Ovulatory Angiogenic Factors and Receptors are Altered by an Environmentally Relevant Phthalate Mixture in Mouse Granulosa and Endothelial Cells *In Vitro*

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Phthalates are known endocrine-disrupting chemicals that people are exposed to daily. Previous work by our group has shown that phthalates decrease mouse ovarian follicular production of prostaglandins, which are key regulators of ovulatory angiogenesis. Angiogenesis is critical for successful ovulation and involves granulosa cell secretion of pro-angiogenic factors that bind to receptors on ovarian endothelial cells. Specifically, the ovulatory cascade induces granulosa cells to produce prostaglandin E_2 (PGE_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), and members of the vascular endothelial growth factor (VEGF) family, while endothelial cells express multiple prostaglandin E receptors (PTGER1-4), prostaglandin F receptor (PTGFR), and vascular endothelial growth factor receptors (FLT1, KDR, FLT4). We hypothesized that phthalates alter both the production of angiogenic factors by granulosa cells and the expression of angiogenic receptors on ovarian endothelial cells. Mouse granulosa cells were isolated from immature mice 48 hours after pregnant mare serum gonadotropin (PMSG) injection. The cells were treated with vehicle control (dimethylsulfoxide, DMSO) or an environmentally relevant phthalate mixture (MPTmix, 1-500µg/mL) that was derived using urinary phthalate metabolite levels in pregnant women. This is considered environmentally relevant considering humans are exposed to a mixture of phthalates daily that are rapidly converted into active metabolites that reach the ovary. Following one hour of MPTmix exposure, the cells received human chorionic gonadotropin (hCG) treatment to induce the ovulatory cascade. The cells and media were collected at 11 hours post-hCG treatment and subjected to gene expression analysis and enzyme-linked immunosorbent assays ($n=5$, $p\leq0.05$). In complementary experiments, mouse ovarian microvascular endothelial cells were treated with DMSO or MPTmix (1-500µg/mL) with or without VEGFA supplementation. The cells were collected at 24 and 48 hours for gene expression analysis ($n=4$, $p\leq0.05$). Exposure to hCG+MPTmix decreased the granulosa cell production of PGE_2 at doses of 1, 10, 100, and 500µg/mL and $PGF_{2\alpha}$ at doses of 10, 100, and 500µg/mL compared to hCG ovulatory controls. Of the prostaglandin receptors on endothelial cells, treatment with MPTmix increased *Ptger4* at 500µg/mL (24hr); and decreased *Ptgfr* expression at 10µg/mL (24hr) and 100µg/mL (48hr) doses when compared to DMSO. For the VEGFs in granulosa cells, exposure to hCG+MPTmix decreased the mRNA levels of *Vegfd* at doses of 100 and 500µg/mL and increased protein levels of VEGFA at a dose of 500µg/mL, while gene expression of *Vegfa* remained unchanged. Of the VEGF receptors, MPTmix decreased endothelial cell expression of *Flt1* at doses of 1µg/mL and 100µg/mL (24hr); decreased *Kdr* expression at doses of 1µg/mL and 10µg/mL (24hr), and at 500µg/mL (48hr); and decreased expression of *Flt4* at doses of 10µg/mL and 100µg/mL (24hr) when compared to DMSO controls. Supplementation with VEGFA restored the MPTmix-induced changes in the expression of all angiogenic receptors to DMSO control levels. These data demonstrate that exposure to an environmentally relevant phthalate mixture altered the production of granulosa cell-derived angiogenic factors.

Additionally, phthalate exposure altered the expression of key angiogenic receptors on endothelial cells, and these toxic effects were restored with VEGFA supplementation. Thus, phthalate exposure may induce dysregulation of the communication network between granulosa cells and endothelial cells necessary for ovulatory angiogenesis. Supported by R01ES033767.