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# Bisphenol A exposure alters release of immune and developmental modulators and expression of estrogen receptors in human fetal lung fibroblasts

Laziyan Mahemuti<sup>1,5</sup>, Qixuan Chen<sup>1</sup>, Melanie C. Coughlan<sup>1</sup>, Min Zhang<sup>1</sup>, Maria Florian<sup>1,5</sup>, Ryan J. Mailloux<sup>1,5</sup>, Xu-Liang Cao<sup>2</sup>, Kylie A. Scoggan<sup>3,4</sup>, William G. Willmore<sup>5</sup>, Xiaolei Jin<sup>1,\*</sup>

1. Regulatory Toxicology Research Division, Bureau of Chemical Safety, Food Directorate, HPFB, Health Canada, Ottawa, Ontario, Canada.

E-mail: [Mahemuti\\_Laziyan@hc-sc.gc.ca](mailto:Mahemuti_Laziyan@hc-sc.gc.ca)

2. Food Research Division, Bureau of Chemical Safety, Food Directorate, HPFB, Health Canada, Ontario, Canada

3. Nutrition Research Division, Bureau of Nutritional Sciences, Food Directorate, HPFB, Health Canada, Ottawa, Ontario, Canada

4. Sector Strategies Division, Risk Management Bureau, Safe Environments Directorate, HECSB, Health Canada, Ottawa, Ontario, Canada

5. Institute of Biochemistry, Departments of Biology and Chemistry, Carleton University, Ottawa, Ontario, Canada

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## ABSTRACT

Bisphenol A (BPA) has been shown to exert biological effects through estrogen receptor (ER)-dependent and ER-independent mechanisms. Recent studies suggest that prenatal exposure to BPA may increase the risk of childhood asthma. To investigate the underlying mechanisms in the actions of BPA, human fetal lung fibroblasts (hFLFs) were exposed to varying doses of BPA in culture for 24 hr. Effects of BPA on localization and uptake of BPA, cell viability, release of immune and developmental modulators, cellular localization and expression of ER $\alpha$ , ER $\beta$  and G-protein coupled estrogen receptor 30 (GPR30), and effects of ERs antagonists on BPA-induced changes in endothelin-1 (ET-1) release were examined. BPA at 0.01–100  $\mu\text{mol/L}$  caused no changes in cell viability after 24 hr of exposure. hFLFs expresses all three ERs. BPA had no effects on either cellular distribution or protein expression of ER $\alpha$ , however, at 100  $\mu\text{mol/L}$  (or 23  $\mu\text{mol/L}$  intracellular BPA) increased ER $\beta$  protein levels in the cytoplasmic fractions and GPR30 protein levels in the nuclear fractions. These paralleled with increased release of growth differentiation factor-15, decreased phosphorylation of nuclear factor kappa B p65 at serine 536, and decreased release of ET-1, interleukin-6, and interferon gamma-induced protein 10. ERs antagonists had no effects on BPA-induced decrease in ET-1 release. These data suggest that BPA at 100  $\mu\text{mol/L}$  altered the release of immune and developmental modulators in hFLFs, which may negatively influence fetal lung development, maturation, and susceptibility to environmental stressors, although the role of BPA in childhood asthma remains to be confirmed in *in vivo* studies.

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\* Corresponding author. E-mail: [Dawn\\_Jin@hc-sc.gc.ca](mailto:Dawn_Jin@hc-sc.gc.ca); [jxnc329@gmail.com](mailto:jxnc329@gmail.com) (Xiaolei Jin).

## Introduction

The 2,2-Bis (4-hydroxyphenyl) propane, more commonly known as bisphenol A (BPA), is a chemical monomer used primarily in the production of polycarbonate plastics and epoxy resins. Polycarbonate has been used in food contact materials such as beverage bottles, infant feeding bottles, and food containers. Epoxy resins are used in protective linings of cans containing foods and beverages, as well as infant formula products. This has raised concern in the scientific community over the possible developmental effects of BPA in humans. It is known that BPA is rapidly transferred to the fetus after maternal uptake (Takahashi and Oishi, 2000), and that repeated maternal exposure elevates BPA in fetus or the newborn (Durando et al., 2007). However, the levels of fetal exposure to BPA in general population remain to be established. Available information on placenta, fetal blood, amniotic fluid, and fetal liver BPA concentrations suggests a wide range of distribution from undetectable to hundreds of ng/g (Vandenberg et al., 2012; Cao et al., 2012; Edlow et al., 2012; Ikezuki et al., 2002). The human fetal lung is exposed to BPA through both blood and amniotic fluid, however, has not been assessed for BPA levels. With limited detoxification capacity and high surface to volume ratio, fetal lung cells may be exposed to many folds higher BPA concentrations than cells of other fetal tissues.

Exposure to BPA has been linked to a number of developmental and reproductive pathologies in both animal models and humans (Golub et al., 2010; Palanza et al., 2008; Tharp et al., 2012). However, the underlying molecular mechanisms remain to be elucidated. BPA is traditionally considered as an estrogenic endocrine disrupter with some effects being estrogen receptor (ER)-dependent (Yoshitake et al., 2008; Buteau-Lozano et al., 2008; Dang et al., 2007). However, some recent studies demonstrated that it can also act through ER-independent mechanisms (Hanet et al., 2008; Asahi et al., 2010). Estrogens and ERs are known to enhance allergic sensitization in animal models and may enhance susceptibility to atopic disorders like asthma in humans (Bonds and Midoro-Horiuti, 2013). ER $\alpha$  polymorphisms are associated with airway hyper-responsiveness and lung function decline, particularly in female subjects with asthma (Dijkstra et al., 2006). Not only do endogenous estrogens play a role in the pathogenesis of lung diseases, but environmental estrogens (xenoestrogens) can also have similar effects. BPA was found to enhance allergic sensitization and bronchial inflammation and responsiveness in a susceptible animal model of asthma (Midoro-Horiuti et al., 2010). Prenatal exposure to BPA was associated with increased odds of wheeze in children at 6 months of age (Spanier et al., 2012), increased risk of developing experimental allergic asthma in mice (Nakajima et al., 2012), and enhanced airway lymphocytic and lung inflammation in mucosal sensitized female offspring of mice (Bauer et al., 2012). Hijazi et al. (2015) demonstrated that prenatal exposure to BPA caused lung immaturity phenotype in mice, which was rescued by maternal administration of dexamethasone, an inducer of glucocorticoid. While these studies suggest that fetal exposure to BPA may delay lung maturation and enhance susceptibility to respiratory

sensitization and responsiveness, the underlying mechanisms of such effects and the role of ERs remain to be elucidated.

Abnormalities of extracellular matrix (ECM) are a key feature of tissue remodeling in lung disease (Fixman et al., 2007). Lung fibroblasts are known to release many types of immune and developmental modulators including cytokines and chemokines in the ECM (Alkhouiri et al., 2014). Thus, it is plausible to hypothesize that exposure of the fetal lung fibroblasts to BPA alters the release of cytokines, chemokines and/or costimulatory molecules, affecting the development, maturation, and susceptibility of fetal lung to environmental stressors, contributing to increased risk of childhood asthma. In this study, therefore, we examined the protein expression and localization of ERs and the effects of BPA on the release of growth differentiation factor-15 (GDF15), ET-1, interleukin-6 (IL-6), and interferon gamma induced protein 10 (IP-10), and phosphorylation of nuclear factor kappa B p65 (NF- $\kappa$ B p65) in cultured normal human fetal lung fibroblasts (hFLFs) and the potential role of ERs in the action of BPA in this cell line.

## 1. Materials and methods

### 1.1. Chemicals and doses

BPA powder (99%) was purchased from Sigma-Aldrich (Oakville, Ontario, Canada). A 1 mmol/L stock solution of BPA was prepared and diluted in distilled and deionized water to obtain 0.1, 1, 10, 100 and 1000  $\mu$ mol/L BPA as working stock solutions. We used 0, 0.01, 0.1, 1, 10, 20, 50 and 100  $\mu$ mol/L BPA doses in our experiments. These BPA doses were chosen because in a previous study, we found that experimental materials, buffer, serum and media are all contaminated with BPA at concentrations ranging from 0.088 to 19 nmol/L (Cao et al., 2010). The Eagle's Minimal Essential Medium (EMEM) with serum contains 1.93 nmol/L of BPA, which reflects the BPA concentration in our control culture. Any concentrations lower than 1.93 nmol/L is not achievable under the conditions used.

### 1.2. Cell culture

Normal human fetal lung fibroblasts (hFLFs) (WI-38, CCL-75) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cell culture medium and fetal calf serum were obtained from Invitrogen-Gibco (Carlsbad, CA, USA). Cells were maintained in phenol red-free EMEM with 10% fetal bovine serum at 37°C and 5% CO<sub>2</sub>.

### 1.3. Cellular BPA content and localization

The hFLFs ( $4 \times 10^4$  cells/mL or  $4 \times 10^5$  cells/dish) were seeded in 10 cm culture dishes overnight, then treated with 0, 0.01, 0.1, 1, 10, 20, 50, or 100  $\mu$ mol/L BPA for 24 hr. Cells were harvested in a graduated micro glass tube for volume measurement, and then lysed in Pierce RIPA buffer (Thermo Scientific, Rockford, IL, USA). Cell lysates were extracted with acetonitrile followed by further extraction and clean-up using Bond Elut C18 solid phase extraction (SPE) cartridges (500 mg, 6 ml capacity) from Agilent (Mississauga, ON, Canada). The

extracts were then derivatized with acetic anhydride and analyzed by an Agilent 6890 gas chromatograph (GC) coupled to a 5973 mass selective detector (MSD) from Agilent Technologies (USA). Cellular contents of BPA were normalized against cell volumes or protein concentrations. The hFLFs were seeded at  $4 \times 10^4$  cells/mL ( $4 \times 10^5$  cells/dish) in 4-well glass culture slides (BD Biosciences, California, USA) overnight, and dosed for 24 hr. After removing media, each chamber was washed twice with ice-cold phosphate-buffered saline (PBS). Cells were then fixed in 4% paraformaldehyde dissolved in PBS (Santa-Cruz Biotechnology, Inc. California, USA) for 20 min at room temperature. The cells were washed three times with PBS, and then permeabilized in PBS containing 0.1% Triton X-100 for 15 min at room temperature. After being rinsed three times with PBS, the cells were blocked in 10% goat serum for 2 hr (Sigma-Aldrich, St. Louis, Missouri, USA), and then incubated with rabbit polyclonal anti-bisphenol a (BPA)-4-carboxymethyl ether (CME)-4-bovine serum albumin (BSA) (Cosmo Bio Co., Ltd., Tokyo, Japan), and Alexa Fluor 488 goat anti-rabbit IgG (Life Technologies, Carlsbad, CA, USA). BPA positive staining was assessed under a Zeiss Axiovert 40CFL inverted microscope (Carl Zeiss Canada, Toronto, ON, Canada) using 40 $\times$  objective and photographed with a SPOT RT3 digital camera and SPOT basic software (Diagnostic Instrument Inc., Sterling Heights, MI, USA).

#### 1.4. Cell viability, proliferation, and membrane integrity

The hFLFs were seeded at  $4 \times 10^4$  cells/mL ( $4 \times 10^5$  cells/dish) in 96 well culture plates overnight, then treated with 0, 0.01, 0.1, 1, 10, or 100  $\mu$ mol/L BPA for 24 hr. Cell viability was determined using the MultiTox-Glo Multiplex Cytotoxicity Assay kit (Promega, Madison, WI, USA), according to the manufacturer's instructions. The live-cell protease activity is restricted to intact viable cells and is measured using a fluorogenic, cell-permeant peptide substrate. The substrate enters intact cells where it is cleaved to generate a fluorescent signal proportional to the number of living cells. This live-cell protease becomes inactive upon the loss of membrane integrity and leakage into the surrounding culture medium. The extracellular protease activity measures protease released from dead cells or cells with membrane damage using a different substrate that generates luminescence upon cleavage. Luminescence is recorded using luminometer in the same well. Cell viability and proliferation were also measured by cell counts, for which cells were seeded in 6-well culture plates. After 24 hr of exposure to BPA, cells were trypsinized using TrypLE Express (Invitrogen-Gibco, Carlsbad, CA, USA) and counted by trypan blue staining using Countess™ (Invitrogen Carlsbad, CA, USA).

#### 1.5. Phosphorylation of NF- $\kappa$ B and release of GDF-15, IL-6, and IP-10

For measuring NF- $\kappa$ B p65 phosphorylation, hFLFs were seeded at  $4 \times 10^4$  cells/mL ( $4 \times 10^5$  cells/dish) in 4-well glass culture chamber (BD Biosciences, California, USA) overnight, and dosed for 24 hr. After removing media, each chamber was washed twice with ice-cold PBS. Cells were then fixed in 4% paraformaldehyde dissolved in PBS (Santa-Cruz Biotechnology, Inc. CA, USA) for 20 min at room temperature. The cells were then washed three times with PBS, and permeabilized in

PBS containing 0.1% Triton X-100 for 15 min at room temperature. After being rinsed three times with PBS, the cells were blocked in 10% goat serum for 2 hr (Sigma-Aldrich, Missouri, USA), and then incubated with Rabbit polyclonal anti-NF- $\kappa$ B p65 antibody and rabbit polyclonal anti-NF- $\kappa$ B p65 (phospho serine 536) (Abcam Inc., Cambridge, MA, USA), diluted 1:50 in IHC-Tek antibody diluent (IHC WORLD Woodstock, MD, USA) at 4°C overnight. After being washed, cells were incubated with Alexa Fluor 488 goat anti-rabbit IgG (Life Technologies, Carlsbad, CA, USA), diluted in the same diluent as the primary antibody for 2 hr at room temperature. After being rinsed in 1% goat serum in PBS three times for 10 min, the cells were incubated with 2  $\mu$ g/mL Hoechst 33342 (Life Technologies Inc., Burlington, ON, Canada) diluted in deionized water in the dark for 10 min. Slides were mounted with mounting media (Dako, Burlington, Canada). Hoechst positive staining was assessed under a Zeiss Axiovert 40CFL inverted microscope (Carl Zeiss Canada, Toronto, ON, Canada) using 40 $\times$  objective and photographed with a SPOT RT3 digital camera and SPOT basic software (Diagnostic Instrument Inc., Sterling Heights, MI, USA). For quantification of the staining, nuclear positive staining was counted for each dose group (0 and 100  $\mu$ mol/L BPA) using Image J software, and NF- $\kappa$ B p65 (p S536) positive staining was normalized against Hoechst positive staining. Group difference was compared using one way ANOVA on data from 3 independent experiments.

For measuring GDF-15, IL6, and IP10 release, hFLFs were seeded at  $4 \times 10^4$  cells/mL ( $4 \times 10^5$  cells/dish) in 10 cm culture dishes overnight and dosed with BPA at 0, 0.01, 0.1, 1, and 100  $\mu$ mol/L. After 24 hr exposure to BPA, supernatants were collected and analyzed for GDF-15 using a Quantikine Immunoassay kit from R&D Systems (Minneapolis, MN, USA), IL-6 using an Immunoassay kit from Invitrogen Corporation (Camarillo, CA, USA), and IP10 using Abcam IP-10 (CXCL10) Human SimpleStep enzyme-linked immunosorbent assay (ELISA) kit (Abcam Inc., Toronto, ON, Canada) according to instruction provided by the manufacturer.

#### 1.6. Cellular localization of ERs

The hFLFs were seeded at  $4 \times 10^4$  cells/mL ( $4 \times 10^5$  cells/dish) in 4-well glass culture chamber (BD Biosciences, California, USA) overnight, and dosed for 24 hr. After removing media, each chamber was washed twice with ice-cold PBS. Cells were then fixed in 4% paraformaldehyde dissolved in PBS (Santa-Cruz Biotechnology, Inc. California, USA) for 20 min at room temperature. The cells were washed three times with PBS, and then permeabilized in PBS containing 0.1% Triton X-100 for 15 min at room temperature. After being rinsed three times with PBS, the cells were blocked in 10% goat serum for 2 hr (Sigma-Aldrich, St. Louis, Missouri, USA), and then incubated with mouse monoclonal anti-ER $\alpha$  (Cell Signaling Technology Inc., Danvers, MA, USA), mouse monoclonal anti-ER $\beta$  (Abcam Inc., Cambridge, MA, USA), or rabbit polyclonal anti-G-protein coupled receptor 30 (Abcam Inc., Cambridge, MA, USA), diluted 1:100 in IHC-Tek antibody diluent, at 4°C overnight. After being washed, cells were incubated with Alexa Fluor 488 goat anti-mouse IgG (H + L) (Life Technologies, Carlsbad, CA, USA) or Alexa Fluor 488 goat anti-rabbit IgG (Life Technologies, Carlsbad, CA, USA), diluted in the same diluent as the primary antibody for 2 hr at

room temperature. After being rinsed in 1% goat serum in PBS three times for 10 min, the cells were incubated with 2  $\mu\text{g/mL}$  Hoechst 33,342 (Life Technologies Inc., Burlington, ON, Canada) diluted in deionized water in the dark for 10 min. Slides were mounted with mounting media (Dako, Burlington, Canada). Positive staining was assessed under a Zeiss Axiovert 40CFL inverted microscope (Carl Zeiss Canada, Toronto, ON, Canada) using 40 $\times$  objective and photographed with a SPOT RT3 digital camera and SPOT basic software (Diagnostic Instrument Inc., Sterling Heights, MI, USA).

### 1.7. Protein expressions of ERs

Protein expression levels of ER $\alpha$ , ER $\beta$  and GPR30 were determined in cytoplasmic and nuclear fractions using ELISA and Western blotting. Cells were collected after being treated with 0, 0.01, 1 and 100  $\mu\text{mol/L}$  BPA in 10 cm culture dish for 24 hr. Three plates of cells were scraped and pooled into one tube. Nuclear and cytoplasmic fractions of proteins were extracted using NE-PER $\text{\textcircled{R}}$  nuclear and cytoplasmic extraction reagents (Thermo Scientific, Pierce Biotechnology, Rockford, USA). A volume of 200  $\mu\text{L}$  ice-cold cytoplasmic extraction buffer I (CERI) containing protease inhibitors (Halt Protease Inhibitor Single-Use Cocktail, Pierce Biotechnology, Rockford, USA) was added to cell pellets, and the samples were sonicated and incubated on ice for 10 min. Then, 11  $\mu\text{L}$  cytoplasmic extraction buffer II (CERII) was added, vortexed and centrifuged at 4 $^{\circ}\text{C}$  and 17,000  $\times g$  for 5 min. The supernatants containing cytoplasmic fractions were transferred to pre-chilled tubes. The pellets were resuspended in 100  $\mu\text{L}$  ice cold NER nuclear extraction buffer with protease inhibitors, and sonicated, followed by vortexing four times with 15 sec/time and 10 min intervals. The tubes were then centrifuged at 17,000  $\times g$  for 10 min. The supernatant (nuclear) fractions were transferred to pre-chilled tubes. Protein concentrations were determined by DC Protein Assay (Bio-Rad Laboratories Ltd., Mississauga, Ontario, Canada). Protein levels of ER $\alpha$ , ER $\beta$ , and GPR30 in cytoplasmic and nuclear fractions were measured using commercial ELISA kits from USCN Life Science Inc. (Wuhan, China) according to manufacturer's instructions. For Western blotting, thirty  $\mu\text{g}$  of protein extracts were resolved by gradient gel (4%–20% Precise Tris-HEPES Gel, Thermo Scientific, ON, Canada) electrophoresis. Separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes, blocked with 5%-milk dissolved in TBST (10 mmol/L Tris base, 150 mmol/L NaCl, and 0.1% Tween 20, pH 7.5), and probed overnight at 4 $^{\circ}\text{C}$  with a rabbit polyclonal anti-human ER $\alpha$  (Epitomics, CA, USA), Rabbit polyclonal anti-human ER $\beta$  (Invitrogen, CA, USA), and rabbit polyclonal anti-GAPDH antibody (Sigma-Aldrich, ON, Canada). GAPDH bands were used as loading controls. After incubation of the membrane with SuperSignal $\text{\textcircled{R}}$ WestFemto reagent (Thermo Scientific, ON, Canada), protein bands were visualized under a digital phosphorimager (FluorChem HD2, Alpha Innotech/ProteinSimple, CA, USA).

### 1.8. Effect of BPA and ERs antagonists on ET-1 release

The hFLFs were seeded at  $4 \times 10^4$  cells/mL ( $4 \times 10^5$  cells/dish) in 10 cm culture dishes overnight and dosed with BPA at 0, 0.01, 0.1, 1, 10, 20, 50 and 100  $\mu\text{mol/L}$ . After 24 hr exposure to

BPA, supernatants were collected and analyzed using ET-1 ELISA kits from Enzo Life Sciences (Farmingdale, NY, USA) according to instruction provided by the manufacturer. For determining effects of ER antagonists on ET-1 release, hFLFs were cultured as described above and pre-treated with ER antagonists including MPP dihydrochloride (1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinyloxy)phenol]-1H-pyrazole dihydrochloride, ER $\alpha$  selective), PHTPP (4-[2-Phenyl-5,7-bis(trifluoromethyl) pyrazolo[1,5-a]pyrimidin-3-yl]phenol, ER $\beta$  selective) and G15 ((3aS,4R,9bR)-4-(6-Bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3H-cyclopenta[c]quinolone, GPER-30 selective) (all from Tocris Biosciences, Bristol, UK). Antagonist stock solutions at 100 mmol/L were prepared in ethanol or DMSO by warming in a 60 $^{\circ}\text{C}$  water bath. The antagonists were added to the culture at a final concentration of 1  $\mu\text{mol/L}$  at 1 hr before addition of 0 (water) or 100  $\mu\text{mol/L}$  BPA and co-incubated for 24 hr. Supernatants were collected and analyzed for ET-1 as described before.

### 1.9. Statistical analysis

Statistical analyses were performed using SigmaPlot 11.0 (Systat Software, Inc. San Jose, CA, USA). All data were evaluated for normality and equality of variance prior to statistical analysis. Variables with skewed distribution were transformed logarithmically or ranked. ANOVA and Tukey's honestly significant difference (HSD) test were used to compare differences among treatment groups. Differences were considered significant when  $p < 0.05$ .

## 2. Results

### 2.1. Cellular content and localization of BPA

Although many *in vitro* studies on BPA have been published, very few, if any, have correlated effects of BPA with intracellular BPA concentrations. We therefore analyzed BPA concentrations in hFLFs treated with or without BPA for 24 hr. The cellular content of BPA increased with BPA concentration applied to culture (Fig. 1). The ratios of intracellular BPA concentrations to BPA concentrations applied to cell culture decreased with increase in BPA doses from 0 to 0.01, 0.1 and 1  $\mu\text{mol/L}$ , but remained relatively constant from 1 to 10, 20, 50 and 100  $\mu\text{mol/L}$ . This suggests that at lower BPA doses, cells were able to take up and accumulate BPA. At 100  $\mu\text{mol/L}$  BPA concentration, only about 23  $\mu\text{mol/L}$  of BPA were taken up by the cells. This suggests that intracellular binding sites for BPA may be saturated at the high BPA concentration or cells have a compensatory capability to eliminate BPA, particularly at high concentrations. Another possibility would be that the cells treated with higher concentrations of BPA had lower uptake activity as a result of suppression of uptake by BPA treatment. Positive staining of BPA was mainly localized in perinuclear region of hFLFs (Fig. 2). We also observed positive BPA staining in the control cells. This is not surprising, because our previous study suggested that the medium used to culture hFLFs contains about 1.93 nmol/L BPA. The control cells accumulated 52 nmol/L BPA from the medium after 24 hr of exposure, similar to the 79 nmol/L BPA concentration found

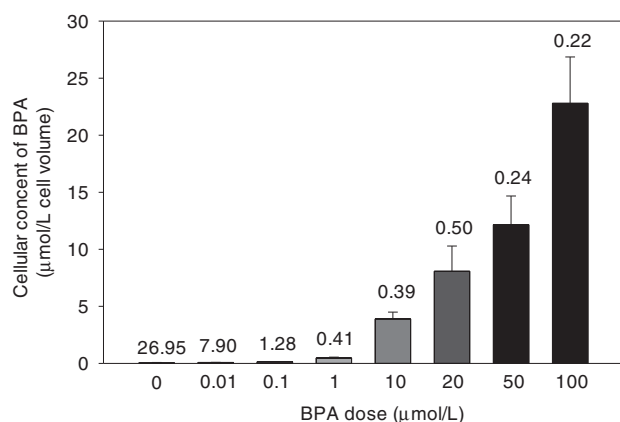


**Fig. 1 – Cellular contents of BPA.** The hFLFs were exposed to different concentrations of BPA for 24 hr. Cellular contents of BPA were measured using GC–MS. Values were normalized against cell volume and expressed as means of four independent experiments. Error bars are the standard errors of the means. The values written on the top of vertical bars are the ratios of cellular BPA contents to BPA doses applied. The value for 0 μmol/L BPA dose group was the ratio of BPA content of the cells in the control group to the background BPA concentration found in the medium. BPA: bisphenol A; GC–MS: gas chromatograph–mass spectrometry.

in the 10 nmol/L BPA treated cells. It is clear that it is impossible to use BPA doses lower than 1.93 nmol/L to treat cells grown in the culture medium used.

## 2.2. Cell viability, proliferation, and membrane integrity

Fetal lung fibroblasts play an important role in fetal lung development. We examined the effects of BPA on the viability and proliferation of hFLFs using multiple techniques. BPA treatment showed no significant effects on the live cell protease activity (Fig. 3a), and the total, viable and dead cell numbers (Fig. 3b), suggesting a lack of influence of BPA on cell viability and proliferation of hFLF under the conditions used. Interestingly, BPA at 100 μmol/L decreased extracellular protease activity, a measurement of protease leakage caused by cell death and membrane damage (Fig. 3a). The intracellular and extracellular protease assays measure two different proteases. It remains to be clarified if BPA at 100 μmol/L decreased expression levels or activity of the type of protease that is measured in the extracellular protease assay.

## 2.3. NF-κB p65 phosphorylation and release of GDF-15, IL-6, and IP-10

NF-κB activation mediated inflammation in preterm infants has been shown to arrests saccular airway formation. Multiple phosphorylation sites including serine 536 (S536) is required for NF-κB p65 activation. We examined effects of BPA treatment on NF-κB p65 phosphorylation at S536 in hFLFs by immunocytochemical staining. NF-κB p65 p S536 positive staining was located mainly in the nuclei of both control and 100 μmol/L BPA-treated cells (Fig. 4a). Quantitative analysis suggested that 24 hr BPA treatment significantly decreased the number of NF-κB p65 p S536 positively stained nuclei (Fig. 4a). We also stained total NF-κB protein and found no

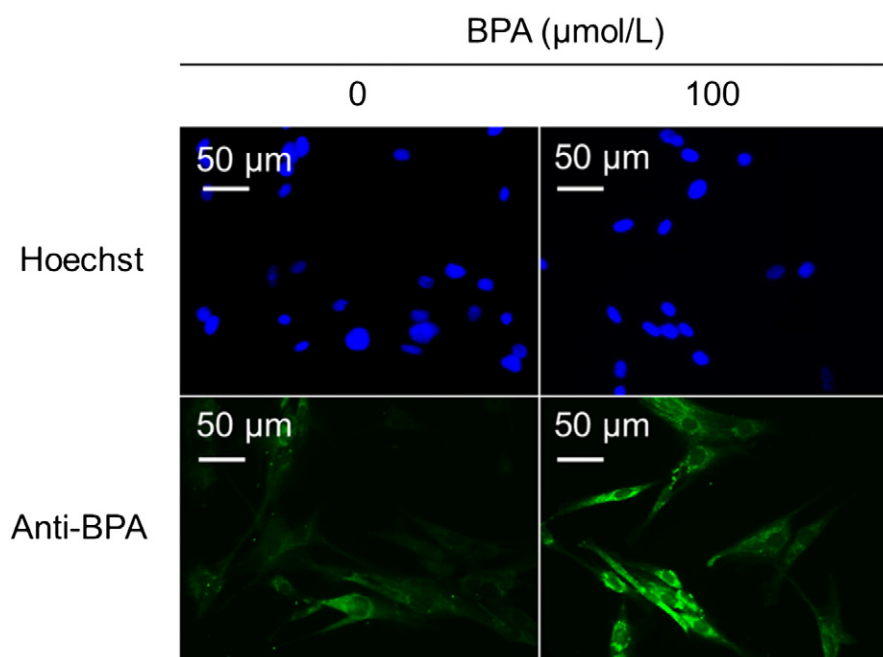


**Fig. 2 – Cellular localization of BPA.** The hFLFs were fixed in 4% paraformaldehyde after 24 hr of exposure to 0 and 100 μmol/L BPA. Fixed cells were incubated with anti-bisphenol A-CME antibody or antibody dilution buffer only (as negative control) for 24 hr, followed by incubation with Hoechst and Alexa Fluor goat anti-rabbit IgG (H + L). Images were obtained at 40× objective lens under fluorescence microscopy. BPA: bisphenol A; hFLFs: human fetal lung fibroblasts.

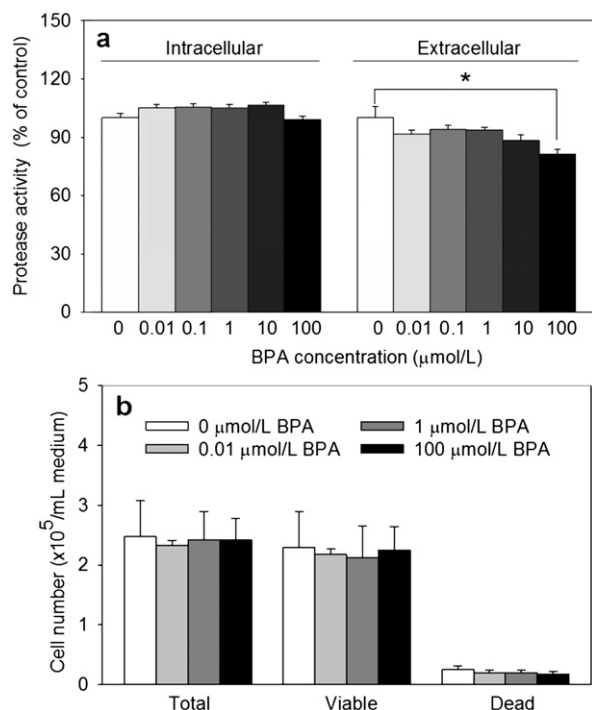


**Fig. 3 – Effects of BPA on cell viability, membrane integrity and proliferation.** The hFLFs were exposed to BPA at 0–100  $\mu\text{mol/L}$  for 24 hr. Cell viability and membrane integrity was measured by intracellular and extracellular protease activity using Multi-Tox assay (a). The total, viable and dead cell numbers were counted using trypan blue after 24 hr BPA treatment (b). Values represent the means of 6–7 independent experiments. Error bars are the standard errors of the means. “\*” indicates a significant difference between the two treatment groups located under the vertical lines at  $p < 0.05$ . BPA: bisphenol A; hFLFs: human fetal lung fibroblasts.

difference between control and BPA-treated cells (data not shown). GDF-15 is known to suppress NF- $\kappa$ B activation and thus has anti-inflammatory function. Thus we examined the effects of BPA on GDF-15 secretion in hFLFs. Treatment with BPA at 0.01 and 1  $\mu\text{mol/L}$  had no effects on the release of GDF-15 from hFLFs after 24 hr of exposure (Fig. 4b). However, at 100  $\mu\text{mol/L}$ , BPA increased GDF15 release after 24 hr of exposure (Fig. 4b). Both IL-6 and IP-10 are NF- $\kappa$ B dependent cytokines. IL-6 is known to play an important role in lung angiogenesis and morphogenesis, while IP-10 is chemotactic for T cells and potent angiostatic agent and may influence immune response and alveolarization in the developing lung. Therefore, we also determined release of IL-6 and IP-10 from hFLFs treated with or without BPA. Treatment with 0.01 and 1  $\mu\text{mol/L}$  BPA for 24 hr showed no effects on IL-6 and IP-10 release from hFLF (Fig. 4b). However, treatment with 100  $\mu\text{mol/L}$  BPA significantly decreased IL-6 and IP-10 release (Fig. 4b).

#### 2.4. Cellular localization of ERs

ERs are known to regulate fetal lung development in a sex-dependent manner. In this study, we examined the

effects of BPA on ERs localization in hFLFs. ER $\alpha$  and ER $\beta$  positive staining was localized in both nuclei and cytoplasm (Fig. 5). GPR30 was found in the plasma membrane and nuclei of hFLFs. In the control cells, all three receptors were stained denser in the nuclei than cytoplasm. BPA at 100  $\mu\text{mol/L}$  did not affect distribution and staining intensity of ER $\alpha$ , but slightly increased ER $\beta$  positive staining in the cytoplasm and had no influence on ER $\beta$  positive staining in the nuclei. The same concentration of BPA also increased GPR30 positive staining mainly in the nuclei. These results suggest that hFLFs, a cell line traditionally viewed as non-estrogen responsive, also express all three estrogen receptors, although their roles in cellular signaling and metabolic responses remain to be established.

#### 2.5. Protein expression of ERs

ELISA quantification of ER protein revealed that 100  $\mu\text{mol/L}$  BPA did not affect ER $\alpha$  protein levels in either cytoplasmic or nuclear fractions (Fig. 6a). However, it increased ER $\beta$  protein levels in the cytoplasmic fractions, with a trend of decrease in the nuclear fractions (Fig. 6b). BPA at 100  $\mu\text{mol/L}$  increased GPR30 protein levels in the nuclear fractions, but had no influence on GPR30 protein levels in the cytoplasmic fractions (Fig. 6c). Western blot analysis of ER protein expression confirmed the results of ELISA analysis (Fig. 6d). All these results are consistent with the findings from immunocytochemical staining.

#### 2.6. Effects of BPA and ER antagonists on ET-1 release

ET-1 is an important regulator in embryo development. When hFLF was treated with 0, 0.01, 0.1, 1, 10, 20, 50, and 100  $\mu\text{mol/L}$  BPA, a trend of decrease in ET-1 release with increasing BPA concentration was observed, however, did not reach statistically significant level until 100  $\mu\text{mol/L}$  BPA (Fig. 7a). Antagonists MPP, PHTPP and G15, which are specific for ER $\alpha$  (Fig. 7b), ER $\beta$  (Fig. 7c), and GPR30 (Fig. 7d), respectively, had little or no effect on BPA-induced decrease in ET-1 release.

### 3. Discussion

In this study, we determined cellular content of BPA after 24 hr of exposure, in order to correlate the effects with the actual intracellular concentrations of BPA in the hFLFs. Our data suggested that the cellular content of BPA increased with increasing BPA concentrations in the medium, but higher proportions of BPA were taken up into the cells exposed to lower, as opposed to higher, concentration of BPA. Only 23  $\mu\text{mol/L}$  in average BPA accumulated in the cells after 24 hr of exposure to 100  $\mu\text{mol/L}$  BPA. This could be partly due to a higher rate of elimination of BPA, reduced BPA uptake, or saturation of BPA uptake by the cells exposed to higher BPA concentrations. The average of 52 nmol/L BPA concentration detected in control cells suggest that it is questionable to claim a low dose effect without measuring cellular content of BPA which may come from exposure to background BPA present in the medium. BPA was mainly localized in the perinuclear region of 100  $\mu\text{mol/L}$  BPA treated

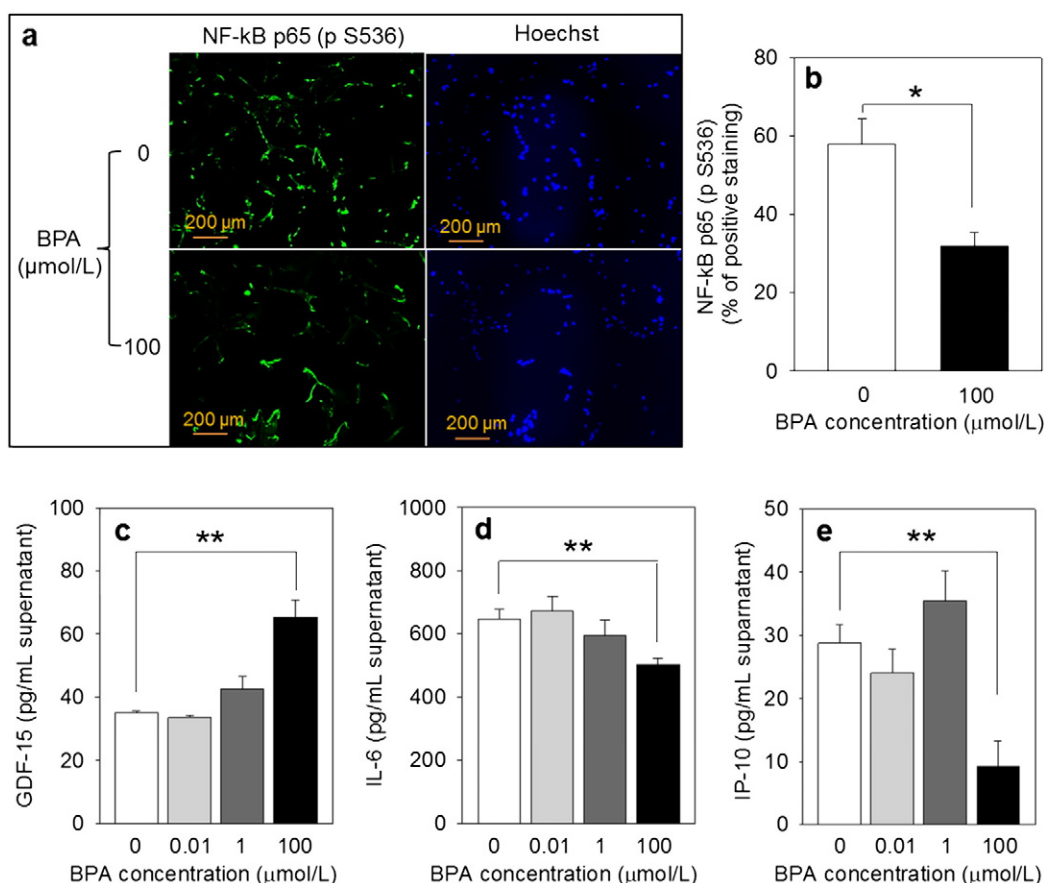


**Fig. 4 – Effects of BPA on GDF-15, IL-6, and IP-10 release and NF-κB phosphorylation.** The hFLFs were treated with 0 or 100 μmol/L BPA for 24 hr and fixed in 4% paraformaldehyde. Fixed cells were stained with rabbit polyclonal anti-NF-κB p65 (p S536) and Alexa Fluor 488 goat anti-rabbit IgG. Images were documented under fluorescent microscope using 10× objective lens. Cell with NF-κB p65 (p S536) positive staining (a) were counted manually, normalized against total number of cells obtained by Hoechst staining, and expressed as % of positive staining (b). The hFLFs culture supernatants were collected after 24 hr exposure to 0, 0.01, 1 or 100 μmol/L BPA. GDF-15 (c), IL-6 (d), and IP-10 (e) concentrations in the supernatants were measured by ELISA. Values represent the means of 3–5 independent experiments. Error bars are the standard errors of the means. “\*” and “\*\*” indicate a significant difference between the two treatment groups located under the vertical lines at  $p < 0.05$  and  $0.01$ , respectively. BPA: bisphenol A; hFLFs: human fetal lung fibroblasts. GDF-15: growth differentiation factor-15; IL-6: interleukin-6; IP10: interferon gamma induced protein 10.

hFLFs, and associated with mitochondria-like structures. This is consistent with our previous finding that more BPA was found in mitochondrial than nuclear and cytoplasmic fractions of HEK293 cells exposed to BPA (Chepelev et al., 2013).

The low dose effects of BPA are actively debated in the literature, particularly the reported effects on embryo development (Hunt et al., 2003; Vandenberg et al., 2007; Palanza et al., 2008; Snijder et al., 2013), which aroused enormous concerns among the public and added challenges to the regulatory agencies. It is unrealistic to use BPA concentrations lower than 1.93 nmol/L in our study. Using different types of cytotoxicity assays, we found no effects of BPA at up to 100 μmol/L dose level on cell viability, proliferation, and membrane integrity after 24 hr of exposure. Rather, we found a decreased extracellular protease activity in hFLFs exposed to 100 μmol/L BPA, which could be a result of decreased protease protein expression and/or activity, or increase resistance to stress by BPA. This correlates to the

observation that 100 μmol/L BPA altered cell arrangement (from a more spread to a more paralleled and bundled distribution) and morphology with increased cell size and more fibrous appearance.

Fetal lung development is a complex biological process which involves temporal and spatial regulation of multiple factors such as growth factors, transcriptional factors, and ECM. Lung fibroblasts play important roles in the development and progression of asthma by secreting ECMs that mediate responses of airway epithelial and smooth muscle cells to effector (Alkhoury et al., 2014; Pain et al., 2014). Activation of lung fibroblasts plays an important role in lung fibrosis (Sivakumar et al., 2012). In order to depict the potential mechanisms involved in the reported developmental effects of BPA, we examined the effects of BPA on NF-κB activation, the release of GDF-15, IL-6, IP-10, and ET-1, and protein expression of ERs in hFLFs, which are all known to have regulatory impacts on cellular processes involved in fetal

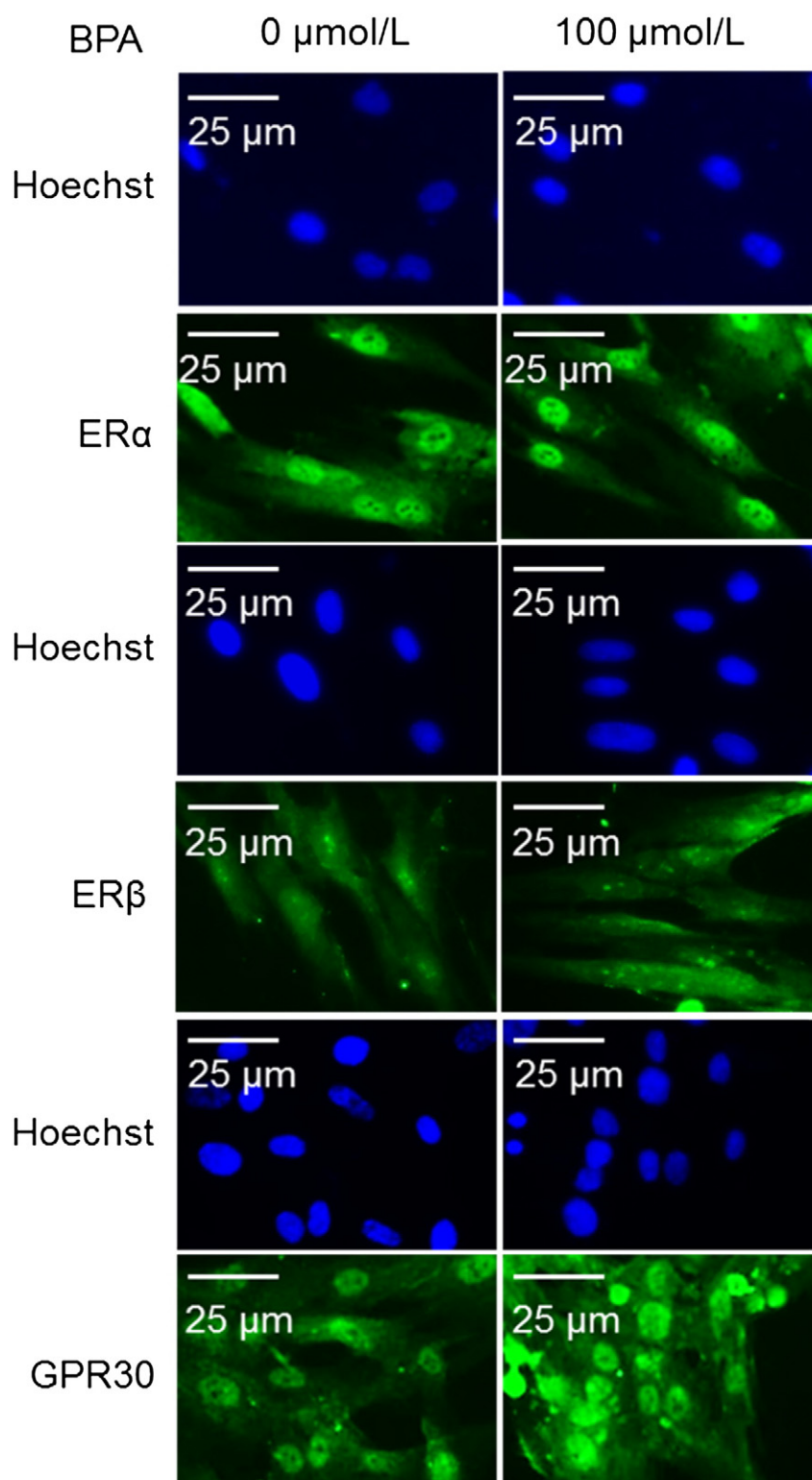


Fig. 5 – Subcellular localization of ER $\alpha$ , ER $\beta$ , and GPR30 in hFLFs. The hFLFs were fixed after 24 hr exposure to 0 or 100  $\mu\text{mol/L}$  BPA and incubated with antibodies against human ER $\alpha$ , ER $\beta$ , or GPR30, or antibody dilution buffer only (as negative control) followed by incubation with Alexa Fluor goat anti-mouse or anti-rabbit IgG (H + L) and Hoechst. Images were obtained at 40 $\times$  objective lens under fluorescence microscopy. ER: estrogen receptor; GPR30: G-protein coupled estrogen receptor 30; hFLFs: human fetal lung fibroblasts; BPA: bisphenol A.

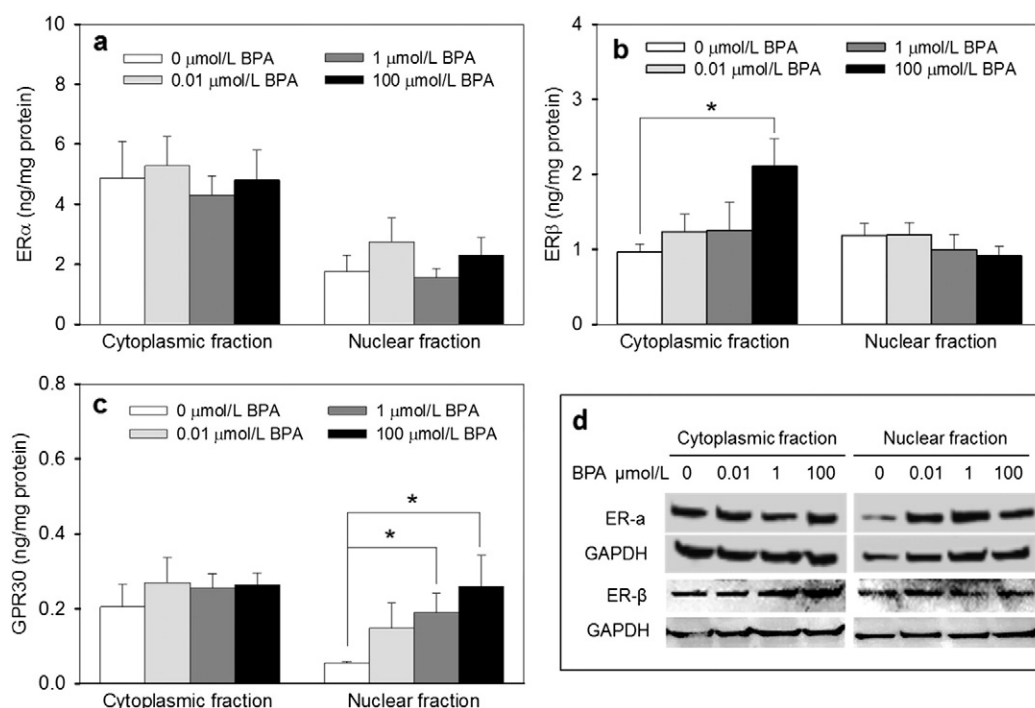


**Fig. 6 – Protein expression of ERs.** hFLFs were exposed to 0, 0.01, 1, and 100  $\mu\text{mol/L}$  BPA for 24 hr. Cellular nuclear and cytoplasmic fractions were obtained from cell lysates using NE-PER nuclear and cytoplasmic extraction buffers. ER $\alpha$  (a), ER $\beta$  (b) and GPR30 (c) protein levels in cytoplasmic and nuclear fractions were measured using ELISA and Western blot (d). Due to the lack of sensitivity of Western blot technique and the low level of protein expression of GPR30, no protein bands for GPR30 were detected. Values were normalized against total protein concentrations. Values represent the means of four independent experiments. Error bars are the standard errors of the means. “\*” indicates a significant difference between the two treatment groups located under the vertical lines at  $p < 0.05$ . ER: estrogen receptor; BPA: bisphenol A; GPR30: G-protein coupled estrogen receptor 30.

lung development and maturation, as well as development and progression of asthma.

NF- $\kappa$ B is a transcription factor regulating many genes involved in inflammation, immunity, cell proliferation, apoptosis, and stress response. Persistent activation of NF- $\kappa$ B has been implicated in inflammatory airway diseases including asthma (Pantano et al., 2008; Schuliga, 2015). Site specific phosphorylation of p65 serves as a code to target NF- $\kappa$ B transcriptional activity to distinct gene subsets (Hochrainer et al., 2013). In this study we observed a decreased phosphorylation of NF- $\kappa$ B p65 at S536, and decreased secretion of downstream genes IL-6 and IP-10, suggesting an anti-inflammatory effect of BPA treatment in hFLFs.

GDF-15, a stress-responsive cytokine, is known to regulate cell proliferation, differentiation, apoptosis, and inflammation and is highly inducible upon exposure to stressors. In GDF-15 deficient mice, GDF-15 expression was induced during fibrosis development and markedly correlated with lung function impairment in this disease (Lambrecht et al., 2014). More interestingly, GDF-15 was found to be upregulated by cigarette smoke and play a critical role in cigarette smoke induced mucin expression in human airway epithelial cells (Wu et al., 2012). GDF15 inhibits macrophage differentiation by suppressing NF- $\kappa$ B activity via delaying I $\kappa$ B degradation (Vanhara et al., 2009). In this study, BPA treatment increased GDF-15 secretion from hFLFs, resulting in decreased NF- $\kappa$ B

activation. This suggests that BPA treatment caused an adaptive response and a suppression of immune reaction.

IP-10 recruits mast cells via chemokine receptor and is increased in the airway smooth muscle cells of asthmatic patients (Carroll et al., 2002). Increased expression of IP-10 was found in the bronchoalveolar lavage fluid and biopsy specimens of subjects with asthma (Miotto et al., 2001). In a murine model of asthma, IP-10 is up-regulated in the lung after allergen challenge. In this study, BPA treatment decreased IP-10 secretion from hFLFs, although its physiological implication in childhood asthma warrants more investigation.

IL-6 plays an important role in lung angiogenesis and morphogenesis (McClintock and Wagner, 2005). IL-6 antibodies significantly reduce the number of peripheral airway buds, epithelial perimeter and epithelial and mesenchymal cellular proliferation rate in rat fetal lung explants (Nogueira-Silva et al., 2006). A lack of IL-6 increases eosinophilia, lung Th2 cytokines and eotaxin as well as airway response to methacholine (DiCosmo et al., 1994; Wang et al., 2000). Clinical and animal studies suggest that antenatal exposure to inflammatory mediators may improve lung volume and compliance as well as accelerate fetal lung maturation (Jobe and Ikegami, 2001). In humans, it was demonstrated that IL-6 elevation in fetuses with chorioamnionitis promoted fetal lung maturation by enhancing surfactant protein A (SP-A) synthesis (Shimoya et al., 2000). In fact, fetal IL-6 is a

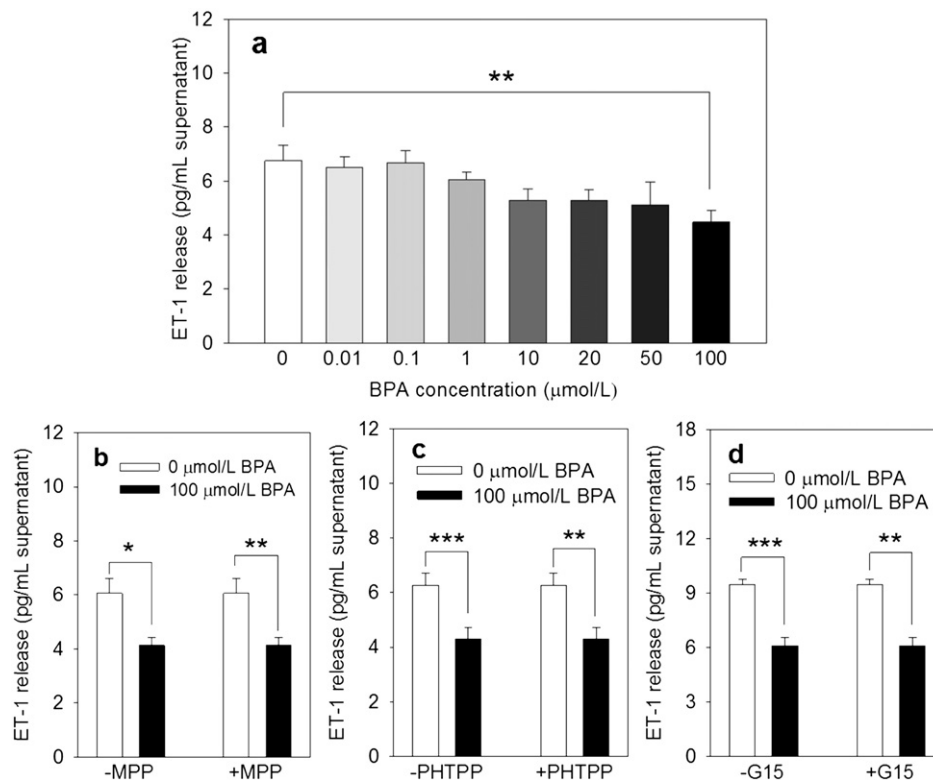


**Fig. 7 – Effects of BPA and ERs antagonists on ET-1 release.** The hFLFs were dosed with 0, 0.01, 0.1, 1, 10, 20, 50 and 100 μmol/L BPA for 24 hr. Supernatants were collected and analyzed for ET-1 release (a) using ELISA kits. Effects of ERα selective antagonist, MPP (b), ERβ selective antagonist, PHTPP (c), and GPR30 selective antagonist, G15 (d) on BPA-induced decrease in ET-1 release from hFLF were determined by adding antagonists at 1 hr before the addition of BPA followed by co-incubation for 24 hr. ET-1 concentrations in the culture supernatants were measured using ELISA kits. Values represent the means of 4 independent experiments. Error bars are the standard errors of the means. “\*”, “\*\*\*”, and “\*\*\*\*” indicate a significant difference between the two treatment groups located under the vertical lines at  $p < 0.05$ , 0.01, and 0.001, respectively. ER: estrogen receptor; BPA: bisphenol A; hFLFs: human fetal lung fibroblasts; PHTPP: 4-[2-Phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl]phenol, ERβ selective; GPR30: G-protein coupled estrogen receptor 30; G15: 3aS,4R,9bR)-4-(6-Bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3H-cyclopenta[c]quinolone, GPR-30 selective; ET-1: endothelin-1; MPP: 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole, ERα selective.

regulatory cytokine of pulmonary surfactant proteins and plays an important role in lung maturity decreasing the incidence of respiratory distress syndrome in preterm neonates. IL-6 was shown to stimulate fetal lung growth and branching in rats (Nogueira-Silva et al., 2006). Van Winkle et al. (2013) demonstrated that fetal exposure of rhesus macaques to BPA alters cellular development of the conducting airway by changing epithelial secretory product expression. In our study, BPA treatment induced a decrease in IL-6 secretion from hFLFs, suggesting a potential negative impact on fetal lung development and maturation, which is consistent with the finding of Hijazi et al. (2015).

ET-1 is an important regulator of embryo development through its regulation of angiogenesis and the migration and proliferation of endothelial cells (Folkman and Shing, 1992). The role of ET-1 in the pathogenesis of asthma has been well documented. In the human respiratory tract, ET-1 mediates airway smooth muscle contraction, growth, and mucus secretion (Goldie and Fernandes, 2000; Hirst et al., 2000). ET-1 deficiency enhanced airway hyperresponsiveness to methacholine in

ET-1(+/-) heterozygous mice (Nagase et al., 1998). In this study, BPA treatment decreased release of ET-1 from hFLFs, which may have an implication in BPA-mediated air way hypersensitivity in experimental animals.

Clinical and experimental evidence suggests that estrogen has significant effects on normal airway function as well as on respiratory disorders, such as asthma (Ticconi et al., 2013). Both ERα and ERβ have been shown to be expressed in the nucleus, cytoplasm and mitochondria of human bronchial epithelial cells (Ivanova et al., 2009) and are involved in the formation and function of normal alveolar units in the female (Massaro and Massaro, 2006). Estrogen has multiple potential effects on inflammation, metabolism and immunity (Gomez et al., 2008), as well as on airway hyperresponsiveness (AHR) (Lim and Kobzik, 2008), that can have a relevant impact on asthma. In this study, we detected protein expression of all three ERs in both cytoplasm and nuclei of both control and BPA treated hFLFs. BPA treatment had no effects on either distribution or protein level of ERα, but increased cytoplasmic expression level of ERβ and nuclear expression level of GPR30. Antagonist

specific for ER $\alpha$ , ER $\beta$ , or GPR30 had little effect on BPA-induced decrease in ET-1 release. These data indicate that although ERs are expressed in hFLFs, the observed effects of BPA on ET-1 release did not depend on direct binding of BPA to these receptors. Resveratrol, with a similar molecular structure to BPA, suppresses cell growth and promotes stress resistance in an ER $\beta$ -dependent fashion in mouse myoblasts (Robb and Stuart, 2014). The increased cytoplasmic expression of ER $\beta$  by BPA in hFLFs may also contributed to BPA-induced stress resistance observed in hFLFs. GPR30 mRNA has been detected in multiple tissues including lung (Owman et al., 1996; Kvingedal and Smeland, 1997). Although the role of GPR30 in pathogenesis of asthma remains to be investigated, in this study, BPA increased GPR30 protein expression in the nuclei in a dose-dependent fashion, and even at 1  $\mu$ mol/L concentration, after 24 hr of exposure. It is interesting that this change in GPR30 expression was not associated with changes in other markers examined. It deserves further investigation to see if the increase in nuclear GPR30 results in other cellular changes if cells are exposed to BPA for longer duration.

Asthma is a major health problem affecting over 300 million people worldwide (WHO, 2010). It is characterized by an exaggerated airway hyperresponsiveness and associated with structural, functional, and inflammatory changes (Ticconi et al., 2013). Human fetal lung may be exposed to environmental endocrine disruptors such as BPA through both fetal blood and amniotic fluid, especially at later stages of development. The exposure levels of human fetal lung to BPA remain to be assessed. Higher exposure levels may occur in the fetal lung than other fetal organs due to the greater surface area to volume ratio and differences in the route of exposure of the lung relative to other organs. Our data suggest that exposure of hFLFs to higher doses of BPA can alter the secretion of important immunological and developmental modulators, such as GDF-15, ET-1 and IL-6, which may have a negative impact on fetal lung development, maturation, and susceptibility to environmental stressors if occurs *in vivo*. As an *in vitro* study, the findings of this study have limited implications since the hFLFs only represent a specific gestational stage of a female fetus. A 24 hr-BPA treatment in culture does not reflect a whole nine month gestational exposure. Whether prenatal exposure to BPA contributes to an increased risk of childhood asthma remains to be confirmed through *in vivo* studies.

### Conflict of interest statement

We declare that there are no conflicts of interest.

### Acknowledgments

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