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Novel cDNA sequences of aryl hydrocarbon receptors and gene expression in turtles (*Chrysemys picta* and *Pseudemys scripta*) exposed to different environments ☆

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ABSTRACT

Reproductive changes have been observed in painted turtles from a site with known contamination located on Cape Cod, MA, USA. We hypothesize that these changes are caused by exposure to endocrine-disrupting compounds and that genes involved in reproduction are affected. The aryl hydrocarbon receptor (AHR) is an orphan receptor that is activated by environmental contaminants. AHR mRNA was measured in turtles exposed to soil collected from a contaminated site. Adult turtles were trapped from the study site (Moody Pond, MP) or a reference site and exposed to laboratory environments containing soil from either site. The red-eared slider was used to assess neonatal exposure to soil and water from the sites. The environmental exposures occurred over a 13-month period. Juveniles showed an age-dependent increase in brain *AHR1*. Juvenile turtles exposed to the MP environment had elevated gonadal *AHR1*. Adult turtles exposed to the MP environment showed significantly decreased brain *AHR2*. The painted turtle AHR is the first complete reptile AHR cDNA sequence. Phylogenetic analysis of the painted turtle AHR showed that it clusters with other AHR2s. Partial AHR1 and partial AHR2 cDNA sequences were cloned from the red-eared slider. MEME analysis identified 18 motifs in the turtle AHRs, showing high conservation between motifs that overlapped functional regions in both AHR isoforms.

1. Introduction

1.1. Aryl hydrocarbon receptor

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor that contains the characteristic motifs basic helix–loop–helix (bHLH) and Per-ARNT-Sim (PAS) (Kawajiri and Fujii-Kuriyama, 2007). AHR was discovered and subsequently cloned using photoaffinity-labeled 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Burbach et al., 1992). TCDD is an environmental contaminant that is known as the classical ligand of AHR. TCDD is a potent inducer of cytochrome P4501A1 (CYP1A1), which is also induced by a wide range of drugs, dietary agents, and environmental contaminants (Tompkins and Wallace, 2007). AHR binds other synthetic compounds, mainly planar hydrophobic halogenated aromatic hydrocarbons and polycyclic aromatic hydrocarbons such as 3-methylcholanthrene (Denison and Nagy, 2003). It has been suggested that vertebrates evolved dioxin sensitivity as an adaptive response to environmental stressors (Hahn, 2002). Evidence suggests that AHR arose in early bilateral metazoans and is present in invertebrate phyla. In vertebrate lineages, it has undergone duplication and diversification, resulting in a family of *AHR* genes (*AHR1, AHR2, AHRR*). It is possible that the varying degrees of sensitivity to dioxin found among animal taxa stems from the diversification of AHR and differential expression of the AHR types.

When bound to TCDD, AHR dimerizes with AHR nuclear translocator (ARNT), enters the nucleus, and activates transcription via binding to xenobiotic response elements (XRE). When CYP1A1 is activated, AHR/ARNT enters the nucleus and binds XRE in the proximal and distal promoter regions of the CYP1A1 gene (Kawajiri and Fujii-Kuriyama, 2007). The binding of the AHR/ARNT heterodimer to the proximal XRE of CYP1A1 results in chromatin remodeling of the CYP1A1 gene and thus increased DNase sensitivity, leading to TCDD-activated gene transcription (Wang and Hankinson, 2002). AHR is degraded rapidly *in vivo* and *in vitro* after ligand binding and is ligand-dependent (Roberts and Whitelaw, 1999). *AHR*-deficient or knockout mice do not show a typical CYP1A1 induction in response to challenge with classical AHR agonists, indicating that CYP1A1 induction is AHR-dependent.

Evidence suggests that AHR plays a key role reproductive development by engaging in cross-talk with genes key to reproduction. Both human and mouse CYP19 P450 aromatase genes possess an XRE that binds AHR and ARNT (Baba et al., 2005). The AHR pathway is also known to interact with the estrogen signaling pathway, inhibiting ER signaling through several mechanisms (Safe and Wormke, 2003). In AHRKO mice, mRNAs of follicle stimulating hormone and lutenizing hormone

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receptor are reduced, resulting in reduced ovarian response to gonadotropin injection (Barnett et al., 2007). AHR response elements have been found in the promoter of the proapoptotic gene marker *Bax*, and *Bax* mRNA was shown to increase in the ovaries of mice after treatment with the AHR ligand dimethylbenz(a)anthracene (Matikainen et al., 2001).

To date, there are few studies on reptile AHR. Hahn et al. (1994) established that AHR was present in the painted turtle (*Chrysemys picta*), via photoaffinity labeling using hepatic cytosolic fraction and [^{125}I]-N_3Br_2DD. A specifically labeled protein was not observed in the American alligator (*Alligator mississippiensis*), although only one individual was examined (Hahn et al., 1994). A complete reptile AHR has not been sequenced before, although partial AHRs have been determined for a phylogenetic study on several species of turtle (Barley et al., 2010).

1.2. Massachusetts Military Reservation (MMR)

The MMR is a military training site on Cape Cod that was declared an Environmental Protection Agency Superfund site in 1989. The site has several sources of contamination that have contaminated the groundwater in the area (AFCEE, 2003). The study site Moody Pond is located close to the MMR, while the reference site Washburn Pond is located away from the path of groundwater flow.

Moody Pond sediment elutriate has been shown to have similar effects to TCDD in zebrafish embryos. Expression of a novel aromatase B splice variant was shown to increase with exposure to TCDD (Novillo-Villajos et al., 2003). A similar expression pattern was induced in embryos exposed to Moody Pond sediment elutriate, but not when embryos were exposed to Moody Pond water. In female painted turtles from Moody Pond, reproductive changes such as reduced ovarian follicular reserve, reduced plasma estradiol and vitellogenin have been described. Exposure to environmental contaminants is the likely cause of these changes (Rie et al., 2005). In male painted turtles from the Moody Pond study site, Kitana et al. (2007) observed a decrease in testicular weight, seminiferous tubule diameter and epididymal sperm number. A previous study examining painted turtles trapped from Moody Pond found hepatic biotransformation enzymes to be elevated, notably CYP1A1 protein, suggesting contamination of Moody Pond with organic compounds such as dioxin-like compounds (Rie et al., 2000). These studies suggest that both male and female turtles are sensitive to EDC present at this site.

In this study, we cloned the turtle AHR in order to pursue a possible role of AHR/estrogenic pathway interactions associated with the previously observed reproductive deficits. Quantitative changes in AHR mRNA were measured under different environmental conditions.

2. Materials and methods

2.1. Adult painted turtles

The specific chemical environment of the contaminated site, Moody Pond, has not been defined, but it is located 500 meters downgrade from at least 8 contaminated sites within the MMR. These include fuel and chemical spills, storm drains, and landfills (AFCEE, 2003). The pond is also immediately to the east of a well-documented contaminant plume from the MMR (Eastern Briarwood). The reference site, Washburn Pond, is to the east of the MMR and is unaffected by groundwater flows from the MMR. Painted turtles (*Chrysemys picta)* were collected from sites in Mashpee, MA for a 13-month laboratory exposure to sediment from the contaminated site or the reference site (Moody or Washburn Pond, respectively) during two 1-week periods in both June and October 2005 using hoop traps baited with canned sardines or cat food. The crossover experiment (Fig. 1A) was done with the painted turtle adults to determine whether 1) the effects of a Moody Pond origin could be mitigated by exposure to the reference environment, Washburn Pond, or 2) if an individual from a Washburn Pond origin would be impacted by a yearlong exposure to the Moody Pond environment. A subset of individual males and females trapped at Moody Pond were placed in the Washburn environment tanks; also a subset of Washburn Pond individuals was placed in the Moody environment tanks. Turtles trapped in Washburn and Moody Ponds were also placed into their native environments. At the beginning of the experiment, 9 adult male and 6 adult female painted turtles of Washburn origin were exposed to the Moody environment; 2 adult male and 4 adult females of Moody origin were exposed to the Washburn environment. Eight adult male and 8 adult female painted turtles of Washburn origin were exposed to the Moody environment; 1 adult male and 3 adult females of Moody origin were exposed to the Moody environment.

2.2. Juvenile red-eared sliders

Due to a lack of commercially available painted turtle neonates, red-eared slider (*Pseudemys scripta*) neonates were purchased from Robert Clark (Hammond, Louisiana, USA). A species of pond turtle that is closely related to the painted turtle, red-eared sliders were used to determine effects of contaminated soil on turtles exposed early in life, starting as neonates (Fig. 1B). To distribute sexes equally between tanks, regression analysis of the ratio between precloacal length and the two posterior lobes of the plastron (PPR) was done. PPR can be used to approximate sex in neonate turtles (Lovich and Gibbons, 1992; de Solla et al., 1998). Males have a relatively longer precloacal length (Lovich and Gibbons, 1992). A group of neonates was autopsied for sample collection at the start of the experiment for assessment of sex dimorphic characteristics and gene expression. At the beginning of the experiment, 30 neonate red-eared sliders were placed in the Washburn environment tank, with the regression analysis assessing 15 female and 15 male red-eared sliders. Twenty-nine red-eared slider turtles were placed in the Moody environment tank, of which 14 were female and 15 were males.

2.3. Animal maintenance and experimental design

Turtles were maintained in the Laboratory Animal Care Facility of the Department of Biology at Boston University. The experimental animals were placed in tanks starting in November 2005 and sacrificed in December 2006 for a 13-month period. Soil for the exposure was collected from Moody and Washburn Pond and stored for later use at 4 °C in doubled plastic bags closed with zip ties.

Painted turtle adults were placed in large floor-standing fiberglass or stainless steel tanks, while neonate red-eared sliders were maintained in Lucite aquaria. The tanks were divided, with standing water in one section and sediment in the other. The water in the tank was pumped onto the surface of the soil to ensure a constant flow of water through the sediment. Water was changed every week in the adult tanks, and every 2 weeks in the neonate tanks. Animals were given a 12-hour day cycle and fed *ad libitum* daily. The soil in both adult and neonate tanks was replaced every two months. Due to the logistical problem of transporting and storing large quantities of collected water from the study site, adults were exposed only to the sediment collected from each site. Neonates were exposed to both soil and water collected from the field sites. Adult tanks were supplied with tap water filtered for chloramines. The water collected from the ponds was filtered prior to storage and stored at 4 °C in doubled plastic bags closed with zip ties.

At sacrifice in December 2006 after 13 months of exposure, all animals were anesthetized in ice slurry and decapitated. Tissue samples were flash-frozen. In neonate turtles, sex can be visually assessed by examining the gonad (Crews et al., 1991). Weight and morphometric measurements were taken at autopsy. Ovarian follicle diameter was measured in adults.