Persistent endocrine-disrupting chemicals in human follicular fluid stimulate proliferation in granulosa tumor spheroids.

Gogola J[1], Hoffmann M[1], Ptak A[1]

[1]Department of Physiology and Toxicology of Reproduction, Institute of Zoology and Biomedical Research, Jagiellonian University, Krakow, 30-387, Poland, justyna.gogola@doctoral.uj.edu.pl, marta.hoffmann@doctoral.uj.edu.pl, anna.ptak@uj.edu.pl

Introduction

Epidemiological studies have shown that women have detectable levels of persistent organic pollutants such as hexachlorobenzene (HCB), 2,2-dichlorodiphenyldichloroethylene (p,p'-DDE), polychlorinated biphenyl 153 (PCB153), perfluorooctanoate (PFOA), and perfluorooctane sulfonate (PFOS) in their follicular fluid (FF) (Petro et al. 2012 and 2014). This evidence suggests that FF is not only rich in steroids, growth factors, and proteins, but also in endocrine-disrupting chemicals (EDCs). Because estrogen mimicry is one of the most commonly reported activities of EDCs, EDCs can impact estrogen-sensitive tissues in the female reproductive system. Moreover, EDCs can act as agonists or antagonists for hormone receptors and may activate pathways involved in the progression of hormone-related cancers such as granulosa cell tumors (GCTs). GCTs account for 5% of all ovarian tumors and arise from the granulosa cells that normally surround the oocytes. GCTs are classified into two subtypes based on clinical presentation and histological characteristics: the juvenile (JGCT) and the adult (AGCT) form. In this study, we analyzed the effect of persistent EDCs on the proliferation of two human GCT cell lines, COV434 and KGN, which represent the juvenile and the adult subtype, respectively. However, based on the actions of individual chemicals the biological effects of mixtures cannot be predicted, we further analysis mixture action on GCTs proliferation.

Materials and methods

Two human GCT-derived cell lines, COV434 (European Collection of Authenticated Cell Cultures) and KGN (Riken Cell Bank, Japan; after approval by Drs. Yoshiro Nishi and Toshihiko Yanase), were cultured in phenol red-free DMEM with 2 mM L-glutamine and 10% fetal bovine serum (FBS), and DMEM/Ham's F12 with 10% FBS, respectively. To form spheroids, 3000 cancer cells were transferred to medium supplemented with 10% FBS and 0.25% methylcellulose, and seeded in 96-well Cellstar U-bottom plates. Cells were incubated for 72 h to allow spheroid formation. COV434 and KGN spheroids were exposed to the following chemicals for 72 h: vehicle (0.1% DMSO), PFOA (0.02–2000 ng/ml), PFOS (0.08–8000 ng/ml), HCB (0.5 pg/ml to 50 ng/ml), p,p'-DDE (0.01–1000 ng/ml), and PCB153 (1 pg/ml to 100 ng/ml), or mixtures of the tested compounds (Mix). Proliferation in the spheroid cultures was determined using the CellTiter-Glo 3D Cell Viability Assay (Promega, France) according to the manufacturer's instructions. Real-time PCR was performed using the TaqMan Gene Expression Cells-to-CT Kit (Applied Biosystems), according to the manufacturer's instructions. The expression of ERα (*ESR1*; assay no.

Hs00174860_m1); ERβ (*ESR2*; assay no. Hs01100353_ml), GPR30 (*GPER1*; assay no. Hs01922715_sl), and IGF1R (*IGF1R*; assay no. Hs00609566_ml) was normalised to the expression of GADPH (assay no. 4310884E), and the relative expression was quantified using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Statistical analysis was carried out by one-way ANOVA followed by Tukey's test (GraphPad Software), and the level of significance was set at $P < 0.05$. Statistical data are presented as the mean ± SEM of three individual experiments performed in triplicate.

Results and discussion

The effects of PFOA, PFOS, HCB, p,p'-DDE, and PCB153 on the proliferation of COV434 and KGN cells were first tested. All the chemicals tested increased COV434 and KGN cell proliferation in a dose-dependent manner. In the juvenile subtype, the strongest increases in cell proliferation were observed after treatment with PFOA and p,p′-DDE (each 1.6-fold vs. control), (Fig 1; $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$). In the adult subtype, the strongest increase in cell proliferation were observed after treatment with PFOA, PFOS, and p,p′-DDE (2.3-, 1.9-, and 1.9-fold vs. control, respectively) (Fig 1; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$). Similar results have been observed for these compounds in various breast cancer cell lines. For example, PCB153 (Ptak et al. 2011) and HCB (García et al. 2010) stimulated proliferation of MCF-7 cells, p,p′-DDE (Aube et al. 2008) induced CAMA-1 cell proliferation, and PFOA (Pierozan et al. 2018a) and PFOS (Pierozan et al. 2018b) induced proliferation of MCF-10A cells. Interestingly, these data indicate that the adult GCT subtype is more sensitive to the proliferative effects of these compounds than the juvenile subtype.

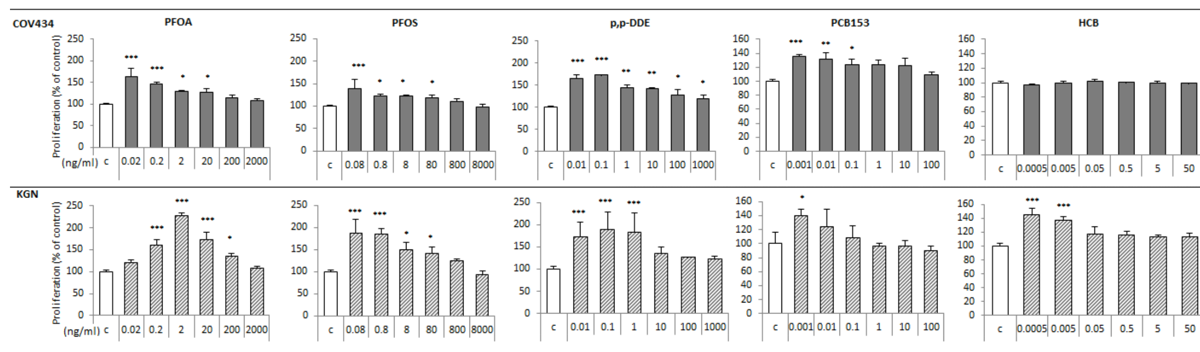

Figure 1. Dose-dependent effect of PFOA (0.02–2000 ng/ml), PFOS (0.08–8000 ng/ml), p,p'-DDE (0.01–1000 ng/ml), PCB153 (1 pg/ml to 100 ng/ml) and HCB (0.5 pg/ml to 50 ng/ml), on COV434 (dark grey bar) and KGN (grey bar) cell proliferation after 72 h of treatment. C, control. The proliferation rate in vehicle-treated cells was set as 100%. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ compared with control cells.

We further analyzed the combined effect of these compounds on granulosa tumor cell proliferation. Treatment of the cells with a mixture of the five compounds strongly stimulated cell proliferation; however, the effect was lower

than predicted (based on the sum of the independent activities of each compound) in both cell lines (Fig 2; *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). Similar to the results with the single compounds, weaker effects were observed in the juvenile GCT subtype than in the adult GCT subtype.

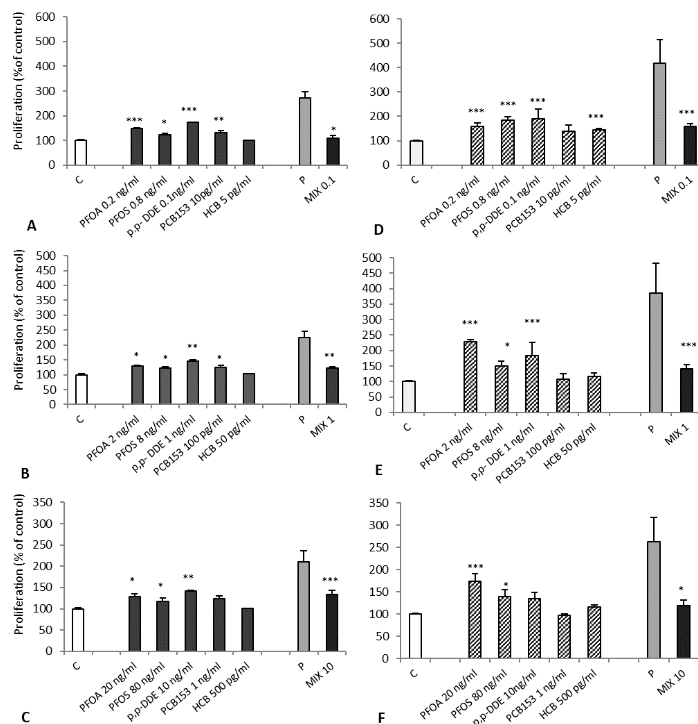

Figure 2. Effects of PFOA (0.2, 2, or 20 ng/ml), PFOS (0.8, 8, or 80 ng/ml), p,p'-DDE (0.1, 1, or 10 ng/ml), PCB153 (10 pg/ml, 100 pg/mL, or 1 ng/mL), HCB (5, 50, or 500 pg/ml) and the mixture (Mix) (same concentrations) on the proliferation of COV434 **(A, B, C)** and KGN **(D, E, F)** cells after 72 hours of culture. C, control. P, predicted effect. The proliferation rate in vehicle-treated cells was set as 100%. *$p < 0.5$, **$p < 0.01$, and ***$p < 0.001$ compared with control cells.

To evaluate the differences in response between the two subtypes of GCTs, the expression of estrogen receptor α and β (ERα and ERβ), the G protein-coupled receptor GRP30, and the insulin-like growth factor-1 receptor **(**IGF1R) were measured by real-time PCR. For each transcript, the relative quantity (RQ) in the COV434 cell line was arbitrarily set to 1. We found that the adult subtype GCT expressed higher levels of the classic estrogen receptors (ERα, ERβ) and IGF1R (Fig. 3, $p < 0.001$). In ovarian tumors and ovarian cancer cell lines, nuclear estrogen receptors and IGF-1 signaling regulate proliferation, invasion, and metastasis (Mungenast et al. 2014, Beauchamp et al. 2010). The basal expression levels of GPR30 were similar in COV434, and KGN cell lines. GPR30 is expressed in multiple tumor types, including ovarian tumors, and regulates cell proliferation and

differentiation (Heublein et al., 2014; François et al., 2015). This evidence is consistent with our observation that the adult GCT subtype is more sensitive to the proliferative effects of EDCs.

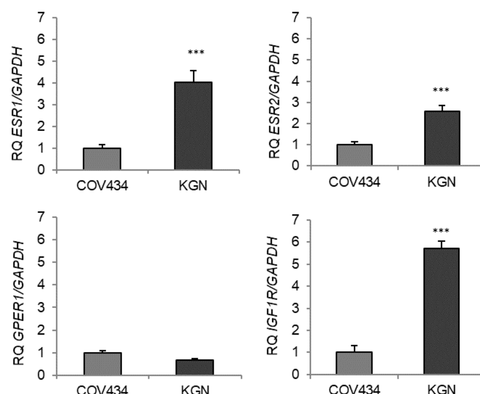

Figure 3. Basal mRNA expression of ERα (*ESR1*), ERβ (*ESR2*), GPR30 (*GPER1*), and IGF1R in COV434 and KGN cells. The mRNA level of each receptor in COV434 cells was set to a relative quantity (RQ) of 1.0. ***$p < 0.001$ in KGN compared to COV434 cells.

Taken together, our results demonstrate for the first time, that mixtures of persistent organic pollutants present in FF may act as mitogenic factors and induce granulosa tumor cell progression.

Acknowledgments:
This study was funded by the National Science Centre (NCN) of Poland (grant number 2016/21/B/NZ7/01080).

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