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Bisphenol A affects human endometrial endothelial cell angiogenic activity *in vitro*

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ABSTRACT

The widespread Bisphenol A (BPA) is classified as an endocrine-disrupting chemical (EDC) with estrogenic properties. Human endometrial endothelial cells (HEECs) play a key role in the endometrial angiogenesis that is under the control of estradiol. The hypothesis was that BPA may affect endometrial angiogenesis by disturbing some functional properties of the HEEC.

To study this, primary HEECs were exposed to environmentally relevant doses of BPA. The HEECs were co-cultured with primary endometrial stromal cells to create conditions as similar to the *in vivo* situation as possible. The effects of BPA were evaluated by proliferation and viability assays, tube-formation assays, quantitative PCRs, Western blots and ELISAs.

BPA slightly increased HEEC tube formation and VEGF-D protein expression compared with vehicle, without affecting HEEC viability or proliferation.

Bisphenol A thus caused changes in HEEC activities *in vitro*, and may therefore have disturbing effects on endometrial angiogenesis.

1. Introduction

Bisphenol A (BPA) is classified as an endocrine-disrupting chemical (EDC) with estrogenic properties [1,2]. It is a selective estrogen receptor modulator (SERM) [3] that has the ability to bind both estrogen receptor alpha (ER-α) and ER-beta (ER-β) [4].

The proportion of BPA that is bound to serum albumin is very low and therefore BPA has an advantage over estradiol, which is to a greater extent bound to sex hormone-binding globulin and serum albumin. The binding capacity of BPA to ERs is approximately 1000-fold less compared with estradiol, which is why BPA is sometimes referred to as a weak estrogenic compound. Others, however, propose BPA to be a strong xenoestrogen [1]. When estrogen is bound to estrogen receptors (ERs), transcriptional activity depends on the recruitment of co-regulators to the estrogen/ER complex. Even though BPA has a 10-fold greater affinity to ER-β, the BPA/ER-α complex has a 500-fold greater potency in recruitment of co-regulators [3], which may contribute to the diversity of effects caused by BPA.

The human endometrium undergoes cyclic changes every month in preparation for embryo implantation. This includes changes in endometrial angiogenic activity and thus also the endometrial vasculature, processes that are under the control of sex steroid hormones. Endometrial angiogenesis is initiated during menstruation to repair the vascular bed and continues during the proliferative phase with endometrial regrowth, and thereafter during the secretory phase with growth and coiling of spiral arteries and formation of a subepithelial capillary network [5]. Endothelial cells cover the luminal surface of all blood vessels. These cells control vascular morphology through angiogenesis and vascular remodeling, and they have functional vascular properties such as the regulation of vascular tone.

Human endometrial endothelial cells (HEEC) are unique endothelial cells, since they express ER-β and possibly also progesterone receptors [6–12]. HEEC activities are therefore to some extent regulated by changes in estrogen and progesterone levels during the menstrual cycle. It has been suggested that ovarian steroids are capable of inducing genes involved in the regulation of human uterine microvascular endothelial cell functions [13]. The presence of ERs on HEECs makes them important targets of EDCs such as BPA. We have previously shown that BPA affects cell proliferation in monocultured HEECs [14]. We have also shown that HEECs grown in co-culture together with stromal cells may react differently from when grown in monoculture [11,12], probably through crosstalk between HEECs and stromal cells. In a recent study it was shown that environmentally relevant doses of BPA had

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a proangiogenic effect on human umbilical vein endothelial cells (HUVECs) [15].

The hypothesis was that BPA may affect endometrial angiogenesis by disturbing normal functional properties of the HEEC. To study this, primary HEECs were exposed to environmentally relevant doses of BPA and the effects were evaluated by proliferation and viability assays, tube-formation assays, quantitative PCRs, Western blots and ELISAs.

2. Materials and methods

The study was approved by the regional Ethics Committee at Uppsala, Sweden. Written informed consent was obtained from all women included in the study.

2.1. Subjects

Endometrial tissue samples were obtained from five premenopausal women with regular menstrual cycles, who underwent hysterectomy for benign medical conditions at Uppsala University Hospital. None of the participants had endometriosis, submucous fibroids, or had received any hormone treatment for a period of at least three months prior to surgery and they were all non-smokers.

2.2. Cell cultures

Endometrial biopsy samples were collected immediately after the uterus was removed, placed in cold sterile phosphate-buffered saline (PBS) (Invitrogen Carlsbad, CA, USA) and transferred to the laboratory. The samples were washed in sterile PBS, followed by a series of brief washings in iodine solution (Apoteket AB, Stockholm, Sweden) and finally three washes in PBS. They were cut into millimeter-sized pieces and exposed to a sterile-filtered solution of digesting enzymes (collagenase type II [2.5 mg/mL; Invitrogen, Carlsbad, CA, USA], deoxyribonuclease II [50 μg/mL; Sigma–Aldrich, St. Louis, MO, USA], hyaluronidase [200 μg/mL; Sigma–Aldrich, St. Louis, MO, USA]) and gentamicin [4 mg/mL; Invitrogen, Carlsbad, CA, USA]) in PBS for 1 h in an atmosphere of 5% CO_2 in humidified air at 37 °C in a Forma Scientific CO_2 incubator (AB Nino Lab, Upplands Väsby, Sweden). After 1 h, the cell suspension was poured through a 40-μm nylon cell strainer (BD Sciences, Franklin Lakes, NJ, USA) into a sterile centrifugation tube containing PBS. Undigested tissue was re-incubated in fresh enzyme solution for an additional hour. The resulting cell suspension was thereafter poured through the cell strainer into the centrifugation tube and the suspension was centrifuged at 400 × *g* for 10 min. The supernatant was discarded and the cell pellet was suspended in 3 mL of sterile PBS containing 0.1% bovine serum albumin (BSA) (Sigma–Aldrich, St. Louis, MO, USA). The suspension was transferred to a smaller sterile tube and 25 μL Dynabeads® CD31 Endothelial Cell (Dynal® Biotech ASA, Oslo, Norway) were added per mL of cell suspension. The tube was placed on a rocker for 30 min at 2–8 °C, after which the suspension was split into two tubes and 2–3 volumes of sterile 0.1% BSA–PBS were added. By placing the tubes in a magnetic holder for 2 min, the endothelial Dynabead®-coated cells were attracted by the magnet and stuck to the test tube wall, while the stromal cells in the supernatant were poured into a separate tube. The tube containing endothelial cells was removed from the magnetic holder, the cells re-suspended in fresh 0.1% BSA–PBS, and the procedure with the magnetic holder was repeated 2–3 times. Both stromal and endothelial cells were suspended in Microvascular Endothelial Cell Medium-2 (EGM™-2MV Bulletkit; Lonza Group Ltd., CH-4002 Basel, Switzerland). Endothelial cells were seeded in 25 cm^2 culture flasks and stromal cells in 75 cm^2 culture flasks (Fischer Scientific GTF, Västra Frölunda, Sweden), and incubated in an atmosphere of 5% CO_2 in humidified air at 37 °C. The culture medium was changed 2–3 times per week and the cells were regularly checked, using an inverted phase-contrast microscope (Nikon Diaphot 300; TeknoOptik AB, Skärholmen, Sweden). At subconfluency, subcultivation of cells was performed by trypsinization with 0.05% Trypsin-EDTA (Gibco, Invitrogen) according to standard procedures. This method of isolation is standard at our lab and careful characterization of isolated endothelial cells has been carried out previously [16]. Each biopsy sample gave rise to one individual co-culture; cells from different women were not pooled. HEECs at passage three to four were grown in 6-well plates (Product No. 92006, TPP Techno Plastic Products AG, CH-8219 Trasadingen, Switzerland) as co-cultures with stromal cells in inserts (24 mm Transwell® with 0.4 μm Pore Polycarbonate Membrane Insert, Sterile [Product No. 3412, Corning, MA, USA]), allowing communication but no direct contact between the two cell types. Both cell types were thus simultaneously exposed to BPA or vehicle (ethanol).

2.3. Exposure to test substances

HEECs and stromal cells were exposed simultaneously to the test substance BPA (Sigma–Aldrich, St. Louis, MO, USA), or vehicle for 24 h. Stock solutions were prepared in ethanol. BPA stock solution was diluted in endothelial cell culture medium EGM-2MV to the final concentrations of 10 μM, 0.1 μM, 1 nM and 0.01 nM used in the test system. The final ethanol concentration was 0.1% (v/v) in all treatment groups and the vehicle-only group.

2.4. Proliferation and viability assays

Cell proliferation (BrdU) and cell viability WST-1 assays (Roche Diagnostics Scandinavia AB, Bromma, Sweden) were carried out according to the manufacturer's instructions, using six replicates from four individual women, as previously described [11]. Briefly, in the proliferation assay treated HEECs were exposed to 16 μL BrdU labeling solution for the 24-h treatment incubation. Labeling was visualized using a peroxidase reagent and absorbance was measured at 450 nm. Viability was assessed by using a WST-1 kit, where 16 μL of WST-1 solution were added to the test substance-treated HEECs for the last hour of a 24-h incubation period. After 1 h incubation at 37 °C, absorbance was measured at 450 nm.

2.5. Tube-formation assay

Endothelial tube-formation assays were performed as described previously [17]. HEECs from five women were co-cultured with stromal cells and treated with BPA (10 μM, 0.1 μM, 1 nM, 0.01 nM) or vehicle for 24 h. Each well of a 96-well cell culture plate was loaded with 70 μL Geltrex reduced growth factor extracellular matrix membrane (Invitrogen Life Sciences, Paisley, UK) and incubated for 30 min at 37 °C until it gelled. Thereafter, the cells were detached by trypsinization and kept as single-cell suspensions in the cell culture medium containing BPA or vehicle. A Bürker chamber was used to determine cell number and cell viability. The cells were plated on top of the extracellular matrix membrane (9000 cells in 100 μL cell culture medium per well) and incubated for an additional 6 h at 37 °C. In total, six wells were used for each treatment. Basic fibroblast growth factor (bFGF) (CAS: 106096-93-9 Sigma–Aldrich, St. Louis, MO, USA) was added to a final concentration of 10 ng/mL to three of the six wells for each treatment. Tube formation was analyzed using a Zeiss Axiovert S100 microscope and a 5×/0.16 objective (Carl Zeiss AG, Germany). The settings for the microscope, camera and software were the same for all images. The images were analyzed by using the Wimtube formation module in WIMASIS Image Analysis (Munich, Germany). The following