Aryl Hydrocarbon Receptor Ablation in Cardiomyocytes Protects Male Mice From Heart Dysfunction Induced by NKX2.5 Haploinsufficiency

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ABSTRACT

Epidemiological studies in humans and research in vertebrates indicates that developmental exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a ubiquitous and biopersistent environmental toxicant, is associated with incidence of early congenital heart disease in the embryo and later in the adult. TCDD-mediated toxicity depends on the aryl hydrocarbon receptor (AHR) but the role of the TCDD-activated AHR in cardiac function is not well-defined. To characterize the mechanisms responsible for AHR-mediated disruption of heart function, we generated several mouse strains with cardiomyocyte-specific *Ahr* gene knockout. Here, we report results on one of these strains in which the *Ahr* gene was deleted by cre recombinase regulated by the promoter of the cardiomyocyte-specific *Nkx2.5* gene. We crossed mice with loxP-targeted *Ahr*^{fx/fx} alleles with *Nkx2.5*^{+/cre} mice bearing a “knock-in” cre recombinase gene integrated into one of the *Nkx2.5* alleles. In these mice, loss of one *Nkx2.5* allele is associated with disrupted cardiac development. In males, *Nkx2.5* hemizygosity resulted in cardiac haploinsufficiency characterized by hypertrophy, dilated cardiomyopathy, and impaired ejection fraction. *Ahr* ablation protected *Nkx2.5*^{+/cre} haploinsufficient males from cardiac dysfunction while inducing a significant increase in body weight. These effects were absent or largely blunted in females. Starting at 3 months of age, mice were exposed by oral gavage to 1 μg/kg/week of TCDD or control vehicle for an additional 2 months. TCDD exposure restored cardiac physiology in aging males, appearing to compensate for the heart dysfunction caused by *Nkx2.5* hemizygosity. Our findings underscore the conclusion that deletion of the *Ahr* gene in cardiomyocytes protects males from heart dysfunction due to NKX2.5 haploinsufficiency.

Key words: aryl hydrocarbon receptor; cardiomyocytes; cardiac hypertrophy; *Nkx2.5*; haploinsufficiency.

The aryl hydrocarbon receptor (AHR), a ligand-activated transcription factor, mediates the teratogenic and carcinogenic effects of environmental planar aromatic xenobiotic hydrocarbons, among which the most potent agent is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). AHR is a member of the basic-region-helix-loop-helix PER/AHR nuclear translocator (ARNT)/SIM

(bHLH-PAS) superfamily of transcription factors. Members of this superfamily function as sensors of extracellular signals and environmental stresses affecting growth and development (Gu et al., 2000). Activation by TCDD causes receptor translocation to the nucleus, dissociation from its cytosolic HSP90 chaperone and heterodimerization with its ARNT partner, also a member

of the bHLH/PAS superfamily (Reyes et al., 1992). Binding of AHR-ARNT complexes to AHR-binding sites in the promoters of target genes recruits transcription cofactors and associated chromatin remodeling proteins and signals initiation of gene transcription (Schnekenburger et al., 2007). Well-known AHR target genes encode drug-metabolizing enzymes belonging to the cytochrome P450 CYP1 family and several phase II detoxification enzymes (Nebert et al., 2004). Emerging evidence indicates that in addition to the well-known roles of the receptor in mediating xenobiotic toxicity there are other AHR transcriptional targets, including genes involved in cell cycle regulation and morphogenetic processes that may play a vital function during embryonic development (Sartor et al., 2009). AHR is also involved in regulation of growth factor signaling, cell cycle proliferation, differentiation, arrest, and apoptosis that result from various forms of cross talk between AHR and other regulatory proteins (Ma et al., 2009).

TCDD-mediated AHR activation disrupts cardiovascular homeostasis in all species studied to date, including fish, avian, and mouse (Ivnitski-Steele et al., 2005). In humans, long-term epidemiologic studies have established a link between occupational exposure to high doses of TCDD and ischemic heart disease (Flesch-Janys et al., 1995). A higher incidence of hypertension, stroke, and coronary heart disease have been found in U.S. Army Chemical Corps veterans and Korean veterans who were occupationally exposed to Agent Orange in Vietnam, and exposed individuals from the accidental exposure in Seveso (Kang et al., 2006; Kim et al., 2003; Pesatori et al., 1998). A systematic analysis of all English-language epidemiologic studies of dioxin exposure and mortality from cardiovascular disease concluded that dioxin exposure in humans was associated with mortality from both ischemic heart disease and all cardiovascular disease (Humblet et al., 2008).

A number of physiological roles of the AHR have been recognized from the study of AHR null mice. Mice with a homozygous ablation of the *Ahr* gene are viable and resistant to TCDD toxicity (Fernandez-Salguero et al., 1996) although numerous age-related pathologies were observed in several organs, including reduced liver size, diminished reproductive capabilities, immunosuppression, epidermal hyperplasia, and an impaired cardiovascular phenotype (Fernandez-Salguero et al., 1995). Whole-body knockout mice showed a gross enlargement of the heart size and histologically demonstrated chronic, focal inflammation, and fibrosis in the ventricular and atrial myocardia (Fernandez-Salguero et al., 1997). Interference with endogenous AHR developmental functions, either by gene ablation or by *in utero* exposure to TCDD, disrupts stem cell differentiation and alters expression of homeobox transcription factors that control cardiomyogenesis (Wang et al., 2013), causing a phenotype of structural, molecular, and physiological cardiac abnormalities that progresses into the adults (Carreira et al., 2015a).

Cardiogenesis is a highly concerted process that requires precise orchestration of multiple gene networks regulating developmental commitments toward cellular differentiation, migration, proliferation, and death (Rana et al., 2013; Srivastava and Olson, 2000; Van Vliet et al., 2012). A key regulator of heart differentiation is the homeodomain-containing transcription factor NKX2.5, an evolutionarily conserved transcription factor that regulates cardiac myogenesis and heart morphogenesis from fly to man (Harvey, 1996). NKX2.5 is the earliest known cardiogenesis marker, expressed as early as E7.5 in mouse embryos (Schwartz and Olson, 1999). Targeted disruption of the *Nkx2.5* gene in mice caused abnormal heart morphogenesis at E8.5 and early embryonic death, whereas hemizygous mice

with only one functional *Nkx2.5* allele suffer from hemodynamic insufficiency (Lyons et al., 1995; Tanaka et al., 1999). NKX2.5 mutations in mice and humans cause congenital cardiac malformations and atrioventricular conduction defects (Biben et al., 2000; Jay et al., 2004; Schott et al., 1998; Tanaka et al., 2002), suggesting that NKX2.5 controls cardiac gene programming and specifies heart lineage while working in combination with other regulators to maintain cardiac homeostasis. Interactions between NKX2.5 and AHR have just begun to be recognized; genome-wide studies in mouse embryonic stem cells have shown that AHR activation by TCDD represses *Nkx2.5* mRNA and protein expression in a dose-dependent manner (Wang et al., 2010). Furthermore, *in vivo* studies have shown a significant decrease in the number of NKX2.5 positive nuclei in embryonic hearts of TCDD-exposed *Ahr*^{+/+} mice (Carreira et al., 2015b). However, *Nkx2.5* regulation by AHR appears to be indirect or dependent on interactions between AHR and other factors, for a screen of the *Nkx2.5* promoter region between – 10,000 and + 1000 nucleotides from the transcription start site failed to find canonical AHR-binding motifs (Wang et al., 2013).

To characterize the mechanisms responsible for AHR-mediated disruption of heart function, we have generated several mouse strains with cardiomyocyte-specific *Ahr* gene knockout. We previously reported on one of these, in which loxP-targeted *Ahr*^{fx/fx} alleles were deleted by cardiomyocyte-specific expression of cre recombinase driven by the promoter of the β Mhc (myosin heavy chain-beta) gene (Kurita et al., 2016). In those mice, AHR expression protected adult female mice from TCDD exposure-induced heart dysfunction, we further wished to test the hypothesis that gene-environment combinatorial interactions between AHR, TCDD exposure, and NKX2.5 might aggravate the cardiac insufficiency resulting from *Nkx2.5* hemizygosity. To this end, we took advantage of the availability of haploinsufficient *Nkx2.5*^{+/-cre} mice, bearing a “knock-in” cre recombinase gene integrated into the *Nkx2.5* locus, to knockout the cardiomyocyte *Ahr* gene simultaneously with the initiation of NKX2.5 expression and heart development at E7.5 in mice. Our findings underscore the conclusion that deletion of the AHR protects males against heart dysfunction due to NKX2.5 haploinsufficiency.

MATERIALS AND METHODS

Mice, genotyping, TCDD treatment, and determination of *Ahr* allele excision. We housed C57BL/6J mice in the Experimental Laboratory Animals Medical Services at the University of Cincinnati under controlled conditions of temperature, humidity, and lighting and provided standard mouse chow and water *ad libitum*. We conducted all experiments following the highest standards of humane care in accordance with the NIH Guide for the Care and Use of Laboratory Animals that have been approved by the University of Cincinnati Institutional Animal Care and Use Committee.

Ahr^{fx/fx}*Nkx2.5*^{+/-} mice with a floxed *Ahr* allele were a generous gift from Dr Christopher Bradfield (University of Wisconsin-Madison) (Walisser et al., 2005). *Ahr*^{+/+}*Nkx2.5*^{+/-cre} mice, bearing a cre recombinase transgene knocked-in in the *Nkx2.5* promoter by homologous recombination were a generous gift of Dr Jeffery Molkentin (Cincinnati Children's Hospital) and Dr R.J. Schwartz (Baylor College of Medicine, Houston, Texas) (Moses et al., 2001). *Ahr*^{fx/fx}*Nkx2.5*^{+/-} mice were initially bred into *Ahr*^{+/+}*Nkx2.5*^{+/-cre} mice to generate *Ahr*^{+/fx}*Nkx2.5*^{+/-cre} mice. Female *Ahr*^{+/fx}*Nkx2.5*^{+/-cre} mice were crossed to male *Ahr*^{fx/fx}*Nkx2.5*^{+/-} mice to generate *Ahr*^{fx/fx}*Nkx2.5*^{+/-} (*Ahr* pseudo-wild type, *Nkx2.5*

wild type), $Ahr^{fx/+}Nkx2.5^{+/+}$ (Ahr pseudo-wild type, $Nkx2.5$ wild type), $Ahr^{fx/fx}Nkx2.5^{+/cre}$ (Ahr floxed, $Nkx2.5$ haploinsufficient), and $Ahr^{fx/+}Nkx2.5^{+/cre}$ (Ahr pseudo-wild type, $Nkx2.5$ haploinsufficient). The Ahr -wild type genotypes $Ahr^{+/+}Nkx2.5^{+/+}$ and $Ahr^{fx/+}Nkx2.5^{+/cre}$ replaced the pseudo- Ahr wild type genotypes $Ahr^{fx/+}Nkx2.5^{+/+}$ and $Ahr^{fx/+}Nkx2.5^{+/cre}$ genotypes in the experiments shown in Figures 2 and 3. All mice used in the experiments reported here were backcrossed for at least 6 generations into a C57BL/6J background. To avoid litter effects, we used 1–2 males and the same number of females from each of several independent litters. The total number of mice in each experiment is indicated in the figure legends. When mice reached 3 months of age, groups of 5–8 mice were treated with corn oil or 1 μ g TCDD/kg/week by oral gavage for 2 additional months, measuring their body weight once per week throughout the duration of the TCDD treatment. After discontinuation of TCDD treatment, mice were examined for echocardiographic parameters at 5, 7, and 12 months of age. Some mice were sacrificed at 9 months to determine organ weights. To genotype the cre transgene and the Ahr floxed, excised, or wild-type alleles, we used PCR analysis of DNA isolated from snipped ear tissue. Analysis of $Ahr^{fx/fx}$ excision was carried out by multiplex PCR of DNA isolated from several tissues and organs including heart ventricles using 2 forward primers and 1 reverse primer. The genotypes of all the mice used in these analyses were verified by PCR amplification. Information of primers used for genotyping and excision are shown in Table 1.

Echocardiography. A full echocardiographic study was carried out to determine functional abnormalities, as previously described (Rubinstein et al., 2014) at the times indicated. Briefly, mice were anesthetized with isoflurane (1.5%–2%), and images were obtained from a parasternal long axis view between 2 and 10 mm in depth in both M-mode and B-mode using the Vevo 2100 Ultrasound system equipped with a MS400 probe (30-MHz centerline frequency). As previously described, data were post-processed using the Vevostain software (Visualsonic, Vevo 2100, v1.1.1 B1455) by an investigator blinded to the experiment working at a separate workstation. The B-mode, color, and Doppler images were analyzed for valvular function and structural abnormalities, while the M-mode images were postprocessed for cardiac functional analysis including ventricular size, ejection fraction, and cardiac output.

Collection of tissue samples. Following euthanasia, tissues were harvested, rinsed in PBS, gently blotted to remove excess fluids, and weighed using a high definition scale. Hearts were longitudinally bisected with one-half used for morphological studies (ie, histological processing) and the other one-half processed for molecular studies (real time RT-qPCR) as detailed later. When so indicated for reporting, organ weights were normalized to body weights.

Statistical analyses. All results are expressed as the mean $\pm$ SEM of biological replicates. Statistical analysis was performed using IBM SPSS Statistics ver. 19.0. A 1- or 2-way ANOVA followed by

Bonferroni's post hoc test was performed to compare means of body and tissue weights, and echocardiography. A P value of < 0.05 was considered statistically significant.

RESULTS

Cardiomyocyte-Specific Excision of the Ahr Gene

To investigate the potential interactions between AHR and the master cardiac transcription factor $NKX2.5$ in the developing heart, we used the cre -loxP system to generate cardiomyocyte-specific Ahr knockout mice. We crossed $Nkx2.5^{+/cre}$, which express the cre recombinase gene driven by the cardiomyocyte-specific $Nkx2.5$ promoter, with $Ahr^{fx/fx}Nkx2.5^{+/+}$ mice that have 2 floxed Ahr alleles. Further mating gave rise to mice of the genotypes described in the Materials and Methods section. To verify the excision of the floxed Ahr allele, we tested 15 different tissues from $Ahr^{fx/fx}Nkx2.5^{+/cre}$ mice for the loss of the Ahr^{fx} allele. Only heart tissue showed significant excision of the floxed Ahr allele with no detectable excision in other tissues, strongly supporting the cardiomyocyte-specificity of the excision (Figure 1). Cardiomyocytes constitute $< 50\%$ of the heart cells (Ali et al., 2014) and noncardiomyocyte cells, such as fibroblasts, do not express $NKX2.5$. Most likely, the unexcised Ahr^{fx} alleles that can still be detected in the heart (Figure 1) are present in these other cell types.

Ahr Ablation in Males Protects From Cardiac Hypertrophy Induced by $Nkx2.5$ Haploinsufficiency

Starting at 7 months of age, $NKX2.5$ haploidy in $Nkx2.5^{+/cre}$ mice caused an increase in the body weight of males compared with wild type (Figure 2A). Knocking out Ahr in $NKX2.5$ haploinsufficient mice significantly increased the body weight of male $Ahr^{fx/fx}Nkx2.5^{+/cre}$ even at an earlier age (5 months) (Figure 2A). In contrast, a significant increase of body weight in female mice of the same genotype was observed only at 9 months of age (Figure 2B), suggestive of a possible protective effect of sex. The bearing of 2 unexcised floxed Ahr alleles in mice of $Ahr^{fx/fx}Nkx2.5^{+/+}$ genotype was sufficient to cause an increase of body weight in male mice from 5 to 9 months of age (Figure 2A) but not in females (Figure 2B). To determine whether genotype also had an effect on the organ weights of these mice, we measured organ weights in a subset of the mice when they reached 9 months of age. Compared with wild type, $Ahr^{+/+}Nkx2.5^{+/cre}$ haploinsufficient males showed a significant increase in mean heart weight relative to body weight, consistent with the cardiac hypertrophy observed by others in these mice (Lyons et al., 1995; Tanaka et al., 2002). Knockout of the Ahr receptor blunted the hypertrophy of the haploinsufficient $Ahr^{fx/fx}Nkx2.5^{+/cre}$ male mice and caused their mean heart weight to drop by 30% and be more similar to the weight of the fully wild-type $Ahr^{+/+}Nkx2.5^{+/+}$ mice (Figure 3-Heart). In contrast, $Nkx2.5$ haploinsufficiency showed no effect on the relative heart weights of females (Figure 3-Heart). All male mice of $Ahr^{fx/fx}Nkx2.5^{+/+}$, $Ahr^{+/+}Nkx2.5^{+/cre}$, and $Ahr^{fx/fx}Nkx2.5^{+/cre}$ genotypes showed

Table 1. Primer List for Genotyping

Forward (5' > 3')	Reverse (5' > 3')	Amplicon size (bp)	Target
GGCGTTTTCTGAGCATACCT	CTACACCAGAGACGGAAATCC	587	$Nkx2.5^{cre}$ allele
CAGTGGGAATAAGGCAAGAGTGA	GGTACAAGTGACATGCCTGC	Ahr wt allele: 106 Ahr fx allele: 140	Ahr^{+} or Ahr^{fx} allele
CAGTGGGAATAAGGCAAGAGTGA	GTCACCTCAGATTACACTTTCTA	180	Excised Ahr^{fx} allele

Figure 1. Determination of tissue-specific *Ahr* excision in cardiomyocyte-specific *Ahr* knockout mice. **A**, PCR genotyping and gel electrophoresis analysis of *Ahr^{fx/fx}Nkx2.5^{+/+}*, *Ahr^{fx/fx}Nkx2.5^{+/+}*, *Ahr^{fx/fx}Nkx2.5^{+/cre}*, and *Ahr^{+/fx}Nkx2.5^{+/cre}* in heart ventricles of 2-day postnatal (PND2) old mice. The panel shows the *cre* allele in *Nkx2.5^{cre}* and wild-type, excised, and unexcised *Ahr* floxed alleles. **B**, Genotyping of several organs in 30-day-old *Ahr^{fx/fx}Nkx2.5^{+/cre}* mice. H, heart; L, liver; K, kidney; SP, spleen; TH, thymus; P, pancreas; ST, stomach; CO, colon; IN, intestine; M, muscle; LU, lung; B, brain; E, eye; and BW, bone marrow.

significantly lower kidney weights than wild-type males, although, surprisingly the presence of the loxP site in the unexcised floxed *Ahr* alleles in *Ahr^{fx/fx}Nkx2.5^{+/+}* was sufficient to cause a reduction in the kidney weight of these mice relative to the wild type (Figure 2-Kidney). We have no explanation for this phenomenon beyond the possibility that the insertion of the loxP site might somehow influence the expression levels of the *Ahr* gene in kidney. As in the heart, genotype had no other effect on the relative kidney weight of females (Figure 3-Kidney). In both males and females, genotype caused no changes in the relative weights of liver, spleen, or fat tissues (data not shown).

Ahr Ablation in the Heart Perturbs Cardiac Function

To determine whether *Ahr* ablation in the heart would hinder adult heart function and aggravate potential responses to TCDD, we sought to determine the extent to which echocardiographic parameters of heart physiology were altered by genetic disruption of the *Ahr* in cardiomyocytes. Resting (conscious) heart rates were determined in male and female mice at 5, 7, and 12 months of age. Echocardiographic assessment revealed no genotype-dependent difference in heart rate of males or females at 5 or 7 months (data not shown) but significant differences at the later age of 12 months. At this time phenotypic differences were evident in both males and females, leading to a significant heart rate depression in haploinsufficient *Ahr^{fx/fx}Nkx2.5^{+/cre}* mice, with 2 deleted cardiac *Ahr* alleles, relative to their *Ahr^{+/fx}Nkx2.5^{+/cre}* counterparts, with just one deleted *Ahr* allele, or to the genotypes with wild-type *Nkx2.5^{+/+}*. This effect of *Ahr* genotype on heart rate was evident in both sexes (Figure 4A).

Loss of AHR in the heart also caused significant genotype- and sex-dependent changes in ventricular volume of *Nkx2.5^{+/cre}* haploinsufficient mice. In males, significant differences due to genotype were observed both in systolic and diastolic volumes. Deletion of both *Ahr* alleles in these mice caused a significant difference in both systolic and diastolic volumes relative to wild-type males and a significant increase relative to the pseudo-wild type *Ahr^{+/fx}Nkx2.5^{+/cre}* with only one *Ahr* allele excised (Figs. 4B and 4C). The effect of genotype on these

Figure 2. Body weight changes in cardiomyocyte-specific *Ahr* knockout mice. Five to eight mice from each sex, male (A) and female (B), were used for body weight measurements. Data are expressed as the mean $\pm$ SEM. * indicates significance in the comparison between *Ahr^{+/+}Nkx2.5^{+/+}* and *Ahr^{fx/fx}Nkx2.5^{+/+}* and # indicates significance in the comparison between *Ahr^{+/+}Nkx2.5^{+/+}* and *Ahr^{fx/fx}Nkx2.5^{+/cre}* (*#P < 0.05).

parameters in females was either strongly blunted (Figs. 4B and 4D–F) or mildly significant (Figure 4C).

We also found significant changes due to genotype in cardiac output, ejection fraction, and stroke volume, but only in the male experimental groups, not in the female. Haploinsufficient mice of *Ahr^{+/fx}Nkx2.5^{+/cre}* had significantly reduced cardiac output, ejection fraction, and stroke volume but ablation of both *Ahr^{fx}* alleles in *Ahr^{fx/fx}Nkx2.5^{+/cre}* males restored these functions to wild type-like levels (Figs. 4D–F).

TCDD Exposure Restores Cardiac Physiology in Aging Males

To determine whether TCDD exposure affected the outcome of gene interactions between *Ahr* and *Nkx2.5*, we focused on mice of 2 *Nkx2.5^{+/cre}* genotypes, bearing either a wild-type *Ahr^{+/fx}* allele combination—*Ahr^{+/fx}Nkx2.5^{+/cre}*—or the fully ablated *Ahr^{fx/fx}* complement—*Ahr^{fx/fx}Nkx2.5^{+/cre}*. Echocardiographic parameters in these mice were determined at the end of a 2-month exposure to 1 μ g TCDD/kg/week, from 3 to 5 months of age, and subsequently after 2 and 7 additional months unexposed to TCDD. No obvious differences of cardiac function in response to TCDD exposure were observed at 5 and 7 months but significant differences from control mice were found in aging 12-month-old males. No change in heart rate was evident in exposed mice relative to controls (Figure 5A) but exposure, terminated 7 months earlier, caused a significant reduction in ventricular volumes in systole and in diastole for both genotypes in 12-month-old males (Figs. 5B and 5C). Accordingly, we found a major TCDD

Figure 3. Organ weight (normalized to body weight) of heart and kidney at 9 months of age. Five to eight mice per group were used for weight determinations of male and female mice from 4 genotypes, *Ahr*^{+/+}*Nkx2.5*^{+/+}, *Ahr*^{f/f}*Nkx2.5*^{+/+}, *Ahr*^{+/+}*Nkx2.5*^{+/cre}, and *Ahr*^{f/f}*Nkx2.5*^{+/cre}. Data are expressed as the mean $\pm$ SEM. (**P* < 0.05, ***P* < 0.01, and ****P* < 0.001).

exposure-dependent difference between 2 genotypes in cardiac output, ejection fraction, and stroke volume. As shown earlier in Figure 4, cardiac function was significantly depressed in male *Ahr*^{+/+}*Nkx2.5*^{+/cre} mice and restored to wild-type levels by full ablation of 2 *Ahr*^{f/f} alleles. TCDD exposure caused no change in the parameters in *Ahr*^{f/f}*Nkx2.5*^{+/cre} mice but increased them to wild-type levels in *Ahr*^{+/+}*Nkx2.5*^{+/cre} males (Figs. 5D–F).

DISCUSSION

In this study, we have investigated the role of the interactions between NKX2.5, AHR, and TCDD on cardiac function and structure. Previous studies on whole-body *Ahr* knockout mice have shown a phenotype of heart hypertrophy and fibrosis, suggesting that the AHR plays an endogenous role in cardiac function and in TCDD-induced cardiotoxicity (Fernandez-Salguero et al., 1997). Similar studies on whole-body *Ahr* knockout mice (Kopf et al., 2008; Kopf and Walker, 2009) and in mice transgenic for a constitutively activated AHR (Brunnberg et al., 2006) demonstrated that adult TCDD exposure induces an increase in blood pressure and heart hypertrophy, supporting the conclusion that

endogenous cardiac AHR functions are responsible for heart toxicity in response to TCDD exposure. Recently, to determine the role of cardiomyocyte-specific *Ahr* in heart function and its disruption by TCDD, we generated another cardiomyocyte-specific *Ahr* knockout mouse in which loxP-targeted *Ahr*^{f/f} alleles were deleted by expression of cre recombinase driven by the promoter of the β Mhc (myosin heavy chain-beta) gene (Kurita et al., 2016). In these mice, AHR expression protected adult female mice from TCDD-exposure induced heart dysfunction, including a significant increase in body weight, blood pressure, and cardiac hypertrophy and a decrease in cardiac ejection fraction (Kurita et al., 2016). Because expression of myosin heavy chain-beta, which starts at E10.5 in mice, is a later event in cardiomyocyte development than expression of NKX2.5 (Ng et al., 1991), we argued that an earlier *Ahr* deletion might have more dire consequences in the development of a heart disease phenotype. Hence, we chose to drive expression of cre recombinase with the promoter of the *Nkx2.5* gene, the earliest known cardiogenesis marker, expressed as early as E7.5 in mouse embryos (Schwartz and Olson, 1999). In addition to the earlier expression time, a second advantage of this choice was that the cre gene is integrated into the *Nkx2.5* locus, such that “knock-in” *Nkx2.5*^{+/cre} mice bear a single functional *Nkx2.5* allele and thus are NKX2.5 haploinsufficient (Lyons et al., 1995; Tanaka et al., 1999). Using these mice as the genetic background of the *Ahr* ablation would help determine whether AHR cooperates with or antagonizes NKX2.5 haploinsufficiency.

Nkx2.5 haploinsufficient mice had significantly increased left ventricular systolic and diastolic volume in both *Ahr*^{+/+}*Nkx2.5*^{+/cre} and *Ahr*^{f/f}*Nkx2.5*^{+/cre} males, with no effect of *Ahr* genotype detected in *Nkx2.5*^{+/cre} females. Cardiac deletion of both *Ahr* alleles in *Ahr*^{f/f}*Nkx2.5*^{+/cre} males restored the loss of cardiac function of *Ahr*^{f/f}*Nkx2.5*^{+/cre}. Females, however, showed no loss of cardiac function, providing strong evidence of sexual dimorphism. For the most part, AHR-mediated responses were absent in females and present in males, albeit at times with a deleterious effect. *Ahr* ablation provided males with significant protection against body weight and heart weight increase, and increase of left ventricle volume, presumably the result of *Nkx2.5* haploinsufficiency. Paradoxically, the interactions between *Ahr* and *Nkx2.5* had in some occasions a detrimental effect on male heart function. Conversely, females showed AHR-independent responses or no response at all to *Nkx2.5* haploinsufficiency in most of the parameters tested. Cardiomyocytes constitute < 50% of the cells in the heart (Ali et al., 2014); for which reason, it is unfeasible to determine whether cardiomyocyte AHR-independent responses in females are truly cardiomyocyte AHR independent or are dependent on the mesenchymal or epicardial AHR, which are not ablated in the *Ahr*^{f/f}*Nkx2.5*^{+/cre} mice. In contrast, the AHR-dependent responses in the males are unambiguously dependent on the cardiomyocyte AHR.

Interestingly, a majority of the findings driven by whole-body AHR disruption that we have observed in the past were generally more pronounced in females than in males (Carreira et al., 2015a). Although our current results do not address sexual dimorphism directly, it is reasonable to surmise that a network of interacting signals regulated by *Ahr* and other genes might be responsible for sex-specific responses that may also be expressed in a tissue-specific manner. Our findings suggest that the compensatory mechanisms at play in the heart may be sufficient in one sex but not the other to cope with environmental stressors. Preeminent among these are dimorphic hormonal responses, such as the complexes formed between the AHR and

Figure 4. Echocardiographic determination of cardiac functions in mice with a cardiomyocyte-specific *Ahr* deletion. Five to eight mice of each sex and genotype, as shown in the figure, were used for echocardiographic determination of heart rate (A), volume in systole (B), and diastole (C); cardiac output (D), ejection fraction (E), and stroke volume (F) at 12 months of age. Data are expressed as the mean $\pm$ SEM. Asterisks indicate significance (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$). Only the indicated comparisons reached statistical significance.

the estrogen receptor, which may modify estrogenic responses (Carlson and Perdew, 2002).

In this work, we set out to test whether interactions between *Ahr* and *Nkx2.5*, in combination with TCDD exposure might aggravate the cardiac insufficiency resulting from *Nkx2.5* hemizygosity. Our data indicate that in this context insufficiency is in fact a function of at least 3 components: *Ahr* genetics, TCDD exposure, and cell lineage identity. The large increase in ventricular volume resulting from *Nkx2.5* hemizygosity is increased

even further by *Ahr* ablation, but regardless of *Ahr* deletion in cardiomyocytes, the increase is suppressed by TCDD, suggesting that the effect is either AHR-independent or -dependent on the unexcised *Ahr* gene present on noncardiomyocyte cardiac cells. In contrast, all 3, cardiac output, stroke volume, and ejection fraction, are largely reduced by *Nkx2.5* hemizygosity, and all 3 are rescued by deletion of the *Ahr* gene, suggesting a detrimental cooperation between NKX2.5 and AHR. It is somewhat surprising that in this case TCDD promotes a beneficial

Figure 5. Echocardiographic analyses of heart function in 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) exposed and control mice with a cardiomyocyte-specific *Ahr* deletion. Five to eight 3-month-old mice of each sex and genotype were exposed for 2 months to 1 $\mu\text{g/kg/week}$ of TCDD or corn oil vehicle by gavage. At 5 months of age, exposure was terminated and the mice maintained until they reach 12 months, at which time they were used for echocardiographic determination of the same parameters analyzed in Figure 4. C, corn oil control and T, TCDD. Data are expressed as the mean $\pm$ SEM. Asterisks indicate significance (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$).

outcome, rescuing the mice from the detrimental effects of the AHR-NKX2.5 interaction.

It is intriguing that the response to TCDD is so protracted, to the extent that at 12 months of age, 7 months after termination of exposure, TCDD- and AHR-dependent disruption of echocardiographic parameters still persists in males. Continued presence of TCDD in circulation is unlikely to be the reason of this persistence because the biological half-life of TCDD in mice is

only a few days (DeVito and Birnbaum, 1995). It is striking that deletion of the *Ahr* gene on embryonic day E7.5, the time of *Nkx2.5* expression, has such protective consequences in adult heart dysfunction and handling of TCDD. Ironically, rather than being detrimental, adult TCDD exposure had no cardiac dysfunction effects on the *Ahr*^{f/f}*Nkx2.5*^{+/cre} mice but instead brought these functions back to normal in mice of *Ahr*^{+/f}*Nkx2.5*^{+/cre} genotype, likely reflecting multiple interconnected

compensatory responses in cardiac function and structure. Possibly, the ones that we observe are just the more evident subset of complex molecular departures from homeostasis that translate into a more severe adult phenotype (Carreira et al., 2015a,b). To identify the sustainable functional signals and long-term biological processes triggered by Ahr ablation would be fundamental to understand the central role that the AHR signaling network plays in cardiovascular function and dysfunction.

ACKNOWLEDGMENTS

The authors thank Dr Ying Xia, Dr Chia-I Ko, Dr Hongxia Zhang, Andrew VonHandorf, and Matthew de Gannes for a critical reading of the article.

FUNDING

This research was supported by National Institute of Environmental Health Sciences (NIEHS) grants (R01 ES06273, R01 ES024744, and R01 ES10807).

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