Toxicology in Vitro 27 (2013) 1649–1658

Contents lists available at SciVerse ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

In vitro microarray analysis identifies genes in acute-phase response pathways that are down-regulated in the liver of chicken embryos exposed *in ovo* to PFUdA

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ARTICLE INFO

Article history:
Received 23 November 2012
Accepted 8 April 2013
Available online 16 April 2013

Keywords:
Chicken
Hepatocyte
Gene-expression
Perfluoroundecanoic acid
Perfluorooctane sulfonate
Mode of action

ABSTRACT

Perfluoroundecanoic acid (PFUdA) is one of the most highly detected perfluoroalkyl compounds in wild bird tissues and eggs. Although PFUdA does not affect hatching success, many PFCs are known to impair post-hatch development and survival. Here we use microarrays to survey the transcriptional response of cultured chicken embryonic hepatocytes (CEH) to PFUdA for potential targets of PFUdA action that could lead to developmental deficiencies in exposed birds. At 1 μM and 10 μM PFUdA significantly altered the expression of 346 and 676 transcripts, respectively (fold-change > 1.5, $p < 0.05$, false discovery rate-corrected). Using functional, pathway and interactome analysis we identified several potentially important targets of PFUdA exposure, including the suppression of the acute-phase response (APR). We then measured the expression of five APR genes, fibrinogen alpha (*fga*), fibrinogen gamma (*fgg*), thrombin (*f2*), plasminogen (*plg*), and protein C (*proC*), in the liver of chicken embryos exposed *in ovo* to PFUdA. The expression of *fga*, *f2*, and *proC* were down-regulated in embryo livers (100 or 1000 ng/g, $p < 0.1$) as predicted from microarray analysis, whereas fibrinogen gamma (*fgg*) was up-regulated and *plg* was not significantly affected. Our results demonstrate the utility of CEH coupled with transcriptome analysis as an *in vitro* screening tool for identifying novel effects of toxicant exposure. Additionally, we identified APR suppression as a potentially important and environmentally relevant target of PFUdA. These findings suggest *in ovo* exposure of birds to PFUdA may lead to post-hatch developmental deficiencies, such as impaired inflammatory response.

1. Introduction

The majority of toxicological research on perfluoroalkyl compounds (PFCs) has focused on the two most environmentally prominent congeners: perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA). Less attention has been given to other PFCs, such as the long-chain compounds. Several long-chain PFCs have been detected at elevated concentrations in the tissue of wild animals in high trophic positions, especially in fish-eating birds (Bustnes et al., 2008; Gebbink et al., 2009, 2011; Lofstrand et al., 2008; Verreault et al., 2005). In some cases, levels of long-chain perfluorocarboxylic acids (PFCAs), such as perfluoroundecanoic acid (PFUdA), are among the highest PFC concentrations detected. Concentrations of PFUdA as high as 184 ng/g wet weight (ww) and 675 ng/g ww were reported in the plasma of glaucous gulls and eggs of parrot bills, respectively (Verreault et al., 2005; Yoo et al., 2008).

We recently demonstrated that long-chain PFCs, such as PFUdA and perfluorodecane sulfonate (PFDS), do not affect the hatching

Abbreviations: *a2m*, alpha-2-macroglobulin; APR, acute-phase response; *cdo1*, cysteine dioxygenase type I; CEH, chicken embryonic hepatocytes; *cpb2*, carboxypeptidase B2; DE, differentially expressed; DMSO, dimethyl sulfoxide; *f2*, thrombin (coagulation factor II); FDR, false discovery rate; *fga*, fibrinogen alpha; *fgg*, fibrinogen gamma; GSH, glutathione; *gsta3*, glutathione-S-transferase alpha 3; *gsta4*, glutathione-S-transferase alpha 4; *gsto1*, glutathione-S-transferase omega 1; HNF4A, hepatocyte nuclear factor 4 alpha; IPA, Ingenuity Pathway Analysis; *isg12-2*, interferon stimulated gene 12 protein-like 2; *lbfabp*, liver basic fatty acid binding protein; *mgst1*, microsomal glutathione-S-transferase 1; PFC, perfluoroalkyl compound; PFCA, perfluorocarboxylic acid; PFDS, perfluorodecane sulfonate; PFNA, perfluorononanoic acid; PFOA, perfluorooctanoic acid; PFOS, perfluorooctane sulfonate; PFUdA, perfluoroundecanoic acid; *plg*, plasminogen; PPARα, perosixome proliferator-activated receptor alpha; PPARγ, peroxisome proliferator-activated receptor gamma; *proC*, protein C; TP53, tumor protein 53.

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http://dx.doi.org/10.1016/j.tiv.2013.04.003

success of chickens (O'Brien et al., 2009). However, the effects of embryonic PFC exposure are known to extend beyond nascency. At high doses, PFOS, PFOA and perfluorononanoic acid (PFNA) severely reduce the postnatal survival of rat and mouse pups (Grasty et al., 2003; Lau et al., 2003, 2004; Luebker et al., 2005; Wolf et al., 2007, 2010). Lower doses cause reduced body weight, delayed eye-opening, impaired motor activity and also reduce neonate survival. Although fewer studies have been conducted on avian species, reports are largely consistent with rodent models, demonstrating effects that include: decreased survival in neonate bobwhite quails following *in ovo* exposure to PFOS (Newsted et al., 2007); increased spleen mass, decreased immunoglobin and increased brain asymmetry in chickens exposed *in ovo* to PFOS (Peden-Adams et al., 2009); and physiological and neurodevelopmental defects in chicks exposed *in ovo* to PFOA (Pinkas et al., 2010; Yanai et al., 2008).

In a previous *in vitro* screening study using chicken embryonic hepatocytes (CEH), (Hickey et al., 2009) showed that exposure to PFUdA altered the mRNA expression levels of specific genes involved in cholesterol, lipid and xenobiotic metabolism. Furthermore, the concentration at which these genes were significantly affected was lower than that observed for PFOS. This suggests that although PFUdA may not affect hatching success, it may be a more potent disruptor of transcriptional regulation than PFOS in CEH, and may present developmental health risks to exposed birds. In the present study, we further characterized the transcriptional response of CEH to PFUdA exposure using DNA microarrays. We then surveyed the expression data to identify possible targets of PFUdA exposure that could lead to developmental deficiencies in exposed birds. Finally, we confirmed the response of a potentially important target pathway in the liver tissue of developing chicken embryos exposed *in ovo* to PFUdA.

2. Materials and methods

All procedures using animals were conducted according to protocols approved by the Animal Care Committee at Environment Canada's National Wildlife Research Centre.

2.1. Chemicals

Linear perfluoroundecanoic acid (PFUdA, purity > 98%) was obtained from Wellington Laboratories (Guelph, ON, Canada). Dosing solutions were prepared by dissolving PFUdA in dimethyl sulfoxide (DMSO).

2.2. Preparation of hepatocyte cultures and dosing

Primary chicken embryonic hepatocyte (CEH) cultures were prepared and exposed to PFUdA as part of a larger study on the *in vitro* transcriptional response of several PFCs. The cell culture preparation and exposure conditions used herein were performed simultaneously with the CEH PFOS exposures described in O'Brien et al. (2011), following the same experimental procedures. The DMSO exposed CEH described in the present study are the same CEH used for the study published by O'Brien et al. (2011). Sixty eggs were obtained from the Canadian Food Inspection Agency (Ottawa, ON, Canada) and artificially incubated for 19 days at 37.5 °C and 60% humidity. On day 19, embryos were euthanized by decapitation. Livers were removed and pooled. Hepatocytes were isolated by collagenase digestion and cultured into 48-well plates. Each well received approximately 780 μg of hepatocytes in 525 μl of medium. Cultured cells were acclimated for 24 h at 37 °C and 5% CO_2. Acclimated cells were dosed with a DMSO solvent control or working solutions of PFUdA. After 24 h of exposure, medium was removed and cells were flash-frozen on dry ice and stored at −80 °C.

Cells for microarray analysis were exposed to solvent control or in-well concentrations of 1 or 10 μM PFUdA ($n = 5$ wells per dose-group, 1 array per well). These concentrations were selected because 10 μM was the lowest concentration at which transcriptional effects were observed without cytotoxicity in Hickey et al. (2009). Dose–response relationships were examined by real-time RT-PCR (qPCR) in a second independent cell culture (prepared using same the method as the first culture). These cells were exposed to solvent control or 0.5, 0.1, 1, 5, 10 or 20 μM PFUdA. Gene expression analysis for this culture was performed using $n = 2$–3 separate wells per dose-group.

2.3. RNA isolation from CEH

Total RNA was extracted from exposed CEH using RNeasy 96 kits (Qiagen) according to manufacturer's directions. The extraction included on-column DNase treatment and an additional DNase treatment after extraction using an Ambion DNA-free kit (Ambion, Austin, TX) as per kit instructions. Quantification of RNA was performed using a NanoDrop 2000 (Thermo Scientific). Quality of RNA samples was assessed using a BioAnalyzer (Agilent). Samples with A_{260}/A_{280} ratios <1.7 and RIN values <8.0 were omitted from further use.

2.4. Microarray hybridization

Microarray hybridizations were performed as described in, and using the same pool of reference RNA as (O'Brien et al., 2011). Five arrays were hybridized per dose, each using RNA isolated from a separate culture well (i.e. $n = 5$ wells per dose). The hybridization procedure, in brief, was as follows: Cy5 (experimental) and Cy3 (reference) labeled complementary RNA (cRNA) were prepared from 150 ng of total RNA using Agilent Quick Amp labeling kits following the manufacturer's instructions. Labeled sample and reference cRNA (825 ng each) was fragmented and hybridized to Agilent 4 × 44k chicken genome arrays (design #015068, Agilent Technologies) using Agilent Hybridization kits as directed by the manufacturer. Samples were hybridized for 17 h, then washed and scanned on an Agilent G2505B scanner at 5 μm resolution. Quantitative data were extracted from the scanned image using Agilent Feature Extraction software version 9.5.3.1.

2.5. Microarray data analysis

Microarray data analysis was carried out as previously described (O'Brien et al., 2011). In brief, analysis was performed using a blocked reference design. Pre-processing was performed in R (http://www.R-project.org). Data were normalized by the global LOWESS method. Differentially expressed genes were identified using an ANOVA model that included the block effect and the main treatment effect. The Fs statistic used for the gene-specific variance components and the associated p-values for all the statistical tests were estimated using the permutation method (30,000 permutations with residual shuffling). These p-values were then corrected for multiple comparisons using a false discovery rate (FDR) of 0.05. Finally, fold-changes for each pairwise comparison were estimated using the least-squares means.

Hierarchical clustering was performed using GeneSpring GX ver. 11.0.2 (Agilent Technologies). Clustering was based on both entities and conditions using the Euclidian distance metric and the centroid linkage rule.

The DAVID Gene ID Conversion Tool (http://david.abcc.ncifcrf.gov/) was used to optimize the number of microarray gene IDs that were mapped as human, rat or mouse orthologs into Ingenuity Path-