Insulin Regulation in AhR-null Mice: Embryonic Cardiac Enlargement, Neonatal Macrosomia, and Altered Insulin Regulation and Response in Pregnant and Aging AhR-null Females


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Received July 1, 2003; accepted August 19, 2003

The aryl hydrocarbon receptor (AhR) was originally characterized because of its high affinity binding of 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin. However, studies using AhR-null mice have demonstrated the importance of this protein in normal physiology and development. Here we demonstrate that AhR-null embryos develop cardiac enlargement, and that this phenotype is dependent, at least in part, on the maternal genotype. Neonates born to AhR-null females had increased heart weights regardless of the neonatal genotype, an outcome also observed in gestational diabetes. The cardiac hypertrophy markers, beta-myosin heavy chain and atrial natriuretic factor, and the cardiac proliferative index were increased in AhR-null embryos, indicating that the cardiac enlargement is associated with myocyte hypertrophy and hyperplasia, which begin prior to birth. Importantly, two- to three-month-old pregnant and seven-month-old nonpregnant females, but not nonpregnant three-month-old AhR-null females had significantly decreased fasting plasma insulin levels and a reduced ability to respond to exogenous insulin compared to controls. Despite these alterations in insulin regulation and responsiveness, pregnant AhR females did not have abnormal glucose tolerance tests and did not develop hyperglycemia, classic characteristics of gestational diabetes. However, twenty-three percent of seven-month-old AhR-null females did have altered glucose tolerance tests, but did not show hyperglycemia or increased hemoglobin A1c concentration under normal feeding conditions. While the ultimate cause of the neonatal phenotype remains unclear, these studies establish that the AhR is required for normal insulin regulation in pregnant and older mice and for cardiac development in embryonic mice.

Key Words: insulin regulation; embryonic cardiac enlargement; neonatal macrosomia; AhR.

The aryl hydrocarbon receptor (AhR) is a cytoplasmic protein, originally characterized because of its high affinity binding for 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD), and similar polyhalogenated aromatic hydrocarbons. Upon binding TCDD, the AhR translocates into the nucleus, dimerizes with the AhR nuclear translocator protein (ARNT), binds dioxin-responsive elements, and upregulates the expression of genes like cytochrome P4501A1 (Denison et al., 1988; Schmidt and Bradfield, 1996). AhR is required for most, if not all, of the toxic effects of TCDD (Fernandez-Salgueiro et al., 1996; Peters et al., 1999; Shimizu et al., 2000), although the mechanisms underlying TCDD-induced toxicity have not been fully elucidated.

The AhR is a member of the basic-helix-loop-helix PAS (Per-ARNT-SIM) transcription family, which also includes its dimerization partner ARNT, hypoxia-inducible factor 1-alpha (HIF1α), PER1-3, and others. Many members of this protein family, including AhR, act as environmental sensors, regulating downstream responses to environmental cues (Gu et al., 2000). For example, HIF1α is involved in the sensing and mediating the response to hypoxia (Wang et al., 1995), while the PER proteins as well as CLOCK and NPAS are involved in the control of circadian rhythmicity in response to day/night cycles (King et al., 1997; Reick et al., 2001; Zheng et al., 1999). Studies using AhR-null mice have identified new roles for the AhR in normal physiology and development, distinct from its role as an environmental sensor of toxicants (Abbott et al., 1999; Fernandez-Salgueiro et al., 1997; Lahvis et al., 2000).

The AhR and its dimerization partner ARNT have been shown to be expressed in the developing mouse and avian heart (Abbott and Probst, 1995; Abbott et al., 1995; Walker et al., 2000). Treatment of chick embryos with TCDD has been demonstrated to result in cardiac malformations, and the expression of AhR and ARNT in the chick cardiovascular system is consistent with a potential role in this toxicity (Walker and...
CATHERINE HOMEOSTASIS IN THE ADULT MOUSE (LUND et al., 2000; WALKER ET AL., 1997). Indeed, TCDD has been demonstrated to be a cardiovascular teratogen in all species tested (HARRIS ET AL., 1973; HASSOUN ET AL., 1984; HORNUNG ET AL., 1999; WALKER AND CATRON, 2000). Interestingly, mice lacking the AhR have been demonstrated to develop cardiac hypertrophy and hypertension, suggesting that the AhR is required for cardiovascular homeostasis in the adult mouse (LUND ET AL., 2000). Given these data, we investigated the role of the AhR in cardiac development using AhR-null mice.

Here we show that AhR-null embryos develop increased heart weight, and that pregnant mice lacking the AhR develop altered insulin regulation and responsiveness as seen by decreased fasting plasma insulin levels and insulin resistance. However, hyperglycemia and altered glucose tolerance, characteristics of gestational diabetes, were not observed. These results suggest that, although the embryonic cardiac enlargement in mice born to AhR-null females was not associated with an overt diabetic condition, alterations in insulin regulation and tissue responsiveness cannot be eliminated as potential causative factors. In addition, we also report that 23% of nonpregnant seven-month-old female AhR–/– mice develop glucose intolerance, and that all AhR-null mice of this age show significantly reduced fasting plasma insulin and tend to exhibit insulin resistance. Despite these alterations in insulin regulation and responsiveness, nonpregnant seven-month-old AhR–/– mice secrete normal amounts of insulin in response to a bolus dose of glucose and do not exhibit hyperglycemia. These data demonstrate that AhR-null females develop altered insulin regulation during pregnancy or as they age, but that these effects are subtle and do not result in overt diabetes.

MATERIALS AND METHODS

Animals. AhR–/– mice were obtained from Dr. Frank Gonzalez (National Cancer Institute) and maintained at the University of Wisconsin-Madison and University of New Mexico (Fernandez-Salguero et al., 1995). Experiments were approved by the appropriate animal care committees. Mice were maintained on a 12-h light cycle and given food and water ad libitum. Atrial natriuretic factor (ANF), beta-myosin heavy chain (β-MHC), myosin light chain 2V (MLC-2V), and 28S ribosomal RNA (28S) were measured by ribonuclease protection assay (RPA), using an RPA II kit from Ambion (Austin, TX). The probe for MLC-2V was supplied by Dr. Gary Lyons (University of Wisconsin-Madison). Probes for murine ANF, β-MHC, and 28S were generated by polymerase chain reaction (PCR), as previously described (THACKABERRY ET AL., 2002). Four litters per genotype were used for each transcript. Blots were quantified using a Cyclone Storage Phosphor System phosphoimager (Packard) with OptiQuant software. Values were normalized against 28S RNA.

Glucose measurements. All mice were maintained on a 12-h dark/light cycle, with lights off at 1800 h. Mice were deprived of food for 15 h prior to collection of blood (fasted), or fed ad libitum. All samples collected from timed-pregnant mice were taken at d14.5 of gestation, and correct timing of the pregnancies was confirmed at parturition. For ad libitum fed glucose measurements, blood samples were taken at 2400 h (n = 4 for both genotypes), 0400 h (n = 4 for both genotypes), 1000 h (n = 19 for AhR–/–, 11 for AhR–/−), 1500 h (n = 19 for AhR–/–, 11 for AhR–/−), and 2000 h (n = 4 for both genotypes). For nonpregnant mice, plasma glucose was measured from seven-month-old female mice following 15 h of fasting (n = 5 for AhR–/–, 8 for AhR–/−, 9 for AhR–/−) or when fed ad libitum at or around 1200 h (n = 4 for all genotypes). Plasma was collected by centrifugation at 2, 750 x g for 10 min at 4°C, and frozen at −20°C until analyzed. Glucose was measured using a Vitros Systems GLU DT enzymatic assay and analyzer (Endocrine Sciences Products, Calabasas Hills, CA). The upper detection limit was 450 mg/dl, and all values that exceeded this limit were reported as 450 mg/dl.

For glucose tolerance tests, two- to three-month-old pregnant mice (n = 4 per genotype), and three- (n = 6 for all genotypes) and seven-month-old (n = 14 for AhR–/–, 13 for AhR–/−, 17 for AhR–/−) nonpregnant mice were fasted for 15 h. An initial blood sample was collected, and mice were then given 2 g/kg glucose by oral gavage. Blood samples were taken at 15, 30, 60, 90, 120, and 150 min after gavage and analyzed for glucose. For insulin tolerance tests, randomly fed two- to three-month-old pregnant females or seven-month-old nonpregnant females were given 0.75 units of porcine insulin (Sigma, St. Louis, MO) per kg of body weight in phosphate buffered saline via ip injection, and blood samples were taken at 15, 30, and 60 min, analyzed for glucose, and compared to preinjection values.

C-Peptide measurements. Two- to three-month-old pregnant (d14.5 of gestation, four mice per genotype), nonpregnant three- month-old (n = 11 for AhR–/–, 6 for AhR–/−, 14 for AhR–/−) and nonpregnant seven-month-old (n = 12 for AhR–/–, 8 for AhR–/−, 15 for AhR–/−) female mice were fasted for 15 h, anesthetized, and blood was collected via cardiac puncture using a heparinized syringe. Correct timing of the pregnancies was confirmed by morphological analysis of the embryos at the time of sacrifice. Plasma was collected by centrifugation at 2, 750 x g for 10 min at 4°C, and blood was then frozen at −20°C until analyzed. C-Peptide was measured using a radioimmunoassay (RIA, Linco Research, St. Charles, MO). For secretory insulin response to glucose, mice were fasted for 15 h, given 2 g/kg glucose via oral gavage, and

### TABLE 1

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of Litters Excluded Per Cross Indicated</th>
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<tr>
<td>AhR–/– Male</td>
<td>12</td>
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<tr>
<td>AhR–/– Female</td>
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<td>AhR–/– Male</td>
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<td>AhR–/– Male</td>
<td>3</td>
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<td>AhR–/– Female</td>
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blood was collected and C-peptide measured 15 min after glucose administration.

**Hemoglobin A1c quantification.** Whole blood from nonpregnant female AhR+/− and wild-type mice at 3 (n = 5 for AhR+/−, 6 for AhR+/+) and 4.5 (n = 3 for both genotypes) and 6.5 (n = 6 for AhR+/−, 4 for AhR+/+) months of age was collected via tail clip. Hemoglobin A1c was isolated using boronate affinity column (Endocrine Sciences) and quantified using a spectrophotometer at 414 nm. Results are expressed as a percent of total hemoglobin concentration.

**Statistics.** For analysis of the nonpregnant glucose tolerance tests, a Bonferroni adjustment of a mean-shift outlier test, accounting for censoring at the upper detection limit of 450 mg/dl, was used to segregate the outlying "glucose intolerant" AhR−/− mice from the "normal" AhR−/− mice (Weisberg, 1985). All other data sets and comparisons of individual time points from the glucose tolerance tests were compared using Students t test. A Kolmogorov-Smirnov (KS-distance) test was used to confirm normal distribution of the data for all other data sets. Statistical significance was set at p < 0.05 for all comparisons.

**RESULTS**

**Neonatal Macrosomia and Increased Cardiac Weight Are Partially Dependent on Maternal Genotype**

Initial data showed that AhR−/− neonates born to AhR−/− females exhibited significantly increased heart weight, body weight, and heart-to-body weight ratios, compared to AhR+/+ neonates born to AhR+/+ females. However, when AhR−/− mice were crossed to each other, AhR−/− neonates failed to show a difference in body, heart weights, or heart-to-body weight ratio compared to AhR+/+ or AhR+/− littersmates. We then performed the following crosses: AhR+/− female × AhR−/− male, AhR−/− female × AhR−/− male and the pure and heterozygous crosses described above. Neonate heart, body, and heart/body weights were grouped by the genotype of the mother and neonate (Fig. 1). Neonates born to AhR+/− females, regardless of the neonatal genotype, showed an increase in heart weight (Fig. 1A). AhR+/− neonates born to AhR−/− females showed an increase in body weight (macrosomia, Fig. 1B), while AhR−/− neonates born to AhR−/− females did not have increased body weights (p = 0.09), but did have an increase in heart-to-body weight ratio (Fig. 1C). In contrast, neonates born to AhR−/− females, regardless of the neonatal genotype, failed to exhibit any differences in heart, body, or heart-to-body weight ratio (Fig. 1).

**Altered Morphology of AhR−/− Embryonic and Neonatal Hearts**

To further investigate the cardiac phenotype of AhR−/− mice born to AhR−/− females, we examined the morphology of embryonic and neonatal hearts. Hearts from AhR−/− neonates exhibited an increase in ventricle wall thickness and an increase in overall size of the heart compared to AhR+/− controls (Fig. 2). This thickening of ventricle walls was evident as early as d14.5 in some embryos, but was not associated with overt cardiac malformations (data not shown).

**Increased Proliferation in AhR-Null Embryonic Hearts**

We next investigated the possibility that the increased ventricular wall thickness seen in embryonic AhR-null hearts may be due to increased proliferation in these hearts. To do this, we used immunohistochemical staining of day 14.5 hearts for PCNA, a marker of S phase. AhR-null embryonic hearts had significantly higher proliferative index compared to control hearts: AhR+/−, 10.3 ± 2.5 proliferating cells per total cell number × 100; AhR−/− 39.6 ± 5.0 proliferating cells per total cell number × 100; n = 5 litters per genotype. The increased proliferation was most evident in the developing ventricular septa, which was the most actively proliferating area of the heart at this stage of development.

**Increased Expression of Cardiac Hypertrophy Genes in the Developing AhR−/− Heart**

To further characterize the cardiac phenotype of embryonic and neonatal AhR−/− mice, we measured the expression of β-MHC, ANF, and MLC-2V transcripts in the hearts of d14.5 and d17.5 embryos, as well as in neonates (Fig. 3). β-MHC was upregulated 10–30-fold at all developmental time points studied (Fig. 3A), while ANF was upregulated significantly on d14.5 and in neonates (Fig. 3B). MLC-2V transcript levels were not significantly increased in null mutant hearts at any time point examined (Fig. 3C).

**Pregnant AhR−/− Females Have Normal Glucose Tolerance Tests**

We then investigated whether pregnant AhR−/− females exhibited changes consistent with gestational diabetes. To determine whether pregnant AhR−/− females could clear plasma glucose normally, we performed glucose tolerance tests on time-pregnant (d14.5) females of all three AhR genotypes. We used d14.5 of gestation for these and all future studies of pregnant female insulin regulation because this is the earliest time point that we observed altered morphology in AhR-null embryos. No differences in glucose clearance were seen among any of the three genotypes (Fig. 4).

**Pregnant AhR−/− Females Exhibit Insulin Resistance**

Insulin tolerance tests were performed to determine if AhR−/− females developed insulin resistance. Pregnant (d14.5) AhR−/− females showed significantly reduced glucose clearance 30 min following insulin injection compared to wild-type females, indicating that these mice exhibited a reduced ability to respond to insulin (Fig. 5). By 60 min, wild-type females mice had largely recovered to their original plasma glucose levels, while AhR-null females appeared to only begin to significantly clear glucose, indicating that these mice also exhibit a delay in their response to insulin.
Pregnant AhR<sup>–/–</sup> Females Show Decreased Fasting C-Peptide Levels

To investigate whether altered insulin regulation may be associated with the macrosomia and cardiac hypertrophy in neonates born to AhR<sup>–/–</sup> female mice, we measured C-peptide levels in pregnant AhR<sup>–/–</sup> females. C-Peptide is an indicator of plasma insulin, since it is produced in equimolar concentrations to insulin, but is significantly more stable in plasma. Pregnant (d14.5) AhR<sup>–/–</sup> females tended to exhibit a reduction in fasting plasma C-peptide (Fig. 6) compared to pregnant AhR<sup>+/+</sup> controls (p < 0.08). To test the insulin secretory response to glucose in AhR-null females, fasted mice were given 2 g/kg glucose by oral gavage, and C-peptide was measured 15 min afterwards. No difference was seen between the three genotypes in their ability to secrete insulin in response to glucose administration (Fig. 6).

Pregnant AhR<sup>–/–</sup> Mice Do Not Experience Hyperglycemia under Normal Feeding Conditions

To determine whether reduced fasting insulin and insulin resistance in pregnant (d14.5) AhR<sup>–/–</sup> females was associated with hyperglycemia, we measured plasma glucose levels of ad libitum fed mice at set hours during a 24-h period. Pregnant AhR<sup>–/–</sup> mice failed to show hyperglycemia (defined as plasma glucose greater than 225 mg/dl) at any time. The average daily plasma glucose levels (average of all time points throughout the 24-h period) were 163.8 ± 8.1 mg/dl for pregnant AhR<sup>+/+</sup> females, and 159.4 ± 8.2 mg/dl for AhR<sup>–/–</sup>.
To determine whether a diabetic phenotype developed with age, we performed glucose tolerance tests on three- and seven-month-old females of all three AhR genotypes. Although the peak levels of glucose were higher on average in AhR-null females at 15 and 30 min following an oral glucose load, no statistical difference was seen in glucose clearance among the three genotypes at three months of age (Fig. 7A). At seven months of age, however, AhR-null mice exhibited elevated but highly variable glucose levels at 15, 30, and 60 min after

**AhR**−/

**Females Develop Glucose Intolerance with Age**

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**FIG. 3.** Expression of cardiac hypertrophy markers in the embryonic and neonatal AhR+/+ and AhR−/− mice. All hearts for this study were obtained from AhR+/+ females mated to AhR+/+ males and AhR−/− females mated to AhR−/− males. (A) Expression of β-MHC, significant upregulation was seen at all developmental time points. (B) ANF expression was upregulated at d14.5 and at birth. (C) MLC-2V was not upregulated at any time point. Expression levels for each individual sample were normalized to 28S controls, and then the means were expressed as a percentage of AhR+/+ control expression. Bars and associated vertical lines represent mean ± standard error. Open bars, AhR+/+; solid bars, AhR−/−. *p < 0.05; n = 4 litters for all transcripts.

**FIG. 4.** Glucose tolerance tests performed on pregnant AhR+/+ and AhR−/− females. Pregnant (d14.5) mice were fasted for 15 h, then given 2 g/kg glucose orally. Blood glucose was measured prior to glucose administration and at 15, 30, 60, 90, 120, and 150 min afterwards. No differences were seen among the three AhR genotypes. Symbols and associated vertical lines represent mean ± standard error. ○ = AhR+/+; △ = AhR+/−; and ● = AhR−/− mice. n = 4 mice per genotype.

**FIG. 5.** Insulin response tests performed on pregnant (d14.5) AhR+/+ and AhR−/− females. Randomly fed pregnant female mice were injected with 0.75 units of insulin ip. Plasma glucose levels were measured prior to injection and at 15, 30, and 60 min afterwards. Results are expressed as percentage of preinjection plasma glucose levels. Pregnant AhR−/− females showed a significant delay in glucose clearance in response to insulin. Symbols and associated vertical lines represent mean ± standard error. ○ = AhR+/+; ● = AhR−/−. *p < 0.05. n = 4 mice per genotype.
glucose administration (Fig. 7B). Using a Bonferroni outlier test, we determined that this variability resulted from 23% (4/17) of the seven-month-old AhR\(^{\text{--/--}}\) females exhibiting an impairment of glucose clearance. When these four AhR-null females were analyzed separately, their ability to clear glucose was significantly impaired compared to wild types, AhR\(^{+/+}\)/\(^{H11001}/\(^{-}\), and the remaining AhR\(^{\text{--/--}}\) female mice (Fig. 7C).

AhR\(^{+/+}\) and AhR\(^{\text{--/--}}\) Females Exhibit Reduced Circulating C-Peptide with Age

To determine if insulin regulation was altered with age, we next investigated C-peptide levels in three- and seven-month-old females of all three AhR genotypes. No differences were seen in plasma C-peptide levels among three-month-old AhR\(^{+/+}\)/\(^{H11001}/\(^{H11001}\), AhR\(^{+/+}\)/\(^{-}\), and AhR\(^{\text{--/--}}\) mice (Fig. 8A); however, seven-month-old AhR\(^{+/+}\) and AhR\(^{\text{--/--}}\) females showed significantly lower fasting plasma C-peptide levels compared to AhR wild-type females (Fig. 8B). The results for both AhR\(^{+/+}\) and AhR\(^{\text{--/--}}\) females were evenly distributed, with no significant outliers representing responders versus nonresponders. In addition, all AhR-null mice were able to secrete insulin normally 15 min following a bolus dose of exogenous glucose (data not shown).

AhR\(^{\text{--/--}}\) Females Exhibit a Delayed Insulin Response

Insulin tolerance tests were performed to determine if seven-month-old AhR\(^{\text{--/--}}\) females developed insulin resistance in association with glucose intolerance. Mice were injected with 0.75 U/kg porcine insulin, and plasma glucose clearance was measured. AhR\(^{\text{--/--}}\) females showed significantly reduced glucose clearance in response to insulin compared to controls at 30 min and no apparent increase in clearance by 60 min (Fig. 9). Glucose clearance in AhR\(^{+/+}\) mice was similar to that of wild-type controls, and the values for all genotypes were normally distributed.

AhR\(^{\text{--/--}}\) Females Do Not Experience Hyperglycemia

The reduced fasting insulin, slower insulin response, and glucose intolerance seen in some AhR-null females could lead...
to hyperglycemia. We therefore measured fasting and ad libitum fed plasma glucose concentrations in wild-type and AhR-null mice. Under fasting conditions, seven-month-old AhR–/– females did not develop hyperglycemia (AhR+/+; 163.0 ± 1.7 mg/dl, n = 5; AhR+/−; 153.0 ± 8.5 mg/dl, n = 8; AhR−/−; 158.6 ± 9.4 mg/dl, n = 9), despite reduced fasting plasma insulin. In addition, ad libitum fed AhR−/− mice did not develop hyperglycemia at midday (AhR+/+; 181.8 ± 10.3 mg/dl; AhR−/−; 181.3 ± 8.7 mg/dl; AhR+−; 167.3 ± 6.5 mg/dl; n = 4 for all genotypes).

**AhR−/− Females Do Not Develop Increased Glycosylated Hemoglobin Concentrations**

We next measured glycosylated hemoglobin A1c concentrations as a measurement of chronic hyperglycemia. Hemoglobin is glycosylated at higher rates in animals experiencing chronic hyperglycemia, and can be quantified as an indicator of hyperglycemia over time (Dan et al., 1997). Our results showed that AhR−/− female mice did not experience increased hemoglobin A1c levels at three, four-and-a-half, or six-and-a-half months of age (Fig. 10). Interestingly, AhR-null females actually have significantly lower hemoglobin A1c levels at six-and-a-half months of age compared to wild types.

![FIG. 8. Fasting plasma C-peptide levels in three- and seven-month-old AhR+/+, AhR−/−, and AhR−/− mice. Mice were fasted for 15 h, blood was collected, and plasma C-peptide was measured by RIA. (A) AhR-null and heterozygous mice had normal fasting C-peptide levels at three months of age. n = 11 for AhR+/+, 6 for AhR−/−, and 14 for AhR−/−. (B) Seven-month-old AhR−/− females had significantly decreased plasma C-peptide levels. Bars and vertical lines represent mean ± standard error. *p < 0.05. n = 12 for AhR+/+, 8 for AhR−/−, 15 for AhR−/−.](image)

![FIG. 9. Insulin tolerance in seven-month-old AhR+/+, AhR−/−, and AhR−/− female mice. Fed female mice were injected with 0.75 units of insulin ip, and plasma glucose levels were measured prior to injection, and 15 and 30 min post injection. Results are expressed as percentage of preinjection plasma glucose levels. AhR−/− females did not clear plasma glucose as quickly as AhR+/+ or AhR−/− mice. Symbols and vertical lines represent mean ± standard error. *p < 0.05. □ = AhR+/+; ▲ = AhR−/−; ● = AhR−/−. n = 4 for all groups.](image)

![FIG. 10. Age-related hemoglobin A1c concentrations in AhR+/+, AhR−/−, and AhR−/− female mice. Hemoglobin A1c levels were measured at 3, 4.5, and 6.5 months of age. No difference is seen between AhR−/− and AhR+/+ at 3 and 4.5 months of age. AhR-null females showed significantly decreased hemoglobin A1c levels at 6.5 months of age compared to wild types. Symbols and vertical lines represent mean ± standard error. *p < 0.05. □ = AhR+/+; ▲ = AhR−/−; ● = AhR−/−. n = 5 for all groups.](image)
DISCUSSION

While the AhR was originally identified because of its high affinity for TCDD and its role in TCDD-induced toxicity, AhR<sup>−/−</sup> knockout mice have demonstrated the importance of this protein in normal development and physiology, including vascular development, reproductive capacity, and adult cardiovascular homeostasis (Abbott et al., 1999; Fernandez-Salgueiro et al., 1997; Lahvis et al., 2000; Thackaberry et al., 2002). We report here for the first time that the AhR also has a role in embryonic cardiac development, and this phenotype is dependent, in part, on the maternal AhR genotype. Furthermore, pregnant AhR<sup>−/−</sup> mice exhibit altered insulin regulation and responsiveness, which may contribute to the embryonic phenotype.

AhR<sup>−/−</sup> mice born to AhR<sup>−/−</sup> females exhibited significantly enlarged hearts. This cardiac enlargement was associated with thicker ventricular walls and upregulation of the cardiac hypertrophy markers β-MHC and ANF. ANF and β-MHC are highly expressed during normal fetal cardiac development and are subsequently downregulated early postnatally (Mercadier et al., 1989). Both β-MHC and ANF are markers of cardiac hypertrophy in adult animals (Sugden and Clerk, 1998), and increased expression of ANF has been reported in neonatal hearts undergoing hypertrophy (Bruneau et al., 2001; Walker and Catron, 2000). To our knowledge, these findings are the first to report upregulation of β-MHC and ANF associated with both embryonic and neonatal cardiac hypertrophy. In contrast, in normal animals MLC-2V is not highly expressed in the fetal heart, but is upregulated in the adult hypertrophied heart (Lee et al., 1988). Thus, it is not inconsistent that MLC-2V was not increased in embryonic and neonatal AhR<sup>−/−</sup> hypertrophic hearts. The increased expression of β-MHC and ANF, taken together with the thickening of the ventricular walls on d14.5 and d17.5 embryos indicate that AhR<sup>−/−</sup> mice experience cardiac hypertrophy prior to birth.

The increase in heart size in AhR-null neonates can also be explained by an increased in proliferation in the hearts of AhR-null embryos. At d14.5, AhR-null embryos had a significantly increased proliferation index, as measured by PCNA staining. This suggests that the cardiac enlargement seen in AhR-null mice may be a result of hyperplasia. While the induction of the cardiac hypertrophy markers β-MHC and ANF suggests that this is a hypertrophic phenotype, it seems likely that both hypertrophy and hyperplasia are involved, since embryonic cardiomyocytes, unlike adult cardiomyocytes, are capable of cell division.

A progressive cardiac hypertrophy occurs in adult male AhR-null mice (Fernandez-Salgueiro et al., 1997; Thackaberry et al., 2002). This adult cardiac hypertrophy appears to be mechanistically distinct from the neonatal hypertrophy, since the adult hypertrophy is manifested beginning at five months of age, while three-month-old AhR-null males have normal heart weights. Thus, embryonic and neonatal cardiac hypertrophy in AhR<sup>−/−</sup> mice may resolve following birth, when the fetus is removed from the maternal environment, and results in normal heart weights by three months of age.

A series of mating experiments revealed that the neonatal cardiac enlargement was dependent, at least in part, on the maternal AhR genotype. Mice born to AhR<sup>−/−</sup> females, including both AhR<sup>−/−</sup> and AhR<sup>+/−</sup> neonates, exhibited significantly increased heart weights compared to mice born to AhR<sup>+/+</sup> females. In contrast, mice born to AhR<sup>+/−</sup> females, including AhR<sup>+/−</sup>, AhR<sup>+/+</sup>, and AhR<sup>−/−</sup> neonates, fail to exhibit cardiac enlargement, compared to mice born to AhR<sup>−/−</sup> females. The effects of maternal AhR genotype on neonatal body weight and heart-to-body weight ratio were more complex. Heterozygous neonates born to AhR<sup>−/−</sup> females had significantly increased body weights, while their AhR<sup>−/−</sup> littermates did not. However, only AhR<sup>−/−</sup> neonates born to AhR<sup>−/−</sup> females showed an increase in heart/body weight ratio. The increase in heart-to-body weight ratio in these mice suggests that the cardiac enlargement seen in AhR<sup>−/−</sup> mice is dependent on a combination of both maternal and neonatal loss of AhR. The AhR<sup>+/−</sup> mice born to AhR<sup>−/−</sup> females show cardiac hypertrophy that is increased in proportion to body weight. In contrast, AhR<sup>−/−</sup> neonates show cardiac hypertrophy that is increased to a larger degree than their body weights. Neonates born to AhR<sup>−/−</sup> dams show no increases in heart weight, body weight, or heart-to-body weight ratio, further suggesting that the cause of the neonatal macrosomia is dependent on both maternal and embryonic AhR status.

Since diabetic pregnancy has been shown to increase neonatal heart and body weight in a manner dependent on maternal genotype alone (Rizzo et al., 1992; Spellacy et al., 1985), we investigated glucose metabolism in pregnant AhR<sup>−/−</sup> females. Two hallmarks of gestational diabetes are glucose intolerance, or the inability to effectively clear a bolus dose of glucose, and insulin resistance, a reduced ability of peripheral tissues to uptake glucose following insulin release. To determine whether pregnant AhR-null females experienced gestational diabetes, we performed tests for glucose tolerance and insulin resistance. Pregnant AhR<sup>−/−</sup> mice are able to clear a bolus dose of plasma glucose normally; however, they exhibited a substantial delay in their ability to clear glucose in response to insulin. This suggests that the peripheral tissues in pregnant AhR<sup>−/−</sup> mice do not uptake plasma glucose in response to insulin as efficiently as controls, but that this insulin resistance was not sufficient to retard glucose clearance following a bolus dose of glucose. Thus, it appears that pregnant AhR-null mice exhibit a partial insulin resistance.

Impaired glucose uptake in response to insulin could be overcome by significantly increased insulin production, or hyperinsulinemia. Indeed, hyperinsulinemia is often seen in insulin resistant gestational diabetes (Buchanan et al., 1990). However, fasting C-peptide levels were significantly reduced, rather than elevated, in pregnant AhR-null mice. This result is contradictory to what would be expected if AhR-null mice...
were overcoming insulin resistance with increased insulin production. Furthermore, when challenged with a bolus dose of glucose, pregnant AhR<sup>−/−</sup> mice secreted normal levels of insulin. Thus, while the pregnant AhR-null mice had an apparent insulin resistance, this was not compensated for by increased insulin production.

While the decreased fasting insulin levels and insulin resistance seen in pregnant AhR<sup>−/−</sup> mice were not associated with abnormal glucose tolerance, we hypothesized that these abnormalities may be correlated with hyperglycemia under ad libitum feeding conditions. To investigate this possibility, we measured plasma glucose concentrations throughout a 24-h period. Our results demonstrate that pregnant AhR-null mice do not experience hyperglycemia at any time, and thus, the altered insulin regulation does not lead to hyperglycemia under any conditions studied.

One form of gestational diabetes, mature onset diabetes of the young-2 (MODY-2) shares many similarities with the phenotype seen in pregnant AhR-null mice. MODY-2 results from a single copy of the glucokinase gene is disrupted, leading to altered glucose sensing and reduced insulin secretion (Froguel <i>et al.</i>, 1993). Thus, like pregnant AhR<sup>−/−</sup> mice, MODY-2 mice have altered basal insulin levels and normal ad libitum fed glucose levels. However, unlike AhR-null mice, MODY-2 mice develop mild hyperglycemia under fasting conditions (Bali <i>et al.</i>, 1995), exhibit abnormal glucose tolerance, and do not develop insulin resistance. While the similarities between the MODY-2 mouse model and pregnant AhR-null mice are intriguing, further studies are needed to characterize the defect leading to reduced insulin production in AhR-null animals.

We next investigated whether the AhR was also required for insulin regulation and responsiveness in nonpregnant mice. Our results demonstrate that nonpregnant female AhR-null mice exhibited decreased fasting plasma insulin levels and decreased sensitivity to insulin-mediated glucose uptake by seven months of age, and that 23% of seven-month-old AhR<sup>−/−</sup> female mice develop overt glucose intolerance. However, none of these effects were observed at three months of age.

While all three-month-old AhR-null females tested had normal glucose tolerances, 23% (4/17) of seven-month-old AhR<sup>−/−</sup> females showed significant glucose intolerance, suggesting that age increases the dysregulation of glucose in AhR-null females. The incomplete penetrance of this phenotype suggests that other factors may be involved, and that the percentage of animals exhibiting this phenotype could continue to increase with age. Glucose intolerance was not seen in pregnant 3- to 4-month-old AhR-null females, indicating that age, but not pregnancy, is one important risk factor for development of this abnormality. Unfortunately, due to the reduced reproductive capacity of AhR-null mice (Abbott <i>et al.</i>, 1999), studies cannot be performed on pregnant seven-month-old AhR-null females.

In addition to glucose intolerance, seven-month-old AhR heterozygous and null females also have decreased fasting plasma insulin levels that were not apparent at three months. This suggests that, like the development of glucose intolerance, the development of reduced fasting insulin production occurs with age. Interestingly, while three-month-old AhR-null females show no alteration in fasting insulin levels, pregnant AhR-null mice of similar age do exhibit decreased fasting insulin. This suggests that both pregnancy and increased age are contributing risk factors in the development of this phenotype. The reduction in fasting plasma insulin seen in AhR<sup>−/−</sup> mice is also significant. This suggests that loss of a single AhR allele is sufficient to alter insulin regulation in mice. The only other reported abnormality seen in AhR<sup>−/−</sup> mice is cardiac hypertrophy (Thackaberry <i>et al.</i>, 2002), which, interestingly, is also seen at seven months of age.

Despite the reduction in fasting plasma insulin, impaired insulin secretion in response to glucose was not observed. These results indicate that AhR<sup>−/−</sup> mice are able to secrete normal amounts of insulin in response to a bolus dose of glucose. Therefore, it seems unlikely that the decreased fasting insulin plays a role in the impaired glucose tolerance in seven-month-old AhR-null females.

Seven-month-old AhR-null female mice also demonstrate a significantly reduced sensitivity to exogenous insulin. A similar insulin insensitivity is seen in pregnant AhR<sup>−/−</sup> mice, implicating age and pregnancy as contributing factors involved in this phenotype. Thus, it seems likely that reduced responsiveness to insulin plays a causative role in the impaired glucose tolerance.

The phenotype seen in AhR-null females resembles some characteristics of type II diabetes, including a reduced ability of peripheral tissues to absorb glucose in response to insulin (decreased insulin response) and impaired glucose tolerance. Furthermore, the reduced insulin sensitivity and glucose intolerant phenotypes in AhR-null females develop with age, while reduced insulin sensitivity also develops with pregnancy. Notably, age and pregnancy have been demonstrated to increase the risk of developing type II diabetes (Bertoni <i>et al.</i>, 2002). This suggests that the AhR-null phenotype and type II diabetes share certain risk factors, and therefore may have some commonalities. Despite the similarities between type II diabetes and the female AhR-null phenotype, type II diabetes typically results in hyperglycemia, increased hemoglobin A<sub>1c</sub>, and hyperinsulinemia. Seven-month-old AhR-null females fail to exhibit any of these characteristics. In fact, they exhibit normal glycemic control, reduced hemoglobin A<sub>1c</sub>, and hypoinsulinemia.

It has been demonstrated that TCDD exposure and AhR activation are linked to alterations in insulin and glucose regulation, but these data fail to provide clear evidence of a mechanism by which the AhR regulates carbohydrate metabolism. TCDD causes hypoinsulinemia and hypoglycemia in animal models (Ebner <i>et al.</i>, 1988; Gorski and Rozman, 1987; Viluksela <i>et al.</i>, 1999), but is associated with hyperinsulinemia, hyperglycemia, insulin resistance, and increased risk for type II diabetes.
diabetes in humans (Bertazzi et al., 2001; Cranmer et al., 2000; Henriksen et al., 1997; Michalek et al., 1999). Thus, the role of the AhR in insulin regulation and tissue responsiveness is unclear.

These data demonstrate that pregnant AhR-null mice have altered insulin regulation, but do not exhibit overt gestational diabetes, and that nonpregnant AhR-null females develop altered insulin regulation by seven months of age, and 23% of these mice develop glucose intolerance. The partial dependence of the neonatal phenotype on the maternal genotype suggests a metabolic abnormality in the mother that promotes the development of macrosomia and cardiac hypertrophy; however, the ultimate cause of the neonatal cardiac hypertrophy and macrosomia is uncertain. In addition, nonpregnant seven-month-old AhR-null female mice do not develop hyperglycemia or increased hemoglobin A1C, indicating that they do not exhibit overt diabetes. These data demonstrate that the AhR is required for normal cardiovascular development, as well as insulin regulation in pregnant and nonpregnant female mice.

ACKNOWLEDGMENTS

We would like to thank Dr. Irena Ivnitski-Steele for conducting the PCNA analysis and Kim Doctorman, Katherine Debelak, and Joel Earnest-DeYoung for their invaluable assistance. Contribution # 348, Molecular and Environmental Toxicology Center, University of Wisconsin, Madison, WI 53726-4087. Supported, in part, by NIH training grant # ES07015, ES10433 to MKW, and New Mexico NIEHS Center (ES12072).

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