MODULATION OF AhR-DEPENDENT GENE EXPRESSION IN LUNG EPITHELIAL A549 CELLS EXPOSED TO 2,3,7,8-TCDD, POLYCYCLIC AROMATIC HYDROCARBONS OR EXTRACTS OF AIRBORNE PARTICLES

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Introduction

Global gene expression studies performed in various mammalian models have indicated that activation of the aryl hydrocarbon receptor (AhR) by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) or other dioxin-like compounds commonly regulates expression of hundreds of genes[1]. However, the specific changes in gene expression seem to be both tissue- and species-dependent, and apparently, there exists only a very limited subset of genes, which are similarly regulated by AhR ligands across different species and cell types[2]. In addition, at least in the *in vitro* studies, the AhR-dependent gene expression seem to be further modulated by cell differentiation status, cell cycle phase, cell confluency etc. Therefore, it is difficult to select general biomarkers of exposure to dioxin-like compounds in particular target cells/tissues and to identify the driving mechanisms regulating expression of genes, which are linked to dioxin toxicity, and which may possibly contribute to processes, such as tumor promotion and progression. At the same time, important targets for dioxin-like compounds, such as lung epithelium, remain to be poorly characterized from the point of view of AhR-induced toxicity.

Apart from TCDD and related persistent dioxin-like compounds, many easily metabolized polycyclic aromatic hydrocarbons (PAHs) are significant and relatively potent transient AhR agonists. It is still a matter of debate, how much does the chronic exposure to PAHs lead to the effects similar to the TCDD-like effects. Nevertheless, we have previously reported that PAHs and not persistent dioxin-like compounds are major contributors to the AhR activation in various cellular models exposed to complex mixtures of extractable organic matter (EOM) from airborne particles[3]. In this study, we compared effects of TCDD, two representative PAHs, benzo[a]pyrene (BaP) and benzo[k]fluoranthene (BkF), and EOM samples on global gene expression in human lung epithelial adenocarcinoma cells A549, using both microarray analysis and RT-qPCR data.

Materials and Methods

Particle matter PM2.5 was collected and extracted by dichloromethane as previously described[4,5]. The A549 cells were grown in Dulbecco´s Modified Eagle´s medium (DMEM) supplemented with 1.0 g/L glucose and pyruvate, 10% FBS, 200 mM glutamine and gentamycin sulfate (10 mg/ml). The cells were cultivated in plastic cell culture dishes (21 cm^2) at 37°C in 5% CO2. After reaching 70 – 80% confluency, the medium was replaced with fresh medium supplemented with 1% FBS. The cells were treated for 6, 24 or 72h, as indicated. RNA isolation, quality control and gene expression profiling were performed according to previous study[4], using Illumina Human-HT12 v3 Expression BeadChips (Illumina, San Diego, CA, USA). RT-qPCR analysis was performed as previously described[4]. For bioinformatics analysis, Genomatix Matrix Library 9.1 was used.

Results and Discussion

A sustained activation of the AhR may affect a wide spectrum of biological processes associated with cancer development and many of these mechanisms still remain to be elucidated[6]. In this study, global gene expression changes were determined in lung epithelial A549 cells after 24h incubation with various EOM samples containing high levels of PAHs and the data were compared to the effects of TCDD, BaP and BkF. For the comparative study, RT-qPCR analysis of selected genes was used. Among the most upregulated genes

modulated by EOMs from all sampling sites were many known AhR-responsive genes participating in xenobiotic metabolism, regulation of AhR and detoxification of aldehydes generated by lipid peroxidation (*CYP1A1*, *CYP1B1*, *TIPARP*, *ALDH1A3* and *ALDH3A1*), the genes involved in inflammatory processes (*TGIF1*, *STAT4*, *STAT1*, *IL8*), regulation of cell proliferation (*VIPR1*, *PADI3*, *ALDH3A1*, *ROR1*, *CALB2*, *NR5A2*, *EREG*), plasma membrane functions and transmembrane transport (*SDPR*, *LAMP3*, *TMEM105*, *TM6SF1*, *ABCA1*), modulation of TGF-β and/or Wnt signaling and cell migration (*GREM1*, *ROR1*, *LMCD1*). Downregulated genes were mostly involved in processes such as embryogenesis and loss of cell viability (*INSL4*), transcription associated with cell proliferation, migration and regulation of cytokines (*NR4A2*), chromatin remodeling complex and early lung morphogenesis (SOX9), TGF-β and Wnt signaling (*BMP6*, *DKK1*, *ID3*), or genotoxic stress response (*TP73L*). Importantly, with exception of TP73L, no deregulation of the genes involved in DNA damage response was found in A549 cells.

A large set of TCDD-deregulated genes belonged to the genes involved in regulation of cell cycle progression/ cell proliferation after the treatment with high doses of EOM, however, effects of TCDD, PAHs and EOM on cell cycle progression were only limited (data not shown). Despite inducing low, but significant levels of DNA adducts[5], the exposure to EOM was not associated with induction of genes involved in DNA damage response and/or apoptosis. It can be speculated that the affected cells with relatively low levels of DNA adducts may thus survive, which may increase a likelihood of fixation of deleterious mutations or potential for further DNA damage. The present data suggest that AhR activation is a key toxicity mechanism of polycyclic aromatic hydrocarbons within lung cells, which could be linked to their non-genotoxic effects in lung epithelium.

The data on global gene expression were further compared with available previous studies performed with A549 cells exposed to TCDD[7], whole airborne particles or BaP[8]. Generally, we found a significant overlap of deregulated genes in A549 cells exposed to TCDD, BaP, or EOM samples (Table 1). The deregulated pathways involved biotransformation of xenobiotics and endogenous compounds, proinflammatory responses and several developmental and cancer-related pathways and genes.

Table 1: Genes similarly regulated after 24h exposure to TCDD, BaP, PM2.5 or EOM in A549 cells.

1) Drug-metabolizing enzymes ("core" AhR target genes) ↑CYP1A1, ↑CYP1B1, ↑TIPARP, ↑AHRR, ↑ALDH1A3
2) Cell cycle, proliferation, apoptosis ↑EREG (induced only after exposure to PM, EOM or TCDD), ↑p21 (BaP), ↑ALDH3A1 (PM, EOM, BaP), ↓CDK6 (TCDD), ↓CCND1, ↓CENPN, ↓CDK2, ↓CDC2, ↓CCNE, ↓AURKB, ↓E2F2 (down-regulation by high doses of EOM only)
3) Proinflammatory reesponses ↑STAT1 and ↑STAT4 (PM, EOM), ↑IL8 (EOM, TCDD)
4) TGF superfamily / Wnt pathways / other morphogenic/cancer pathways ↓CDH1 (PM, BaP, TCDD), ↑GREM1, ↓BMP6, ↓ID3 (PM, EOM), ↑WISP2 (PM), ↑AXIN2 (TCDD), ↑GDF15 and ↑NKD2 (PM, high doses of EOM, BaP)

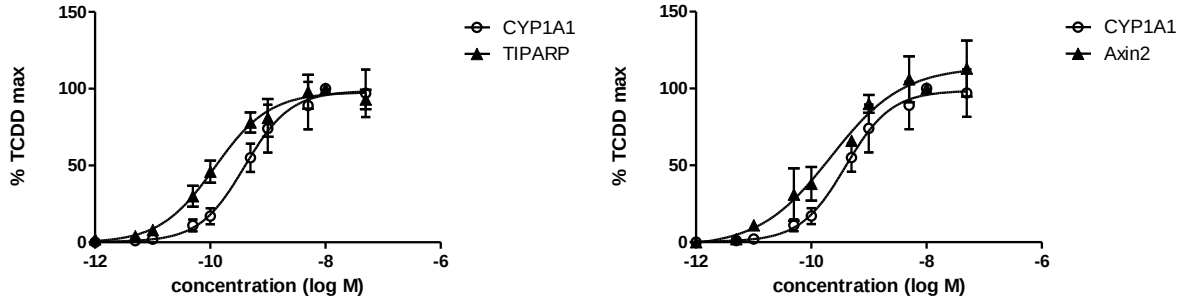
The genes involved in TGF-β signaling pathway were similarly deregulated after TCDD, BaP, BkF and EOM complex mixture treatment. TGF-β is a multifunctional cytokine regulating a diversity of cellular processes including morphogenesis, motility, differentiation, cell proliferation, apoptosis, and invasion in various cell types. TGF-β signaling includes two major pathways activated by TGF-β or bone morphogenetic protein (BMP) ligands, which activate signal transduction pathways involving distinct SMAD proteins. We also observed up-regulation of the BMP antagonist (GREM1) and down-regulation of BMP6 and ID protein inhibitors (ID1, ID2, ID3). Also TGIF1, a transcriptional co-repressor of SMAD2, was significantly up-regulated in A549 cells. Our data seem to suggest that TGF-β/BMP pathways could be suppressed in cells treated with both single AhR

ligands and complex mixtures thereof, and that the short-term exposure of A549 to EOM cells may paradoxically contribute to their epithelial status maintenance.Nevertheless, any possible mutual interactions among GREM1, BMP6, ID genes and TGF-β-dependent transcription machinery in lung epithelial cells are still not fully clear; therefore, the implications for AhR signaling in their deregulation should be interpreted with caution.

Several other individual genes involved in early lung morphogenesis and tumor promotion were deregulated as well (e.g. suppression of SOX-9 and DKK1), suggesting that TCDD, PAHs and EOMs may affect additional pathways associated with lung morphogenesis. Nevertheless, although we have observed a deregulation of some genes associated with lung morphogenesis or tumorigenesis, we did not observe any immediate effects on cell phenotype, including possible EMT.

We hypthesized that at least some of identified genes, associated with tumor promotion, which were deregulated in A549 cells after 24h exposure, might be used as potential biomarkers of exposure to environmental AhR agonists, both persistent dioxin-like compounds and PAHs, in lung cells. Based on this we next determined relative effective potencies (REPs) of TCDD, BaP and BkF to modulate the expression of selected AhR target genes and we tested their use as candidate novel AhR-regulated candidate genes. We used exposure to TCDD and chemical inhibitor of AhR activation (CH223191), to confirm that modulation of selected target genes was AhR-dependent. We focused on the genes potentially involved in TGF-beta, Wnt and other pathways linked to both lung morphogenesis and development of cancer. The 24h and 72h exposure to TCDD or PAHs revealed up-regulation of GREM1, serpin E1 and down-regulation of BMP6, ID1, cyclin D1, E-cadherin and other mRNAs. Both BaP and BkF showed a similar pattern of AhR-inducing effects, albeit with lower inducing potencies. Interestingly, using some of these candidate gene targets, we obtained significantly higher REP values in comparison with a model AhR target gene – *CYP1A1* (Figure 1).

Figure 1: Dose-response sensitivity of selected canonical and novel AhR target genes in A549 cells after exposure to TCDD.

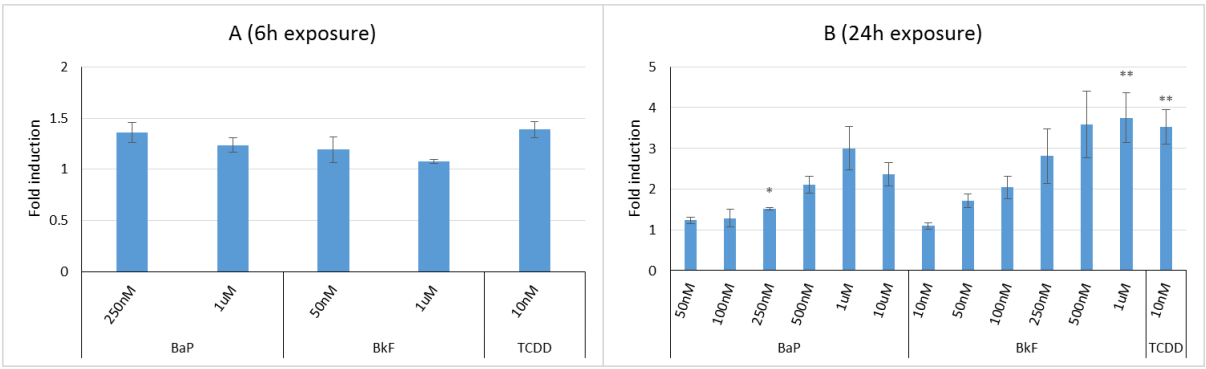


However, although many of selected candidate genes were regulated in an AhR-dependent manner after 24 or 72h exposure, their expression has not been signicantly altered after a shorter (6h) exposure time (a representative example of such gene expression, see Figure 2). This indicated that induction of these genes is mediated by another, yet unidentified AhR-dependent factor(s). A possible mechanism(s) responsible for this inidrect regulation by activated AhR should be elucidated in the next future studies. One possible approach is to attempt to identify common regulatory/response elements (matrices and modules) present in promoter/enhancer parts of the aforemetioned genes. Using the Genomatix software, we are currently comparing reported regulatory sequences of selected genes, evaluating candidate transcription factors involved in their regulation.

In conclusion, global gene expression studies were found to a useful tool enabling to identify AhR-dependent genes deregulated by complex mixtures of airborne pollutants in lung epithelial studies. Such an approach may

serve to identify biological processes deregulated by AhR ligands, which could be potentially linked to lung carcinogenesis, as well as to define future sensitive biomarkers for studies on lung cells exposed to toxic AhR ligands.

Figure 2: Modulation of GREM1 mRNA in A549 cell line exposed to BaP, BkF or TCDD; A) 6h exposure; B) 24h exposure.



Acknowledgements
This study was supported by the Czech Science Foundation, project no. 14-22016S.

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