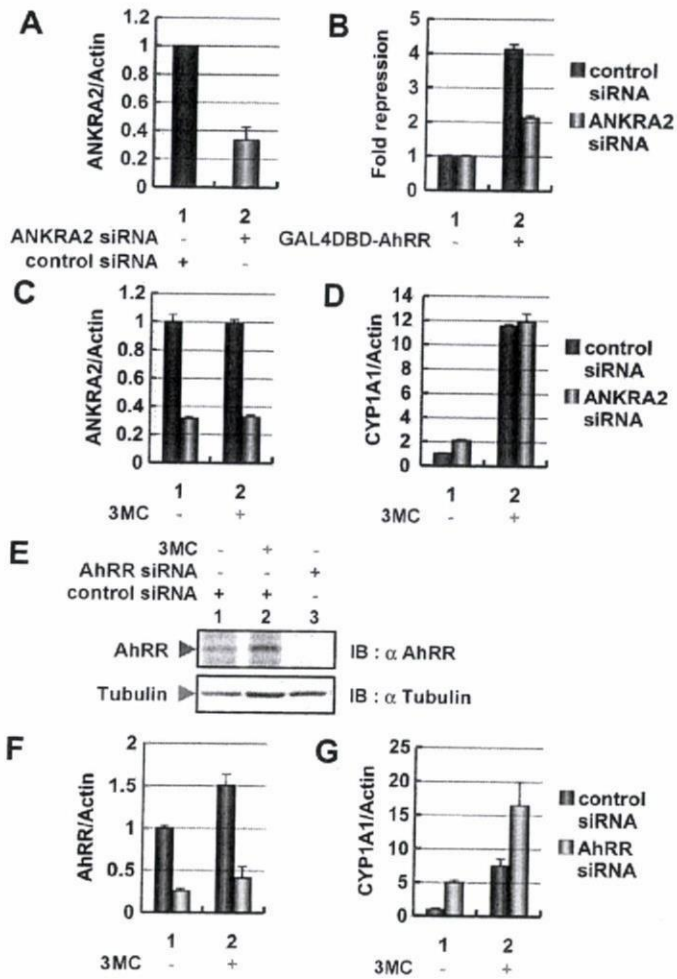

Fig. 3. Effects of ANKRA2 and AhRR siRNAs on the transcriptional repression activity of AhRR. (A) Hepa-1 cells were transiently transfected with ANKRA2 or control siRNA and 48 h after transfection, RNA were prepared from the transfected cells. ANKRA2 mRNA was quantified by RT-PCR method. (B) Hepa-1 cells were transiently transfected with GAL4DBD-AhRR and a reporter gene of GAL-TK-Luc, along with ANKRA2 siRNA or control siRNA. Cell extracts were prepared 48 h after transfection and used for luciferase assays. (C–G) MEF cells were transiently transfected with ANKRA2 siRNA (C and D) or AhRR siRNA (E–G) or control siRNA. After 48 h of transfection, cells were treated with 2 μM of 3MC or Me_2SO and then, 24 h later, cell extracts were prepared and indicated mRNA expression level was quantified using real time RT-PCR (C, D, E, and F) or immunoblot analysis (E).

conditions to even a higher extent than ANKRA2 siRNA treatment (Fig. 3G, lane 1). Interestingly, AhRR siRNA also further increased the induced expression of CYP1A1 mRNA in response to 3MC, as compared with the expression in cells treated with control siRNA, suggesting the existence of an ANKRA2-independent repression mechanism by AhRR (Fig. 3G, lane 2). As reported previously, AhRR may prevent AhR from forming a heterodimer with Arnt in a competitive manner, thereby blocking the binding of AhR to the XRE sequence in the absence of ANKRA2. Taken together, these results indicate that in a silent state of CYP1A1 expression under normal conditions, AhRR represses the expression of *CYP1A1* gene by binding the XRE sequence and recruiting ANKRA2, HDAC4 and/or HDAC5.

Discussion

We previously reported that AhRR repressed AhR transcription activity by competing with Arnt in AhR/Arnt heterodimerization, as well as by binding the XRE sequence in the promoter of CYP1A1 gene. Since Arnt has a weak transcription activity at its C-terminal end, it is possible that AhRR could have some inhibitory activity to compensate for Arnt transcription activity. In this report, we first demonstrated that the C-terminus (555–701 a.a.) of AhRR shows a transcription inhibitory activity which was sensitive to the HDAC inhibitor, TSA, suggesting that AhRR which binds the XRE sequence recruits a HDAC protein. To isolate factors that interact with the C-terminal sequence of AhRR, we utilized the Cytotrap yeast two-hybrid screening method using the C-terminal sequence of AhRR as bait, resulting in isolation of a fragment (113–312 a.a.) of ANKRA2 consisting of 312 amino acids and containing three ankyrin (ANK) repeats. The isolated ANKRA2 fragment was shown to physically interact with AhRR (Fig. 2B). ANK repeats are one of the most common protein sequence motifs mediating protein–protein interactions, but they have not been clarified to bind any specific amino acid sequence or structure. Rather, they are thought to bind a variety of proteins through adaptive alterations in their binding surface features and in the domain size of the ANK repeat by sequence duplication or deletion [20]. Recent studies have demonstrated that ANKRA2 also interacts with megalin [21] and the α-subunit of rat large-conductance Ca^{2+}-activated K^+ channel (rSlo) [22]. According to Rader et al. [21], the C-terminus (177–312 a.a.) of ANKRA2 interacts with a proline-rich motif (PXXPXXP) within the 19 amino acid sequence of the magalin tail, and Lim and Park [22] have shown that 52–150 a.a. of ANKRA2 interacts with the C-terminal end (1119–1210 a.a.) of rSlo Channel. Since there is no apparent sequence similarity in the ANKRA2-interacting domains of megalin, rSlo and AhRR, the precise molecular mechanisms how ANKRA2 interacts with these proteins remain to be investigated.

Our coimmunoprecpitation experiments have revealed that HDAC4 interacts with AhRR in an ANKRA2-dependent manner. HDAC5 may interact with AhRR either directly or via ANKRA2, when the previous report is taken into account [18]. It remains to be studied how HDAC4 and HDAC5 are recruited on the surface of the AhRR and ANKA2 complex, reciprocally or simultaneously. ANKRA2 is also abundantly and ubiquitously expressed in various tissues of mice [21] and cultured cells such as Hepa-1, HeLa and MEF cells used in this study (data not shown). For functional analysis of ANKRA2 and AhRR, we used siRNA to knock down gene expres-

sion. In Hepa-1 cells, repression of TK promoter-driven luciferase activity by GAL4DBD-AhRR was reversed by the addition of ANKRA2 siRNA, indicating that the repression activity of AhRR required ANKRA2. Treatment of MEF cells with ANKRA2 siRNA significantly activated the expression of *CYP1A1* under normal conditions. In contrast, the siRNA treatment had apparently no effect on the enhanced CYP1A1 expression in response to the inducer. These results suggest that under normal conditions, a silent state of *CYP1A1* gene expression is not merely due to the lack of a transcription activator, but resulted from negative regulation by a heterodimer of AhRR and Arnt, which recruits ANKRA2. This observation was substantiated by the experiments using AhRR siRNA, which significantly enhanced the expression of *CYP1A1* gene under normal conditions to even a higher level than did ANKRA2 siRNA under the non-inducing conditions. The greater effect of AhRR siRNA on CYP1A1 expression becomes more pronounced under inducing conditions. These observations could be explained by a two step inhibitory mechanism. First, AhRR inhibits the transcription activity of AhR in an ANKRA2-independent manner, by competing with AhR for forming a heterodimer with Arnt and binding the XRE sequence, as reported previously. Next, AhRR bound to the XRE sequence recruits ANKRA2 and HDAC4 and/or HDAC5 for more efficient repression. In the presence of the inducer 3MC, AhRR synthesis is accelerated so that the AhRR siRNA treatment displays a greater effect on the inducible expression of CYP1A1 than ANKRA2 siRNA. Recently, we have found that the silent state of CYP1A1 is actually negatively regulated by the AhRR system in macrophages (unpublished data). We will be investigating how this silencing mechanism involving AhRR and ANKRA2 functions in different cell types and how it affects target genes other than *CYP1A1* under normal conditions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.09.131.

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Original Article

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Disruption of Aryl Hydrocarbon Receptor (AhR) Induces Regression of the Seminal Vesicle in Aged Male Mice

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Key Words
AhR(–/–)males · Aryl hydrocarbon receptor (AhR) · 3βHsd · Mice · Seminal vesicle regression · Testes · Testosterone

Abstract
The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that mediates diverse dioxin toxicities. Despite mediating the adverse effects, the *AhR* gene is conserved among animal species, suggesting important physiological functions for AhR. In fact, a recent study revealed that AhR has an intrinsic function in female reproduction, though its role in male reproduction is largely unknown. In this study, we show age-dependent regression of the seminal vesicles, probably together with the coagulating gland, in *AhR*(–/–) male mice. Knockout mice had abnormal vaginal plugs, low sperm counts in the epididymis, and low fertility. Moreover, serum testosterone concentrations and expression of steroidogenic 3βhydroxysteroiddehydrogenase (3βHsd) and steroidogenic acute regulatory protein (StAR) in testicular Leydig cells were decreased in *AhR*(–/–) males. Taken together, our results suggest that impaired testosterone synthesis in aged mice induces regression of seminal vesicles and the coagulating glands. Such tissue disappearance likely resulted in abnormal vaginal plug formation, and eventually in low fertility. Together with previous findings demonstrating AhR function in female reproduction, AhR has essential functions in animal reproduction in both sexes.

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor belonging to the basic helix-loop-helix (bHLH)-PAS (Per-AhR/Arnt-Sim) super-gene family [Burbach et al., 1992; Ema et al., 1992]. Since AhR can bind with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin) [Poland et al., 1976; Ema et al., 1992], the molecular properties of AhR as a transcription factor have been extensively studied, especially focusing on the transactivation of a series of drug-metabolizing enzyme genes including *Cyp1a1* [Fujisawa-Sehara et al., 1987; Hankinson, 1995; Mimura and Fujii-Kuriyama, 2003]. In addition to these in vitro studies, in vivo gene disruption studies have revealed that AhR mediates a variety of toxicological effects of dioxin including teratogenesis, immunosuppression, tumor promotion, and estrogenic function [Poland and Knutson, 1982; Gibbons, 1993; Mimura et al., 1997; Brown et al., 1998; Shimizu et al., 2000]. Despite promoting these multiple adverse effects, the *AhR* gene is conserved across a variety of animal species from invertebrates to vertebrates [Hahn, 2002], suggesting that in addition to mediating the response to xenobiotics, there are intrinsic functions for AhR in physiological processes.

Recently, the intrinsic functions of AhR have been investigated with regards to animal reproduction and liver vasculogenesis. Indeed, recent studies in *AhR*(–/–) mice demonstrated that AhR is involved in female reproduction by regulating estradiol synthesizing *Cyp19* (P450

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aromatase) gene expression [Baba et al., 2005] and vessel remodeling in the liver [Lahvis et al., 2005]. Based on the essential functions of estradiol in the female reproductive process such as folliculogenesis, ovulation, and implantation [Fisher et al., 1998; Dupont et al., 2000; Curtis Hewitt et al., 2002], it was concluded that AhR plays an indispensable function in female reproduction. Moreover, in the case of male reproduction, dioxins were reported to reduce epididymal and ejaculated sperm number [Gray et al., 1995; Sommer et al., 1996], implicating that AhR is involved in the male reproductive process. However, there is no direct evidence for the involvement of AhR in this process.

The accessory internal reproductive systems, derived from the Wolffian duct for males and from the Mullerian duct for females, are clearly different between the two sexes. The male internal reproductive system consists of multiple tissues such as the epididymis, the deferens duct, the seminal vesicle, the coagulating gland, and the ejaculatory duct. Developmentally, all these tissues are known to be regulated by androgen signaling [Cunha, 1972; Cooke et al., 1991]. The mature seminal vesicle consists of numerous outpouchings of alveolar glands that empty into the ejaculatory duct. Although semen mostly contains materials secreted from the seminal vesicle, a definite functional relationship linking the seminal vesicle to male fertility has yet to be elucidated. The coagulating gland secretes a substance that, when mixed with the secretions from the seminal vesicle, forms a vaginal plug, and it has been thought that the vaginal plug is required for efficient pregnancy after insemination.

In this study, we define a novel phenotype of *AhR*(–/–) male mice. Interestingly, the seminal vesicle and probably the coagulating gland regressed in an age-dependent manner in the *AhR*(–/–) mouse, and such regression is possibly due to a low level of serum testosterone. These abnormalities possibly produce an abnormal vaginal plug and decrease the fertility of the male mice. This finding together with the previous finding in female reproductive processes [Baba et al., 2005] strongly suggests that AhR greatly influences animal reproduction regardless of the sex.

Materials and Methods

Mice

Targeted disruption of the *AhR* gene was performed as described previously [Mimura et al., 1997]. AhR knockout mice used in this study were backcrossed to C57BL/6J for more than eight generations in order to avoid experimental variation due to genetic background.

Antibodies

A full-length cDNA for mouse 3βHsd1 was kindly provided by Dr. A. Payne (Stanford University). A prokaryotic expression vector for 3βHsd was constructed by insertion of the 3βHsd cDNA into pET-28a (Novagen, San Diego, CA). Preparation of recombinant 3βHsd protein and immunization of rabbits were described previously [Morohashi et al., 1993]. Rabbit antibodies for AhR and Cyp19 were generously provided by Dr. R. Pollenz (University of South Florida) and Dr. N. Harada (Fujita Health University), respectively. Rabbit antibody for androgen receptor (AR) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Fertility Assessment

Thirty *AhR*(+/+) wild-type males, three *AhR*(–/–) males harboring the seminal vesicle, and nine *AhR*(–/–) males lacking the seminal vesicle were mated with *AhR*(+/+) females for 5 days. Twelve days after mating, the female mice were sacrificed to determine whether they became pregnant or not. The presence of the seminal vesicle in each *AhR*(–/–) male was determined both one week prior to the mating and just after the mating was completed. Statistical analysis was performed by Fisher's exact test. All protocols for animal experimentation were approved by the Institutional Animal Care and Use Committee of the National Institute for Basic Biology.

Immunohistochemistry and Western Blot

To detect AhR and AR, cryosections (10 μm) were prepared from the seminal vesicle treated overnight with 4% paraformaldehyde at 4°C. After washing with phosphate-buffered saline (PBS), the sections were boiled for 10 min in 10 mM sodium citrate (pH 7.0) to unmask antigen epitopes, followed by treatment with 0.3% H_2O_2 in methanol for 20 min at –20°C. The sections were incubated overnight at 4°C with anti-AhR or anti-AR antibody, washed with PBS, and then incubated with biotinylated donkey anti-rabbit IgG for 3 h at room temperature. After washing, the sections were incubated with horseradish peroxidase-conjugated streptavidin, and then visualized with diaminobenzidine at room temperature. To detect AhR and 3βHsd in the testes, 5 μm paraffin sections were prepared from 4% paraformaldehyde-fixed testes. After deparaffinization, antigen epitopes were unmasked by treatment with 20 μg/ml proteinase K (Sigma Chemical Co., St. Louis, MO) for 10 min at room temperature for AhR or unmasked by boiling for 10 min in 10 mM sodium citrate (pH 7.0) for 3βHsd, followed by treatment with 0.3% H_2O_2 in methanol for 20 min at –20°C. The sections were incubated overnight at 4°C with the anti-AhR or anti-3βHsd antibody, washed with PBS, and then incubated with biotinylated donkey anti-rabbit IgG or Cy-3 conjugated goat anti-rabbit IgG for 3 h at room temperature. After washing, sections immunoreacted with biotinylated antibodies were incubated with horseradish peroxidase-conjugated streptavidin, and then visualized with diaminobenzidine. The sections immunoreacted with Cy-3-conjugated antibody were counterstained with DAPI (2-(4-amidinophenyl)-1H-indole-6-carboxamidine), and then 3βHsd-positive cells were counted under fluorescence microscope.

To prepare whole tissue lysate for Western blot analysis, tissues were lysed with a cell-lysis buffer containing 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 1 mM EDTA (pH 8.0), and 1% SDS. Next, 10 μg of whole tissue lysates were subjected to SDS-PAGE followed by Western blot analyses using the antibodies for AhR, AR, Cyp19, Ad4BP/SF-1 and 3βHsd, as described previously [Morohashi et al., 1994].

Determination of Serum Testosterone Concentrations

Four *AhR*(+/+) and three *AhR*(–/–) 24-week-old and eight *AhR*(+/+) and ten *AhR*(–/–) 52-week-old male mice were anesthetized with diethyl ether for collection of blood samples. After isolating the serum fraction, serum testosterone concentration was determined by enzyme immunoassay (Cayman Chemical Company, Ann Arbor, MI) according to the protocol provided by the manufacturer.

Sperm Count

Count of epididymal sperm number was performed as reported previously [Bell et al., 2007]. Briefly, the cauda epididymis was dissected and pierced three times with a scalpel blade. Then, the tissue was incubated in 5 ml of PBS containing 0.57% (w/v) BSA at 37°C for 90 min. After incubation, the number of sperms was counted under a microscope.

Leydig Cell Count

Serial sections of the testes prepared from eight *AhR*(+/+) and ten *AhR*(–/–) 52-week-old mice were stained with anti-3βHsd antibody and DAPI. The number of 3βHsd-positive Leydig cells was counted under a fluorescence microscope in 32 sections (4 sections for each animal) of *AhR*(+/+) and 40 sections of *AhR*(–/–).

Quantitative RT-PCR

Quantitative RT-PCR was performed with a 7500 real-time PCR system (Applied Biosystems, Foster City, CA) using Power SYBR Green PCR master mix (Applied Biosystems). The thermal-cycling condition was 50 cycles of 15 s at 95°C and 1 min at 60°C. Primer pairs used for quantitative RT-PCR were as follows: 3βHsd (fwd), 5′-CAG ACC ATC CTA GAT GTC-3′; 3βHsd (rev), 5′-ACT GCC TTC TC GCC ATC-3′; StAR (fwd), 5′-CCG GAG CAG AGT GGT GTC A-3′; StAR (rev), 5′-GCC AGT GGA TGA AGC ACC AT-3′; Insl3 (fwd), 5′-CCT GGC TAT GTC ATT GCA ACA-3′; Insl3 (rev), 5′-TGG TCC TTG CTT ACT GCG ATC T-3′ [Cederroth et al., 2007]; and P450scc (fwd), 5′-CAG AAC TAA GAC CTG GAA GGA CCA-3′; P450scc (rev), 5′-TGG GTG TAC TCA TCA GCT TTA TTG AA-3′.

Results

Regression of Seminal Vesicles in Aged AhR(–/–) *Males*

We have previously reported that *AhR*(–/–) female mice had defective reproductive activity. In the present study, we examined the effect of *AhR* on the reproductive activity in *AhR*(–/–) males. Although the defect was milder than that observed in females, we found suppression of reproductive activity in aged *AhR*(–/–) males. Therefore, we examined whether the reproductive tissues are also affected in *AhR*(–/–) males. The seminal vesicle was completely regressed in certain population of *AhR*(–/–) males (fig. 1A). At the same time, we noticed that this tissue regression was rare in the young adult. Therefore, the regression was examined in terms of animal age. Regression of the seminal vesicle was identified in 53.8% of the 24-week-old, 66.7% of the 32-week-old, and 50.0% of the 52-week-old *AhR*(–/–) males, whereas no such regression was observed in the 8-week-old knockout males (fig. 1B). No such tissue regression was observed in age-matched *AhR*(+/+) mice, strongly suggesting that AhR is essential for the maintenance of seminal vesicle in aged mice.

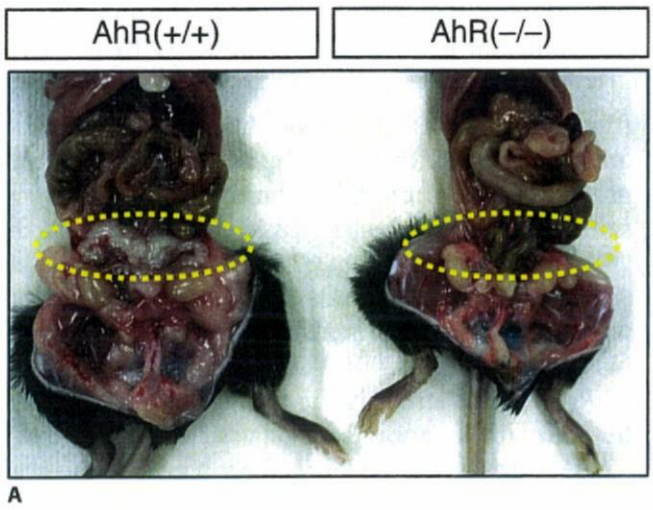
Next, we quantified the regression process by measuring tissue weight. The weight of the seminal vesicles was similar in *AhR*(+/+) and *AhR*(–/–) at 8 weeks after birth (fig. 1C). However, the weight of the *AhR*(–/–) seminal vesicles did not increase after 24 weeks while that of *AhR*(+/+) increased in an age-dependent manner. We expected to observe apparent tissue regression in some of the knockout animals, but no such tissue was observed, suggesting that the regression occurs and is completed rapidly.

Since AhR is implicated in maintenance of the seminal vesicle, the expression of AhR in the seminal vesicle was analyzed by immunohistochemistry (fig. 1D) and Western blotting (fig. 2B). As shown in figure 1D, AhR was expressed in the epithelial cells of the seminal vesicle and accumulated in the cytoplasm rather than in the nuclei of these cells. This cytoplasmic localization was similar to that observed in the liver [Poland et al., 1976].

Testosterone Synthesis in AhR(–/–) *Males*

Since proliferation of the seminal vesicle epithelial cells is controlled by an androgen-mediated signal [Neubauer et al., 1981], we subsequently investigated the expression of androgen receptor (AR) immunohistochemically (fig. 2A). Similar to AhR, AR was expressed in the epithelial cells of the seminal vesicle. To assess whether regression of the seminal vesicle is due to low expression of AR in *AhR*(–/–) animal, whole tissue extracts were prepared from the seminal vesicles of *AhR*(+/+) and *AhR*(–/–) males at 8 and 32 weeks of age, and then subjected to Western blot analyses. Unexpectedly, however, no decrease in the expression of AR was observed in the absence of AhR at both 8 and 32 weeks (fig. 2B).

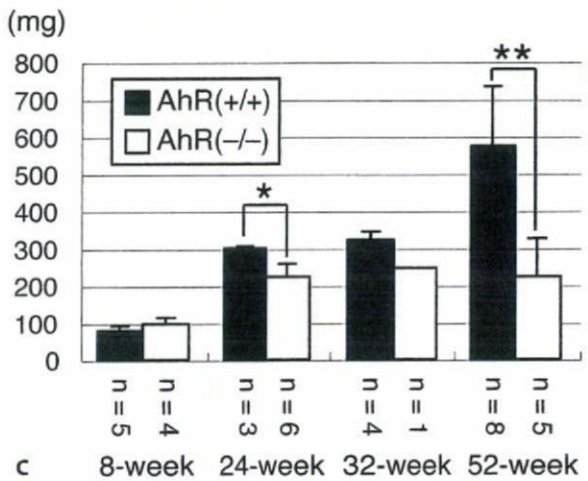
In addition to AR, testosterone is required for AR signaling. Therefore, we were interested in determining whether testosterone production is affected in *AhR*(–/–)



A

	AhR(+/+)	AhR(−/−)
8-week	5/5 (100%)	4/4 (100%)
24-week	8/8 (100%)	6/13 (46.2%)
32-week	4/4 (100%)	1/3 (33.3%)
52-week	8/8 (100%)	5/10 (50.0%)

B



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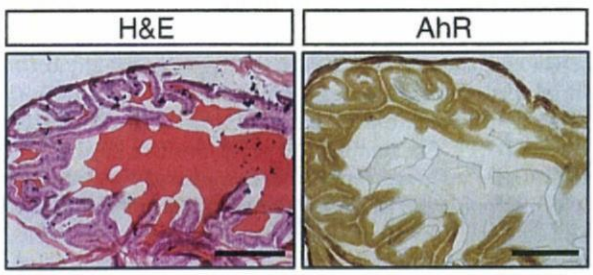

D

Fig. 1. Seminal vesicle regression in aged *AhR*(−/−) mice. **A** The reproductive tracts of 20-week-old *AhR*(+/+) and *AhR*(−/−) males. The seminal vesicle in the *AhR*(+/+) male is indicated by yellow dotted circles while this tissue is absent in the *AhR*(−/−) male. **B** Seminal vesicle regression in *AhR*(−/−) males is age-dependent. *AhR*(+/+) and *AhR*(−/−) males at 8, 24, 32, and 52 weeks of age were analyzed for the presence of seminal vesicles. Data are numbers of mice with intact seminal vesicles per total number of mice. **C** Comparison of seminal vesicle wet weight between *AhR*(+/+) and *AhR*(−/−) males. The seminal vesicles isolated from 8-, 24-, 32-, and 52-week-old *AhR*(+/+) and *AhR*(−/−) males were weighed. Numbers of the mice examined are indicated. Values are mean ± SD, * $p < 0.025$, ** $p < 0.005$. Statistical analysis was not performed with the 32-week-old mice because the number of *AhR*(−/−) males harboring the seminal vesicle was small. **D** Expression of AhR in the seminal vesicle. Ten-micrometer cryosections were prepared from a 10-week-old *AhR*(+/+) seminal vesicle. The sections were stained with hematoxylin and eosin (H&E) or immunohistochemically with anti-AhR antibody (AhR). Scale bars = 200 μm.

males. The testicular weight of *AhR*(−/−) males was compared with that of age-matched *AhR*(+/+) males at 8, 24, 32, and 52 weeks after birth. As shown in fig. 3A, the weights were mostly similar in *AhR*(+/+) and *AhR*(−/−) males at the above ages, although a slight difference was observed in 52-week-old mice. We then measured serum testosterone concentration in the 24- and 52-week-old mice, and found that it had clearly decreased in *AhR*(−/−) mice to approximately one third, and half of 24- and 52-week-old *AhR*(+/+) mice (fig. 3B). This result suggested that low testosterone concentrations cause, at least in part, the defect of seminal vesicles of aged *AhR*(−/−) males.

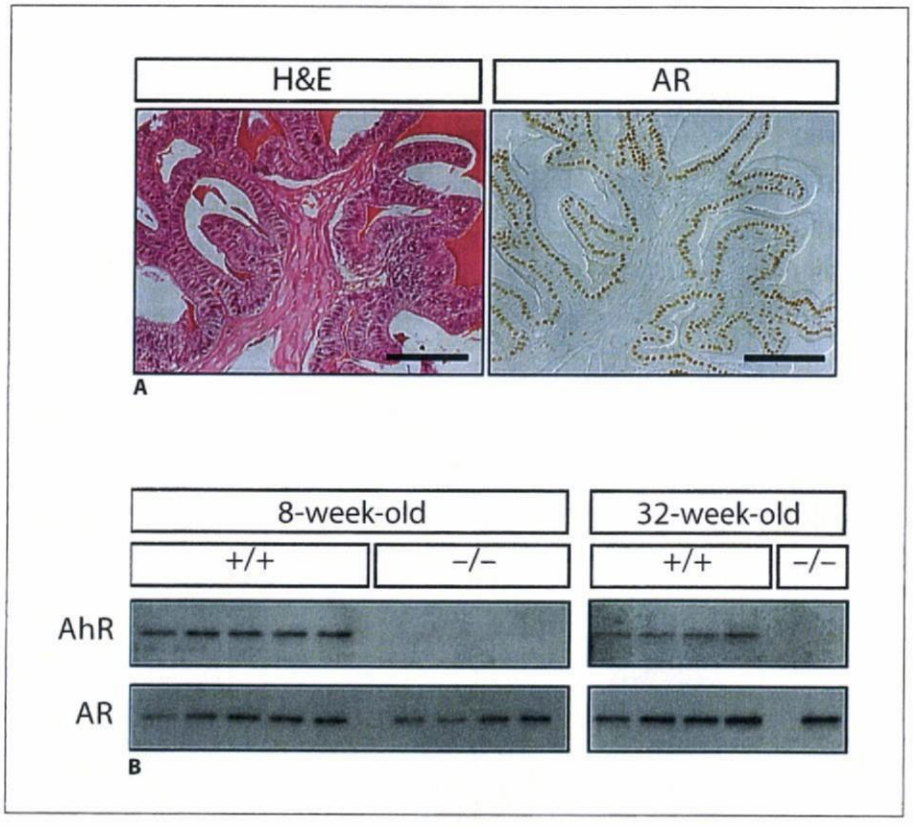

Fig. 2. Unaffected expression of androgen receptor in seminal vesicle of *AhR*(−/−). **A** Expression of AR in the seminal vesicle. Cryosections (10 μm thick) were prepared from seminal vesicles of 10-week-old *AhR*(+/+) and stained with hematoxylin and eosin (H&E) or anti-AR antibody (AR). Scale bars = 200 μm. **B** Expression of AR in seminal vesicles of 8- and 32-week-old *AhR*(+/+) and *AhR*(−/−) males. Whole tissue extracts (10 μg) prepared from seminal vesicles were subjected to Western blot analyses with antibodies for AhR and AR. Five 8-week-old *AhR*(+/+) seminal vesicles, four 8-week-old *AhR*(−/−) seminal vesicles, four 32-week-old *AhR*(+/+) seminal vesicles, and one 32-week-old *AhR*(−/−) seminal vesicle were used.

Since spermatogenesis is one of the physiological events in the testis and is regulated by testosterone, we also determined whether sperm production is affected in the *AhR*(−/−) testes. We counted epididymal sperm numbers in *AhR*(+/+) and (−/−) mice (fig. 3C). The number was decreased in the *AhR*(−/−) mice to approximately two thirds of the *AhR*(+/+), suggesting that low concentrations of serum testosterone affect spermatogenesis in the *AhR*(−/−) testes. To investigate the process of spermatogenesis, serial sections of the *AhR*(+/+) and *AhR*(−/−) testes were prepared. Morphologically, a substantial number of elongated spermatids were differentiated in the *AhR*(−/−) testes as well as *AhR*(+/+) testes (fig. 3D). Moreover, histological examination of the caudae epididymidis revealed the presence of abundant sperm cells even in the *AhR*(−/−) males. No evidence of any histological abnormality that would cause the reduced number of the sperm was observed in the *AhR*(−/−) testes.

Low Expression of 3βHsd in AhR(−/−) *Testes*

Testosterone is synthesized in testicular Leydig cells, and therefore coincident AhR expression was determined in Leydig cells. Consistent with previous reports [Schultz et al., 2003], anti-AhR immunoreactivity was detected in Leydig cells (fig. 4A). The low serum testosterone concentration suggested the potential involvement of AhR in the development and/or function of Leydig cells. Therefore, we performed immunohistochemical staining with antibody for 3βHsd, a Leydig cell marker [Dupont et al., 1990]. As shown in figure 4A, Leydig cells were present in the testes of both genotypes. We then performed fluorescent immunohistochemical examination followed by cell counting to determine if the number of Leydig cells is decreased in the *AhR*(−/−) testes (fig. 4B). There was no significant difference in Leydig cell number between the testes of *AhR*(+/+) and *AhR*(−/−) (fig. 4C), suggesting that the low level of serum testosterone is not due to a decrease in Leydig cell number but rather reduced ability to produce testosterone. To investigate the possibility of suppression of steroidogenic function of Leydig cells, we compared the expression of 3βHsd in *AhR*(+/+) and *AhR*(−/−) testes by Western blot analyses. Comparable expression of 3βHsd was observed in 10-week-old *AhR*(+/+) and *AhR*(−/−) mice, but the expression was less in 24-, 32-, and 52-week-old *AhR*(−/−) testes compared to age-matched *AhR*(+/+) mice (fig. 5A).

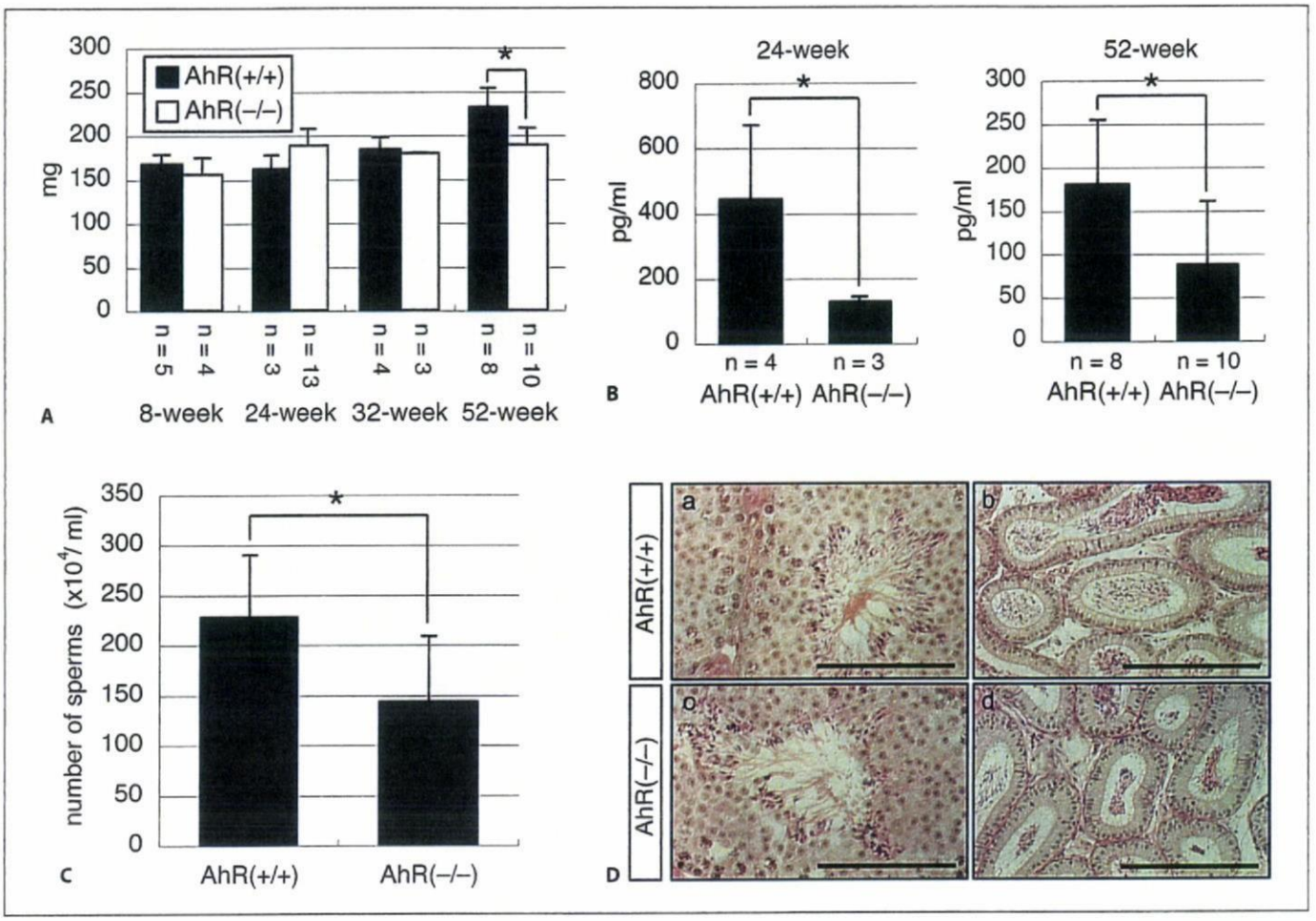

Fig. 3. Low serum testosterone levels in *AhR*(–/–) males. **A** Comparison of testicular weight between *AhR*(+/+) and *AhR*(–/–) mice. The testes isolated from 8-, 24-, 32-, and 52-week-old *AhR*(+/+) and *AhR*(–/–) mice were weighed. Numbers of mice examined are indicated. Values are mean ± SD, * $p < 0.005$. **B** Serum testosterone concentrations in 24- and 52-week-old *AhR*(+/+) and *AhR*(–/–) mice determined by enzymatic immunoassay. Numbers of mice examined are indicated. Values are mean ± SD, * $p < 0.05$. **C** Comparison of epididymal sperm number between 52-week-old *AhR*(+/+) and *AhR*(–/–). Sperm cells were recovered from eight *AhR*(+/+) and ten *AhR*(–/–) 52-week-old mice. * $p < 0.025$. **D** Histological analysis of the testes (**a**, **c**) and caudae epididymidis (**b**, **d**) of *AhR*(+/+) and *AhR*(–/–) mice. Five-micrometer paraffin-embedded sections of the testes and caudae epididymidis from 20-week-old *AhR*(+/+) and *AhR*(–/–) mice were stained with hematoxylin and eosin. Scale bars in **a** and **c** = 100 μm, **b** and **d** = 200 μm.

Our previous study demonstrated that AhR regulates *Cyp19* (aromatase P450) gene expression in the ovary [Baba et al., 2005]. Since aromatase P450 is capable of converting testosterone to estradiol, increased expression of the enzyme would lead to decrease in serum testosterone and thus Cyp19 expression was examined in 24-week-old mice. However, there was no discernible difference in Cyp19 expression between the *AhR*(+/+) and *AhR*(–/–) testes (fig. 5B). Moreover, the expression of StAR (steroidogenic acute regulatory protein), Insl3 (insulin like-3), and P450scc (side chain cleavage) necessary for the endocrine function of Leydig cells was examined by quantitative RT-PCR. As shown in figure 5C, the expression of the *StAR* gene was decreased as well as *3βHsd* in the *AhR*(–/–) testes. In contrast, the expression of *Insl3* and *P450scc* in the *AhR*(–/–) testes was comparable to *AhR*(+/+), indicating that not all the steroidogenic genes are regulated by AhR. Further, we investigated the expression of Ad4BP/SF-1, which is known to regulate the expression of all steroidogenic genes; however, the expression of Ad4BP/SF-1 protein was not affected in the *AhR*(–/–) testes (fig. 5D).

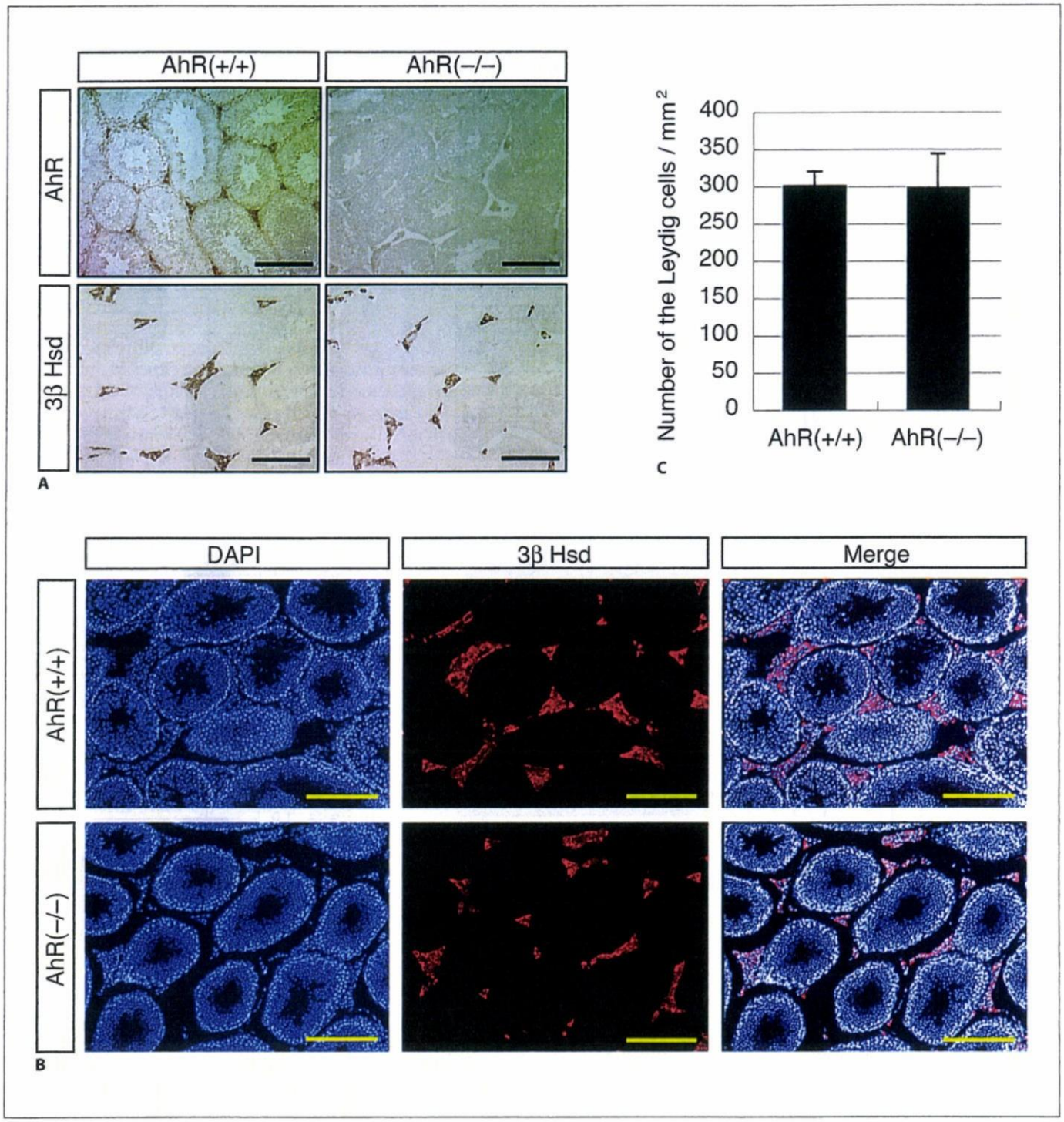

Fig. 4. No difference in number of testicular Leydig cells between *AhR*(+/+) and *AhR*(–/–) mice. **A** Expression of AhR and 3βHsd in Leydig cells. Five-micrometer paraffin sections were prepared from the testes of 24-week-old *AhR*(+/+) and *AhR*(–/–) mice and subjected to immunohistochemical analyses using anti-AhR and anti-3βHsd antibodies. Scale bars = 200 μm. **B** Immunohistochemical staining of testes of 52-week-old *AhR*(+/+) and *AhR*(–/–) mice using anti-3βHsd antibody (red). Nuclei were stained with DAPI (blue). Sections were prepared from eight *AhR*(+/+) and ten *AhR*(–/–) mice. Representative results are shown. Scale bars = 200 μm. **C** Comparison of Leydig cell number between *AhR*(+/+) and *AhR*(–/–). Numbers of 3βHsd-positive cells in testes of eight *AhR*(+/+) and ten *AhR*(–/–). 3βHsd-positive cells were counted in four sections for each animal. Values are average numbers of Leydig cells ± SD per mm^2.

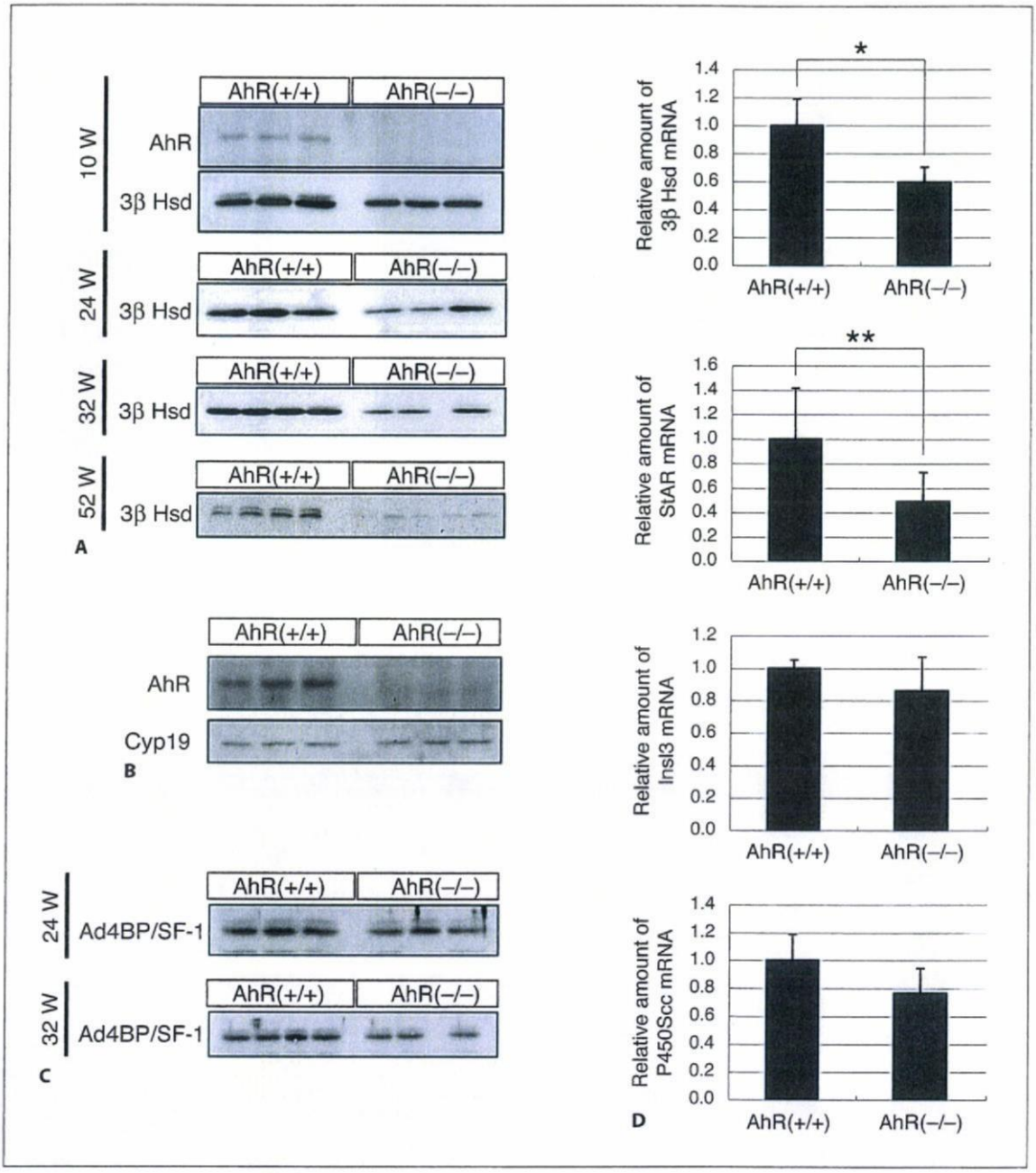

Fig. 5. Low expression of 3βHsd in *AhR*(–/–) testes. **A** Age-dependent differential expression of 3βHsd in testes of *AhR*(+/+) and *AhR*(–/–) mice. Whole tissue extracts (10 μg) prepared from testes of 10-, 24-, 32-, and 52-week-old *AhR*(+/+) and *AhR*(–/–) mice were subjected to Western blot analyses using anti-AhR and anti-3βHsd antibodies. Three or four males were used for each blotting. **B** Expression of Cyp19 in testes of *AhR*(+/+) and *AhR*(–/–) mice. Whole tissue extracts (10 μg) prepared from testes of 24-week-old *AhR*(+/+) and *AhR*(–/–) mice were subjected to Western blot analyses using anti-AhR and anti-Cyp19 antibodies. Three *AhR*(+/+) and three *AhR*(–/–) males were used. **C** Expression of 3βHsd, StAR, Insl3, and P450scc mRNA in testes of *AhR*(+/+) and *AhR*(–/–) mice. Total RNA was prepared from testes of 52-week-old *AhR*(+/+) and *AhR*(–/–), and then the amount of the mRNA was quantified by real-time RT-PCR, * $p < 0.025$, ** $p < 0.1$. **D** Expression of Ad4BP/SF-1 in testes of *AhR*(+/+) and *AhR*(–/–) mice. Whole tissue extracts (10 μg) prepared from testes of 24- and 32-week-old *AhR*(+/+) and *AhR*(–/–) mice were subjected to Western blot analyses using anti-Ad4BP/SF-1 antibodies. Three *AhR*(+/+) and three *AhR*(–/–) males were used.

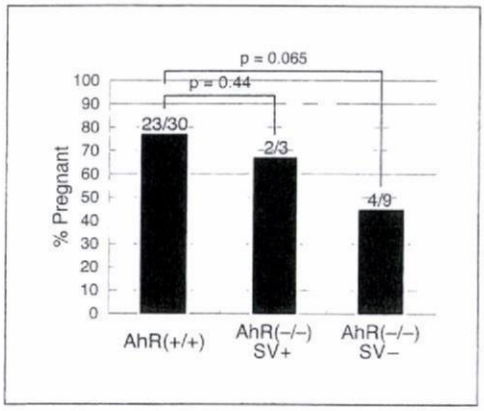

Fig. 6. Low fertility of *AhR*(–/–) males lacking seminal vesicles. Thirty *AhR*(+/+) and three *AhR*(–/–) mice with seminal vesicles, and nine *AhR*(–/–) mice lacking seminal vesicles were mated with *AhR*(+/+) females. Data represent the percentages of successful pregnancies. Numbers on bars represent the number of pregnant females per total number of female mice.

Reduced Fertility of AhR(–/–) *Males*

Lastly, we examined how the reproductive activity is affected in *AhR*(–/–) males. In order to determine reproductive activity, 21- to 33-week-old *AhR*(+/+) and *AhR*(–/–) males were mated with wild-type females. Before mating, the *AhR*(–/–) males were surgically examined to determine whether they still possess the seminal vesicles or not. Three of these males still had their seminal vesicles while nine of them did not. These two groups, together with wild-type males, were then subjected to mating experiments. *AhR*(–/–) males lacking any seminal vesicles showed less reproductive activity than *AhR*(+/+) males and *AhR*(–/–) males harboring the seminal vesicle (fig. 6). The reproductive activity of *AhR*(–/–) males harboring seminal vesicles was not statistically different from that of *AhR*(+/+) males (fig. 6).

During this experiment, the presence of seminal vaginal plugs was checked every morning, and frequently these plugs showed abnormal characteristics with the female mice mated with the *AhR*(–/–) males. Small amounts of white-colored and non-fixed plugs were observed in females mated with males lacking the seminal vesicles (data not shown). Since the vaginal plug is considered to be critical for successful pregnancy, the rate of pregnancy was compared between females with normal and those with abnormal plugs. As expected, successful pregnancies were counted in 4 of the 5 females with normal plugs, while 5 of the 6 females with the abnormal plug had unsuccessful pregnancies.

Discussion

Through the analyses of *AhR*(–/–) males, we demonstrated a novel function for AhR: maintenance of the seminal vesicle. Although the weight of the seminal vesicle was reported previously to be decreased by *AhR* gene disruption [Lin et al., 2002], we showed for the first time a complete regression of the seminal vesicle in *AhR*(–/–) males. At the same time, we noticed that the regression occurs preferentially in aged adult animals. Since the previous study only examined mice that were younger than 90 days old [Lin et al., 2002], it seems perhaps unlikely to encounter any mice lacking the seminal vesicle at that young age. In order to explain the mechanism underlying tissue regression, it was important to examine if apoptosis is increased while cell proliferation is decreased during and just prior to the regression. However, since this regression is considered to occur randomly among individuals, we could not find seminal vesicles in which regression was apparently in progress, suggesting that the process of regression proceeds in a very short period. Because of this regression feature, we neither can predict precisely when the regression starts in each animal, nor determine whether this regression is caused by increased apoptosis or decreased cell proliferation.

Developmentally, the seminal vesicle is derived from the caudal region of the Wolffian duct as a male sex-accessory gland. Likewise, the coagulating gland is derived from the same duct, and thereafter it is fused to the posterior margin of the seminal vesicle. Therefore, the coagulating gland may disappear simultaneously with the seminal vesicle in *AhR*(–/–) males. Functionally, the coagulating gland secretes a substance required for the formation of a vaginal plug to guarantee efficient pregnancies. In this study, we observed a decrease in successful reproductive activity and abnormal vaginal plugs when *AhR*(–/–) males were used in the mating. Therefore, we assumed that the abnormal vaginal plug formation caused by the disappearance of the seminal vesicle together with coagulating gland is a possible reason accounting for the decreased reproductive activity of *AhR*(–/–) males. In addition to vaginal plug formation, sperm number is another factor to guarantee efficient pregnancies. Therefore, we counted the number of epididymal sperms and found that it was reduced in *AhR*(–/–). Although a definite relationship between fertility and sperm number has yet to be determined, this observation raises another possibility: the low sperm count explains the decreased reproductive activity of *AhR*(–/–) males.

The implication of AR in seminal vesicle development was elucidated by *Ar*-knockout mice in which the seminal vesicles failed to develop from the fetal stage [De Gendt et al., 2004]. In addition, castration at adulthood led to regression of the seminal vesicle while administration of dihydrotestosterone rescued such castration-induced regression [Neubauer et al., 1981]. Moreover, administration of androgen antagonists decreased the weight of the seminal vesicle [Vinggaard et al., 2002]. These observations indicated that androgen signaling is indispensable for the maintenance of the adult seminal vesicle as well as for the development of fetal tissue. Considering the importance of androgen signaling in the development and maintenance of the seminal vesicle, we reasoned two possibilities for tissue regression; one is the low expression of AR in the seminal vesicle while the other is reduced testosterone production in testicular Leydig cells. Since AhR is expressed in both seminal vesicles and Leydig cells, any disruption of the *AhR* gene would potentially affect both or either of them. Eventually, examination of the two possibilities strongly suggested that the decreased expression of 3βHsd and StAR in testicular Leydig cells leads to a concomitant decrease in serum testosterone and thus the regression of the seminal vesicle in the *AhR*(–/–) male. Androgen is known to mediate a variety of male functions, and spermatogenesis is one representative event. In fact, sperm production was affected in *AhR*(–/–) males. This differential tissue effect in the decrease of testosterone is possibly due to the sensitivity to testosterone concentration. In fact, administration of androgen antagonist demonstrated that the seminal vesicle is the most sensitive tissue among the male reproductive accessory tissues [Vinggaard et al., 2002].

The expression of AhR in the seminal vesicle implies specific functions of this receptor in the tissue. Although AhR does not regulate AR expression, AhR possibly regulates genes essential for the proliferation of seminal vesicle epithelial cells. In fact, an AhR-defective variant of mouse hepatoma Hepa 1c1c7 cells exhibited a prolonged doubling time caused by G_1 cell-cycle arrest [Ma and Whitlock, 1996]. Embryonic fibroblasts prepared from *AhR*(–/–) tissue grow slower because of accumulation of cells in G_2/M-phase due to an altered expression of G_2/M kinases Cdc2 and Plk [Elizondo et al., 2000]. These observations suggest that AhR promotes cell proliferation. However, opposing functions of AhR in cell cycle regulation have also been demonstrated. For example, in rat 5L hepatoma cells, G_1 arrest was induced by TCDD, an AhR ligand, which resulted in overexpression of CDK2 inhibitor, $P27^{kip1}$ [Kolluri et al., 1999; Sherr and Roberts, 1999]. It was also revealed that AhR forms a protein-protein complex with RB [Ge and Elferink, 1998; Puga et al., 2000; Elferink et al., 2001]. Taken together, it has been well-established that AhR regulates cell proliferation. Thus, in addition to the non-cell-autonomous effects observed in response to decreased testosterone production, the seminal vesicle is potentially regressed cell-autonomously through an abnormal cell cycle regulation in *AhR*(–/–) cells.

Our study demonstrated that the seminal vesicle regressed in the aged *AhR*(–/–) males and that this regression is likely caused by a decrease in testosterone production. In fact, low expression levels of 3βHsd and StAR were found in the *AhR*(–/–) testes. Likewise, our previous study [Baba et al., 2005] demonstrated that AhR activates aromatase *P450* (*Cyp19*) gene transcription in the steroidogenic granulosa cells when the ovaries are at preovulatory phase in the estrous cycle [Lynch et al., 1993]. However, no alteration of Cyp19 expression was observed in the *AhR*(–/–) testes. These results clearly demonstrated that AhR is involved in sex steroid synthesis in both sexes although the affected sites in the steroidogenic process are different between males and females. The mechanism for the sex differences in AhR action remains to be resolved, however, these observations strongly suggested that, similar to the female reproductive activity, AhR has a critical function in the male reproduction as well.

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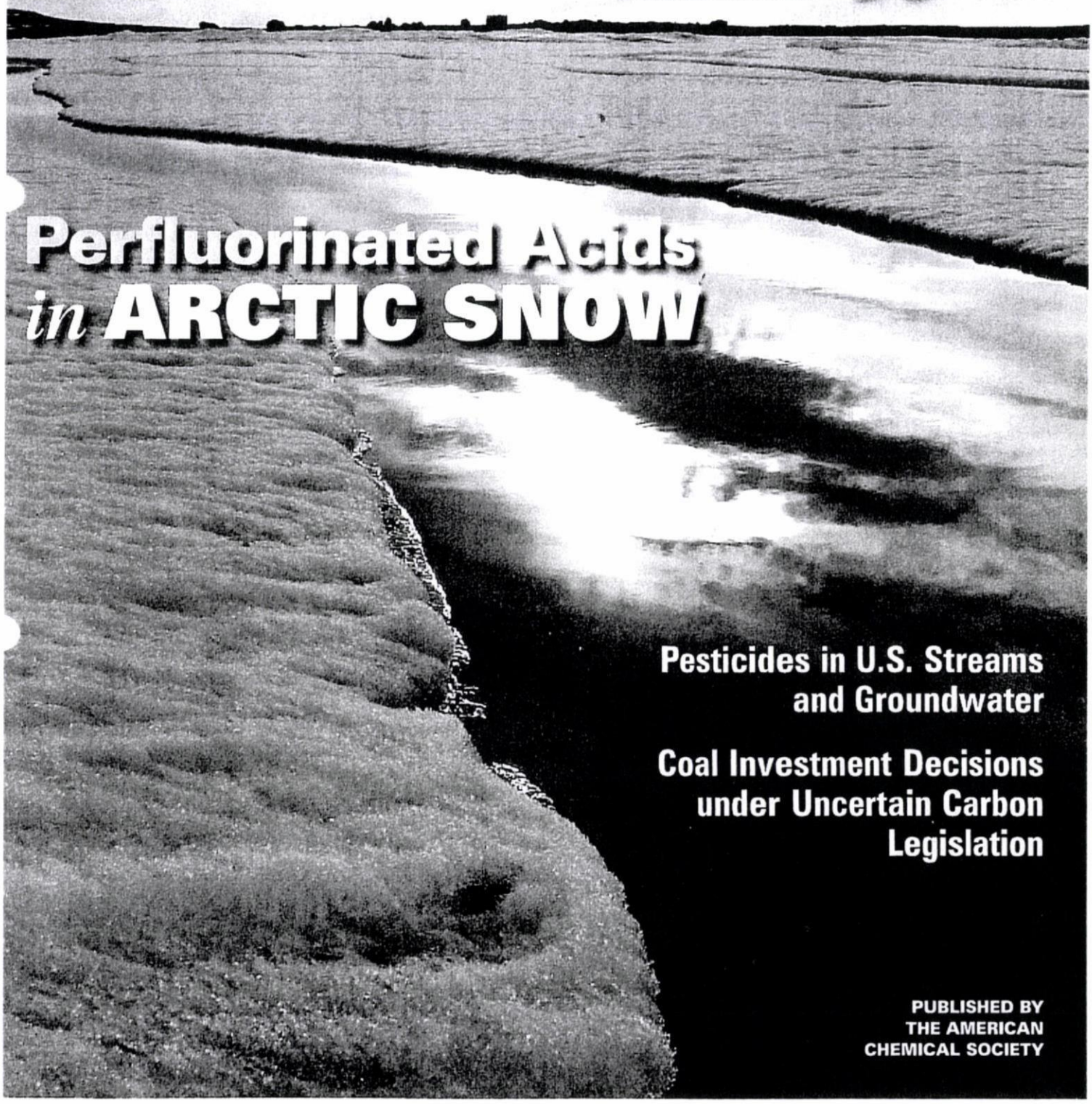

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Aryl Hydrocarbon Receptor Plays a Significant Role in Mediating Airborne Particulate-Induced Carcinogenesis in Mice

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Urban particulate air pollution is associated with an increased incidence of cancers, and especially lung cancer. Organic extracts of airborne particulate matter (APM) cause cancer in mice, and PAHs adsorbed to APM are associated with particle-induced carcinogenesis. PAHs are agonists for AhR and are predominantly responsible for lung cancer through induction of highly carcinogenic metabolites. PAH metabolization requires CYP1A1 induction through activation of AhR, and therefore we hypothesized that carcinogenesis due to PAHs in APM would be reduced in AhR−/− mice. To examine this hypothesis, we performed a long-term continuous-application study of carcinogenesis in AhR−/− mice using airborne particulate extract (APE) of APM collected in Sapporo. Tumor development (squamous cell carcinoma) occurred in 8 of 17 AhR+/+ mice (47%), but no tumors were found in AhR−/− mice, and CYP1A1 was induced in AhR+/+ mice but not in AhR−/− mice. These results demonstrate that AhR plays a significant role in APE-induced carcinogenesis in AhR+/+ mice and CYP1A1 activation of carcinogenic PAHs is also of importance. Therefore, measurement of CYP1A1 induction in vitro may be useful for assessment of APM-induced carcinogenesis in humans. We also show that PAH-like compounds are major contributors to AhR-mediated carcinogenesis, whereas TCDD and related compounds make a smaller contribution.

Introduction

Urban air particulate matter (APM) are mutagenic in short-term genetic bioassays (*1*, *2*), and many exhibit carcinogenic activity in vitro and in vivo (*3*). Epidemiological studies show that urban particulate air pollution is a risk factor for lung cancer (*4*, *5*). However, the biological mechanisms underlying APM carcinogenicity remain unknown.

Among the numerous genotoxic and carcinogenic compounds adsorbed onto urban APM, polycyclic aromatic hydrocarbons (PAHs) are the most prominent because of their known carcinogenic and/or mutagenic properties (*6*, *7*). The carcinogenicity of PAHs occurs through metabolic activation by cytochrome P450 and epoxide hydrolase, which results in formation of highly carcinogenic diol-epoxide metabolites that form DNA adducts that initiate the carcinogenic process (*8*).

Among the cytochrome P450s, CYP1A1 and CYP1B1 have important roles in metabolic activation of carcinogenic PAHs (*9*). Induction of drug-metabolizing enzymes including CYP1A1 and CYP1B1 by PAHs and other environmental contaminants is mediated by a ubiquitous intracellular receptor called the aryl hydrocarbon receptor (AhR) (*10*). AhR is a ligand-activated transcription factor that occurs in many cells and tissues and mediates PAH-induced toxicity, teratogenicity, and carcinogenicity (*11*). Increased expression of AhR occurs in human lung carcinoma compared to normal human lung tissues (*12*). APM extracts induce CYP1A1 and CYP1B1 in the human lung-derived cell line CL5 (*13*) and show significant AhR-mediated activity in vitro in ethoxyresorufin-*O*-deethylase (EROD) induction and in an AhR luciferase reporter system (*14*).

Previously, we have shown that the skin carcinogenicity of BaP, a prototypical PAH, is lost in AhR-deficient (AhR−/−) mice, suggesting that AhR-mediated induction of CYP1A1 is important in BaP-induced skin carcinogenesis in mouse (*15*). We also reported that the skin carcinogenicity of dibenzo[*a*,*l*]pyrene (DB[*a*,*l*]P), a powerful carcinogenic PAH (*16*), was dramatically suppressed in AhR−/− mice, suggesting that the AhR-induced CYP1A1 expression may correlate with susceptibility to DB[*a*,*l*]P carcinogenesis (*17*). These findings imply that AhR-mediated induction of P450s including CYP1A1 is important in activation of PAHs in mouse carcinogenesis.

For evaluating health risks, an understanding of the role of AhR in carcinogenesis caused by environmental mixtures is of importance since people are exposed to such mixtures in daily life. To date, there is no direct proof that AhR plays a significant role in vivo as a mediator of carcinogenesis of environmental mixtures, including APM. However, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds as well as PAHs can bind to AhR to elicit induction of P450s, and urban APM is a complex mixture of substances such as PAHs, TCDD, and related compounds. Therefore, the immunotoxic and carcinogenic reactions elicited by PAHs and TCDD in APM may be mediated by AhR.

The main objective of this study was to examine whether AhR signaling has a net potentiating effect on APM carcinogenicity in mice. Skin tumorgenesis was investigated by long-term treatment with an APM extract (airborne particulate extract; APE) collected by hi-volume samplers, through topical application to the skin of wild type AhR+/+ and AhR−/− mice. A second aim was to evaluate the AhR-mediated biological activity of APE and to differentiate the effect on this activity of PAHs from that of TCDD and related compounds. For this purpose, AhR-mediated activity was determined with crude and cleaned APE, using a reporter-gene assay based on chemically activated luciferase expression (the CALUX assay).

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Materials and Methods

Air Particulate Samples. APM samples were collected on the roof of the Hokkaido Research Institute of Environmental Sciences, which is situated in a residential area about 2 km from the center of Sapporo and entirely surrounded by fields and grounds and not affected directly by vehicle exhaust PM. APM was collected on glass or tissue quartz-filters using high-volume samplers at a rate of 80 m^3/h during the cold season (October–March) from 1973 to 1986. Exposed filters were replaced with new filters daily. A total of 910 24-h filter samples were obtained by filtering 1 770 000 m^3 of air. After weighing the filter samples, they were placed in plastic sacks, vacuum sealed, and maintained in the refrigerator room at a constant −20 °C. The extraction procedure was conducted immediately before the chemical analysis, in vitro bioassay, and the beginning of the animal experimentation (April 2001). To ensure storage of filter samples was suitable, comparative studies were made for the 19-year period. During the storage of the filter samples from 1988 to 2007, no significant changes in mutagenic activity and PAHs concentration were detected. Thus, we presume that there was little or no degradation of the stored samples over time (1973–2001).

Extraction of Organic Matter. Organic material was extracted from the filter samples by ultrasonication using dichloromethane. After filtration through paper to remove undissolved matter, the APE samples were combined. A portion was dried, dissolved in hexane or dimethyl sulfoxide, and used for analysis of PAHs and TCDD and related compounds or for mutagenicity testing and the CALUX assay, respectively. Another portion of APE was used in a skin-painting experiment.

Chemical Analysis and Mutagenicity Test of APE. Sixteen PAHs including six carcinogenic PAHs according to IARC evaluation in APE (Table 1) were analyzed on an Agilent 6890 gas chromatograph (GC) with a 5973 mass spectrometer (MSD) using selected ion monitoring. A DB-5 MS column (J&W Scientific; 30 m × 0.25 mm i.d.; film thickness, 0.25 μm) was used to separate the PAHs. Dioxin-like compounds (17 polychlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzo-*p*-furans (PCDFs)) and 12 PCBs were analyzed by high-resolution gas chromatography-high-resolution mass spectrometry (HRGC-HRMS). A toxicity equivalent (Eq) value for each compound was calculated using the WHO-toxicity equivalent factor (TEF). The total Eq concentration based on HRGC-HRMS analysis (Chemical TCDDEq) for APE was yielded by summation of the calculated Eq concentration for each dioxin-like compound. Mutagenicity of APE was examined by preincubation in the Ames mutagenicity test (*18*) using *Salmonella typhimurium* TA98 and TA100 with (+S9 mix) and without metabolic activation (−S9 mix).

Determination of AhR Mediated Activity of APE. AhR-mediated activity of APE was determined by the CALUX assay, which is based on a genetically engineered rat H4IIE hepatoma cell line with an AhR-controlled firefly luciferase reporter gene construct for detection of CYP1A1-inducing compounds in APE. The assays were performed in 96-well plates as described previously (*19*). Briefly, 24 h after seeding the cells were dosed with crude APE or cleaned APE. Using a sulfuric acid silica column, the APE was cleaned by removing PAHs and PAH-related compounds (PAH-like compounds), including PAHs, nitroarenes, aza-arenes, aminoarenes, methyl-
arenes, etc. The exposure time was either 3 h to measure most compounds (especially PAHs) or 24 h to measure TCDD and related compounds, which are resistant to biotransformation in the cells. The final results are expressed as toxicity equivalents (Eq) in the CALUX assay for BaP (CALUX BaPEq) or TCDD (CALUX TCDDEq), based on the CALUX assay concentration–response curve of BaP or TCDD.

Detection of CYP1A1 by RT-PCR. APE (6.4 mg) was applied to the shaved back of mice once a week for 4 weeks. Six days after the last application, aliquots of RNA (1 μg) were extracted from the dorsal skin of control and APE-treated mice of the two genotypes, and the expression of CYP1A1 mRNA was determined by RT-PCR.

Preparation of Sample Extracts for skin Painting. The mutagenic activities in TA100 with S9 mix per unit of APE and BaP were 4840 and 310 000 revertants/mg, respectively. The amount of extract equivalent to mutagenicity of 1 mg of BaP in the Ames assay was 64 mg (310 000 ÷ 4840 = 64) of APE. Extract for skin painting was adjusted to a concentration equivalent to the mutagenic toxicity equivalent of BaP (M BaPEq) 100 μg (= APE 6.4 mg)/200 μL of acetone.

Animals Procedures. AhR−/− mice were developed by Mimura et al. (*20*). AhR+/+ (n = 17) and AhR−/− (n = 15) female mice aged 6–8 weeks old were used in the study. All mice were genotyped by PCR screening of DNA from the tip (*15, 20*). The animals were housed in clean racks in a filtered-air environment under controlled conditions of temperature (22 ± 1°C), relative humidity (50 ± 5%), and a 12-h light-dark cycle. Sterilized diets and water were available ad libitum throughout the study.

Treatment and Tumor Induction. The dorsal skin of AhR+/+ and AhR−/− mice was shaved 2 days before treatment. Acetone suspensions of APE at 32 mg/mL (equivalent to 500 μg/mL M BaPEq) prepared from the combined APE were epicutaneously dropped onto the shaved backs in a volume of 200 μL. A single application of 200 μL containing 6.4 mg of APE is equivalent to 100 μg M BaPEq. This dose of APE was chosen with toxicity in mind and to obtain data for comparison with the results of the continuous application test (*15*), in which 200 μg of BaP was used once a week. After the application, the mice were restrained until the acetone had completely evaporated. Treatment was repeated continuously once a week until a skin tumor was detected. Animals were inspected weekly for tumor development, and the numbers of skin tumor lesions of larger than 2 mm were counted. The mice were sacrificed and dissected in the 58th week. The main organs were fixed in 10% neutral-buffered formalin, embedded in paraffin as tissue slices, and sectioned and stained with hematoxylin and eosin for microscopic histopathological evaluation.

Statistical Analysis. The statistical significance of the difference in tumor incidence between AhR+/+ and AhR−/− mice was evaluated by Student t-test. A p value of less than 0.05 was considered significant.

Results

PAH Concentrations in APE. Quantitative chemical analysis of APE showed the presence of many carcinogenic PAHs, including BaP, dibenzo[*a,h*]anthracene (DahA), and indeno-[1,2,3-*cd*]pyrene (IND) (Table 1). Measured PAH concentrations were corrected for biological activity and expressed as BaP toxicity equivalent (BaPEq) concentration. The BaPEq concentration for each PAH was calculated by multiplying the PAH concentration by the corresponding TEF (*21*), using the TEFs given by Nisbet and LaGoy (*22*).

The BaPEq concentration and the relative contribution to carcinogenic activity of each PAH, expressed as a percentage of the total BaPEq concentration of the mixture, and the measured air concentration of each PAH are given in Table 1. Benzo[*b+j*]fluoranthene (BbjF) had the highest measured air concentration, followed by chrysene (Chr), benzo[*ghi*]-perylene (BghiP), IND, BaP, and BaA. A total BaPEq concentration of 10.2 ng/m^3 was calculated for APE (Table 1), and the BaPEq concentration of 5.6 ng/m^3 for BaP accounted for 55% of the total; therefore, BaP contributed most to the total calculated BaPEq concentration, in agreement with literature data (*23, 24*).

TABLE 1. Measured and TEF[a]-Adjusted (BaPEq) PAH Concentrations in APE

compound (abbreviation)	TEF[a]	measured concentration ng/m³, air	measured concentration (ng/mg, APE)	BaPEq concentration ng/m³, air	BaPEq concentration (ng/mg, APE)	rel contribution in BaPEq (%)
naphthalene (Naph)	0.001	0.23	(25)	0.0002	(0.025)	0.0
acenaphthylene (Aceny)	0.001	0.11	(12)	0.0001	(0.012)	0.0
fluorene (Flu)	0.001	0.13	(14)	0.0001	(0.014)	0.0
phenanthrene (Phen)	0.001	1.6	(173)	0.0016	(0.173)	0.0
anthracene (Ant)	0.01	0.38	(41)	0.0038	(0.41)	0.0
fluoranthene (Fluor)	0.001	4.2	(453)	0.0042	(0.45)	0.0
pyrene (Pyr)	0.001	4.6	(496)	0.0046	(0.50)	0.0
benzo[*a*]anthracene (BaA)[b]	0.1	5.5	(593)	0.55	(59)	5.4
chrycene (Chr)	0.01	9.1	(982)	0.091	(9.8)	0.9
benzo[*b*+*j*]fluoranthene (BjbF)[b]	0.1	11	(1187)	1.1	(119)	10.8
benzo[*k*]fluoranthene (BkF)[b]	0.1	4.6	(496)	0.46	(50)	4.5
benzo[*a*]pyrene (BaP)[b]	1	5.6	(604)	5.6	(604)	54.8
indeno[1,2,3-*cd*]pyrene (IND)[b]	0.1	6.2	(669)	0.62	(67)	6.1
dibenzo[*a*,h]anthracene (DahA)[b]	1	1.7	(183)	1.7	(183)	16.6
benzo[*ghi*]perylene (BghiP)	0.01	8.7	(939)	0.087	(9.4)	0.9
total				10.2	(1103.0)	100

[a] Data from ref *22*. [b] Probably or possibly carcinogenic to humans according to IARC evaluation.

TABLE 2. CALUX BaPEq, CALUX TCDDEq, and Chemical TCDDEq Concentrations in Crude or Cleaned APE

	CALUX BaPEq[a] ng/m³, air (ng/mg, APE)	CALUX TCDDEq[b] pg/m³, air (ng/mg, APE)	chemical TCDDEq[c] pg-TEQ/m³, air (pg-TEQ/mg, APE)	
crude APE	979 (105634)	21 (2266)		
cleaned APE	7.87 (849)	0.613 (66)		0.13 (14)
			PCDDs TEQ	0.036 (3.9)
			PCDFs TEQ	0.091 (9.8)
			Co-PCBs TEQ	0.0072 (0.78)

[a] CALUX BaPEq : BaP equivalent based on CALUX assay using a BaP standard curve. [b] CALUX TCDDEq : TCDD equivalent based on CALUX assay using a TCDD standard curve. [c] Chemical TCDDEq :TCDD equivalent based on HRGC-HRMS analysis.

Mutagenic Activity of APE. The TA100 strain was more sensitive to the mutagens in APE than the TA98 strain, both with and without metabolic activation. Addition of S9 produced an increase in mutagenic response in both strains, which indicates the presence of promutagens in APE (Data are not shown.).

AhR-Mediated Activity (CALUX Assay) and TCDD Concentration in APE. Luciferase expression induced by crude and cleaned APE was transformed into BaP or TCDD equivalent concentration using a BaP or TCDD standard curve based on response in the CALUX assay after a 3-h or 24-h exposure time. These data (CALUX BaPEq, CALUX TCDDEq) and the results of HRGC-HRMS analysis of TCDD and related compounds in cleaned APE (Chemical TCDDEq) are shown in Table 2. The CALUX BaPEq concentration of crude APE was 979 ng/m³ air, or 105634 ng/mg APE, and the measured BaP concentration (5.6 ng/m³) (Table 1) accounted for only 0.57% of CALUX BaPEq. The CALUX BaPEq of cleaned APE (7.87 ng/m³) accounted for only 0.80% of CALUX BaPEq for crude APE, suggesting that most of the CALUX BaPEq for crude APE was derived from PAH-like compounds in APE. Therefore, the small amount of CALUX BaPEq derived from TCDD and related compounds in cleaned APE could be ignored. Similarly, CALUX TCDDEq for cleaned APE (0.613 pg/m³) accounted for only 2.9% of CALUX TCDDEq for crude APE, showing that the contribution of TCDD and related compounds to CALUX TCDDEq for crude APE was very small. The concentration of TCDD and related compounds (Chemical TCDDEq) in the HRGC-HRMS analysis of cleaned APE was 0.13 pg/m³, and the relative rate of luciferase induction of TCDD for BaP (=1) after a 3-h exposure was 3.85×10^4. Based on these numbers, it was calculated that 0.13 pg/m³ of Chemical TCDDEq would be equivalent to 5.01 pg/m³ of CALUX BaPEq and that the contribution of this value to CALUX BaPEq for crude APE was as low as 0.51%. The contribution of 0.13 pg/m³ of Chemical TCDDEq to CALUX TCDDEq for crude APE was also low (0.62%).

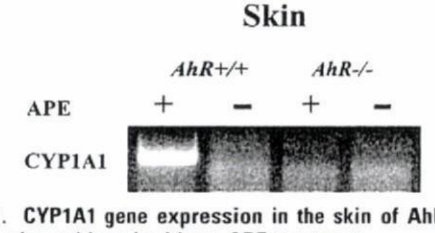

FIGURE 1. CYP1A1 gene expression in the skin of AhR+/+ and AhR−/− mice with and without APE treatment.

Induction of CYP1A1 by APE. Expression of CYP1A1 in the skin of AhR+/+ and AhR−/− mice was investigated using RT-PCR. Following APE treatment, CYP1A1 was induced in AhR+/+ mice but not in AhR−/− mice. No induction of CYP1A1 was apparent without APE treatment, regardless of the genotype (Figure 1). Therefore, the results show that APE induces CYP1A1 through an AhR-dependent pathway.

Tumor Incidence in Mice. APE suspended in acetone was continuously applied once weekly to the dorsal skin of 17 female AhR+/+ mice and 15 female AhR−/− mice. No tumors and hypertrophic changes were observed by the naked eye in AhR−/− mice, whereas AhR+/+ mice showed gradual depilation and inflammatory changes in the skin. In AhR+/+ mice, the first subcutaneous tumor appeared 29 weeks after initiation of treatment, and tumors were present in 3 mice after 41 weeks and 5 mice after 49 weeks. After 58 weeks, 8 of the 17 mice (47%) had papillomatous tumors of larger than 2 mm. Of the 8 induced tumors, 6 were solitary, but multiple tumors occurred in two mice: one having two and one having three small papillomas. In tissue examination under a microscope, it was found that all of the 2-mm or larger tumors in AhR+/+ mice were squamous cell carci-

TABLE 3. Incidence of Skin Tumors Induced in Two Mouse Genotypes after Repeated Application of APE

	AhR genotype	
	+/+	−/−
no. of mice	17	15
squamous cell carcinoma	8	0
papilloma	0	0
keratocanthoma	0	0
total no. of tumor-bearing mice (%)	8 (47%)	0 (0%)

noma, which showed infiltrative growth into muscular tissues in parts of the whole tumor (histopathological image). In addition, erosion and bleeding, which were thought to be caused by extract toxicity, were observed in the anal region of a few AhR+/+ mice. In AhR−/− mice, no tumors developed in the experimental period of 58 weeks, giving a statistically significant difference in tumor incidence between AhR+/+ and AhR−/− mice (8/17 vs 0/15, $p < 0.01$; Table 3). The gross appearance of back skins in AhR+/+ and AhR−/− mice after 58 weeks following repeated application of APE is illustrated in Figure 2. No tumors were evident in internal organs, and there were no other remarkable side effects or observations.

Discussion

PAHs in APE are important environmental carcinogens that pass through the cell membrane and bind to AhR, leading to induction of drug-metabolizing enzymes such as CYP1A1. PAHs are metabolically activated by these enzymes and transformed to DNA-binding carcinogenic substances. Therefore, carcinogenesis caused by PAHs in APE should be decreased in AhR−/− mice, since transactivation of the drug-metabolizing enzymes will not occur. A study in AhR−/− mice showed that acute toxicity or teratogenicity of dioxin is AhR-dependent (*20*, *25*), and our previous study of BaP in AhR−/− mice suggested that induction of CYP1A1 via AhR and metabolic activation of BaP by this enzyme are important in BaP carcinogenesis (*15*). Therefore, the current study was performed to examine the potential AhR dependence of carcinogenesis caused by APE derived from APM, an environmental mixture of compounds collected in Sapporo, in AhR+/+ and AhR−/− mice.

Tumor Incidence and CYP1A1 Expression in AhR+/+ Mice. APE treatment induced CYP1A1 gene expression in AhR+/+ mice and tumors were observed in 47% of these mice over 58 weeks. In contrast, CYP1A1 was not induced, and tumor formation was completely suppressed in AhR−/− mice. These results provide strong support for the hypothesis that the carcinogenic action of APE is mediated primarily by AhR. The primary route of metabolic activation of PAHs involves induction of CYP1A1 mediated by AhR (*10*), which leads to enhanced turnover of PAHs and increased production of highly carcinogenic metabolites. The expression of CYP1A1 in the skin of APE-treated AhR+/+ mice (Figure 1) is in agreement with our earlier study showing CYP1A1 gene expression induced by BaP (*15*). Therefore, it is likely that AhR plays an important role in conversion of APE into carcinogenic compounds through induction of CYP1A1. In the present study, Ahr −/− mice were resistant to APE-induced skin carcinogenesis as well as BaP. On the other hand, there has been a report indicating that Ahr −/− mice were not less susceptible to BaP induced adduct formation when BaP was administered orally (*26*). In addition, the toxicity of BaP is augmented in AhR nonresponsive (*27*) and CYP knockout mice (*28*). Further long-term carcinogenesis studies using oral administration are needed to address the paradoxical effect regarding carcinogenicity and genotoxicity.

Following APE application to skin, tumors occurred in 47% of AhR+/+ mice over about 14 months but not at all in AhR−/− mice, suggesting that APE causes AhR-dependent carcinogenesis. In an application test performed with BaP only, tumors developed in 94% of AhR+/+ mice in 6 months with administration of 200 μg of BaP per week (*15*), compared to 100 μg of APE M BaPEq in the current study. We note that the correlation between mutagenesis and carcinogenesis is complex (*29*), and use of the value of M BaPEq dose, instead of BaP dose, may not necessarily be appropriate; thus, care should be taken regarding interpretation of data using the applied amount of APE based on M BaPEq, as discussed below.

Contribution of PAHs to the Carcinogenic Effect. The contribution of BaP alone to the total BaPEq concentration of APE was 55% (Table 1). BaP is the most studied PAH compound and is thought to be representative of the 16 PAHs in the APE in this study; however, BaP is just one of at least 100 PAHs that have been identified in APM and just one of many carcinogenic compounds in the atmosphere. In our previous application test using only BaP, the applied amount was 200 μg. Since the 6.4 mg dose of APE per week in the current study contained only 3.87 μg BaP, the tumor incidence in the APE-treated AhR+/+ mice does not appear to be solely due to BaP in the mixture. The contribution of BaP to total carcinogenesis capacity is reported to be 6−7.4% and 2.4% for gasoline-powered vehicles (*30*, *31*) and 1.4% for flue gas-condensed substances in coal-heating furnaces (*32*). Based on these data, the contribution of BaP to APE carcinogenesis is estimated to be about 5% or lower.

The contribution to the total carcinogenicity of the PAH fraction comprising compounds with 3, 4, or more rings has been estimated to be 84−91% and 81% for gasoline-engine exhaust (*30*, *31*), and the PAH fraction seems to contribute predominantly to the total carcinogenicity of diesel exhaust (*33*) and hard-coal combustion flue gas condensate (*32*). The major sources of air pollution in Sapporo from 1973 to 1986 were gasoline-powered vehicles, diesel-powered vehicles, and coal firing for home heating, suggesting that most carcinogenesis caused by APE is due to PAHs from such pollution sources. However, the contribution of carcinogenic compounds in APM other than PAHs, such as nitroarenes (NO_2-PAHs), aza-arenes, and polycyclic aromatic compounds (*34*), should also be taken into account. Continuous application of gas condensed substances from home heating furnace to

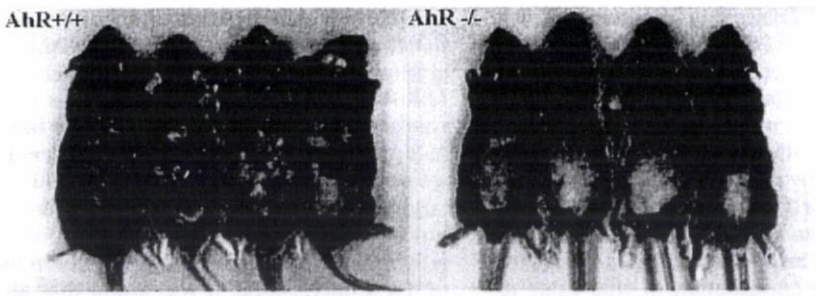

FIGURE 2. Gross appearance of skin tumors in AhR+/+ and AhR−/− mice after repeated application of APE.

the back of mice has shown that fractions including nitroarenes and aza-arenes account for only 4−7% of total carcinogenesis capacity (*35*). However, nitroarenes are an important subgroup of PAHs found in extracts from diesel and gasoline engine exhausts. 3-Nitrobenzanthrone (3-NBA) is an extremely potent mutagen and suspected human carcinogen that is one of several nitroarenes identified in urban PM. Recently, it has been reported that CYP1A1/2 could play an important role in the oxidative metabolism of 3-NBA and the main metabolite of 3-NBA, 3-aminobenzanthrone, to reactive DNA adducts, thereby enhancing their own genotoxic potential (*36, 37*). Thus, for accurate risk assessment of nitroarenes including 3-NBA, further studies on the carcinogenic effect after metabolic activation by P450s are required.

BaPEq Concentration. The carcinogenic potency of a PAH can be assessed based on its BaPEq concentration, and the total BaPEq concentration in mixtures is obtained from the sum of the BaPEq concentrations of components, assuming additive carcinogenic effects in the mixture. BaP had the highest contribution to the total calculated BaPEq concentration (55%), indicating the importance of BaP as a surrogate compound for PAHs mixtures in air; BaP contributions of 42−50% and 50−67% in urban air have also been reported (*23, 24*).

AhR Mediated Activity (CALUX BaPEq and CALUX TCDDEq). Evaluation of AhR-mediated activity of urban APM is important toxicologically for characterization of its carcinogenic potential. In the current study, AhR-mediated activity of APE was determined using the CALUX assay, for crude APE and cleaned APE, to differentiate the contribution to this activity of PAH-like compounds and TCDD and related compounds. A significant induction of AhR-mediated activity was observed for crude extract, expressed as CALUX BaPEq 979 ng/m^3 and CALUX TCDDEq 21.0 pg/m^3. The health consequences of these data are unknown, but this activity might contribute to adverse health effects of APM. The health risks of APM including PAH like compounds and dioxins will be assessed in further in vivo studies.

The CALUX assay response of crude APE may be due to both easily biodegradable and persistent AhR agonists, such as PAHs and TCDD, respectively. To test whether TCDD and related compounds contribute to the AhR-mediated activity of crude APE, cleaned APE was tested in 3-h and 24-h CALUX assays. A reduction in AhR-mediated activity due to depletion of PAH-like compounds in cleaned APE was observed for both CALUX BaPEq and CALUX TCDDEq, suggesting that PAH-like compounds are responsible for most AhR-mediated activity in crude APE. As PAHs, PAH-related compounds may be able to bind to and activate AhR. These compounds may include possible human and/or animals carcinogens. The Chemical TCDDEq concentration of 0.13 pg/m^3 indicates that TCDD and related compounds make relatively small contributions to this activity.

Finally, Revel et al. have demonstrated in an animal model that a natural AhR antagonist, resveratrol, inhibits BaP-induced CYP1A1 enzyme activity and subsequent formation of DNA adducts (*38*). Given the importance of AhR in APM-induced carcinogenesis, a competitive AhR inhibitor may provide a chemopreventive effect against development of cancer. Therefore, long-term exposure to APM in mice would be useful for evaluation of prevention of cancer by AhR antagonists.

In conclusion, our results show that APE induces carcinogenesis in AhR+/+ mice and provide the first direct evidence that AhR plays an essential role in APE-induced carcinogenesis. Our data also indicate that PAH-like compounds are significant contributors to AhR-mediated activity, whereas TCDD and related compounds make an almost negligible contribution.

Acknowledgments

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