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Bisphenol A modulates inflammation and proliferation pathway in human endometrial stromal cells by inducing oxidative stress

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ABSTRACT

Bisphenol A (BPA) has been implicated in altered human reproductive function. The oxidative stress or change of inflammatory signaling may appear a key factor in the biological changes of the human endometrium. Using MTT assay we assessed BPA mediated modulation of oxidative stress and inflammation responses in human endometrial stromal cells (ESCs). According to the results, reactive oxygen species (ROS) generation was highest upon exposure to 1000 pmol BPA. Increased mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB) were demonstrated. Gene expression and release of inflammatory cytokines were increased. Upon BPA exposure, elevated estrogen receptor (ER)-α expression levels in ESCs correlated with changes in oxidative stress, inflammatory gene expression and signal changes in cellular proliferation signaling. These findings support that BPA induces oxidative stress and activates inflammatory signals in cultured ESCs via ER-α. Together, this result may provide insight into the association between BPA exposure and endometrium-related disorders.

1. Introduction

Endocrine disrupting chemicals may have deleterious effects on the human reproduction [1]. Bisphenol A (BPA, 4,4′-(propane-2,2-diyl)diphenol) is a well-known endocrine disrupting chemical used in a wide range of products, including food and beverage containers, water piping, safety equipment, coatings, adhesives, high-performance composites, and automotive and aircraft parts. BPA has received considerable attention for its potential adverse health effects in both animals and humans because of its weak estrogenic-like effects [2]. BPA may be a causative agent of some human chronic diseases such as diabetes, obesity, reproductive disorders, birth defects, and breast cancer as well as cardiovascular, chronic respiratory, and kidney diseases [3]. Recent studies focusing on female reproductive disorders suggested that BPA is associated with recurrent or spontaneous miscarriage [4,5], endometrial hyperplasia [6], and endometriosis [7]. BPA is known to be associated with shorter luteal phase in human [8], and in vitro studies revealed BPA impairs decidualization of human uterine stromal fibroblasts and human endometrial stromal cells [9,10].

Oxidative stress is defined as an imbalance between the production of reactive oxygen species (ROS) and antioxidant defenses that results in a series of events including damage to cellular lipids, proteins, or DNA. In the Chinese population, BPA exposure has been linked to increased oxidative stress [11]. In hippocampal neuronal cells, BPA exposure induced ROS and mitogen-activated protein kinases (MAPKs) in response to oxidative stress [12]. Increased oxidative stress is observed in reproductive disorders involving the endometrium, such as endometriosis [13] and miscarriage [14]. Pro-inflammatory signaling also plays an important role in the progression of diseases such as endometriosis. BPA affects inflammatory signals in human adipocytes and macrophages [15,16]. The effect of BPA on the oxidative stress response of human endometrial cells or change of inflammatory signaling, which may cause biological changes in the human endometrium, has not been investigated.

In this study, we evaluated whether BPA induces oxidative stress in human endometrial cells and investigated the effects of BPA on changes in inflammatory modulators in the human endometrium to determine the pathogenesis of endocrine-related diseases involving the endometrium. As estrogen receptor (ER)-α is the primary mediator of estrogenic actions in the human endometrium [17], we explored the potential mechanism associated with the ER and nuclear factor-κB (NF-κB) pathways in the human endometrium.

2. Materials and methods

2.1. Patient and sample collection

For endometrial stromal cell (ESC) cultures, endometrial samples were obtained from premenopausal women (n = 5) who underwent hysterectomy for carcinoma in situ, who had no evidence of

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endometrial abnormalities, intramural myomas, adenomyosis, or pelvic endometriosis, and did not receive any hormonal medications in the preceding 3 months. All patients were of reproductive age with normal menstrual cycles. All endometrial samples were confirmed histologically as disease-free and in the early proliferative phase. Written informed consent was obtained from each patient using consent forms and the protocols were approved by the Review Board for Human Research of Dong-A Medical Center.

2.2. Isolation and culture of human ESCs, proliferation assay

ESCs were separated from fresh endometrial tissues. The tissue was minced and the cells were dispersed in HBSS containing 25 mM HEPES, 1 × antibiotics, 2 mg/ml collagenase, and 0.2 mg/ml DNase, followed by incubation for 20 min at 37 °C with agitation. Dispersed endometrial cells were separated by filtration through a sieve (70 μm). Endometrial glandular epithelium was retained by the sieve, whereas dispersed stromal cells passed into the filtrate. Stromal cells were suspended in Ham's F-12/Dulbecco's minimal essential medium containing antibiotics–antimycotics (1%, v/v) and fetal bovine serum (10%, v/v) and maintained at 37 °C in a humidified atmosphere (5% CO_2 in air). Cells between passages 3 and 9 were used. The effects of BPA on stromal cells were assessed using the MTT assay. The cells (5×10^3 cells/mL) were incubated in a 96-well plate, along with 1–100000 pmol BPA and cultured at 37 °C under a humidified atmosphere with 5% CO_2 for 1–3 days. Subsequently, 20 μL MTT solution (5 mg/ml in PBS) was added to each well and the plates were incubated at room temperature for 4 h. Absorbance at a wavelength of 495 nm was measured on an ELISA Reader (BioTek Instruments, Inc., Winooski, VT, USA). Data were expressed in optical density units. For the subsequent experiments, 10–1000 pmol BPA was used. The ER antagonist ICI (0.01 μM) was used with 1000 pmol BPA.

2.3. ROS assay

ROS generation was monitored by flow cytometry using the peroxide-sensitive fluorescent probe 2,7-dichlorofluorescein diacetate (DCF-DA). DCF-DA is converted by intracellular esterases into DCFH, which is oxidized to dichlorofluorescein (DCF) in the presence of a proper oxidant. Cells were seeded in 6-well plates and the media was replaced with serum-free media after 18 h. Cells were harvested, after which a single-cell suspension was ensured by detaching adherent cells followed by staining with 10 μM DCF-DA in PBS for 30 min at 37 °C. After staining, the cells were treated with 1 M H_2O_2, 1000 pmol BPA, and 1000 pmol BPA plus ICI (0.01 μM) in culture media for 30 min at 37 °C. Total free radical abundance was assessed by spectrofluorimetry using flow cytometry (Beckman Coulter, Inc., Brea, CA, USA) at 488/535 nm excitation/emission wavelengths, respectively. Free radical levels were calculated. For Hoechst staining, cells were incubated with DCF-DA for 30 min at 37 °C, washed three times with PBS, and exposed to H_2O_2 and BPA for 30 min as described above. The number of attached cells was estimated after washing with PBS and 4 μg/mL Hoechst 33342 staining for 5 min. Stained cells were observed under a flexible confocal microscope (Zeiss, Oberkochen, Germany).

2.4. Reverse transcription-polymerase chain reaction (RT-PCR) and quantitative PCR (q-PCR)

Total RNA was isolated using TRIzol® reagent. cDNA was generated from 0.2 μg total RNA using the RevertAid First-strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany). Primers used for gene amplification are shown in Table 1.

Real-time PCR was performed on an Applied Biosystems 7000 Real-time PCR System (Foster City, CA, USA) with SYBR green premix. Ct (threshold cycle) values were calculated, and the ΔΔCt method was used for relative mRNA quantification. The expression levels of genes were normalized to that of GAPDH.

Table 1
Primers for gene expression.

Gene	Primers
Estrogen receptor-alpha	S : 5′-AAG AGC TGC CAG GCC TGC C-3′ AS : 5′-TTG GCA GCT CTC ATG TCT CC-3′
Estrogen receptor-beta	S : 5′-GTC AGG CAT GCG AGT AAC AA-3′ AS : 5′-GGG AGC CCT CTT TGC TTT TA-3′
Cu/Zn SOD	S : 5′-GTGGGG AAG CAT TAA AGG ACT GAC-3′ AS : 5′- CAATTA CAC CAC AAG CCA AAC GAC-3′
Catalase	S : 5′- TCG AGC ACG GTA GGG ACA GTT CAC-3′ AS : 5′- TCC GGG ATC TTT TTA ACG CCA TTG-3′
HO-1	S : 5′- TTC TTC ACC TTC CCC AAC-3′ AS : 5′- GCA TAA AGC CCT ACA GCA AC-3′
GPx	S : 5′- GCG GCG GCC CAG TCG GTG TA-3′ AS : 5′- GAG CTT GGG GCT GGT CAT AA-3′
IL-1β	S : 5′- TGA TGG CTT ATT ACA GTG GCA ATG -3′ AS : 5′- GTA GTG GTG GTG GGA GAT TCG -3′
IL-6	S : 5′- GGTACATCCTCGACGGCATCT -3′ AS : 5′- GTG CCT CTT TGC TGC TTT CAC -3′
TNF-α	S : 5′- CCC AGG CAG TCA GAT CAT CTT C -3′ AS : 5′- AGCTGCCCCTCAGCTTGA -3′
GAPDH	S : 5′-TGA ACG GGA AGC TCA CTG G-3′ AS : 5′-TCC ACC ACC CTG TTGCTG TA-3′

2.5. ELISA

The levels of tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-1β after exposure of 4 h and 48 h were measured in the supernatants of the primary stromal cell culture with a commercially available enzyme-linked immunosorbent assay kit (Quantikine, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Measurements were conducted in three independent experiments

2.6. NO assay

NO was measured using the Griess reagent. The amount of stable nitrite was determined with a colorimetric assay after BPA treatment for 0, 24, 48 or 72 h. Briefly, 50 μL of culture supernatant was mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthyl ethylenediamine dihydrochloride, and 2.5% H_3PO_4) and incubated at room temperature for 10 min. Absorbance was read at 540 nm using an ELISA reader (BioTek Instruments, Inc.). Nitrite concentrations were determined by extrapolation of a sodium standard curve.

2.7. Nuclear protein extraction and western blot

After BPA treatment for the indicated times, the cells were washed with 1 ml ice-cold PBS, centrifuged at 3000 × g for 5 min, and resuspended in 100 μL of ice-cold hypotonic buffer (10 mM HEPES/KOH, 2 mM $MgCl_2$, 0.1 mM EDTA, 10 mM KCl, 1 × protease inhibitor, pH 7.9). After 10 min on ice, the cells were vortexed and centrifuged at 15,000 × g for 30 s. Pelleted nuclei were washed with PBS three times and gently resuspended in 50 μL ice-cold saline buffer (50 mM HEPES/KOH, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol, 1 × protease inhibitor, pH 7.9). After 2 h on ice, the nuclei were vortexed, sonicated for 30 s, and centrifuged at 15,000 × g for 5 min at 4 °C. Aliquots of the supernatant containing nuclear proteins were frozen in liquid nitrogen and stored at —70 °C. For immunodetection, cells were harvested and lysed in buffer containing 20 mM Tris, 137 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Triton X-100, protease inhibitors (Sigma–Aldrich Chemical Co., St. Louis, MO, USA), and Phosphatase