM-DM; Zenbio) and changed every other day for 8 days. Cells were harvested for RNA using TRIzol; cDNA synthesis and real-time PCR were performed as described above. The stromal vascular fraction (SVF) was harvested at 100% confluence prior to addition of the adipocyte differentiation medium, and then again at day 8 after differentiation (Adipo fraction).

## Statistical analysis

Data are represented as mean  $\pm$  SEM. Data were log transformed prior to statistical analysis. A two-way analysis of variance (ANOVA; SigmaPlot, version 12.0; Systat Software Inc., Chicago, IL) was used to determine statistical significance, which was defined as  $p < 0.05$ . Glucose and insulin tolerance tests were analyzed using repeated measure, two-way ANOVA. Holm-Sidak method was used for post-hoc analyses.

## Results

### Generation and characterization of mice with adipocyte AhR deficiency

To confirm effective and specific deletion of exon 2 of AhR in adipocytes, AhR mRNA abundance was quantified in adipose tissues (SubQ, RPF, EF, BAT), liver, brain, heart, and kidney from mice fed standard laboratory diet (2 months of age). AhR mRNA abundances were not significantly different in liver, kidney, or brains from *AhR<sup>fl/fl</sup>* compared to *AhR<sup>AdQ</sup>* mice (Figure 1A). In heart, AhR mRNA abundance was reduced in *AhR<sup>AdQ</sup>* compared to *AhR<sup>fl/fl</sup>* mice, but did not meet criteria for statistical significance (Figure 1A;  $p = 0.17$ ). In retroperitoneal (RPF) white and brown adipose tissue (BAT) of *AhR<sup>AdQ</sup>* mice, AhR mRNA abundance was significantly decreased compared to *AhR<sup>fl/fl</sup>* mice (Figure 1A;  $P < 0.05$ ). Since adipose tissue is heterogeneous in cell types, preadipocytes were differentiated from the SVF of subcutaneous adipose tissue of mice from each genotype. AhR mRNA abundance was not significantly altered in preadipocytes from *AhR<sup>AdQ</sup>* mice prior to differentiation (SVF, Figure 1B). In contrast, AhR mRNA abundance was significantly reduced in fully differentiated adipocytes from *AhR<sup>AdQ</sup>* mice compared to *AhR<sup>fl/fl</sup>* controls (Adipo fraction, Figure 1B;  $P < 0.05$ ).

Unexpectedly, two month old male *AhR<sup>AdQ</sup>* mice fed standard mouse diet exhibited significantly increased fat mass and reduced lean mass compared to age-matched *AhR<sup>fl/fl</sup>* controls (Table 1). Additionally, *AhR<sup>AdQ</sup>* mice had significantly larger visceral adipose depots (epididymal and retroperitoneal fat;  $P < 0.05$ ) and moderately larger subcutaneous adipose depots ( $p = 0.45$ ; Table 1). However, these effects did not result in significant differences in body weights between genotypes (Table 1). Because differences in body fat deposition could potentially affect glucose homeostasis, we conducted baseline glucose and insulin tolerance tests. There was no significant difference in glucose or insulin tolerance between genotypes in *AhR<sup>AdQ</sup>* mice compared to *AhR<sup>fl/fl</sup>* controls (see Supplemental Material, Figure S1A,B).

#### **Effect of adipocyte AhR deficiency on insulin tolerance in lean mice acutely exposed to PCB-77**

We previously demonstrated that lean mice exposed to 4 divided doses of PCB-77 develop impaired insulin tolerance within 48 hours after the last dose (Baker et al. 2013b). Thus, we defined effects of adipocyte AhR deficiency on acute PCB-77-induced dysregulation of insulin tolerance in mice fed a LF diet (Figure 2A,B). Body weights were not significantly different between groups at study endpoint (*AhR<sup>fl/fl</sup>*, VEH,  $28.1 \pm 0.5$ ; *AhR<sup>fl/fl</sup>*, PCB-77,  $27.9 \pm 0.5$ ; *AhR<sup>AdQ</sup>*, VEH,  $25.5 \pm 0.5$ ; *AhR<sup>AdQ</sup>*, PCB-77,  $27.1 \pm 0.5$  g;  $P > 0.05$ ). In mice administered VEH, deficiency of AhR in adipocytes resulted in a modest, but significant improvement in insulin tolerance, as indicated by a significant reduction in blood glucose concentrations at 90 and 120 minutes post-insulin administration, and by a significant reduction in the AUC compared to *AhR<sup>fl/fl</sup>* controls (Figure 2A,B;  $P < 0.05$ ). Notably, administration of PCB-77 significantly impaired insulin tolerance in *AhR<sup>fl/fl</sup>*, but not in *AhR<sup>AdQ</sup>* mice (Figure 2A,B;  $P < 0.05$ ).

## Effect of adipocyte AhR deficiency on the development of obesity, body fat distribution, and glucose homeostasis

Since obesity has been reported to increase the total body burden of lipophilic PCBs (Brown and Lawton 1984; Fukano and Doguchi 1977; McFarland and Clarke 1989), we examined effects of adipocyte AhR deficiency on the development of HF diet-induced obesity and glucose homeostasis in mice of each genotype administered VEH or PCB-77. When challenged with a HF diet, adipocyte AhR deficient mice administered VEH had significantly increased body weights, reduced lean mass, and increased fat mass compared to *AhR<sup>fl/fl</sup>* controls (Figure 3A-C;  $P < 0.05$ ). Moreover, excess adiposity in HF-fed *AhR<sup>AdQ</sup>* mice administered VEH was deposited subcutaneously, with significant increases in subcutaneous adipose tissue mass (*AhR<sup>fl/fl</sup>*,  $3.8 \pm 0.5$ ; *AhR<sup>AdQ</sup>*,  $5.5 \pm 0.4$ ;  $P < 0.05$ ; representative images in Figure 3D) and adipocyte size compared to *AhR<sup>fl/fl</sup>* controls (Figure 4A,B). However, these effects were not observed in *AhR<sup>AdQ</sup>* mice administered PCB-77 (Figure 4A). Moreover, in mice administered VEH, mRNA abundance of F4/80, a macrophage marker, was significantly increased in epididymal adipose tissue of *AhR<sup>AdQ</sup>* mice compared to *AhR<sup>fl/fl</sup>* controls, regardless of treatment group (VEH groups: *AhR<sup>fl/fl</sup>*,  $1.62 \pm 0.67$ ; *AhR<sup>AdQ</sup>*,  $3.52 \pm 0.68$ ;  $P < 0.05$ ; PCB-77 groups: *AhR<sup>fl/fl</sup>*,  $1.29 \pm 0.49$ ; *AhR<sup>AdQ</sup>*,  $2.12 \pm 0.68$   $\Delta\Delta\text{Ct}$ ;  $P < 0.05$ ). In livers or soleus muscle from HF-fed mice administered VEH, there was no significant effect of genotype on AhR mRNA abundance (Supplemental Figure S2;  $p = 0.06$ ). However, administration of PCB-77 resulted in a significant increase in AhR mRNA abundance in livers, but not in soleus muscle from HF-fed mice of each genotype, with no significant differences between genotypes (Supplemental Figure S2;  $P < 0.05$ ).

Consistent with previous findings (Baker et al. 2013b), PCB-77 had no significant effect on glucose tolerance in obese *AhR<sup>fl/fl</sup>* or *AhR<sup>AdQ</sup>* mice (Supplemental Figure S3A). At 12 weeks of

HF feeding in mice of each genotype administered PCB-77, adipose tissue concentrations of PCB-77 were considerably higher than those of its metabolite, hydroxy-PCB-77 (weight gain phase, Supplemental Figure S3B and S3C). Moreover, adipose concentrations of PCB-77 were significantly increased in *AhR<sup>AdQ</sup>* mice compared to *AhR<sup>fl/fl</sup>* controls. In contrast, adipose concentrations of hydroxy-PCB-77 were not significantly different between genotypes (Supplemental Figure 3C).

### **Effect of adipocyte AhR deficiency on PCB-77-induced impairments of glucose and insulin tolerance in obese mice exhibiting weight loss**

Previous studies demonstrated that when obese mice lost weight, benefits of weight loss to improve glucose and insulin tolerance were diminished in mice exposed to PCB-77 during the weight gain phase of diet-induced obesity (Baker et al. 2013b). Thus, obese *AhR<sup>fl/fl</sup>* and *AhR<sup>AdQ</sup>* mice administered VEH or PCB-77 during the weight gain phase (12 weeks of HF feeding) were made to lose weight by switching to a LF diet (4 weeks). Despite larger body weights of HF-fed *AhR<sup>AdQ</sup>* mice administered VEH compared to *AhR<sup>fl/fl</sup>* controls prior to weight loss (Figure 3), after 4 weeks of feeding the LF diet there were no significant differences in body weights between groups (Supplemental Figure S4).

As previously demonstrated (Baker et al. 2013b), upon weight loss *AhR<sup>fl/fl</sup>* mice administered PCB-77 exhibited significantly impaired glucose and insulin tolerance compared to VEH (Figure 5A-D;  $P < 0.05$ ). In contrast, PCB-77 had no effect on glucose or insulin tolerance in *AhR<sup>AdQ</sup>* mice experiencing weight loss. In response to weight loss, concentrations of PCB-77 in adipose tissue decreased significantly in both genotypes (Supplemental Figure S3B;  $P < 0.05$ ). While concentrations of hydroxy-PCB-77 increased in adipose tissue of both genotypes of mice that

experienced weight loss, levels of the PCB metabolite were significantly decreased in adipose tissue from *AhR<sup>AdQ</sup>* mice compared to *AhR<sup>fl/fl</sup>* controls (Supplemental Figure S3C; P<0.05).

Previous studies demonstrated that administration of PCB-77 during the weight gain phase of diet-induced obesity had no effect on adipose inflammation or glucose homeostasis presumably due to sequestration of the lipophilic toxin in expanded adipose lipids (Baker et al. 2013b).

However, when obese mice previously exposed to PCB-77 lost weight, the benefits of weight loss to reduce adipose mRNA abundance of TNF- $\alpha$  and to improve glucose tolerance were mitigated (Baker et al. 2013b). These results suggest that upon weight loss, PCB-77 is released from expanded adipose lipids of obese mice to promote AhR-mediated adipose inflammation and to impair glucose tolerance, blunting beneficial effects of weight loss. We quantified mRNA abundance of AhR, CYP1A1 (as a marker of AhR activation) and TNF- $\alpha$  (as a marker of inflammation) in adipose tissue of obese mice experiencing weight loss (mice were exposed to PCB-77 during the weight gain phase of diet-induced obesity). In mice administered PCB-77, AhR mRNA abundance in adipose tissue was significantly decreased in *AhR<sup>AdQ</sup>* mice compared to *AhR<sup>fl/fl</sup>* mice (Figure 6A; P<0.05). Similarly, in adipose tissue of *AhR<sup>fl/fl</sup>* mice administered PCB-77, CYP1A1 mRNA abundance was markedly increased, indicative of AhR activation (Figure 6B; P<0.05). In contrast, PCB-77 had no effect on CYP1A1 mRNA abundance in adipose tissue from mice with adipocyte AhR deficiency. Finally, mRNA abundance of TNF- $\alpha$  was significantly increased in adipose tissue of *AhR<sup>fl/fl</sup>* mice administered PCB-77 compared to VEH, with no effect of the toxin on TNF- $\alpha$  mRNA abundance in adipose tissue of *AhR<sup>AdQ</sup>* mice (Figure 6C; P<0.05).

## Discussion

Results from these studies demonstrate a pivotal role for adipocyte AhRs in the regulation of adiposity and regional adipose deposition, and in the effects of lipophilic coplanar PCBs to impair glucose homeostasis in mice. Due to their lipophilicity, PCBs bio-accumulate in adipose lipids. Our results support the concept that adipose tissue is not simply an inert reservoir of these toxins. Rather, bio-accumulation of PCBs within adipocyte lipids, with dynamic re-distribution of PCBs upon lipolysis (e.g., with weight loss), results in activation of cytoplasmic AhR to impair glucose utilization and/or insulin signaling. Indeed, adipocyte AhR deficiency totally abolished acute effects of coplanar PCBs to impair insulin tolerance in lean mice, and also abolished effects of PCBs to impair glucose homeostasis in obese mice experiencing weight loss. Moreover, we report an unexpected phenotype of increased body weight and adiposity of HF-fed mice that lack AhR expression in adipocytes. Interestingly, with increased adiposity, mice with adipocyte AhR deficiency exhibited significant subcutaneous adipose deposition. Despite subcutaneous adipose tissue deposition, overall increases in adiposity resulted in pronounced adipose inflammation and more significant impairments of glucose homeostasis in HF-fed adipocyte AhR deficient mice. Since these effects were observed in HF-fed adipocyte AhR mice that were not exposed to environmental toxins, these results suggest that an endogenous ligand(s) of AhR plays a role in the regulation of regional adiposity, adipose inflammation and glucose homeostasis. Moreover, these results demonstrate that adipocyte AhRs mediate effects of coplanar PCBs to impair glucose homeostasis in lean and in obese mice experiencing weight loss.

Previous studies in our laboratory demonstrated that coplanar PCB ligands of the AhR promoted inflammation of mature murine 3T3-L1 adipocytes (Arsenescu et al. 2008; Baker et al. 2013a; Baker et al. 2013b). Similarly, exogenous AhR ligands such as dioxin, as well as coplanar PCBs, promoted expression of a variety of inflammatory genes in human adipocytes (Kim et al. 2012). Moreover, administration of dioxin to C57BL/6 mice increased adipose tissue inflammation (Kim et al. 2012). Results from this study demonstrate that deficiency of adipocyte AhR promotes adipose inflammation, including infiltration of macrophages into adipose tissue (as detected by F4/80 expression). Moreover, these results extend previous findings by demonstrating that a coplanar PCB acts at adipocyte AhR to increase expression of the proinflammatory cytokine, TNF- $\alpha$ , in adipose tissue as well as to impair insulin resistance in C57BL/6 mice. Notably, acute effects of PCB-77 to impair glucose homeostasis in lean mice, and in obese mice experiencing weight loss, were abolished in mice with adipocyte AhR deficiency. These results demonstrate that PCB-induced alterations in glucose homeostasis are mediated through adipocyte AhR. Selective effects of lipophilic toxins such as PCBs at adipocyte AhR may relate to their marked bio-accumulation within adipose lipids (Bourez et al. 2012; Bourez et al. 2013).

An unexpected finding of the present study was an increase in body weight, adiposity, and impaired glucose homeostasis in adipocyte AhR deficient mice fed a HF diet (e.g., vehicle group). Anorexia and/or body wasting have been described as toxic manifestations of dioxin exposure (Tuomisto et al. 1995). In addition, dioxin has been demonstrated to reduce differentiation of 3T3-L1 adipocytes in an AhR-dependent manner (Alexander et al. 1998). However, effects of AhR activation appear to be concentration-dependent, as previous studies in our laboratory demonstrated that low concentrations (< 0.1 nM) of dioxin, as well as coplanar

PCBs (3.4  $\mu$ M), promote differentiation of 3T3-L1 adipocytes while higher concentrations of these AhR ligands (10 nM and 34  $\mu$ M, respectively) inhibited adipocyte differentiation (Arsenescu et al. 2008). In addition to regulation of adipocyte differentiation, mouse embryonic fibroblasts (either as preadipocytes or differentiated to adipocytes) from mice with whole body AhR deficiency exhibited elevated triglyceride synthesis (Alexander et al. 1998). Results from the present study demonstrate that in the setting of diet-induced obesity, deficiency of adipocyte AhR results in increased adipocyte size and adiposity. It is unclear if adipocyte AhR deficiency stimulated differentiation of new adipocytes (Arsenescu et al. 2008), or enhanced triglyceride synthesis to promote adiposity. Moreover, AhR-mediated regulation of body weight may result from the combined effects of activation of AhR in various cell types, as congenic mice with high AhR signaling activity were more susceptible to diet-induced obesity compared to those with low AhR activity (Kerley-Hamilton et al. 2012). Further studies are warranted to define cell-specific mechanisms for AhR regulation of adipose mass and body weight.

In addition to an increase in overall adiposity and body weight when challenged with a HF diet, adipocyte AhR deficient male mice exhibit enhanced deposition of subcutaneous adipose tissue. Overall increases in adiposity of obese adipocyte AhR deficient mice resulted in more pronounced impairments of glucose homeostasis. Estrogen has been associated with a gynoid lower body distribution of adipose tissue (subcutaneously) in females, while androgen may contribute to more upper body (visceral) adipose deposition in males. The AhR interacts with estrogen and its receptors through various mechanisms, including potential estrogenic effects in the absence of estrogen, versus anti-estrogenic effects in the presence of estrogen (Brunnberg et al. 2011). It is possible that alterations in adipose deposition in HF-fed adipocyte AhR deficient mice may result from either an estrogenic or anti-androgenic effect to promote gynoid adipose

deposition. Further studies are required to define the basis of changes in regional adiposity in adipocyte AhR deficient mice fed a HF diet.

Coplanar PCBs are substrates for CYP1A1, a target gene of AhR activation, resulting in the production of water soluble hydroxylated metabolites that are more readily excreted (Toborek et al. 1995). Results from this study demonstrate that during the weight gain phase of diet-induced obesity, PCB-77 is not readily metabolized to hydroxy-metabolites in adipocytes. This is most likely the result of sequestration of the parent toxin to adipocyte triacylglycerol droplets, where CYP1A1 does not have ready access to the substrate. Similar to previous reports (Kim et al. 2011) levels of PCB-77 in adipose tissue were increased in adipocyte AhR deficient mice exhibiting increased body weight and adiposity. It is possible that elevations in adipose levels of the parent toxin, PCB-77, contributed to an ability of the toxin to act at other target sites important in the control of body weight, adipose mass and the regulation of glucose homeostasis. For example, in the present study adipocyte AhR deficient mice exposed to PCB-77 did not exhibit increased adiposity or body weight, suggesting that increased levels of the parent toxin acted at AhR on other cell types to protect against obesity and impaired glucose homeostasis. In support, exposure of C57BL/6 mice fed a very HF diet to persistent organic pollutants that included AhR ligands resulted in paradoxical improvements in insulin and glucose tolerance (Ibrahim et al. 2011). As previously indicated (Ibrahim et al. 2011) the interplay between dietary nutrients and environmental pollutants is complex; however, our results suggest that actions of PCBs at adipocyte AhR mediate harmful effects of the toxin to impair glucose homeostasis. In the present study, upon weight loss, levels of the hydroxy-PCB-77 metabolite in adipose tissue increased markedly in *AhR*<sup>fl/fl</sup> mice, and were associated with a marked induction of adipose

CYP1A1. In contrast, adipose tissue from mice with adipocyte AhR deficiency experiencing weight loss did not exhibit increased abundance of CYP1A1, which most likely contributed to reduced adipose levels of the hydroxy-PCB-77 metabolite. These results demonstrate that CYP1A1 induction by PCB-77 in adipose tissue, mediated by adipocyte AhR, are associated with increased levels of hydroxy-PCB-77.

## **Conclusions**

Results from this study demonstrate that coplanar PCBs act at adipocyte AhR to promote adipose inflammation and impair glucose homeostasis in lean mice and in obese mice experiencing weight loss. Moreover, results demonstrate a previously unappreciated role for adipocyte AhR to regulate adiposity, adipose inflammation, body weight, and glucose homeostasis in mice with diet-induced obesity. These results suggest that bio-accumulation of lipophilic coplanar PCBs to adipose tissue contributes to the development of adipocyte insulin resistance. Moreover, endogenous AhR ligands, present in the setting of diet-induced obesity, may play a pivotal role in regulating adipose mass and deposition.

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**Table 1.** Characteristics of *AhR<sup>fl/fl</sup>* mice fed standard diet.

<b>Parameter/Genotype</b>	<b><i>AhR<sup>fl/fl</sup></i></b>	<b><i>AhR<sup>AdQ</sup></i></b>
Body Weights (g)	24.6 ± 0.4	23.6 ± 0.5
Lean mass (% body weight)	81.0 ± 0.5	77.2 ± 1.2*
Fat mass (% body weight)	8.9 ± 0.3	11.2 ± 0.9*
Epididymal fat (g)	0.89 ± 0.12	1.27 ± 0.07*
Retroperitoneal fat (g)	0.16 ± 0.02	0.26 ± 0.03*
Subcutaneous fat (g)	0.56 ± 0.08	0.74 ± 0.03

Data are mean ± SEM from n = 6-8 mice/group.

\*P<0.05 compared to *AhR<sup>fl/fl</sup>*.

## Figure Legends

**Figure 1.** Development of mice with adipocyte deficiency of AhR. A, AhR mRNA abundance in liver, kidney, brain, heart, retroperitoneal fat (RPF), and brown adipose tissue (BAT). Data are mean  $\pm$  SEM from n = 5 mice/genotype. \*, P<0.05 compared to *AhR<sup>fl/fl</sup>*. B, AhR mRNA abundance in SVF (preadipocytes) and after 8 days of differentiation to mature adipocytes (adipo fraction) in cells from *AhR<sup>fl/fl</sup>* and *AhR<sup>AdQ</sup>* mice fed standard mouse diet. Data are mean  $\pm$  SEM from n = 3 mice/genotype.

**Figure 2.** Effect of adipocyte AhR deficiency on insulin tolerance in lean mice administered PCB-77. A, Blood glucose concentrations following administration of insulin in mice of each genotype administered VEH or PCB-77 (week 3). \*, P<0.05 compared to VEH within genotype. \*\*, P<0.05 compared to *AhR<sup>fl/fl</sup>* within treatment. B, Area under the curve (AUC) for data in A, above. Data are mean  $\pm$  SEM from n = 6-8 mice/group. \*, P < 0.05 compared to *AhR<sup>fl/fl</sup>* within treatment; \*\*, P < 0.05 compared to VEH within genotype.

**Figure 3.** Effect of adipocyte AhR deficiency on the development of obesity and increased adiposity. A, Body weights of mice in each treatment group and of each genotype during 12 weeks of HF feeding. Data are mean  $\pm$  SEM from n = 6-8 mice/treatment/genotype. \*, P<0.05 compared to *AhR<sup>fl/fl</sup>* within treatment. B, Lean mass and fat mass as a percentage of body weight after 12 weeks HF feeding. Data are mean  $\pm$  SEM from n = 6-8 mice/group. \*, P < 0.05 compared to *AhR<sup>fl/fl</sup>* within treatment group. \*\*, P<0.05 compared to VEH within genotype. C, A representative mouse fed the HF diet from each genotype. D, Subcutaneous adipose tissue removed from representative *AhR<sup>fl/fl</sup>* (left) and *AhR<sup>AdQ</sup>* (right) mice (n = 2/group).

**Figure 4.** Effect of adipocyte AhR deficiency on the size of subcutaneous adipocytes of HF-fed mice. A, Quantification of adipocyte size in tissue sections of subcutaneous adipose tissue. Adipocyte size was calculated on three measurement frames within each section of adipose tissue (n = 3 sections/mouse) from mice in each group (n = 3 mice/group). B, Representative sections of subcutaneous adipose tissue from mice of each genotype administered VEH. Boxes denote a single adipocyte within each representative section. \*, P<0.05 compared to *AhR<sup>fl/fl</sup>* within treatment.

**Figure 5.** Effect of adipocyte AhR deficiency on PCB-77-induced impairment of glucose and insulin tolerance in obese mice exhibiting weight loss. After 12 weeks of HF feeding, mice of each genotype and treatment group were switched to a LF diet for 4 weeks to induce weight loss. (A), Blood glucose concentrations following a bolus of intraperitoneally administered glucose in mice of each genotype and treatment group following 4 weeks of weight loss. (B), Blood glucose concentrations following a bolus of intraperitoneally administered insulin in mice of each genotype and treatment group. \*,  $P < 0.05$  compared to  $AhR^{fl/fl}$  within treatment group. (C), Area under the curve (AUC) for data in A, above. (D), AUC for data in B, above. Data are mean  $\pm$  SEM from  $n = 6-8$  mice/genotype/treatment group. \*,  $P < 0.05$  compared to VEH within genotype. \*\*,  $P < 0.05$  compared to  $AhR^{fl/fl}$  within treatment group.

**Figure 6.** Effect of adipocyte AhR deficiency on PCB-77-induced elevations in mRNA abundance of CYP1A1 and TNF- $\alpha$  in adipose tissue following weight loss. Abundance of mRNA for AhR (A), Cyp1A1 (B) and TNF- $\alpha$  (C) in epididymal adipose tissue from mice of each genotype and treatment group following 4 weeks of weight loss. Data are mean  $\pm$  SEM from  $n = 5$  mice/group. \*,  $P < 0.05$  compared to VEH within genotype. \*\*,  $P < 0.05$  compared to  $AhR^{fl/fl}$  within treatment group.

Figure 1.

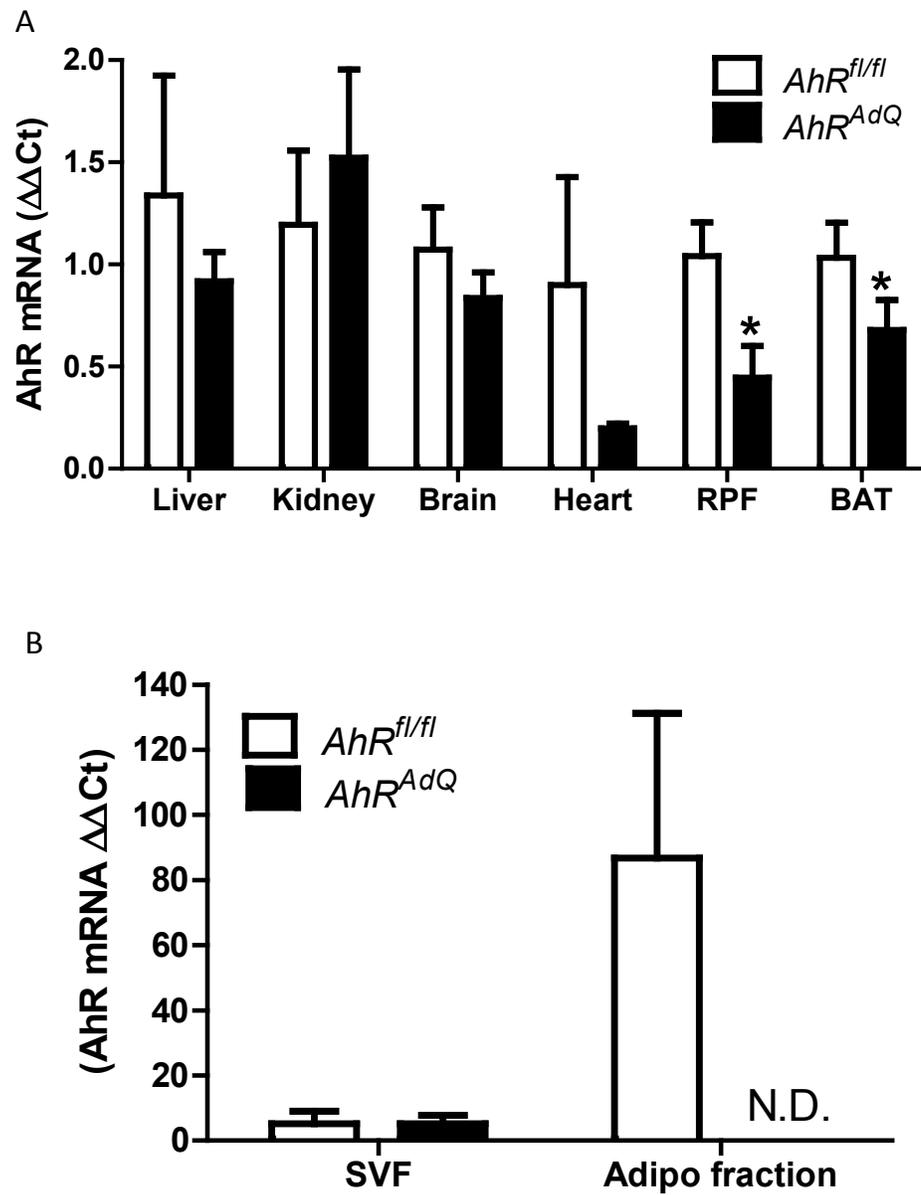


Figure 2.

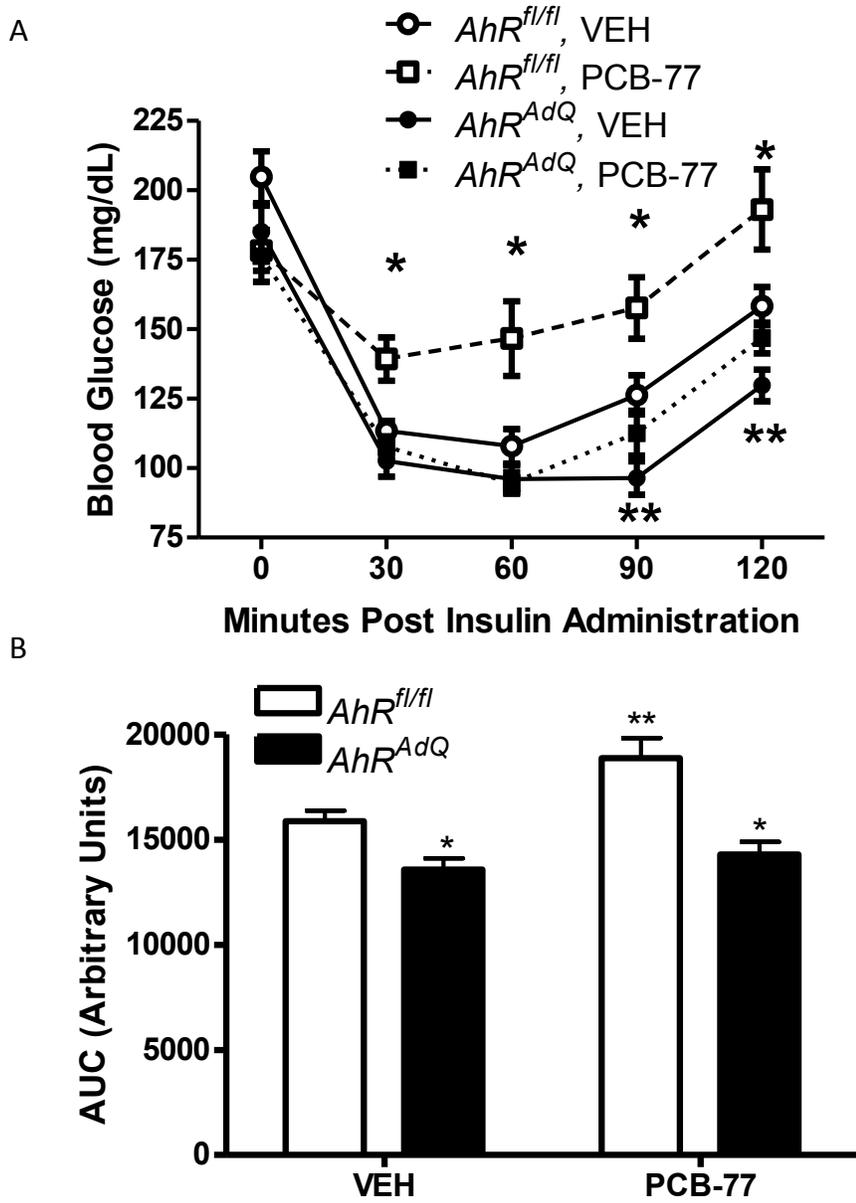


Figure 3.

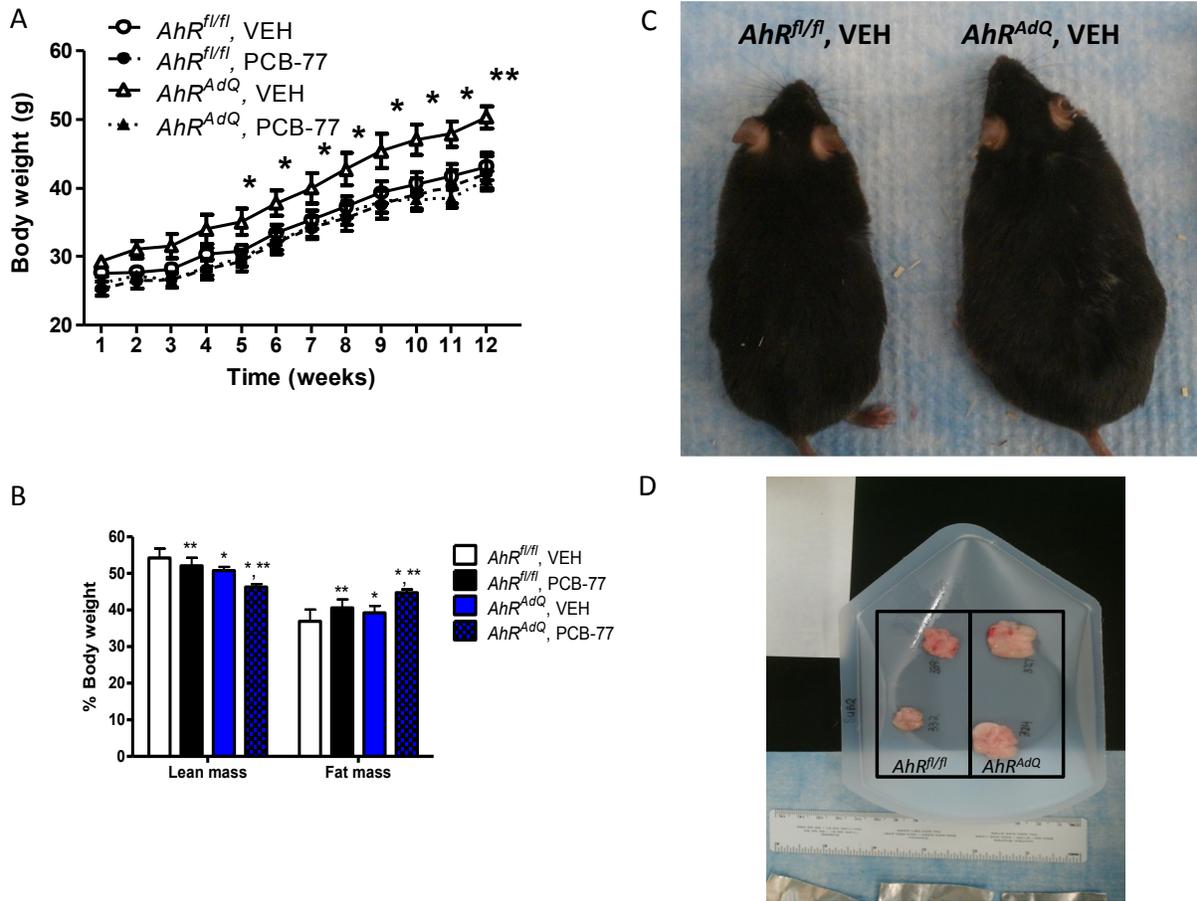
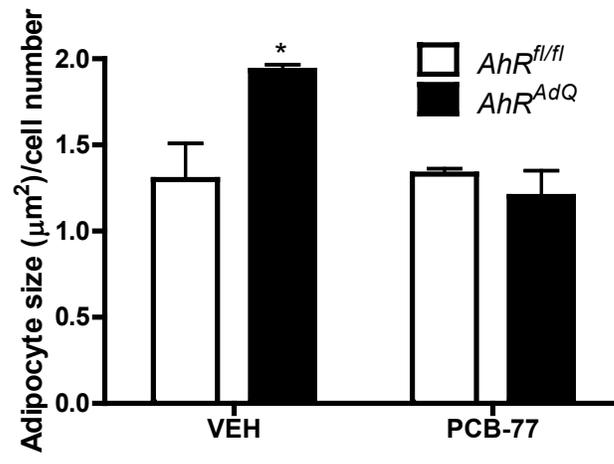


Figure 4.

A



B

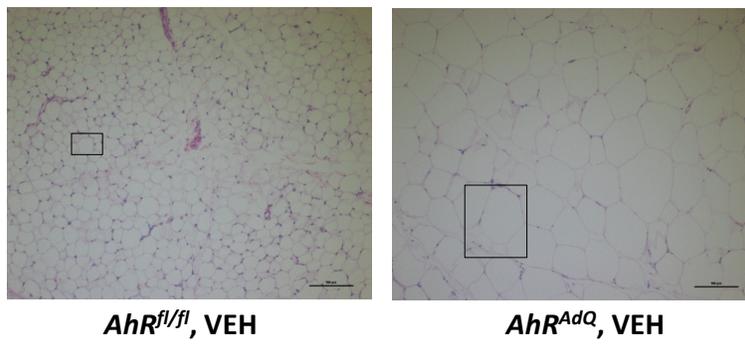


Figure 5.

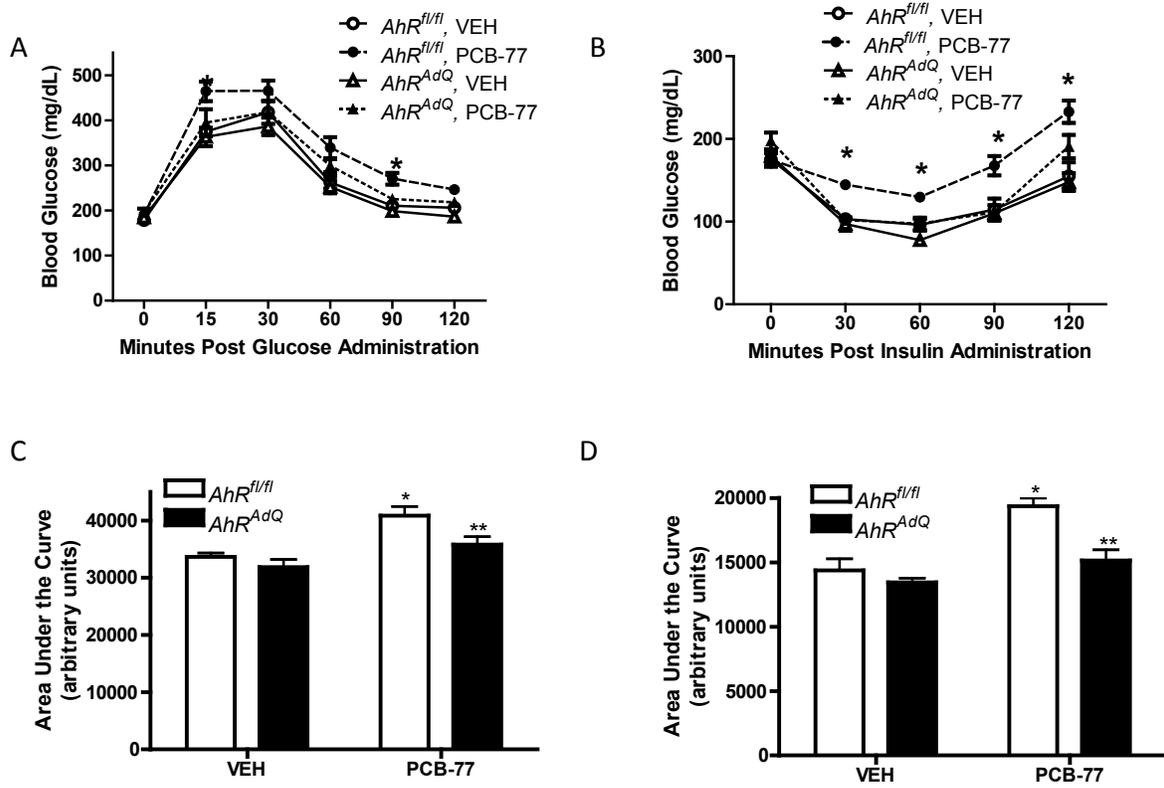


Figure 6.

