

Patent Ductus Venosus and Dioxin Resistance in Mice Harboring a Hypomorphic *Arnt* Allele*

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The *Ah* receptor nuclear translocator (ARNT) is the dimeric partner of hypoxia-inducible factors and thus plays a pivotal role in cellular adaptation to low oxygen environments. ARNT is also a dimeric partner for the *Ah* receptor (AHR), and this complex is essential in regulating the adaptive metabolic response to polycyclic aromatic hydrocarbons. Because of the essential role of ARNT in hypoxia-driven developmental events, it has been difficult to study the physiological significance of AHR-ARNT heterodimers *in vivo*. To address this issue, we developed a hypomorphic *Arnt* allele that displayed normal development and allowed the examination of the role of ARNT in AHR biology. In this regard, the AHR is also known to mediate two additional biological processes: the toxicological response to compounds such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (dioxin) and the developmental closure of a fetal vascular structure known as the ductus venosus. Although the mechanism of the adaptive pathway has been well described, the mechanism of AHR-mediated signal transduction in the toxic and developmental pathways is not well understood. Liver perfusion studies demonstrated that ARNT hypomorphs have a patent ductus venosus, identical to that observed in the *Ahr* null mice. Parallel dioxin toxicity studies demonstrated that the ARNT hypomorphs exhibited resistance to the end points of dioxin exposure. Moreover, we observed that toxicity could be segregated from the classical adaptive responses such as P4501A induction. Taken in sum, these experiments demonstrate that ARNT is an essential component of AHR developmental signaling and shed light on the mechanism of dioxin toxicity.

The aryl hydrocarbon receptor nuclear translocator (ARNT)¹ and the *Ah* receptor (AHR) are founding members of the PAS

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¹ The abbreviations and trivial names used are: ARNT, aryl hydrocarbon receptor nuclear translocator; *Arnt*^{neo}, hypomorphic *Arnt* allele; AHR, aryl hydrocarbon receptor; ALT, alanine aminotransferase; bHLH, basic helix-loop-helix; dioxin, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; DV, ductus venosus; EROD, ethoxyresorufin *O*-deethylase; HIF, hypoxia-inducible factor; *Neo*, neomycin resistance cassette; PAH, polycyclic aromatic hydrocarbon; PAS, Period-ARNT-Single-minded.

superfamily of transcriptional regulators (1). These proteins were originally identified as the result of their involvement in the regulation of an adaptive metabolic response to certain xenobiotics, such as polycyclic aromatic hydrocarbons (PAHs) (2, 3). In this pathway, PAH molecules bind to the AHR, which then translocates to the nucleus and heterodimerizes with its transcriptional partner, ARNT. The AHR-ARNT complex then interacts with specific response elements to up-regulate a battery of xenobiotic metabolizing enzymes that include the cytochrome p450 enzymes, *Cyp1a1*, *Cyp1a2*, *Cyp1b1* as well as the phase II enzymes *Gst-a1* and *Ugt1-06* (2, 4). Given that each of the up-regulated enzymes metabolize PAHs, we refer to this process as the "adaptive pathway" of AHR.

In addition to its role in the adaptive metabolism of xenobiotics, the AHR also mediates two other biological pathways that we refer to as "toxic" and "developmental." In the toxic pathway, exposure to potent agonists such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (dioxin) results in a response that includes end points such as hepatotoxicity, thymic involution, epithelial hyperplasia, and cleft palate (5). A compelling body of genetic and pharmacological evidence has demonstrated that the AHR mediates most, if not all, of these toxic responses (5–7). With regard to the developmental pathway, a highly reproducible phenotype arising in *Ahr* null mouse models is a reduced liver size. We have proposed that the smaller liver size is the result of a persistent fetal vascular shunt known as the ductus venosus (DV), and furthermore, that the failure of the DV to close at parturition decreases the portal blood supply to the liver (8). As a consequence, nutrient supply is limited, and the liver size is decreased.

We view AHR signal transduction as encompassing three physiological processes: 1) the adaptive pathway ensuring metabolism of PAHs, 2) the toxic pathway mediating the deleterious effects of dioxin exposure, and 3) the developmental pathway guiding the resolution of fetal liver vascular architecture. We hypothesize that the fundamental mechanisms behind these three distinct biological processes are dependent upon AHR-ARNT heterodimerization, interaction with cognate responsive elements, and up-regulation of transcriptional targets. The ability to produce three distinct biological events from one signal transduction mechanism may lie in either the transcriptional up-regulation of the same battery of genes with some temporal and/or spatial specificity or the up-regulation of gene sets unique to each biological event.

Although ARNT has a well defined role in adaptive metabolism, its role in mediating the developmental signaling and dioxin-induced toxicity of AHR has not been fully addressed. Although it may be assumed that all features of AHR signal transduction are dependent upon ARNT heterodimerization, a formal demonstration of this hypothesis is lacking. In this regard, it is important to note that at least three putative

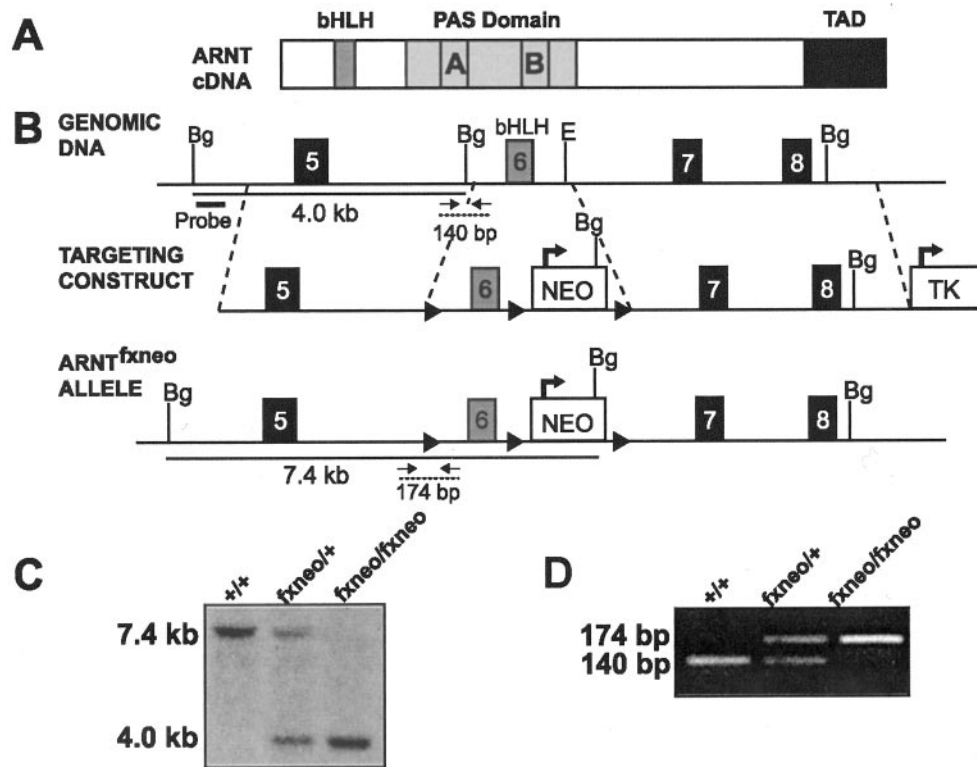


FIG. 1. Generation of *Arnt*^{fxneo} mice. *A*, a schematic diagram of the *Arnt* cDNA identifying important functional domains including the bHLH, the PAS domain with A and B repeats, and the transactivation domain (*TAD*). *B*, a schematic diagram illustrating the region surrounding the bHLH domain of the murine *Arnt* locus, the targeting construct, and the resulting hypomorphic allele. *Exon numbers* reflect known coding exons. The *Neo* cassette was inserted into an endogenous *EcoRI* site (*E*) 3' to the bHLH. *Dashed lines* indicate regions of homology used for homologous recombination. *Solid lines* indicate fragment sizes detected by the probe following digestion of genomic DNA with *BglII* (*Bg*). *Dotted lines* represent the fragment sizes generated by PCR genotyping of the wild-type and hypomorphic alleles using OL2300 as the forward primer and OL2301 as reverse primer. *TK*, thymidine kinase. *C*, a Southern blot of mouse tail biopsies showing bands of 4.0 and 7.4 kb, indicating the presence of the wild-type and mutant alleles. *D*, PCR genotyping of tail biopsies showing bands of 140 and 174 bp, indicating the presence of the wild-type and mutant alleles.

homologs of ARNT have been identified in the human genome, ARNT2, MOP3, and MOP9, (9–12). Furthermore, the ARNT2 protein has been shown to form transcriptionally active partnerships with the AHR in transient transfection experiments (9). In an effort to examine the possibility that the toxic and developmental aspects of AHR biology are dependent upon ARNT heterodimerization, we used gene targeting to generate a hypomorphic or low-expressing *Arnt* allele, designated *Arnt*^{fxneo}. This novel allele allowed us to bypass the known embryonic lethality of the *Arnt* null mutation, and therefore, to assess the effect of limiting ARNT on AHR-dependent toxicity and development (13, 14). Through this examination, we provide evidence to support the idea that heterodimerization is an essential feature of adaptive, toxic, and developmental AHR signaling.

EXPERIMENTAL PROCEDURES

Generation of *Arnt*^{fxneo} Mice—A bacteriophage (P1) clone containing the murine *Arnt* locus was obtained from Genome Systems (St. Louis, MO) (13). The strategy for creation of the targeting construct (PL910) involved insertion of a neomycin resistance cassette (*Neo*), flanked by *loxP* recombination sequences, into the *EcoRI* site 3' of exon 6 (Fig. 1B). A third *loxP* site was inserted into the intron 5' of exon 6, replacing the internal *BglII* site. Our homologous recombination protocols using GS1 embryonic stem cells (Genome Systems) have been described previously (15). Clones were screened for homologous recombination by Southern blot of *BglII*-digested genomic DNA using a 600-bp probe 5' to the end of the targeting construct (PL1220). Targeted clones were injected into 3.5-day postcoital C57BL/6J blastocysts, and resulting chimeras were backcrossed to C57BL/6J mice to determine germ line transmission of the targeted allele. Prior to experimental analysis, transmitting animals were backcrossed to C57BL/6J for at least five generations. Genotyping was performed by PCR on DNA isolated from tail biopsies using

the forward primer, OL2300 (5'-GCAACTTTGACAAGGCAGCATTTA-3'), and the reverse primer, OL2301 (5'-GGCAGGGGAATCTCT-GAGTTCT-3'). These primers amplified a 140-bp band from the wild-type allele and a 174-bp band from the targeted allele due to the insertion of the *loxP* site. The PCR was carried out for 30 cycles (92 °C/30 s; 58 °C/30 s; 72 °C/30 s) in a reaction mixture containing 2.5 units of Taq polymerase (Promega), 50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25 °C), 1.5 mM MgCl₂, 1% Triton® X-100, 200 μM dNTPs, and 0.2 μM of each primer.

Animals—Mice were housed in a selective pathogen-free facility on corn cob bedding with food and water *ad libitum* according to the rules and guidelines set by the University of Wisconsin. ARNT expression was evaluated in untreated adult wild-type and *Arnt*^{fxneo/fxneo} mice by Western blot analysis of cytosolic fractions prepared from various tissues including the liver, spleen, heart, lung, thymus, and uterus (15, 16). In toxicology studies, 6-week-old mice were dosed once by oral gavage with 64 μg/kg of dioxin in corn oil or with corn oil alone. After 16 days, animals were deeply anesthetized with an intraperitoneal injection of urethane (Sigma, 200 μg/g of body weight). Whole blood was obtained by retro-orbital puncture for serum analysis of alanine aminotransferase (ALT). After liver perfusion to determine DV status (see below), organs were dissected and weighed. Liver sections were fixed in 10% formalin and embedded in paraffin for sectioning and histological staining. Western blots and ethoxyresorufin *O*-deethylase (EROD) assays were performed on cytosolic and microsomal liver fractions, respectively (15, 16). In situations where multiple comparisons could be made, an analysis of variance was performed, and Tukey's test was used to determine differences with a *p* ≤ 0.05.

Ductus Venosus Status—The status of the DV was assessed by perfusion of the liver with trypan blue (8). Briefly, livers were cannulated via the portal vein, and the inferior vena cava was incised to permit outflow. After flushing the liver with phosphate-buffered saline, ~0.5 ml of trypan blue was injected into the liver. Lack of blue coloration in the liver following perfusion indicated a patent (open) DV. To confirm this analysis, time-lapsed angiography was performed to visualize the

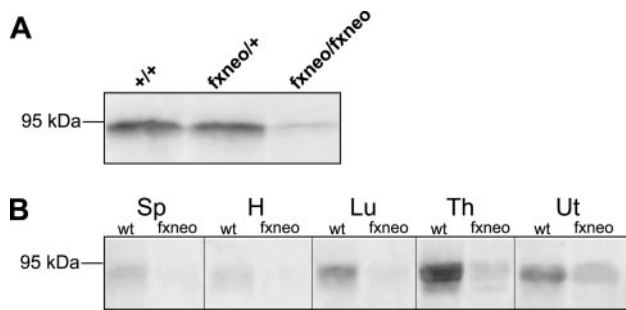


FIG. 2. The $Arnt^{fxneo/fxneo}$ allele has hypomorphic expression in a variety of mouse tissues. **A**, Western blot analyses comparing protein levels in cytosolic liver extracts from wild-type (+/+), $Arnt^{fxneo/+}$, and $Arnt^{fxneo/fxneo}$ mice. **B**, Western blot analyses showing protein levels in cytosolic extracts from wild-type (wt) and $Arnt^{fxneo/fxneo}$ ($fxneo$) mice prepared from spleen (Sp), heart (H), lung (Lu), thymus (Th), and uterus (Ut).

flow of Omnipaque 300 (Nycomed Inc., Princeton, NJ) after injection into the portal vein. To visualize liver vascular architecture, continuous x-ray images were obtained over 10 s (8).

RESULTS

Generation of $Arnt^{fxneo}$ Mice—Given that a targeted disruption of the *Arnt* locus results in developmental lethality at embryonic day 10, we examined the idea that the generation of a hypomorphic allele would provide sufficient ARNT to bypass the developmental block and produce animals that were limited in the amount of ARNT available for various signal transduction pathways (13, 14). Previous targeting events in our laboratory demonstrated that insertion of the *Neo* gene proximal to the exon encoding the basic helix-loop-helix (bHLH) domain often resulted in the generation of a hypomorphic allele (15). Therefore, we inserted a *Neo* cassette flanked by *loxP* sites (“floxed”) adjacent to exon 6, which encodes the bHLH of *Arnt* (Fig. 1B). A third *loxP* site was inserted upstream of exon 6 for later creation of the conditional null. A map of the *Arnt* allele, which we designate as $Arnt^{fxneo}$, is shown in Fig. 1B. Also shown is a map of the *Arnt* structural gene as well as the construct used for embryonic stem cell targeting. Following homologous recombination in embryonic stem cells and both positive and negative selection, surviving clones were screened by Southern blot (Fig. 1C). Correctly targeted clones were used to generate chimeras, and these mice provided germ line transmission of the $Arnt^{fxneo}$ allele. A PCR-based protocol was developed to genotype pups based on detection of the *loxP* sequence upstream of the exon encoding the bHLH (Fig. 1D).

The $Arnt^{fxneo}$ Allele Is Globally Hypomorphic in Expression—The influence of this mutation on ARNT protein expression was examined by Western blot of protein extracts derived from various tissues prepared from wild-type mice and mice homozygous for the $Arnt^{fxneo}$ allele (i.e. $Arnt^{fxneo/fxneo}$). This analysis revealed that ARNT expression was significantly diminished in all tissue samples analyzed, including liver, spleen, heart, lung, thymus, and uterus (Fig. 2, A and B). We estimate that expression from $Arnt^{fxneo/fxneo}$ in mouse liver was reduced to ~10% of wild-type levels, based on comparison with liver protein extracts obtained from wild-type animals. The expression from $Arnt^{fxneo/fxneo}$ in other tissues was estimated to be between 5 and 15%. This experiment establishes that the $Arnt^{fxneo}$ allele is globally hypomorphic for ARNT protein expression. To examine the relationship between ARNT expression and the copy number of *Arnt* alleles, we also examined protein extracts from wild-type, $Arnt^{fxneo/+}$, and $Arnt^{fxneo/fxneo}$ mouse livers (Fig. 2A). When a single hypomorphic allele is combined with a wild-type allele (i.e. $Arnt^{fxneo/+}$), expression is decreased when compared with the wild-type allele. As noted above, homozygosity results

in a further, but not complete, reduction of protein expression. This observation is consistent with the idea that protein expression is a linear sum of expression from all alleles present and is an indication that locus compensation is not occurring.

Embryonic Development of $Arnt^{fxneo}$ Hypomorphs—Due to the embryonic block described previously for the *Arnt* null alleles, we examined the influence of our hypomorphic $Arnt^{fxneo}$ allele (also referred to as the “ARNT hypomorph”) on fetal development. When dams heterozygous for $Arnt^{fxneo}$ allele (i.e. $Arnt^{fxneo/+}$) were crossed with homozygous $Arnt^{fxneo/fxneo}$ males, the resulting progeny were 63% (84/143) $Arnt^{fxneo/+}$ heterozygotes and 35% (50/134) $Arnt^{fxneo/fxneo}$ homozygotes. The lack of conformation to the expected Mendelian ratio (50:50) indicated partial embryonic lethality arising from the homozygous allele. The fact that a portion of homozygous $Arnt^{fxneo/fxneo}$ pups survive indicates that expression of *Arnt* from the hypomorphic allele is above the threshold to bypass the developmental block. Male and female homozygote survival rates were approximately equal.

The outcome of our ARNT hypomorph breeding program also demonstrated that the hypomorphic $Arnt^{fxneo}$ allele appeared to have a significant effect on the ability of the dam to maintain a pregnancy since homozygous $Arnt^{fxneo/fxneo}$ dams failed to produce live litters even when crossed to wild-type males. The fact that homozygous pups are born to heterozygous $Arnt^{fxneo/+}$ dams when the sire is homozygous $Arnt^{fxneo/fxneo}$ provides evidence that the embryonic lethality in hypomorphic $Arnt^{fxneo/fxneo}$ dams is maternal in origin. Although considered preliminary, the gestational failure in $Arnt^{fxneo/fxneo}$ dams appears to occur around the time of implantation (data not shown). For the purpose of the following studies, the salient point is that hypomorphic animals can be generated by crosses of the heterozygous $Arnt^{fxneo/+}$ dams with homozygous $Arnt^{fxneo/fxneo}$ males. All ARNT hypomorphs surviving from these crosses are outwardly normal, and the male hypomorphs are fertile.

ARNT Is Required for Resolution of the Ductus Venosus—Previous studies from our laboratory have demonstrated that the *Ahr* null animals are viable but display a rare vascular phenotype where the fetal DV fails to close. Because AHR signaling is important in vascular resolution and because ARNT is the heterodimeric partner of the AHR, we asked whether our ARNT hypomorphs would display a similar vascular phenotype to that seen in the $Ahr^{-/-}$ mice. Time-lapse radiographs of the liver vascular system illustrate the distinct vascular phenotypes of wild-type and $Arnt^{fxneo/fxneo}$ mice. Upon injection of contrast agent in wild-type mice, both primary and secondary portal branches of the liver vascular network fill quickly and completely (Fig. 3, A–C). When the vascular pattern of the $Arnt^{fxneo/fxneo}$ mice was evaluated, we found that the contrast agent entered the liver via the portal vein and immediately flowed through the DV into the inferior vena cava (Fig. 3, IVC), where it exited the liver (Fig. 3, D–F). As a direct comparison, we also examined the vascular structure of the *Ahr* null mouse. As observed previously, contrast agent also flowed through the DV in *Ahr* null mice, thus demonstrating that the ARNT hypomorphs and the *Ahr* null mice have an identical liver vascular phenotype (Fig. 3, G–I) (8). Liver perfusion studies using a colored dye to visualize filling of the hepatic vascular system also documented the DV phenotype and revealed that the DV persists in ~70% (17/24) of the $Arnt^{fxneo/fxneo}$ mice. Moreover, this phenotype is independent of sex of the animal (data not shown).

The $Arnt^{fxneo}$ Allele Maintains the Adaptive AHR Signaling Pathway—In an effort to understand the implications of the hypomorphic $Arnt^{fxneo}$ allele on the adaptive and toxic signal-

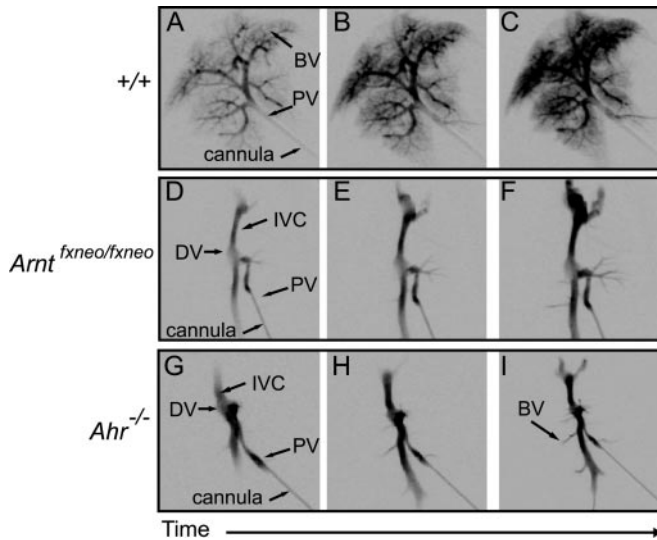


FIG. 3. Persistent ductus venosus in the $Arnt^{fxneo}$ hypomorph recapitulates the Ahr null liver vascular phenotype. Time-lapsed radiographs of portal vein-injected contrast agent entering the liver of wild-type (+/+), $Arnt^{fxneo/fxneo}$ (D–F), and $Ahr^{-/-}$ mice (G–I) are shown. Arrows indicate key anatomical features of the liver. PV, portal vein; IVC, inferior vena cava; BV, branching vessels; DV, ductus venosus.

ing pathways of AHR, we examined the response of $Arnt^{+/+}$, $Arnt^{fxneo/+}$, and $Arnt^{fxneo/fxneo}$ mice to a single dose of 64 $\mu\text{g}/\text{kg}$ of dioxin. Sixteen days after dioxin treatment, liver microsomes were prepared, and the P450 arm of the adaptive metabolic response was measured. For liver, EROD activity is a composite measure of CYP1A1 and CYP1A2 enzymes, which are classic AHR-induced adaptive responses to dioxin exposure. Microsomes from all groups showed negligible EROD activity in the absence of the AHR ligand, dioxin. In response to dioxin, microsomes from wild-type, $Arnt^{fxneo/+}$, and $Arnt^{fxneo/fxneo}$ mice are similarly induced, demonstrating that the adaptive AHR signal transduction pathway is functional in ARNT hypomorphs at this dose (Fig. 4A). Consistent with the idea that increased liver weight following dioxin exposure is at least partially the result of proliferation of the smooth endoplasmic reticulum, liver weight in dioxin-treated wild-type and $Arnt^{fxneo/+}$ mice was increased to approximately the same extent when compared with their vehicle-treated controls (Fig. 4B). Liver weights were also increased proportionally in the $Arnt^{fxneo/fxneo}$ mice treated with dioxin when compared with their vehicle-treated controls. Although the presence of the DV correlates with a smaller liver, the proportional increase in liver weight in ARNT hypomorphs is independent of the presence of the DV (Fig. 4B, inset) (8).

The $Arnt^{fxneo}$ Allele Confers Resistance to Dioxin Toxicity—In an effort to better understand the role of ARNT in the toxic pathway of AHR signaling, we employed the ARNT hypomorph to examine two classical end points of dioxin toxicity. Following exposure of $Arnt^{+/+}$, $Arnt^{fxneo/+}$, and $Arnt^{fxneo/fxneo}$ mice to 64 $\mu\text{g}/\text{kg}$ of dioxin for 16 days, tissues were harvested, weighed, and prepared for histological and clinical examination. One of the classical end points of dioxin toxicity is thymic involution (5, 17). Thymus weight in both wild-type and $Arnt^{fxneo/+}$ mice was reduced by ~80% in response to dioxin exposure (Fig. 5A). In contrast, thymic involution in $Arnt^{fxneo/fxneo}$ hypomorphs was about half that observed in the wild-type and $Arnt^{fxneo/+}$ mice. The attenuated dioxin toxicity in the thymus of $Arnt^{fxneo/fxneo}$ mice demonstrated that the hypomorphic allele is resistant to this end point. The resistance of the $Arnt^{fxneo/fxneo}$ hypomorphs to dioxin-induced thymic involution concurs with the findings

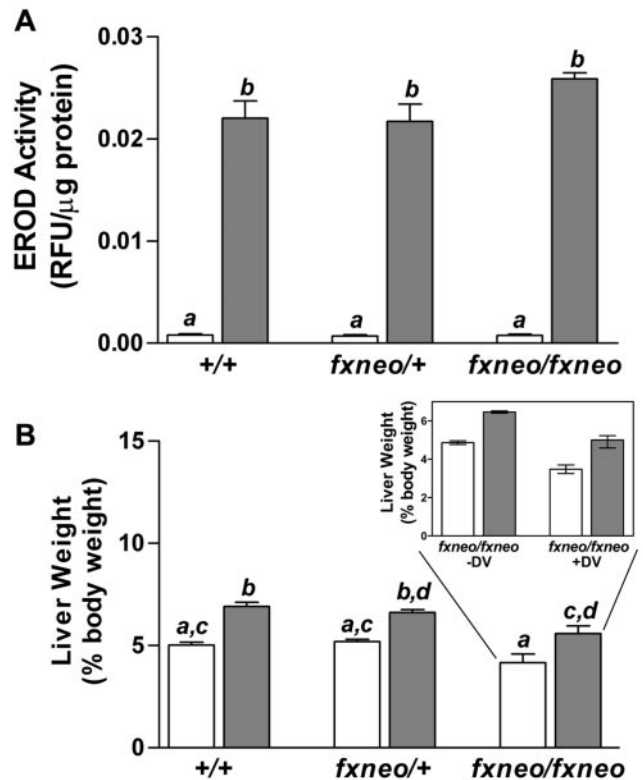


FIG. 4. $Arnt^{fxneo}$ hypomorphs retain P450 inductive capacity and hepatomegaly following dioxin exposure. Six-week-old wild-type (+/+), $Arnt^{fxneo/+}$, and $Arnt^{fxneo/fxneo}$ mice were administered a single oral dose of dioxin (64 $\mu\text{g}/\text{kg}$) dissolved in corn oil (vehicle) or an equivalent volume of corn oil alone. After 16 days, livers were excised and weighed, and microsomes were prepared. Each experimental group contains five animals, with the exception of the vehicle-treated $Arnt^{fxneo/fxneo}$ group, which contains four animals. White bars, vehicle-treated animals; gray bars, dioxin-treated animals. Error bars, standard error. Those groups not sharing a superscript letter differ significantly at $p \leq 0.05$. A, P450 induction is intact in ARNT hypomorphs. EROD activity in wild-type (+/+), $Arnt^{fxneo/+}$, and $Arnt^{fxneo/fxneo}$ microsomal preparations following dioxin treatment. Microsomal isolations were incubated with ethoxyresorufin in the presence of NADPH. EROD activity was measured from vehicle and dioxin-treated animals at an excitation of 510 nm and emission of 590 nm. Fluorescent values were normalized to total protein levels. B, Liver weight increased proportionally in wild-type (+/+), $Arnt^{fxneo/+}$, and $Arnt^{fxneo/fxneo}$ mice following dioxin treatment. Inset, a patent DV confers a smaller liver to ARNT hypomorphs but does not alter the proportional increase in liver weight following dioxin treatment. For the graph in the inset, each experimental group contains two animals, with the exception of the dioxin-treated +DV $Arnt^{fxneo/fxneo}$ group, which contains three animals.

of a recent publication describing the absence of thymic involution in mice where $Arnt$ was specifically inactivated in thymic T-cells (18).

To assess the influence of the $Arnt^{fxneo}$ allele on dioxin-induced hepatotoxicity, we examined two classic pathological measures. As our first measure of hepatotoxicity, we examined the levels of serum ALT in animals exposed to 64 $\mu\text{g}/\text{kg}$ of dioxin. Sixteen days after dosing, wild-type animals treated with dioxin displayed ALT values that were 10-fold higher than in controls (Fig. 5B). This elevation in ALT was significantly reduced in mice carrying two hypomorphic alleles ($Arnt^{fxneo/fxneo}$), indicating a resistance to dioxin-induced hepatotoxicity. In dioxin-exposed animals, we observed a trend toward a graded response as a function of the number of hypomorphic alleles present. However, these serum ALT values from dioxin-treated $Arnt^{fxneo/+}$ or $Arnt^{fxneo/fxneo}$ animals are not significantly different from untreated controls. As our second measure of hepatotoxicity, a histological examination was per-

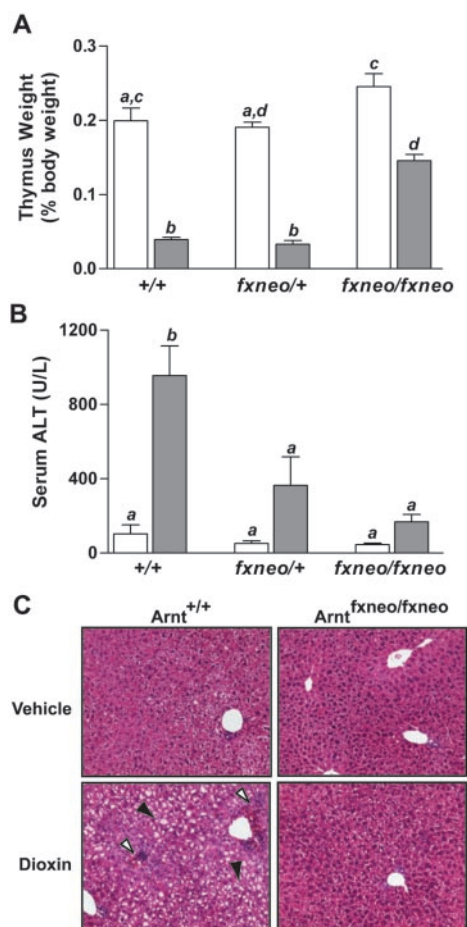


FIG. 5. Attenuation of dioxin-induced thymic involution and hepatotoxicity in $Arnt^{fxneo}$ hypomorphs. Six-week-old wild-type (+/+), $Arnt^{fxneo/+}$, and $Arnt^{fxneo/fxneo}$ mice were administered a single oral dose of dioxin (64 $\mu\text{g}/\text{kg}$) dissolved in corn oil (vehicle) or an equivalent volume of corn oil alone. Tissues and sera were harvested after 16 days. Each experimental group contains five animals, with the exception of the vehicle-treated $Arnt^{fxneo/fxneo}$ group, which contains four animals. White bars, vehicle-treated animals; gray bars, dioxin-treated animals. Error bars, standard error. Those groups not sharing a superscript letter differ significantly at $p \leq 0.05$. Attenuation of thymic involution (A), serum ALT levels (B), and hepatotoxicity (C) in dioxin-treated $Arnt^{fxneo/fxneo}$ hypomorph mice when compared with wild-type controls is measured. Liver sections were prepared and stained with hematoxylin-eosin for assessment of liver pathology. Black arrowheads, areas of hydropic vacuolation and degeneration; black and white arrowheads, region of granulocyte infiltration concomitant with congestion.

formed. This analysis also indicated a reduction in the extent of hepatotoxicity in ARNT hypomorphs. When compared with their vehicle-treated controls, wild-type mice treated with dioxin displayed extensive hydropic vacuolation and degeneration throughout all zones of the liver as well as massive granulocyte infiltration associated with areas of mild congestion (Fig. 5C). In contrast, liver sections from $Arnt^{fxneo/fxneo}$ mice treated with dioxin displayed minimal hydropic degeneration and limited granuloma formation in the parenchyma (Fig. 5C).

DISCUSSION

It is difficult to assess the role of ARNT in whole animal models because the null allele at this locus results in embryonic lethality at embryonic day 10 (13, 14). This developmental block is believed to be due to the role of ARNT as the transcriptional partner to the various HIFs (19–22). Based on current technology, there are two approaches to overcome the essential nature of ARNT in development. The first is to generate a tissue-specific excision model, and the second is to develop a

hypomorphic allele that has globally reduced ARNT expression. Based upon previous work in the laboratory, we devised a strategy that had the potential to yield both such models from a single targeting event in embryonic stem cells. The application of tissue-specific *Arnt* alleles will be addressed in later work. In this report, we describe the utility of the ARNT hypomorph in understanding the role of this protein in AHR-mediated toxicity and development.

The Role of ARNT in AHR-mediated Toxicity—There is little evidence to demonstrate that ARNT is directly involved in the classical end points of dioxin toxicity. Demonstrating a role for ARNT is an extremely important issue because a number of models have been proposed that do not necessarily require the ARNT protein. Such models include the signaling of AHR through cellular factors such as c-Src kinase, the retinoblastoma protein, ceramide, steroid receptors, and NF- κ B (23–29). Therefore, we set out to understand the role that ARNT plays in AHR-mediated dioxin toxicity. In this regard, we postulated that if the toxic pathway mechanistically resembles the adaptive pathway, then toxic events would be diminished in ARNT hypomorphic animals. That is, we are testing a model where toxicity is the direct result of AHR·ARNT-dependent transcriptional events. Additionally, we are also testing a “cross-talk” model of toxicity. In that model, it is proposed that toxicity may result from the depletion of free ARNT upon dioxin-induced activation of the AHR (1). As a consequence of this depletion, other ARNT-dependent pathways could be compromised (*i.e.* HIF1 α) (30–32). If cross-talk is at play, then ARNT hypomorphic animals should demonstrate an exacerbated response to dioxin. Finally, we are testing the idea that ARNT is directly involved in the AHR pathway and not its potential homologues, ARNT2, MOP3, or MOP9 (9–12).

Examination of the response of the ARNT hypomorph to dioxin exposure now permits us to more clearly define the mechanism of the toxic pathway signaling of AHR. In response to dioxin, our composite data show that the ARNT hypomorphs maintain their inductive metabolic capacity while demonstrating a resistance to two classic toxic end points, thymic involution and hepatotoxicity. Based on these observations, we can make a number of conclusions regarding the role of ARNT in toxicity. The first is that ARNT homologs are not a factor in hepatotoxicity and thymic atrophy. If ARNT homologs had played a role, we would have expected the manifestation of the toxicological end points to be unaltered by the presence of the hypomorphic $Arnt^{fxneo}$ allele. The second conclusion is that the AHR is dependent on ARNT for both the toxic and the developmental pathways. If the AHR were acting independently of ARNT, then the hypomorphic $Arnt^{fxneo}$ allele should not diminish the outcome of dioxin exposure. The final conclusion is that the cross-talk mechanism of toxicity is not at play. Our prediction was that if cross-talk was operational, ARNT hypomorphs should be more sensitive to toxic end points. Our observations clearly show attenuation in the extent of toxicity in the ARNT hypomorphs treated with dioxin, and therefore, render the cross-talk model unlikely for these end points.

The above analysis of dioxin toxicity suggests that the mechanisms by which the AHR mediates adaptive metabolic and toxic responses both require the ARNT protein. These data are consistent with a model where hepatotoxicity and thymic involution are dependent on AHR·ARNT dimerization. Although not formally addressed in this study, the most obvious possibility is that toxicity is dependent upon AHR·ARNT-mediated transcription. This idea is supported by our previous observation that mice with a mutation in the DNA binding domain of AHR are resistant to these same toxic end points (15). Another important observation that must be considered in the develop-

ment of any model of toxicity is the observation that EROD activity was similar in ARNT hypomorphs when compared with wild-type mice. This result suggests that the induced P450 activity of these tissues is independent of the toxic end points measured in this study. This conclusion can be supported or refuted by direct examination of the corresponding *Cyp* null models developed by other laboratories (33–35).

The Role of ARNT in AHR-mediated Development—Examination of the ARNT hypomorph also provides insight into the mechanism by which the AHR regulates liver development and vascular maturation. Given that ARNT is the common heterodimeric partner to the AHR, we set out to test the idea that the AHR-ARNT partnership is important in development. Based upon their highly reproducible nature, we focused on DV closure and regulation of adult liver size as relevant developmental end points. Similar to the toxicity models described above, a number of models exist that could explain the mechanism by which the AHR might act during development in an ARNT-independent manner. It is our working hypothesis that the AHR-ARNT heterodimer functions in a manner that is mechanistically similar to its role in adaptive metabolism and toxicity. Based on this model, we predict that ARNT hypomorphs would display liver phenotypes that mirror those seen in the *Ahr* null mice.

Upon investigation of the vascularity of livers from ARNT hypomorphs, we observed a 70% incidence of patent DV, as well as a reduced liver size in the corresponding animals (Figs. 3 and 4). A patent DV is a rare occurrence in both humans and rodents. Until now, the only previous documentation of a patent DV in adult mice was reported for the *Ahr* nulls (8). The observation that the ARNT hypomorph phenotypically mimics the *Ahr* null is a strong indication of genetic cooperativity between these two loci. That is, mutations in either partner of this heterodimeric transcription factor result in an identical vascular phenotype. This observation strongly supports the hypothesis that AHR and ARNT are acting cooperatively in the resolution of the DV and that ARNT is an essential component of the developmental pathway of AHR signal transduction.

Given that ARNT is a partner to multiple PAS proteins, coupled with our evidence that ARNT is involved in all aspects of AHR biology, it is interesting to consider the relative requirements of ARNT for each pathway. In this regard, generation of the ARNT hypomorph has revealed pathway-dependent thresholds of this protein for different signaling outcomes. Under the conditions of reduced ARNT protein generated by this hypomorph, the HIF-dependent embryonic lethality is bypassed, and the adaptive up-regulation of P450s is preserved. In contrast, under these limiting conditions, there is significant failure in DV closure, and dioxin-induced thymic involution and hepatotoxicity is attenuated. Taken in sum, these observations suggest a different quantitative requirement for the ARNT protein partnership by different biological pathways.

In conclusion, we have provided evidence to demonstrate that a hypomorphic *Arnt*^{cxneo} allele can be generated by insertion of the neomycin gene adjacent to exon 6 of the *Arnt* locus. The observation that ARNT expression in this mouse model is globally down-regulated allowed us to investigate the role of this protein in those aspects of its biology requiring AHR. In future experiments, this animal model should have equal power to elucidate hypoxia signal transduction. Using this model, we have provided evidence that both the toxic and the

developmental pathways of AHR signal transduction depend on cooperation with ARNT and imply a direct role for dimerization in these processes. This AHR-ARNT cooperativity appears to be an essential factor for both the toxic end points of dioxin exposure as well as the AHR-dependent closure of the DV.

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