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Polychlorinated biphenyl exposure alters the expression profile of microRNAs associated with vascular diseases

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ABSTRACT

Exposure to persistent organic pollutants, including polychlorinated biphenyls (PCBs) is correlated with multiple vascular complications including endothelial cell dysfunction and atherosclerosis. PCB-induced activation of the vasculature subsequently leads to oxidative stress and induction of pro-inflammatory cytokines and adhesion proteins. Gene expression of these cytokines/proteins is known to be regulated by small, endogenous oligonucleotides known as microRNAs that interact with messenger RNA. MicroRNAs are an acknowledged component of the epigenome, but the role of environmentally-driven epigenetic changes such as toxicant-induced changes in microRNA profiles is currently understudied. The objective of this study was to determine the effects of PCB exposure on microRNA expression profile in primary human endothelial cells using the commercial PCB mixture Aroclor 1260. Samples were analyzed using Affymetrix GeneChip® miRNA 4.0 arrays for high throughput detection and selected microRNA gene expression was validated (RT-PCR). Microarray analysis identified 557 out of 6658 microRNAs that were changed with PCB exposure ($p < 0.05$). *In-silico* analysis using MetaCore database identified 21 of these microRNAs to be associated with vascular diseases. Further validation showed that Aroclor 1260 increased miR-21, miR-31, miR-126, miR-221 and miR-222 expression levels. Upregulated miR-21 has been reported in cardiac injury while miR-126 and miR-31 modulate inflammation. Our results demonstrated evidence of altered microRNA expression with PCB exposure, thus providing novel insights into mechanisms of PCB toxicity.

1. Introduction

MicroRNAs (miRNAs) are a class of small, endogenous, non-coding RNAs (~22–25 nucleotides long) that have recently been shown to play a critical role in regulating gene expression and protein production (Bartel, 2004). Ever since their discovery in the early 90s, over 1000 miRNAs have been identified in the human genome, and these miRNAs are predicted to regulate approximately 30% of the human protein-coding genome (Ardekani and Naeini, 2010). Traditionally, miRNAs were thought to be negative regulators of gene expression (RNA silencing) that act either by base pairing with complementary sequences on the mRNA transcript and promoting its degradation or by repressing translation (Bartel, 2004). However, emerging studies have demonstrated that miRNAs can also positively regulate gene expression by targeting promoter sequences of specific genes (Place et al., 2008). Because of their regulatory role, miRNAs have the ability to regulate biological and physiological processes such as cell proliferation, differentiation, metastasis, inflammation and apoptosis. In fact, the pivotal role of miRNAs in influencing the development and progression of diseases is becoming increasingly recognized (Li and Kowdley, 2012). Changes in miRNA profile can be induced by pathophysiology or exposure to environmental stimuli that eventually results in altered cellular responses, making these molecules potential biomarkers for disease prognosis and diagnosis as well as targets for therapeutic implications (Duong Van Huyen et al., 2014; Srinivasan et al., 2013). Advances in oligonucleotide microarrays and next generation sequencing technologies have enabled the entire miRNAome to be analyzed. Altered miRNA expression profile has been implicated in almost all cancer types (Pichler and Calin, 2015); however, the role of these noncoding molecules is not limited to neoplasia but extends to other pathologies including vascular diseases and cardiac complications, neurodevelopmental diseases and liver diseases (Chang et al., 2016; Fernandez-Hernando and Baldan, 2013; Kim and Kim, 2014; Pirola et al., 2015).

Cardiovascular diseases (CVD) are the primary cause of mortality and morbidity in developed countries and include atherosclerosis,

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vascular inflammation and endothelial cell dysfunction among other complications (Pagidipati and Gaziano, 2013). These pathological states are accompanied by modified expression profile of genes associated with cardiac function/inflammation such as vascular cell adhesion molecules e.g. (VCAM-1, ICAM-1) and cytokines. Interestingly, with the advent of miRNA research, multiple studies have shown that cellular homeostasis of these CVD-related genes is regulated by specific miRNAs (Fernandez-Hernando and Baldan, 2013; Ono et al., 2011; Small et al., 2010). Our laboratory has previously demonstrated that exposure to environmental toxicants such as polychlorinated biphenyls (PCBs) caused endothelial cell dysfunction by activating the aryl hydrocarbon receptor (AhR), subsequently leading to induction of pro-inflammatory cytokines, adhesion proteins and cytochrome P450s (Arzuaga et al., 2007; Petriello et al., 2014).

PCBs are persistent organic pollutants that were commercially manufactured and used mainly as dielectric fluids in electrical transformers. Epidemiologic studies have correlated PCB exposure with multiple health complications such as liver injury, hypertension and vascular inflammation (Cave et al., 2010; Perkins et al., 2016). PCBs have been shown to interact with a myriad of receptors in the body including the AhR and hepatic nuclear receptors, and this has been attributed as a mechanism of PCB-induced toxicity (Luthe et al., 2008; Wahlang et al., 2014). Furthermore, these receptors are intimately involved in maintaining energy homeostasis, cell proliferation, differentiation and inflammation by activating their target gene battery. Importantly the gene expression of these receptors and their target genes may also be under the regulatory control of miRNAs. However, literature regarding which miRNAs are affected with exposure to environmental chemicals such as PCBs is still scarce. We therefore hypothesize that PCBs can induce changes in miRNA profiles in endothelial cells that potentially affect downstream protein turnover. In order to test our hypothesis, we utilized microarray technology for evaluation and the MetaCore database for data interpretation. This could potentially be a new mechanism of action for PCB-induced toxicity in the vasculature.

2. Materials and methods

2.1. Materials

Aroclor 1260 was purchased from AccuStandard (New Haven, CT, USA). Dimethyl sulfoxide (DMSO) and ethyl alcohol were purchased from Sigma Aldrich (St. Louis, MO, USA). Chloroform and 2-propanol were obtained from Fisher Scientific (Hampton, NH, USA).

2.2. Cell culture

Human umbilical vein endothelial cells (HUVECs, 10-donor pool) were obtained from Lifeline Cell Technology (Walkersville, MD, USA). Cells were maintained in VascuLife Basal Medium supplemented with components of the VascuLife VEGF LifeFactors Kit (containing Vascular Endothelial Growth Factor, 2% fetal bovine serum and other appropriate life factors) and 1% antimicrobial supplement (penicillin, streptomycin and amphotericin B). The cells were incubated in a 5% carbon dioxide atmosphere and 95% humidity at 37 °C in T75 flasks and fresh media was added every 2 days. Cells were passaged at 80% confluence. Before passaging, cells were washed with 10 mL of sterile phosphate-buffered saline (PBS) and incubated with 6 mL of Lifeline's 0.05% Trypsin/0.02% EDTA for 3 min. Then, 6 mL of Lifeline's Trypsin Neutralizing Medium was added to the cells. The entire 12 mL was collected and centrifuged at 150 ×*g* for 5 min. The liquid above the cell pellet was aspirated, and cells were resuspended in fresh medium and seeded in 6-well plates.

In order to choose an incubation time for the study, HUVECs were initially exposed to Aroclor 1260 for either 12, 16 or 24 h and assessed for Cyp1A1 (an AhR target gene) as well as miR-21 induction. The concentrations of Aroclor used for these preliminary studies were 1, 5 and 10 μM. The 12 h incubation did not significantly induce miR-21 even at 10 μM while the 24 h incubation elicited a biphasic concentration–response with CYP1A1 induction. Because the study primarily focused on miRNA induction with PCB exposure, without taking into account factors such as time and concentration dependence, the 16 h time point was chosen.

2.3. RNA isolation

For microarray analysis, cells were harvested after a 16 h exposure to Aroclor 1260. RNA was extracted using the mirVana™ miRNA Isolation Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) to obtain high quality intact RNA that includes the low molecular weight RNA, using an efficient glass fiber filter-based method. The RNA integrity was determined on the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). All samples had an RNA integrity number (RIN) >9. For real time polymerase chain reaction (RT-PCR), cells were harvested and RNA was isolated using TRIzol reagent (Thermo Fisher Scientific Inc). RNA purity and quantity were assessed with the NanoDrop 2000/2000c (Thermo Fisher Scientific Inc) using the NanoDrop 2000 (Installation Version 1.6.198) software.

2.4. Microarray studies

500 ng of total RNA was labeled using Affymetrix Flashtag Biotin HSR RNA Labeling kit (P/N 901910, Affymetrix, Santa Clara, CA, USA) according to the manufacturer's instructions. The labeling process begins with a brief tailing reaction followed by ligation of the biotinylated signal molecule to the target RNA sample. 450 ng of biotin labeled RNA was hybridized to Affymetrix GeneChip® miRNA 4.0 Arrays at 48 °C and 60 rpm for 16–18 h. The chip contains miRNAs and pre-miRNAs that cover 100% of the miRBase version 20 database. Each array contains 30,434 total mature miRNA probe sets. The arrays were washed and stained using the Hybridization Wash and Stain Kit in the 450 Fluidics Station (Affymetrix). Chips were scanned using the Affymetrix GeneChip 7G scanner to acquire fluorescent images of each array. Data were collected using Affymetrix Command Console Software. The data discussed in this study have been deposited in NCBI's Gene Expression Omnibus which is accessible through GEO Series accession number GSE79005.

2.5. Real-time PCR

Complementary DNA (cDNA) was synthesized from total RNA using the QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA, USA). PCR was performed on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using the Taqman Fast Advanced Master Mix (Thermo Fisher Scientific Inc). Primer sequences from Taqman Gene Expression Assays (Thermo Fisher Scientific Inc) were as follows: actin, beta (ACTB); (Hs01060665_g1), miR-21; (Hs04231424_s1), miR-31; (Hs04231431_s1), miR-126; (Hs04273250_s1), miR-221; (Hs04231481_s1), and miR-222; (Hs04415495_s1). The levels of mRNA were normalized relative to the amount of ACTB mRNA, and expression levels in cells that were not exposed to PCBs were set at 1. Gene expression levels were calculated according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.6. In-silico network analysis

The miRNAs that were determined to be statistically altered due to PCB treatment were filtered by selecting only the annotated miRNAs using miRBase v21 database. Target gene information was obtained using TargetScan, miRDB (predicted targets) and TARBASE (validated targets). The mir2disease database and Human microRNA Disease Database (v2.0) were initially utilized to identify miRNAs related to vascular diseases by using the search term 'vascular diseases' or 'cardiovascular diseases'. The miRNAs that were correlated to vascular diseases were