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Effects on the hepatic transcriptome of chicken embryos *in ovo* exposed to phenobarbital

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ABSTRACT

This work aimed at evaluating the toxic effects of *in ovo* exposure to phenobarbital (PB) and unveiling the mode of action by transcriptome analysis in the embryonic liver of a model avian species, chicken (*Gallus gallus*). Embryos were initially treated with saline or 1 μg PB /g egg at Hamburger Hamilton Stage (HHS) 1 (1st day), followed by 20 days of incubation to HHS 46. At 21st day, chicks that pipped successfully were euthanized and dissected for assessing the PB caused effects on phenotypes and the liver transcriptome in both genders. In the PB treatment group, a 7% attenuation in tarsus length was found in females. While no adverse phenotypic effect on the liver somatic index (LSI) was observed, PB caused significant changes in the expressions of 52 genes in males and 516 genes in females (False Discovery Rate < 0.2, p value < 0.05, and absolute fold change > 2). PB exposure modulated the genes primarily enriched in the biological pathways of the cancer, cardiac development, immune response, lipid metabolism, and skeletal development in both genders, and altered expressions of genes related to the cellular process and neural development in females. However, mRNA expressions of chicken xenobiotic receptor (CXR)-mediated CYP genes were not induced in the PB treatment groups, regardless of males and females. On the contrary, PB exposure repressed the mRNA expressions of *CYP2AC2* in males and *CYP2R1*, *CYP3A37*, and *CYP8B1* in females. Although transcription factors (TFs) including SREBF1 and COUP-TFII were predicted to be commonly activated in both genders, some TFs were activated in a gender-dependent manner, such as PPARa in males and BRCA1 and IRF9 in females. Taken together, our results provided an insight into the mode of action of PB on the chicken embryos.

1. Introduction

Phenobarbital (PB), an anticonvulsant commonly used for treatment of epilepsy, is a prototypical activator of the mammalian nuclear receptor, constitutive androstane receptor (CAR). This receptor transcriptionally induces hepatic xenobiotic metabolizing enzymes such as cytochrome P450 (CYP) genes; *CYP2B10* in the mouse and *CYP2B6* in the human (Geter et al., 2014). PB is a potent non-genotoxic liver carcinogen in the mouse. The mechanism underlying PB-induced tumor formation has been characterized by a cascade of events including CAR activation, increased hepatocellular proliferation, and eventually the formation of liver foci, adenomas, and carcinomas (Deguchi et al., 2009). In contrast with the mouse study, epidemiological data from epilepsy patients treated with PB indicated no explicit correlation between PB and human liver cancer risk, though their PB plasma levels were close to those found in susceptible rodents (Deguchi et al., 2009). This suggests that species differences in the tumor induction may therefore attribute to divergent functions of CAR between mice and humans (Braeuning et al., 2014).

Prenatal exposure to PB has been reported to increase the risk of teratogenicity, such as cleft palate and cardiovascular anomalies in the human newborn (Bath and Scharfman, 2013). Similar morphological anomalies were also observed in rodent studies (e.g. mice), where maternal PB exposure negatively shortened the litter size and decreased birth weight of the offspring (Abel et al., 1987). Besides, prenatal exposure to PB hindered the brain development as well as cognition and behaviour of the offspring. In rats, prenatal PB exposure was known to impair the cognitive functioning such as working memory and spatial learning (Bath and Scharfman, 2013). However, to date few studies have addressed the effects of prenatal exposure to PB on the liver in terms of the mode of action, regardless of humans or other animals.

Since PB-induced signaling pathway is believed to be evolutionarily conserved in avian species (Watanabe et al., 2013; Handschin et al., 2000), prenatal exposure to PB may exert deleterious effects on avian embryos. In the chicken hepatocytes, the response to PB is mediated by the chicken xenobiotic receptor (CXR) that might be an ancestral gene

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of both CAR and pregnane X receptor (PXR) in mammals (Handschin et al., 2000). Transcriptional regulation of several hepatic CYP genes mediated by CXR has been found in the chicken. For example, *CYP2C23a* (previously known as *CYP2H1*) and *CYP3A37* are orthologs to the mammalian *CYP2B* and *CYP3A*, respectively (Matis et al., 2013). Compared with the studies on rodents, there are a very limited number of investigations evaluating the toxic effects of PB on the chicken. In the case of in vivo tests, PB administrated at the dose of 80 μg/g into 8 weeks old chickens for three days induced the hepatic mRNA expressions of four CXR-mediated CYP genes with statistical differences; *CYP2C45* were significantly induced in both males and females with the fold changes of 2.8 and 3.6, respectively, whereas *CYP2C23a*, *CYP2C23b*, and *CYP3A37* with the fold changes of 7.6, 12.6 and 2.5, respectively, were merely up-regulated in females (Watanabe et al., 2013). Besides, in a recent test of chicken embryos *in ovo* exposed to PB solutions at the doses of 0.34–3.4 μg/g egg, PB shortened the vertebral column and the limbs during embryonic skeletogenesis probably due to the impairment of angiogenesis and osteogenesis (Yan et al., 2016). Although the negative effects of PB on chicken xenobiotic metabolism and skeletogenesis have been clarified, it remains to be undetermined whether PB affects CXR signaling in the liver of chicken embryos and what the mode of action involved in the effects is.

Transcriptome techniques, such as microarray and RNA-sequencing by next-generation sequencer (NGS), allow to simultaneously measure the mRNAs of a large number of genes whose expression levels are changed in response to xenobiotic exposure and perturbed biological networks. To date, a range of studies to evaluate the effects of PB has primarily been dedicated to rodents, which revealed the role of CAR in activating the xenobiotic metabolism and other biological pathways, including immune response, cellular growth and proliferation, lipid metabolism etc. (Ross et al., 2009; Phillips et al., 2009). Thus, we hypothesize that PB may also activate CXR in chicken embryos and interfere with these pathways.

Although the environmental concentrations of PB detected in the avian embryos has not been reported, CAR/CXR ligands (e.g., polychlorinated biphenyls and triclosan) have been detected in avian species. In addition, effects mediated by CAR/CXR have been recently found in chicken embryos treated with chemicals, e.g. triclosan at the dose of 1 μg/g egg (Guo et al., 2018). To characterize the CXR-mediated effects and evaluate the hazard of CXR ligands, we therefore performed this study by exposing PB to chicken embryos at the dose of 1 μg/g egg. The aim of this study was to characterize the effects of PB on the signaling pathways consisting of gene-gene networks in the liver of chicken embryos. Our hypothesis is that in concert with the effects of PB on rodents, *in ovo* exposure to PB in chicken embryos would interact with CXR, induce the mRNA expressions of target CYPs (e.g. *CYP3A37*), and interfere with several signaling pathways in relation to immune response, cellular growth and proliferation, lipid metabolism and skeletal development. As far as we know, this is the first comprehensive study on the hepatic transcriptome profile in chicken embryos treated with PB.

2. Materials and methods

2.1. Chemical and solution

Sodium chloride (99.5%; CAS no. 7440–23-5) used for preparation of vehicle saline (0.9%) and phenobarbital sodium (referred to as PB, 98% purity; CAS no. 57–30-7) were purchased from Wako Pure Chemical Industries, Ltd (Japan). Working solution was prepared to yield nominal dose of 1 μg PB /g egg.

2.2. Egg injection and tissue collection

White leghorn chicken (*Gallus gallus*) eggs at Hamburger Hamilton Stage (HHS) 1 (1st day) were purchased from Niinobe Hatchery Co., Ltd. (Kagawa, Japan). After confirming the developmental stage of each embryo by candling, chemical injections were implemented. Briefly, the injection volume was adjusted to approximate 10 μL per egg to achieve the final concentrations of chemicals in two groups: vehicle control saline and PB solution (1 μg/g egg). Solutions were initially injected into the air sac at the blunt end of the egg using a micro-syringe (n = 10 / group; Fig. 1A), followed by 20 days of incubation to HHS 46 under the constant conditions (temperature 38 ± 1 °C; humidity 55 ± 2%; rotation 1 cycle per hour). At 21st day, chicks that pipped successfully were euthanized and dissected according to the regulation of animal experiments of Ehime University. Liver stored with Trizol (ZYMO) and muscle samples were flash-frozen in liquid nitrogen and stored at −80 °C until total RNA, and genomic DNA isolation, respectively. We employed five parameters to assess the phenotypic effects caused by PB, including mortality (the number of deaths / the number of total embryos X 100%), embryo mass (g), liver somatic index (LSI = liver mass / embryo mass X 100%), tarsus length (mm), and bill ratio (bill ratio = upper bill length / lower bill length X 100%). To obtain the enough samples in two genders, the *in ovo* injection experiment was re-conducted twice with five embryos in each treatment.

2.3. Genetic gender determination

The gender of individual embryos was determined by amplifying chromo-helicase-DNA (CHD) binding genes on the chicken Z and W chromosomes with Gene Amp polymerase chain reaction (PCR) system 9700 (Guo et al., 2018). In brief, we initially extracted the genomic DNA from 22 to 25 mg muscle using NucleoSpin Tissue kit according to the manufacturer's protocol (Macherey-Nagel, Japan). PCR reactions with special cycling conditions (Table S1) were then performed using Go Taq® Green Master mix kit (Promega, US). Primers concentrations were optimized as follows: CHD-fwd: 5′ – ATCGTCAGTTTCCCTTTCAG −3′ (5 μM); CHD - rev: 5′ – GATCCAGTGCTTGTTTCCTC −3′ (5 μM). The PCR products were resolved via agarose gel electrophoresis (3% agarose), where a single band of the CHD gene (CHD-Z) was observed for male but two bands (CHD-Z and CHD-W) for female. Four male and female embryos in each treatment were randomly selected and applied for assessment of the phenotypic effects. Similarly, embryonic livers of the selected four males and females in each treatment were applied for further transcriptome analysis.

2.4. RNA sequencing

Total RNA was isolated from approximate 25 mg of liver from each experimental group using Direct-zol RNA miniPrep kit in accordance with the manufacturer's instructions (Zymo research, Japan). RNA concentration and purity were assessed using a ND-1000 NanoDrop spectrophotometry (NanoDrop Technologies, Inc., USA), followed by determination of the sample integrity using Agilent 2011 bioanalyzer (Agilent Technologies, Inc., USA). Only samples with an A260 /A280 > 1.8 and a RNA Integrity Number (RIN) > 7.0 were used for RNA sequencing. Total RNA samples were submitted for next-generation sequencing analysis service using lllumina HiSeq. 2500 (Hokkaido System Science, Japan). Image analysis, base-calling, filtering based on fluorescence purity and output of filtered sequencing files were performed using Illumina Analysis Pipeline.

2.5. RNA-seq data analyses

For the raw reads in FastQ format, we employed cutadapt (v 1.1; Martin, 2011) and Trimmomatic (v 0.32; Bolger et al., 2014) to trim Illumina adapter sequences and low quality region in sequencing files, respectively. Afterwards, the trimmed mRNA reads were mapped to reference genome *Gallus gallus* 5.0 (Ensembl Genes 87) using mapping programs Bowtie1 (v 1.1.1) and SAMtools (v 0.1.18) embedded in Tophat (v 2.0.14; Trapnell et al., 2009). Based on the above mapping