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Effects of *in ovo* exposure to benzo[*k*]fluoranthene (BkF) on CYP1A expression and promoter methylation in developing chicken embryos

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are toxic environmental pollutants that are potent teratogens. Recent research suggests that early life exposure to PAHs can affect health outcomes later in life. Some of these latent responses may be mediated by epigenetic mechanisms such as DNA methylation. The role of DNA methylation in regulating responses to PAHs in birds is currently unknown. Here, we assess the effect of *in ovo* exposure to the model PAH, benzo[*k*]fluoranthene (BkF), on aryl hydrocarbon receptor (AHR) mediated cytochrome P4501A (*CYP1A*) gene expression and promoter methylation in chicken embryos. Fertilized chicken eggs were injected with BkF (0–100 μg/kg) prior to incubation. BkF exposure was associated with an increase in *CYP1A4* and *CYP1A5* mRNA levels at mid-incubation (embryonic day 10), which dropped to baseline levels towards the end of the incubation period (embryonic day 19). The transient induction in *CYP1A* expression was accompanied by small but significant increases in *CYP1A* promoter methylation, which persisted until after shortly after hatching. Methylation within the *CYP1A* promoter was correlated with levels of *CYP1A5*, but not *CYP1A4* mRNA. Characterization of the role of DNA methylation in the AHR response pathway may increase our understanding of the effects of early life exposure to PAHs in birds.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are environmental contaminants of concern for humans and wildlife. Of the thousands of different congeners that belong to this class, 16 have been classified as priority contaminants by the US EPA (Douben, 2003). PAHs can be introduced into the environment through natural sources (e.g. forest fires, volcanic activity) or human activity (e.g. oil spills or industrial activity) (Douben, 2003). Birds are receptors of concern for PAHs. For example, during oil spills, birds can be exposed to PAHs through ingestion of contaminated prey, dermal contact or inhalation (Albers, 2006; Broman et al., 1990).

The principle route of exposure of avian embryos to PAHs is maternal deposition. During laying, female birds deposit lipophilic contaminants into the egg, potentially exposing avian embryos to significant levels early in life (Albers, 2006). Concentrations of total PAHs measured in eggs of wild birds are generally found in the range of 1 to 10 μg/kg wet weight (Pereira et al., 2009; Shore et al., 1999). Levels can be substantially higher following exposure to point sources of PAHs. For example, after the Prestige oil spill in 2003, total PAHs in bird eggs reached up to 461 μg/kg wet weight (Zuberogoitia et al., 2006). Much of this chemical burden will be metabolized by the developing embryo; 94% of a mixture of PAHs injected into fertilized chicken eggs at embryonic day 4 was metabolized before hatching (approximately 2 weeks later) (Näf et al., 1992).

In birds, as in other vertebrates, cytochrome P4501A (CYP1A) is the main enzyme responsible for transforming PAHs to more soluble metabolites which can be excreted (Nebert et al., 2013). The avian CYP1A family of mixed function oxidases is represented by two isoforms; CYP1A4 and CYP1A5 (Gilday et al., 1996). Expression and activity of these isoforms is highly induced by exposure to PAHs such as benzo[*k*]fluoranthene (BkF) and similar compounds (Clark et al., 2014; Head et al., 2015). This induction leads to rapid metabolism, limiting the potential for accumulation within tissues. However, the continuous presence of PAHs in the environment means that adult birds can be chronically exposed.

In adult birds, exposure to PAHs can be associated with impaired immune and liver function, reduced liver mass, cancer, and reproductive effects. In avian embryos, exposure to PAHs has been shown to cause developmental abnormalities (e.g. hepatic lesions, edema and microphthalmia) and embryomortality (Brunström et al., 1990; Albers, 2006). Early-life exposure to PAHs can also cause latent effects that first become apparent during later life stages and can sometimes persist into future generations. For example, exposure of mice to PAHs in utero

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resulted in greater body weight and fat mass later in life. This phenotype was observed 60 days after the end of exposure and persisted in the F2 generation (Yan et al., 2014). Similarly, in utero exposure of mice to environmental tobacco smoke (a well-known source of PAHs), resulted in airway inflammation and changes in cytokine expression six weeks after the end of exposure (Lee et al., 2015). In a fish model, exposure of adult zebra fish to benzo[*a*]pyrene (BaP) caused developmental abnormalities up to and including the F2 generation (Corrales et al., 2014b). A growing body of research suggests that epigenetic marks (such as DNA methylation) may sometimes be involved in these types of latent or multi-generational effects (Lee et al., 2015; Yan et al., 2014).

The role of DNA methylation in the molecular response to PAHs in birds is unknown, but the response pathway itself is well described. The aryl hydrocarbon receptor (AHR) is a nuclear transcription factor that is activated by PAHs, and other contaminants such as dioxin-like compounds. Activation of the AHR leads to its translocation to the nucleus and binding of the AHR complex to xenobiotic response elements (XREs, 5′-GCGTG-3′) within the DNA. These motifs are commonly found in the promoters of AHR target genes, such as *CYP1A* (Guyot et al., 2013), and *CYP1A* isoforms are dramatically induced by AHR ligands in birds and other vertebrates (Clark et al., 2014; Head et al., 2015). The presence of CpG sites within the XRE of *CYP1A* and other genes suggest that AHR binding can be regulated through DNA methylation (Kikuchi et al., 2003).

Patterns of DNA methylation are established during development, making early life stages most susceptible to environmental stressors that affect the epigenome. DNA methylation generally acts as a repressor of the expression of many genes, including *CYP1A* (Siegfried and Simon, 2010; Suter et al., 2010). Furthermore, PAHs and similar compounds have been reported to interfere with normal DNA methylation (Bollati and Baccarelli, 2010; Head et al., 2012). For example, in zebrafish, larval exposure to BaP resulted in changes in gene-specific DNA methylation in 10 genes at 96hpf, some of which are known AHR target genes (e.g. nqo1, cyp1b1, gstp1) (Corrales et al., 2014a). In human, prenatal exposure to tobacco smoke correlated with hypomethylation of the *AHR* Repressor (*AHRR*), a negative regulator of the AHR pathway and hypermethylation of *CYP1A1*, an AHR target gene (Joubert et al., 2012).

In the current study, we hypothesized that *in ovo* exposure of chicken to PAHs results in transient changes in gene expression (as the PAHs are metabolized), but persistent changes in DNA methylation of *CYP1A* genes. To test this hypothesis, we exposed chicken embryos to the potent AHR ligand, BkF, and assessed gene expression and methylation state of the promoter of AHR target genes *CYP1A4* and *CYP1A5*.

2. Materials and methods

2.1. Chemicals

BkF (99.6% purity) was purchased from Sigma (St-Louis, MO). Stock solutions were prepared by dissolving BkF in corn oil (Selection brand) sterilized with a 0.22 μm syringe filter (Fisher Scientific) to final concentrations of 0.6 mg/mL, 0.06 mg/mL and 0.006 mg/mL. The solutions were stored at room temperature, and protected from light until use for egg injections. The concentration of the most concentrated stock solution was verified analytically by GC/MS to be 0.71 mg/mL (Axys Analytical, Sidney BC, Canada). Nominal concentrations are reported here.

2.2. Egg injection and tissue sampling

All animal studies were approved by McGill University's animal care committee. Fertile unincubated White Leghorn chicken (*Gallus gallus*) eggs were purchased from Couvoir Simetin (Mirabel, QC) and stored at room temperature for up to six days prior to injections. Eggs were injected directly into the air-cell prior to incubation, as previously described with minor modifications (Rutkiewicz and Basu, 2013). Briefly, eggs were weighed and candled on embryonic day 0 (ED0, preincubation) and the location of the air cell was marked in pencil. Eggs were sprayed with ethanol, and a single hole was drilled through the shell above the air cell using a hobby drill (Dremel). A volume of 10 μL of BkF stock solutions or corn oil was injected into each egg, for nominal concentrations of approximately 1, 10, and 100 μg/kg BkF. After injection, holes in the shell were sealed with hot glue. A non-injected control group was also included.

Eggs were incubated for 7, 10, 19, or 21 (hatch) days at 37.5 °C with 60% relative humidity and 45 minute rotations in a poultry incubator (Ova-Easy Advanced Series II cabinet incubators type 190, Brinsea Products, Titusville, FL). Once a week, eggs were candled and dead embryos and infertile eggs were removed from the incubator. For hatching studies, rotations were stopped and relative humidity was increased to 70% on day 19 of incubation. Eggs were placed in individual compartments in preparation for hatching. Upon hatching, chicks were labeled with a leg tag, allowed to dry and then placed in a brooder where they were kept with free access to water and food for 2 days.

Chicks were euthanized by decapitation on day 7, day 10 and day 19 of embryonic development, or 2 days post hatching (ED7, ED10, ED19 and D2 respectively). Livers were removed, placed in RNAlater (Qiagen) and stored at 4 °C overnight. The next day, livers were transferred to − 20 °C storage. The number of samples analyzed for each time point and dose group varied due to the presence of infertile and dead eggs in the experiment. Sample sizes for each treatment group and time point analyzed ranged between six and 15. Details are presented in Supplementary data (Table S1).

As described in the following sections, *CYP1A* gene expression, global DNA methylation, and *CYP1A* promoter methylation were analyzed in liver tissue from the BkF-treated embryos and chicks. Quantification of gene expression was performed in samples from every time point (ED7, ED10, ED19, and D2) whereas the DNA methylation analyses mainly focused on the latest time point (D2). The rationale behind this approach was that we were most interested in detecting changes in DNA methylation that persisted beyond the initial exposure. As described in the Introduction, PAHs injected on ED0 are expected to be nearly completely metabolized by ED19. Changes observed at D2 could therefore be considered to be persistent.

Of the three doses tested, the lower two (1 and 10 μg/kg egg weight) are in a range that could be considered to be environmentally relevant. Concentrations of BkF in eggs of various species of wild birds was below 0.25 μg/kg egg weight in several studies (Pereira et al., 2009; Zuberogoitia et al., 2006), however, concentrations as high as 28.87 μg/kg egg weight have been reported following an oil spill (Vidal et al., 2011). Total PAH levels in eggs are often detected within the range of 1 to 10 μg/kg egg wet weight (Pereira et al., 2009; Shore et al., 1999). The highest dose tested, 100 μg/kg egg weight, is approximately 10 times a level that could be considered environmentally typical (for total PAHs) and resulted in significant mortality to injected embryos (35% at ED19, 57% at D2). Chicks that survived to D2 were not measurably different from control chicks in terms of weight and incidence of deformities. Mortality of embryos in the two lower treatment groups was below or similar to corn oil injected controls (Supplementary data, Table S2).

2.3. DNA/RNA isolation

DNA and RNA were isolated from liver tissue with either the Allprep DNA/RNA 96 DNA isolation kit, or the DNeasy Blood & Tissue kit and the RNeasy Mini Kit (Qiagen). Briefly, 5–15 mg of RNAlater-stabilized tissue was disrupted using a TissueLyser (Qiagen). Purification of DNA and RNA was carried out according to manufacturer's recommendations. To ensure that RNA isolated from tissues was free of genomic DNA contamination, on-column DNA digestion was performed using