Composition dependent mechanisms of PAH mixtures action as tumor promotor and progressor in non-cancer and cancer ovarian granulosa cells.

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Introduction

Ovarian tumors may be caused by changes in gene expression involving number of signaling pathways. Some of these genes may be involved in responses to environmental factors such as polycyclic aromatic hydrocarbons (PAHs). In female reproduction, a biological sensor that responds to the signals of many toxic chemical compounds seems to be the aryl hydrocarbon receptor (AhR) (Cavallini et al., 2016). AhR forms a heterodimer with the aryl hydrocarbon receptor nuclear translocator (ARNT), its cofactor. After translocation from the cytoplasm to the nucleus, the AhR/ARNT complex binds to the dioxin-responsive element (DRE), an eight-nucleotide motif located on the promoter of several target genes. Two well-studied AhR regulated genes, cytochrome P450 1A1 (CYP1A1) and COMT, a marker of phase II of PAHs detoxification, are involved in the detoxification of harmful toxicants (Denison et al., 2011). There are several mechanisms by which AhR signaling may be downregulated. One may be AhRR, an inhibitor of AhR (Mimura et al., 1999) that is induced by a variety of AhR agonists (Evans et al., 2005). The mechanisms of action of these toxic compounds are not always clear because the members of a group of pollutants do not operate through the same mechanism of action. We measured the concentrations of all 16 PAHs which were identified as priority pollutants by both the US Environmental Protection Agency and the European Commission, in maternal and cord blood (Zajda et al., 2017). The analysis showed similar content of these compounds, suggesting that exposure to PAHs, both during fetal life and in the postnatal period may contribute to abnormal ovarian function. To explore the mechanism of PAHs action in tumor promotion or progression, we used two granulosa cell lines: non-cancer granulosa cells (HGrC1) and granulosa cancer cells (COV434). The cells were exposed to the mixture 1 composed of all cancerogenic and non-cancerogenic compounds (M1) and mixture 2 (M2) composed of the five non-cancerogenic PAHs observed at the highest levels in maternal and cord blood. We measured the effects of two different PAHs mixtures on AhR, AhRR, ARNT, CYP1A1, COMT and NFkB protein expression. To confirm the hypothesis that PAHs are involved in AhR-dependent signaling mechanism in ovarian cells, we also silenced AhR, AhRR and NFκB gene expression and investigated the effects of PAHs on CYP1A1 protein expression. The cell proliferation was end point.

Materials and methods

Cell lines and treatment: two different mixtures were prepared: mixture 1 (M1) composed of all 16 priority PAHs: naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benz(a)anthracene, chrysene, benzo(a)fluoranthene, benzo(k)fluoranthene, BaP, indeno(1,2,3-cd)pyrene, dibenzo(ah)anthracene and benzo(ghi)perylene at the levels observed in maternal blood 57.5; 2.1; 1.7; 3.9; 71; 79; 34; 26.5; 4.7; 7.5; 2.2; 1.2; 0.75; 1.65; 1.85; 2.2 (ng/ml), respectively, and mixture 2 (M2), composed of naphthalene, phenanthrene, anthracene, fluoranthene, and pyrene in the same reactive proportions.

Western blotting: protein expression was assessed and quantification was performed using standard procedures. Antibodies and a horseradish peroxidase-conjugated secondary antibody and Western blotting luminol reagent were obtained from Santa Cruz Biotechnology. The blots were stripped and probed with anti-β-actin.
siRNA transfection: siRNAs were introduced to cells using DharmaFECT 3 Transfection Reagent (GE Healthcare Dharmacon Inc., Lafayette, CO, USA), according to the manufacturer's instructions. The efficacy of gene knockdown was evaluated by real-time PCR analysis.
Cell proliferation: the alamarBlue assay (Invitrogen, Carlsbad, CA, USA) is designed to quantify the proliferation of various human cell lines.

Results and discussion:

1.1 *Action of real life mixtures on AhR, AhRR, ARNT, CYP1A1 and COMT protein expression in human non-cancer granulosa cell line HGrC1 (Fig.1A) and effect of PAH mixtures on protein expression of CYP1A1 after gene silencing with siAhR (Fig. 1B).*

In non-cancer granulosa cells, M1 composed of all 16 priority compounds, increased AhR and ARNT expression ($p < 0.05$), decreased AhRR expression ($p < 0.05$) in parallel with increased CYP1A1 expression ($p < 0.05$). There was no effect on the enzymes of phase II metabolism (COMT). Lack of activation of COMT suggest an incomplete detoxification and possible action of harmful metabolites. It is in accordance with Hahn et al. (2009) who showed that activation of AhR is required for optimal CYP1A1 induction, the production of mutagenic metabolites and tumor initiation. It was also confirm by our data showing that AhR silencing reversed the stimulatory effect of M1 on CYP1A1 expression. Cytochrome P450 enzymes metabolize PAHs successively into epoxides and diol-epoxides. Activity of CYP1A1 may also result in cellular damage due to the formation of DNA adducts. Opposite, M2 composed of only five low molecular weight compounds had no effect on AhR and AhRR, increased ARNT and the enzymes of phase II metabolism (COMT) ($p < 0.05$) suggesting complete detoxification and protection from exposure to harmful metabolite.

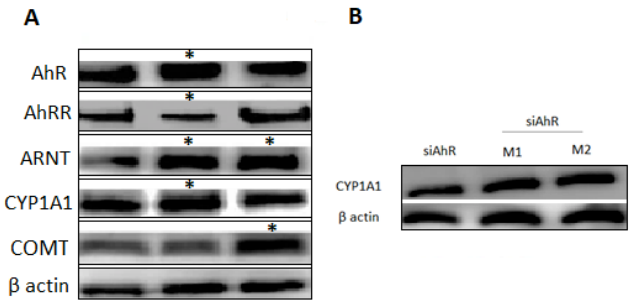

Fig.1 Action of human blood levels mixtures on protein expression. Protein levels were densitometrically scanned and normalized against the β-actin (42 kDa) signal. Statistically significant differences between bars on the graph are indicated by *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

1.2 *Action of real life mixtures on AhR, AhRR, ARNT, CYP1A1, COMT and NFkB protein expression in human cancer granulosa cell line COV434 (Fig.2A) and effect of PAH mixtures on protein expression of CYP1A1 after gene silencing with siAhR, siAhRR siNFkB (Fig. 2B).*

In cancer granulosa cells both M1 and M2, composed of all 16 priority compounds, decreased AhR and CYP1A1 ($p < 0.01$) expression and had no effect on the enzymes of phase II metabolism (COMT). There was a differences in the activation of AhRR expression. M1 increased ($p < 0.05$), while M2 had no effect. Interestingly, AhR silencing reversed the inhibitory effect of both M1 and M2 on CYP1A1 protein expression while AhRR silencing reversed the inhibitory effect of M1 on CYP1A1 protein expression but the inhibitory effect of M2 was still observed ($p < 0.01$). Taking into consideration the fact that M1 down-regulated AhR expression in parallel with increased AhRR and ARNT expression, we suggest that the formation of AhRR and ARNT dimers could be responsible for the inhibitory effect of PAHs on CYP1A1 expression. Inhibitory effect on CYP1A1 expression should be also due by activation of NFkB and interaction of NFkB with AhRR (Baba et al., 2001). Presented results showed that M1 and M2 significantly increased NFκB protein expression, with a greater effects of M1 ($p < 0.001$). NFκB silencing reversed the inhibitory effect of both M1 and M2 on CYP1A1. In the case of exposure to M2, composed of only non-cancerogenic compounds, the inhibitory effect on AhR and CYP1A1 expression was not correlated with AhRR expression and despite AhRR silencing, the inhibitory expression of CYP1A1 was still visible. We suggest that, after exposure to M2, NFκB alone decreased the expression of CYP1A1. The consequences of inhibiting AhR signaling by NFκB is the attenuation of ligand-induced CYP1A1 expression (Dietrich and Kaina, 2010).

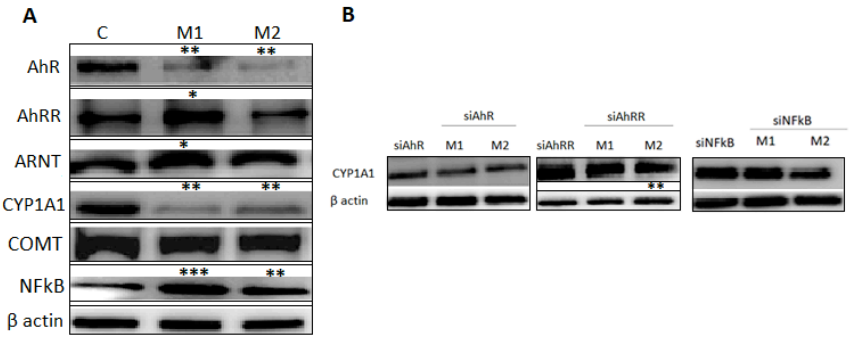

Fig.2 Action of human blood levels mixtures on protein expression. Protein levels were densitometrically scanned and normalized against the β-actin (42 kDa) signal. Statistically significant differences between bars on the graph are indicated by *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

1.5 Effect of the PAH mixtures on cell proliferation in HGrC1 and COV434 cells.

Interestingly no composition, but cell line dependent action on cell poliferation was noted. In HGrC1 cells M1 and M2 increased cell proliferation. In COV434 only M1 increased cell proliferation after 24h of exposure (we observed no effect after longer time of incubation), while M2 had no effect. Rodriguez and Potter (2013) showed that increased expression of CYP1A1 can be correlated with proliferation and it is in agreement with our results. Activation of CYP1A1 parallel with increased cell proliferation in HGrC1 cells and inhibition of CYP1A1 expression consequently no effect on proliferation in COV434 cells was observed. Noted in the presented data M1

action on cancer cell proliferation was probably due to fact that M1, unlike M2, contained carcinogenic compounds such as B(a)P. Wei et al. (2016) suggested that B(a)P promotes the proliferation and metastasis of granulosa cells via the AhR signaling pathways.

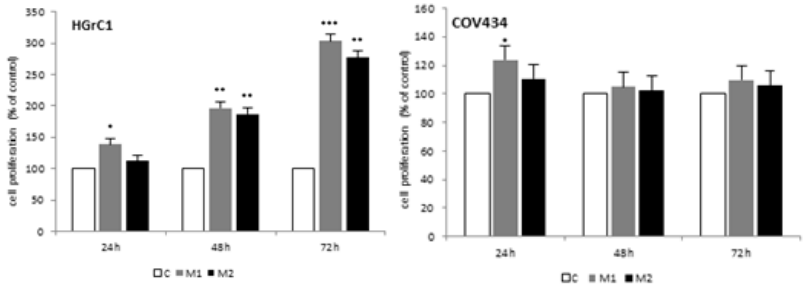

Fig.3 Action of human blood levels mixtures on cell proliferation. Statistically significant differences between bars on the graph are indicated by *p < 0.05, **p < 0.01 and ***p < 0.001.

Based on the results of our study, it seems that both cell phenotype and the PAHs composition are critical parameters in determining whether AhR dependent signaling pathway will activate phases of detoxification and promote or inhibit proliferation. We showed different canonical mechanism: up-regulation AhR and CYP1A1 protein by M1, up-regulation COMT by M2, with stimulatory effect of both mixtures on proliferation in HGrC1 cells. Different non-canonical mechanisms of action for these two PAH mixtures by down-regulation CYP1A1 and no effect on proliferation in granulosa cancer cells: the activation of AhRR and NFκB expression by M1 and only NFκB by M2.

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