

***In Vitro* Study of Mechanisms Underlying the
Developmental Effects of Bisphenol A Using Human
Fetal Lung Fibroblasts**

by

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ABSTRACT

Both experimental and/or epidemiological studies suggest that prenatal exposure to bisphenol A (BPA) may delay fetal lung development and maturation and increase the susceptibility to childhood respiratory disease. However, the underlying mechanisms remain to be elucidated. In our study with cultured human fetal lung fibroblasts (HFLF), we demonstrated that 24 h exposure to 1 and 100 μ M BPA increased nuclear expression of GPR30, at 100 μ M, also increased cytoplasmic expression of ER β and release of GDF-15, as well as decreased release of IL-6, ET-1, and IP-10 through suppression of NF κ B phosphorylation, with no effects on cell viability. By performing global gene expression and pathway analyses, we identified molecular pathways, gene networks, and key molecules that were affected by 100, but not 0.01 and 1, μ M BPA in HFLF. Using multiple genomic and proteomic tools, we confirmed these changes at both gene and protein levels. Our data suggest that 100 μ M BPA increased CYP1B1 and HSD17B14 gene and protein expression and release of endogenous estradiol, which was associated with increased ROS production and DNA double strand breaks, upregulation of genes and/or proteins in steroid synthesis and metabolism, and activation of Nrf2-regulated stress response pathways. In addition, BPA also activated ATM-p53 signaling pathway, resulting in increased cell cycle arrest at G1 phase, senescence, and autophagy in HFLF. Fetal lung development and maturation requires paracrine interaction between fetal lung alveolar type II epithelial cells and fibroblasts. The results from our studies suggest that prenatal exposure to BPA at high enough concentrations may affect fetal lung development and maturation, by altering the release of developmental, immune, and hormonal modulators from fetal lung fibroblasts, and thereby affecting susceptibility to childhood respiratory disease.

Preface

This thesis is written in integrated thesis format, summarizing the work that, at the time of thesis submission, has been published already (Chapter 2) and submitted (Chapter 3). Three publications were co-authored.

Status of manuscripts at the time of thesis submission

Chapter 2:

Mahemuti L, Chen Q, Coughlan MC, Florian M, Mailloux RJ, Cao XL, Scoggan K, Willmore WG, Jin X, 2016. Bisphenol A exposure alters release of immune and developmental modulators and expression of estrogen receptors in human fetal lung fibroblasts. *J Environ Sci.* 48, 11-23.

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Statement of contributions

I have contributed towards the research described in this thesis as follows:

1. Jointly with Drs. Jin and Willmore, I formulated the hypotheses.
2. I planned and executed the experiments and collected the data.
3. I analyzed the data, jointly with Drs. Jin and Willmore.
4. In collaboration with Dr. Jin, I trained and co-supervised two undergraduate students; Dilion Dong, who helped with the immunocytostaining of Cyp1B1, Western blot analysis of LC-3B and autophagy detection, and Robert Woodworth who participated in some preliminary data collection.
5. Individuals who contributed to the research presented in Chapters 3 and 4 include Melanie Coughlan (Technician in Dr. Jin's lab) contributed to the DNA microarrays sample preparation; Dr. Charlie Chen performed the real-time qPCR experiments; Dr. Maria Florian who helped to generate the CYP1B1 Western blot data; Dr. Xu Liang Cao who helped to determine BPA uptake in cells by GC-MS; Mrs Cun Ye Qiao performed statistical analysis for DNA microarray.
6. With the assistance of Drs. Jin and Willmore, I prepared the manuscripts that form the basis of this thesis.
6. The mRNA extraction and real-time qPCR analysis were done in the laboratory of Dr. Kylie Scoggan at Health Canada
7. With the help of Drs. Jin and Willmore, I designed and performed the experiments described in Chapters 3 and 4.
8. In collaboration with the Health Canada Animal Care and Pathology lab, and under Dr. Jin's supervision, I participated in a rat study of a Northern Contaminant Mixture (NCM) project, by contributing in the daily animal husbandry, animal necropsy and sample and data collection, for all three co-authored manuscripts mentioned above.

I would like to formally acknowledge the contributions of my co-authors of the manuscripts that form the basis of this thesis. As stated above, Drs. Jin and Willmore's contributions included formulating hypotheses, experimental design, data analysis and manuscript preparation for all manuscripts included in this thesis. The contribution of other co-authors, including postdoctoral fellows and undergraduate students from the Dr. Jin's lab, is acknowledged above.

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In addition, I have obtained the permission from each of my collaborators to present our collaborative work in the thesis.

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Abbreviations

AhR: Aryl hydrocarbon receptor

ATM: Ataxia telangiectasia, mutated

β – Gase: β -glucuronidase

BPA: Bisphenol A

BPACB: Creatinine-Based BPA

CCA: Cell cycle arrest

Cdk2: Cyclin dependant kinase 2

Ccnd2: Cyclin D2

Ccne1: Cyclin E1

CYP1B1: Cytochrome p450 1B1

DDIT4: DNA damage inducible transcription factor 4

DMEM: Dulbecco's Modified Eagle's Medium

DMSO: Dimethylsulfoxide

DSB: double strand break

E2: 17 β -estradiol

EGFR: Epidermal growth factor receptor

ER: Estrogen receptor

ET-1: Endothelin-1

FDA: Food and drug administration

G15: (3aS, 4R, 9bR)-4-(6-Bromo-1, 3-benzodioxol-5-yl)-3a, 4, 5,9b-3H-cyclopenta[c]quinolone

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

GC-MS: Gas chromatograph-mass spectrometry

GDF-15: Growth differentiation factor-15

GPR30: G-protein coupled estrogen receptor 30

H2A.X: H2A histone family, member X

γ - H2A.X: gamma H2A.X

H3: Histone 3

HFLF: Human fetal lung fibroblast

HO-1: Heme oxygenase

HRP: Horseradish peroxidase

HSD17b14 : 17-beta hydroxysteroid dehydrogenase type 14

IL-6 : Interleukin-6

IP10: Interferon gamma induced protein 10

IPA: Ingenuity Pathway Analysis

kDa: kiloDalton

LC3B: Microtubule-associated proteins 1A/1B light chains 3B

MPP: 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole

mRNA: Messenger RNA

MAPK: Mitogen-activated protein kinase

MTT: Methylthiazolyldiphenyl-tetrazolium bromide

MW: Molecular weight

NTP: National toxicology program

NF- κ B: Nuclear factor kappa-light-chain-enhancer of activated B cells

NQO1: NAD(P)H quinone dehydrogenase 1

Nrf1: Nuclear respiratory factor 1

Nrf2: Nuclear respiratory factor 2

p53: Tumor suppressor p53

PBS: Phosphate buffered saline

PC: Polycarbonate

PCR - Polymerase chain reaction

PHTPP: 4-[2-phenyl-5,7-bis(trifluoromethyl) pyrazolo[1,5-a]pyrimidin-3-yl]phenol

qPCR: Quantitative PCR

REDD1: Regulated in development and DNA damage responses 1

RNA: Ribonucleic acid

ROS: Reactive oxygen species

SA- β -gal: Senescence-associated- β -galactosidase

SULT: Sulfotransferase

TGF- β 1: Transforming growth factor beta 1

TBARS: Thiobarbituric acid reactive substances

UGT: UDP – glucuronyltransferase

UDP: Uridine 5'-diphosphate

UGT2B15: UDP-glucuronosyltransferase 2B15

1 Chapter: General Introduction

Bisphenol A (BPA) is widely used in the manufacture of products containing polycarbonate and epoxy resin including food and drink containers. Human exposure to BPA occurs mainly through ingestion of contaminated food and drink, such as canned food (Kang et al., 2006, Gao et al., 2016, Cao et al., 2015), although thermos paper, dust, cosmetics and BPA manufacturing are other potential exposure sources (Russo et al., 2017, Gao et al., 2016, Hines et al., 2017). More than 90% of the U.S. population has detectable levels of BPA in urine samples, with the highest concentration found in children, followed by adolescents (Calafat et al., 2005, Calafat et al., 2008). In both humans and animals, BPA is metabolized mainly by uridine 5'-diphosphoglucuronosyltransferases (UGTs) into glucuronide (Hanioka et al., 2008, Yokota et al., 1999). However, maternal exposure to BPA can lead to fetal exposure to BPA via placental transfer of maternal BPA-glucuronide conjugate that can be deconjugated to form free BPA by β -glucuronidase in the fetus (Nishikawa et al., 2010; Takahashi and Oishi, 2000). In fact, BPA has been detected in human placenta, fetal blood, amniotic fluid, and fetal liver at a wide range of concentration up to ng/g (Vandenberg et al., 2012; Cao et al., 2012; Edlow et al., 2012; Ikezuki et al., 2002, Nahar et al., 2015). With much lower expression of metabolic enzymes, the fetus has limited capability to detoxify xenobiotics comparing with the adult. As compared to fetal liver, fetal lung has much lower expression of UGTs (2B7/2B15 in human, 2B1 in rat), but higher expression of β -glucuronidase (Lucia et al., 1977), higher surface to volume ratio, and exposure through both cord blood and amniotic fluid, which renders it more susceptible to BPA toxicity

(Ekström et al., 2013, Tokota et al., 1999). Recent studies revealed that fetal exposure to BPA may delay lung maturation and increase childhood susceptibility to respiratory disease in both animal model and human subjects (Spanier et al., 2012, Hijazi et al., 2015). However, the underlying mechanisms remain to be elucidated.

BPA is considered as a weak estrogen, and can act through both estrogen receptor (ER)-dependent and ER-independent pathways (Yoon et al., 2014). In a previous study with human fetal lung fibroblasts (HFLF), a cell type important in regulating fetal lung differentiation and development, we found that BPA at a concentration and duration of exposure used caused no change in cell viability, but decreased release of two important regulators of embryo development and immune response, endothelin-1 (ET-1) and interleukin-6 (IL-6), suppressed phosphorylation of nuclear factor kappa b (NFκB), while also increased cytoplasmic expression of ERβ and nuclear expression of G-protein coupled estrogen receptor 30 (GPR30) (Mahemuti et al., 2016). However, ERα, ERβ and GPR30 antagonists could not block the effect of BPA on ET-1 release. The exact molecular pathways leading to these effects of BPA remain to be investigated.

In addition to BPA glucuronide, BPA sulfate and ortho-quinone metabolites also occur *in vivo*, especially when high dose of BPA is used (Ye et al. 2011, Adkinson and Roy, 1995). Bisphenol o-quinone, potentially formed via oxidation of BPA by cytochrome P450 (CYP) enzymes, can bind DNA and form DNA adducts (Adkinson and Roy, 1995). CYP1B1 is a major CYP450 enzyme expressed in human extrahepatic tissues including lung (Bièche et al., 2007). CYP1B1 expression in fetal lung is increased by maternal cigarette smoking (Vyhlidal et al., 2013). In fact, fetal CYP1B1 was shown to bioactivate dibenzo (a,l) pyrene leading to transplacental carcinogenesis in

mice (Castro et al., 2008). It was demonstrated that deletion of CYP1B1 (Gao et al., 2005) and microsomal epoxide hydrolase (mEH) (Gao et al., 2007) protects mice against spleen cell immunosuppression produced by 7,12-dimethylbenz[a]anthracene (DMBA) *in vivo*. DMBA-3,4-dihydrodiol-1,2-epoxide, the metabolic product of DMBA by CYP1B1 and mEH, was found to be responsible for immunosuppression. More interestingly, DMBA-induced splenic immunosuppression is p53-dependent, but not aryl hydrocarbon receptor (AhR)-dependent, which is associated with activation of ataxia telangiectasia, mutated (ATM) and occurs in the absence of cytotoxicity (Gao et al., 2008).

CYP1B1 is capable of oxidizing catechol estrogens to their respective quinones that can further react with DNA (Zhang et al., 2007). It is reasonable to speculate that BPA may be oxidized to quinones by CYP1B1 in human fetal lung cells where CYP1B1 is constitutively expressed or induced by BPA exposure, which can increase ROS production, cause DNA damage/repair and cell cycle arrest, which then may lead to immunosuppression. Benzo[a]pyrene increases production of reactive oxygen species (ROS) in immortalized human bronchial epithelial cells, which is associated with increased DNA adduct formation, CYP1B1 mRNA, phosphorylation of p53 at Ser15, and cell cycle arrest at G2/M (Zhu et al., 2014). In fact, both *in vivo* and *in vitro* studies have demonstrated that BPA at a range of concentrations induces oxidative stress shown as increased ROS, malendialdehyde MDA, 8-hydroxydeoxyguanosine (8-OHDG) levels, as reviewed by Gassman (2017), however, the sources and pathways involved were not identified. In contrast, some studies showed no effects of BPA on ROS production (Berger et al., 2016), while others revealed antioxidant activities of BPA (Chepelev et al., 2013; Ponniah et al., 2015). This leads one to speculate that differences in CYP enzyme

expression may be one of the factors contributing to the discrepancy. It is not surprising that BPA induced greater genotoxicity in human renal epithelial cells, ACHN than human hepatoma cells, HepG2, with ACHN showing greater increase in DNA double strand breaks (DSB) evaluated by phosphorylated histone H2AX (γ -H2AX) and also higher expression of CYP1B1 than HepG2 (Audebert et al., 2011). CYP1B1 is extensively studied in the lung tissue and known as important regulator of lung development (Shimada et al., 1997; Dinu et al.2016; Uppstad et al., 2010). There is a lack of information as to the effect of BPA on CYP1B1 expression in fetal lung cells. With increasing evidence suggesting adverse effects of prenatal exposure to BPA on fetal lung development, the molecular mechanisms underlying the effects of BPA warrants in depth investigation.

Therefore, the aims of this thesis project were to investigate the effects and mechanisms of action of BPA in fetal lung development using Human Fetal Lung Fibroblast (HFLF) as an *in vitro* model.

There is no published data to show which genes are involved and which pathways and functions are affected by BPA in human fetal lung. To reveal the key molecular pathways involved in the developmental effects of BPA in human fetal lung and their potential implications in the reported link between prenatal exposure to BPA and increased sensitivity to childhood respiratory diseases, we examined the gene expression profiles in HFLF treated with non-cytotoxic concentrations of BPA. Using Ingenuity Pathway Analysis (IPA), we identified molecular pathways, gene networks, and key molecules that were affected by BPA in HFLF. Using multiple genomic and proteomic tools, we confirmed these changes at both gene and protein levels. Our data suggest that

BPA exposure increases CYP1B1 protein expression and release of endogenous estradiol, which is associated with increased ROS production, upregulation of genes and/or proteins in steroidogenesis, and activation of Nrf2-regulated stress response pathway, in addition to activation of ATM-p53 signaling pathway that parallels with increased cell cycle arrest at G1 phase, senescence, autophagy in HFLF. These findings are consistent with our previous finding that BPA induces immune suppression under the same condition.

The findings of my PhD research will provide some mechanistic explanation for the reported effects of prenatal exposure to BPA on fetal development and risk of childhood asthma.

1.1 History of BPA

Bisphenol A (2,2-bis (4-hydroxyphenyl) propane) was discovered in 1891 by Russian chemist Aleksandr Dianin. In the early 1930s, the British biochemist Edward Charles Dodds tested BPA as an artificial estrogen, but found to be much less effective in estrogenic activity than estradiol (Dodds and Lawson, 1936; Dodds et al., 1938). However, the first mention of BPA was made in a scientific paper in 1905 by Thomas Zincke of the University of Marburg, Germany (Zincke, 1905). BPA has been used since the 1950s to harden polycarbonate (PC) plastics and make epoxy resin, which is contained in the lining of food and beverage containers (Walsh, 2010). In 1960s, the FDA approved the use of BPA in consumer products like PC water bottles, baby bottles, food containers and epoxy linings for metal-based food and beverage cans. In 1992, Dr. David Feldman of Stanford University discovers BPA has migrated from his PC test tubes into a

test specimen and appears to be mimicking estrogen (Krishnan et al., 1993). In 1997, adverse effects on the endocrine system of low-dose BPA exposure in laboratory animals were first proposed (Nagel et al., 1997). It was at this time that bans on the use of BPA were initiated in some countries. In 1998, due to consumer concern about the toxic effects of BPA, Japanese industries voluntarily reduced the use of BPA between 1998 and 2003. In 2009, Canada became the first country to take regulatory action against BPA. Canada announced that it was moving forward with proposed regulations to prohibit the advertisement, sale and importation of polycarbonate plastic baby bottles that contain BPA, to reduce newborn and infant exposure to this substance (Canada, 2009).

The FDA states on its website that it "supports the industry's actions to stop producing BPA-containing baby bottles and infant feeding cups for the U.S. market, along with facilitating the development of alternatives to BPA for the linings of infant formula cans (Szabo L, 2008). Between the Health Canada announcement and the NTP findings, a majority of retailers and bottle manufacturers have switched from PC to alternatives.

1.2 Production and use of BPA

BPA is used primarily to make plastics, and products using BPA-based plastics have been in commercial use since 1957 (BPA INFO, 2002). World production capacity of this compound was 1 million tons in the 1980s (Fiege et al., 2002). At least 3.6 million tonnes (8 billion pounds) of BPA are used by manufacturers yearly. It is a key monomer in production of epoxy resins (Replogle, 2009) and in the most common form of PC

plastic. BPA production volume in a U.S. estimated at 2.4 billion pounds in 2007, and an estimated value of almost \$2 billion. It is a monomer used in manufacturing most or all polycarbonate plastics, the majority of epoxy resins, and certain other products such as flame retardants (Mannsville, 2008a). Epoxy resins containing BPA are used as coatings on the inside of almost all food and beverage cans (Erickson 2008). BPA is a preferred color developer in carbonless copy paper and thermal point of sale receipt paper (Raloff, 2009).

1.3 Human exposure to BPA

Various studies have examined specific products or activities for potential for human exposure to BPA, including canned foods, microwave containers (Mariscal-Arcas et al., 2009), soft drinks (Cao et al., 2009), polycarbonate bottles including baby bottles (Carwile et al., 2009), smoking, alcohol consumption (He et al., 2009), medical procedures/products including cardiopulmonary bypass and hemodialysis (Calafat et al., 2009), dental sealants, and plastic tubing (Vandenberg et al., 2007). Although, most human exposure appears comes from food packaging materials (Willhite, 2008).

The degree to which BPA leaches from PC bottles into liquid may depend more on the temperature of the liquid or bottle, than the age of the container. BPA can also be found in breast milk (Mendonca et al., 2012). A study found higher urinary concentrations in young children than in adults (Edginton et al., 2009). Infants fed with liquid formula are among the most exposed, and those fed formula from PC bottles can consume up to 13 µg/kg/day (EFSA, 2006). In US and Canada, BPA has been found in

infant liquid formula in concentrations varying from 0.48 to 11 ng/g (Ackerman et al., 2010). A Canadian total diet study estimated dietary intakes of 0.33 µg/kg bw/d in children <1 month of age compared with 0.075 µg/kg bw/d in all Canadians (Cao et al., 2011). Human exposure to BPA can also occur from handling thermal paper receipts (Biedermann et al., 2010), or exposure to household dust (Wilson et al., 2007; Loganathan and Kannan, 2011). International dietary exposure estimates comprised of BPA concentrations in food and consumption data indicate that dietary exposure to BPA is orders of magnitude greater than that from non-food sources (WHO, 2010; Morgan et al., 2011). BPA is rapidly transferred to the fetus after maternal uptake (Takahashi and Oishi, 2000). BPA was found to accumulate in the human maternal-fetal-placental unit (Schonfelder et al., 2000). Some studies suggest that repeated maternal exposure could elevate BPA exposure of the fetus *in utero* or in the newborn (Durando et al., 2007).

Cao et al (2012) found that the average BPA concentrations in human placental samples were 12.6 ng/g for free BPA, 17.2 ng/g for BPA-glucuronide (BPA-glu), and 30.2 ng/g for total BPA. Other metabolites such as BPA sulphate and hydroxylated BPA made up the 0.4 ng/g residual concentration. The highest concentrations in placental samples were 165 ng/g for free BPA, 178 ng/g for BPA-glu, 280 ng/g for total BPA. In general, samples with higher levels of BPA-glu had higher levels of free BPA. Fetal age was observed to have a significant effect on BPA-glu levels in placental samples, but not on free or total BPA. The percentages of free BPA relative to total BPA, for the placental samples, varied considerably from 4.2 to 100%, suggesting that the ability of maternal liver and/or the placenta to conjugate BPA is highly variable during early to mid-gestation. The average concentrations in fetal liver samples were 9.02 ng/g for free BPA,

19.1 ng/g for BPA-glu, and 25.8 ng/g for total BPA. The highest concentrations in fetal liver samples were 37.7 ng/g for free BPA, 93.9 ng/g for BPA-glu, and 123 ng/g for total BPA. The percentages of free BPA level relative to total BPA for all fetal liver samples varied from 12.4 to 99.1%, indicating extensive variability in the ability of the human fetal-placental unit to BPA-glu. Ikezuki et al (2002) determined BPA concentrations in human biological fluids and found that BPA was present at approximately 1-2 ng/mL in serum and follicular fluid, as well as in fetal serum and full-term amniotic fluid, and at 8.3 ng/mL in amniotic fluid of 15-18 weeks gestational age. Edlow et al (2012) detected BPA in second trimester amniotic fluid samples with total BPA levels ranging from non-detectable to 0.75 ng/mL, and free BPA from 0.31 to 0.43 ng/mL. BPA was also detected in third trimester in amniotic fluid samples. When detected, free BPA comprised 83 and 91% of total BPA in second and third trimester amniotic fluid respectively.

The National Toxicology Program determined that the lowest adverse effect level (LOAEL) for BPA in laboratory animals is 1,000 parts per million (ppm), equivalent to 50 milligrams of BPA per kilogram of body weight per day (50 mg/kg/d) (NTP, 1982). This study became the basis for EPA's 1988 safety standard which has remained in place for decades, but has not been in agreement with scores of low-dose BPA toxicity studies published in the interim.

The U.S. EPA, which sets a safety standard (reference dose) for BPA based on crude, high-dose BPA studies which showed reduced body weight of exposed animals, establishing the standard in 1988 and reaffirmed it in 1993. The "safe" exposure level established by EPA, at 50 μ g/kg/d, is 1,000 times lower than amounts found to affect the growth of animals in high-dose industry studies (EPA, 1993).

1.4 Pharmacokinetics of BPA

1) Chemical Properties of BPA

Although the estrogenic properties of BPA were reported as early as 1936 by Dodds and Lawson, it was first synthesized in 1905. It is obtained by the condensation of phenol with acetone in the presence of a strongly acidic ion-exchange resin, in the gel form, as a catalyst. BPA is a less water-soluble compound (120 mg/L) and it dissociates in an alkaline environment (pKa 9.9–11.3). The chemical formula of BPA is $C_{15}H_{16}O_2$. Its molecular weight is 228.29 g/mol. The BPA molecules are bound with an ester bond, which is sensitive to high temperatures, as well as acidic and basic conditions, and can result in the leaking of BPA under these conditions into the food from the linings of food containers.

BPA has two large phenyl groups, as well as two electron-rich hydroxyl (or alcohol) groups and two methyl groups. BPA in its free form is somewhat lipophilic and tends to associate with lipids. However, BPA is made more water-soluble through conjugation to BPA-glu. The free form is typically found in adipose tissue and in breast milk. The hydrophilic form is usually seen in urine and excrement. The log P (octanol-water partition coefficient) value for BPA is 3.32. In addition, BPA has a low vapor pressure of about 5.00 to 5.32 Pascals (Groshart et al., 2001). These characteristics of BPA give it the propensity to partition in water, and the rate of evaporation from soil and water is low. Given this information, BPA has a moderate potential of bioaccumulation and is not readily biodegradable (Groshart et al., 2001).

2) Metabolism of BPA

Many molecules require metabolic bioactivation to express their toxic potential. Conversely, others are efficiently inactivated through metabolic processes. Therefore, the capacity of cells or biological systems to metabolize BPA molecules determines the consequence of BPA exposure.

Studies show that BPA does not bioaccumulate and has a very short half-life in humans with elimination of the conjugated BPA in about 6 hours in the urine (Völkel et al., 2002). Whereas BPA possesses aqueous solubility of 0.5–1.3 mmol/L, its metabolism to more water-soluble conjugates is extensive. Metabolism of BPA is dominated by Phase II conjugation reactions in the gastrointestinal tract and liver (Hanioka et al., 2008). Conversion of BPA to its primary Phase II metabolite, which the monoglucuronide conjugate (BPA-glu), is critical to prevention of its binding to the estrogen receptors (ERs) (Matthews et al., 2001) (Fig 1.1).

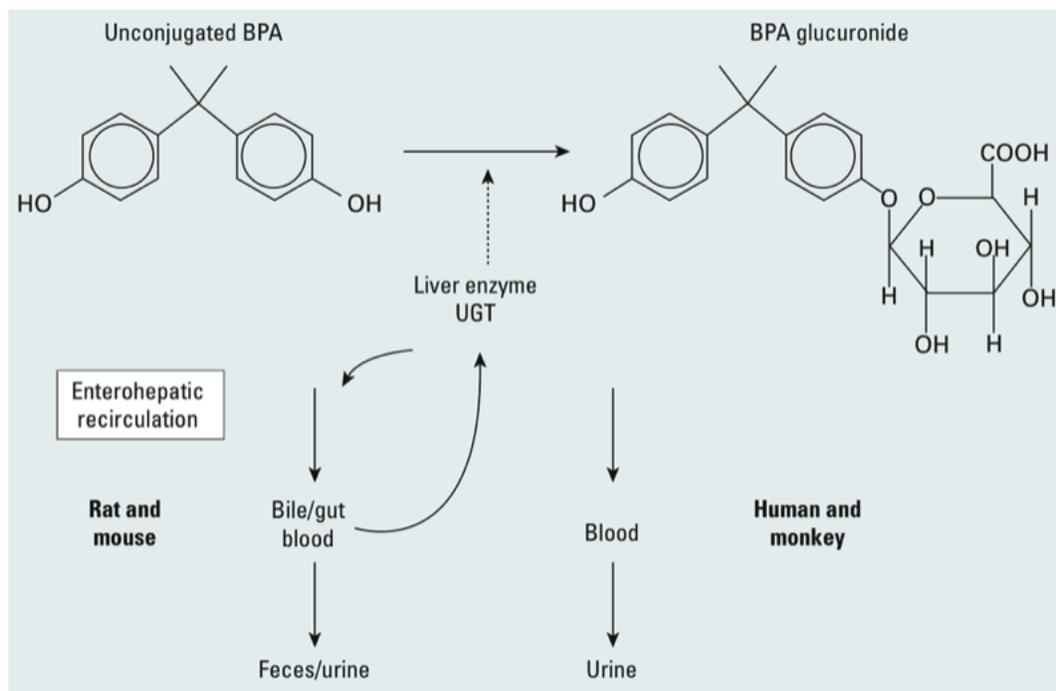


Fig. 1.1 The Metabolism of BPA. The glucuronidation of BPA in the liver and the route of elimination of unconjugated BPA from serum in rodents and primates after initial absorption from the gut and transport to the liver (Taylor et al., 2011. Reproduced with permission from Environmental Health Perspectives).

Other minor metabolites of BPA, including BPA sulfate and BPA-3,4-quinone, have also been observed (Zalko et al., 2003). The quantity of quinone metabolites in placenta, fetus, amniotic fluids were 1 to 8% and BPA sulfate metabolites in these tissues were 3 to 20% (Zalko et al., 2003). BPA-glu is devoid of estrogenic activity (Matthews et al., 2001).

In humans, glucuronidation was described to be catalyzed by the uridine 5'-diphospho (UDP)-glucuronosyltransferase (UGT2B15) (Hanioka et al., 2008). Sulfation

is mediated most probably by the sulfotransferase isoform SULT1A1, as SULT1A1 preferentially conjugates phenols (Campbell et al., 1987a, 1987b).

The hepatic biotransformation of BPA depends on phase I oxidation/reduction involving glutathione and phase II glucuronidation, and sulfate conjugation (Kurebayashi et al., 2003). Despite proper hepatic biotransformation of BPA, the accumulation of BPA in body reservoirs may set the stage for adverse effects. Healthy humans exposed to BPA appear to have an accumulated body burden of BPA and monitoring studies that measure urinary BPA have shown that it stored in lipid reservoirs (Galloway et al., 2010). Galloway et al. demonstrated that higher urinary excretion of BPA was associated with increased waist circumference, BMI (body mass index) and body weight, which is consistent with the fact that BPA is lipophilic with a logP values of 3.32, and it may partition to lipid-rich tissues, as its half-lives is significantly longer than previous predictions of 6 hrs (Stahlhut et al., 2009).

UGT isoforms are localized in the endoplasmic reticulum and catalyze the conversion of small lipophilic compounds, such as BPA, to charged, water-soluble glucuronides (Tukey & Strassburg. 2000). Hepatocytes from mice, rats and humans (Pritchett et al., 2002) and microsomes from rat and human liver and intestine have been shown to catalyze BPA glucuronidation (Hanioka et al., 2008). *In vitro* studies have shown that numerous UGT isoforms catalyze glucuronidation of BPA. UGT2B15 appears to be the major isoform associated with BPA glucuronidation in human liver, although 2B7 is another liver-specific isoform (Hanioka et al., 2008). Similarly, UGT1A9, 1A1 and 1A3 are expressed in both human gastrointestinal tract and liver, and UGT1A8 is expressed only in the gastrointestinal tract (Tukey & Strassburg. 2000).

Furthermore, the ontogeny of UGT isoforms during human development is consistent with efficient glucuronidation of BPA throughout postnatal life stages (i.e. whereas hepatic UGT 1A9 increases with postnatal age, 2B15, 1A1 and 2B7 do not change). In human neonates, some metabolic pathways, e.g. glucuronidation (2-5-fold lower in premature neonates), and some excretory functions, e.g. glomerular filtration (1.7-fold lower), have a lower efficiency compared to adults; these functions reach their full capacities only within one and seven months after birth, respectively (Benedetti et al., 2007; Dorne, 2007). The presence of UGT isoforms has been observed in the fetal liver of rats, humans, (Coughtrie et al., 1988) and monkeys (Leakey et al., 1983), even though at levels below those in neonates and adults. For example, UGT1A1 appears to be absent in human fetal liver. UGT1A6 is present in human fetal liver at levels 1–10% of those in adult human liver. UGT1A3 is present in fetal and neonatal liver at levels 30% of those observed in adults. UGT2B7 is present in fetal liver at levels 10–20% of those of adults and increases to adult levels after 2–3 months of age. UGT2B17 is expressed in human fetal liver at levels less than 10% of those of adults (Hines, 2008). These observations suggest that the human fetus can detoxify BPA through glucuronidation, but at much lower levels than in neonates and adults.

Sulfonation of BPA has been investigated *in vitro* and is catalyzed by human sulfotransferase (SULT) isoform 1A1, with lesser activities for 2A1 and 1E1 (Nishiyama et al., 2002). The human SULT expression does not change during the postnatal period for SULT 2A1 or SULT 1A1 and decreases for SULT 1E1 (Duanmu et al., 2006). Several tissues, including human liver and kidney, contain β -glucuronidase in the membranes of lysosomes and the endoplasmic reticulum (Sperker et al., 1997).

It has been proposed that β -glucuronidase activity in tissues, especially placenta, could reverse the putative detoxification of BPA by UGTs at the tissue level (Ginsberg & Rice, 2009). Many molecules require higher metabolic bioactivation concentrations to express their toxic potential. As compared to adult liver UGT, SULT and steroid sulfatase genes exhibited lower expression whereas β -glucuronidase mRNA expression remained unchanged in the fetal tissues (Ginsberg & Rice, 2009). This study provides evidence that there is considerable exposure to BPA during human pregnancy and that the capacity for BPA metabolism is different in the human fetal liver from adult liver.

In the fetus with no or limited expression of the enzymes required to metabolize BPA, free BPA may accumulate to concentrations that were associated with abnormal liver function (Lee et al., 2013). Animal studies demonstrate that BPA has the ability to reduce cytochrome P450 (CYP) enzymes that are critical for hepatic phase I and II biotransformation (Hassan et al., 2012). Schmidt et al (2013) confirmed that BPA and its structural analogs form hydroxylated metabolites and electrophilic species during bioactivation in human liver. Metabolites of BPA may have stronger activities than the parent compound itself (Nakamura et al., 2011, Yoshihara et al., 2004).

A number of BPA metabolites have been identified including BPA-glu, several double conjugates, and conjugated methoxylated compounds, demonstrating the formation of potentially reactive intermediates. Recently, it was reported that BPA inhibits human hepatic CYP-mediated drug-metabolizing activities including aminopyrine N-demethylation, especially by CYP2C8 and CYP2C19 (Niwa et al., 2000). These CYP monooxygenases play a crucial role in the liver and various other tissues and are involved with oxidation of organic substances and the bioactivation of drugs and

xenoestrogens (Wang et al., 2011). CYP activity is necessary for the conversion of xenoestrogens into inactive metabolites that are both non-inflammatory and biologically inactive.

However, environmental xenoestrogens such as BPA also have the potential to be metabolized into more reactive and proinflammatory metabolites (Namazi et al., 2009). It is known that BPA can be converted to monoquinones and bisquinones by chemical radical oxidants (Yoshida et al., 2001) or by enzymatic oxidation (Yoshida et al., 2002). In rats, BPA is metabolized to DNA-reactive bisphenol-o-quinone through 5-hydroxybisphenol and bisphenol semiquinone (Atkinson et al., 1995).

Recently, the internal concentrations of free BPA and conjugated BPA were measured by Nahar et al (2013) and the gene expression of biotransformation enzymes specific for BPA metabolism were evaluated in 50 first- and second-trimester human fetal liver samples. Both free BPA and conjugated BPA concentrations show wide variations, with free BPA exhibiting three times higher concentrations than conjugated BPA concentrations. As compared to adult liver controls, UDP-glucuronyltransferase, sulfotransferase, and steroid sulfatase genes exhibited lower expression whereas β -glucuronidase mRNA expression remained unchanged in the fetal tissues (Nishikawa et al., 2010) (Fig 1.2). These suggest that fetal organs may be more vulnerable to BPA intoxication than adult organs.

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 fold higher BPA concentrations than cells of other fetal tissues.

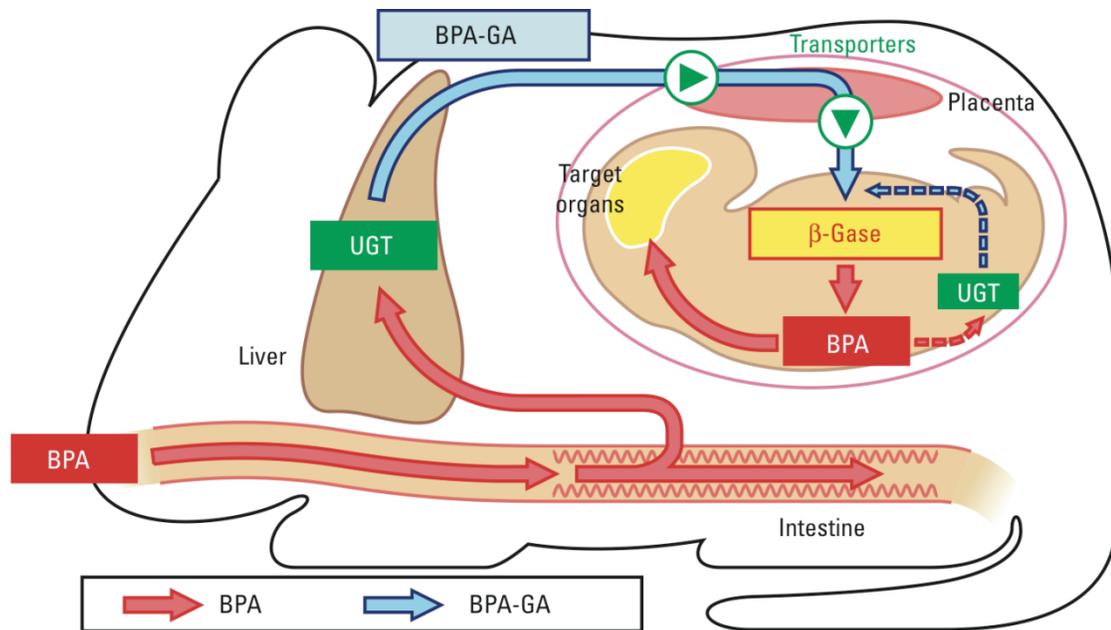


Fig. 1.2 The fetal bio-transformation of Bisphenol A (BPA). BPA, converted to BPA glu in the liver by the enzyme UGT (uridine 5'-diphospho-glucuronosyltransferase), is subsequently transferred from maternal blood to the placenta, and then to the fetus, where it is then deconjugated by β -glucuronidase (β - Gase) to free BPA. The free BPA may remain in the fetus because of a deficiency in fetal UGT activities. (Nishikawa et al., 2010). Reproduced with permission from Environmental Health Perspectives).

1.5 Developmental Toxicity of BPA

Biomonitoring studies have detected BPA at nanomolar concentrations, in human body fluids (such as serum, milk, saliva and urine) collected from all over the world (Vandenberg et al., 2007; Geens et al., 2012). Importantly, BPA has been shown to

accumulate in human body tissues; thus, exposure levels are much higher than previously thought (Michałowicz, 2014). BPA exposure causes meiotic aneuploidy in the female mouse (Hunt et al., 2003). Exposure of pregnant CF-1 mice to 2.4 µg/kg BPA on days 11 to 17 of gestation increased the rate of postnatal growth in males and females and also advanced the timing of puberty in females (Howdeshell et al., 1999). Fetal exposure to BPA increased the risk of developing childhood asthma in experimental animals (Midoro-Horiuti et al., 2009). It was also shown that maternal exposure to BPA affected neurobehavioral development in mice (Palanza et al., 2008). Tharp et al. (2012) assessed the effects of BPA on fetal mammary gland development in nonhuman primates. Pregnant rhesus monkeys were fed 400 µg BPA/kg bw/day BPA from gestational day 100 to term, which resulted in 0.68 ± 0.312 ng/mL of unconjugated BPA in maternal serum, a level comparable to that found in humans. Although BPA did not affect ER expression, it significantly increased density of mammary buds and promoted mammary gland development. However, the underlying molecular mechanisms remain to be elucidated. The effects of BPA on mammary gland development in females and early onset of puberty were also reported (Richter et al., 2007). Gioiosa et al (2013) investigated the behavioral effects of developmental exposure to a low dose of BPA with respect to the timing of the exposure, maternal environment, sex and age at testing. At both juvenile and adulthood testing ages, pre- and post-natally exposed females showed evidence of increased anxiety and were less prone to explore a novel environment relative to the control females, showing a behavioral profile more similar to control males than females. Snijder et al. (2013) examined associations between urinary concentrations of creatinine-based BPA (BPACB) and intrauterine growth in 219 Dutch women. Among 80

women with three BPA measurements, women with BPACB > 4.22 µg/g creatinine had lower growth rates for fetal weight and head circumference than did women with BPACB < 1.54 µg/g creatine. Chou et al. (2011) observed an elevated risk of lower birth weight, smaller size for gestational age, and adverse action of high leptin and low adiponectin secretion in male neonates in the highest quartile of maternal BPA exposure in women from Taiwan.

1.6 Immunotoxicity of BPA

BPA can modulate the immune activity by its effect on estrogenic receptors (ER), aryl hydrocarbon receptor (AhR) and peroxisome proliferator-activated receptor (PPAR) (Rogers et al., 2013). Moreover, it was shown that BPA is capable of both stimulating and inhibiting the activity of immune system cells. Youn et al (2002) showed that T-lymphocytes, isolated from mice previously treated with BPA, produced an increased amount of interferon- γ and a decreased amount of interleukin 4 (IL-4), whereas Lee and Lim (2010) observed that BPA increased the levels of IL-4 and IL-8 in mouse T-lymphocytes (*in vitro* study). It was also found that exposure of mouse splenic lymphocytes to 1 µM BPA concentration inhibited mitogenesis of these cells, particularly B-lymphocytes (Sakazaki et al., 2002). In another study, it was shown that BPA modulated proliferation of B cells as well as production of some cytokines and antibodies (Wetherill et al., 2007). Similarly, Goto et al (2007) noticed that mice treated with BPA produced lymphocytes with higher amounts of immunoglobulin A (IgA) and IgG2a. In addition, BPA was shown to affect non-specific immune defenses. Sugita-Konishi et al

(2003) observed that BPA depleted neutrophils activity and inhibited IL-6 formation in mice infected with non-pathogenic *Escherichia coli*. In a recent study, Roy et al (2012) showed that offspring of male mice exposed to BPA were slightly susceptible to infection by influenza A virus, which was connected with some modulation of their innate immunity. But, they did not observe impairment by BPA of antiviral adaptive immune responses, which are critical for virus clearance and survival after influenza virus infection. In an epidemiological study conducted within National Health and Nutrition Examination Survey (USA) it was shown that, in the groups below the age of 18, higher urinary BPA levels were associated with higher cytomegalovirus (CMV) antibody titers, and in the group above 18 years of age, lower BPA urinary levels were correlated with higher CMV antibody titers, which suggested that BPA could negatively affect human immune function (Clayton et al., 2011).

Ritcher et al. (2007) found an alteration of immune functions in male offspring of pregnant mice fed with 30 µg/kg/day of BPA. Recently, it was reported that maternal exposure to BPA enhanced airway lymphocytic and lung inflammation in mucosal sensitized female offspring of mice (Stephen et al., 2012). Spanier et al (2012) found that prenatal exposure to BPA was associated with increased odds of wheeze (whistling sound produced in the respiratory airways during breathing) at 6 months of age, but the effects did not persist over time. They found that the window of vulnerability was only during early pregnancy (16 weeks gestation). Spanier et al. (2014) also found maternal urinary BPA concentration was marginally associated with a significant increase in the odds of wheezing and abnormal lung function during the first 5 years of age, but child urinary BPA concentrations were not. However, the influence of prenatal exposure did not

continue into adulthood. In a birth cohort study conducted in The Columbia Center for Children's Environmental Health, prenatal urinary BPA concentrations were found to be associated inversely with wheeze at an age of 5 years (Donohue et al., 2013). Urinary BPA concentrations at an age of 3 years were associated positively with wheeze at ages 5 to 6 years. BPA concentrations at an age of 7 years were associated with wheeze and BPA concentrations at ages 3, 5, and 7 years were associated with asthma.

1.7 BPA and Estrogen Receptors (ERs)

BPA is a xenoestrogen, a compound that disturbs function of endocrine system (Vandenberg et al., 2007; Flint et al., 2012). It was shown that BPA behaved similarly to the natural estrogen 17 β -estradiol. BPA induced DNA damage as 17 β -estradiol, but in the concentrations about one thousand times higher (10^{-6} to 10^{-4} M) in comparison to estradiol (10^{-7} M), and at concentrations that are not found in human blood (Iso et al., 2006). Initially, BPA was considered as a weak environmental estrogen, whose activities towards classical nuclear ER- α and ER- β receptors were over 1000 lower than 17- β -estradiol. Nevertheless, further investigations showed that BPA, even at very low (pico- and nanomolar) concentrations exerted multidirectional effects on physiological functions of cells and tissues by binding with receptors such as non classical membrane estrogen receptor (GPR30) present outside of the nucleus (Wetherill et al., 2007). Moreover, in some studies it was shown that most of hydroxylated BPA metabolites exerted stronger estrogenic activities than BPA itself (Alonso-Magdalena et al., 2012).

1.7.1 The estrogen receptor alpha (ER- α) and beta (ER- β) and estrogen-related receptors (ERRs)

The ERs, including ER- α and ER- β , are ligand-dependent transcription factors regulating numerous important physiological processes such as development, reproduction, behaviour, metabolism, bone homeostasis and regulation of the cardiovascular system (Gustafsson, 2003, Chakraborti et al., 2007, Heldring et al., 2007). ERs may form both homodimers and heterodimers, depending upon whether one or both receptors are present in the cell. ERs can activate gene expression by genomic mechanisms through binding to specific recognition sites in the regulatory regions of target genes or protein-protein interactions with other transcription factors like Sp1 or AP-1 (Jakacka et al., 2001). ERs can also mediate rapid signalling by non-genomic mechanisms through interacting with cytoplasmic molecules like mitogen activated protein kinase (Suzuki et al., 2008). BPA has been considered as an endocrine disruptor that can exert effects through ER-dependent mechanisms, which is defined as mechanisms involved in direct binding of BPA to the ER- α and ER- β , resulting in activation of down-stream gene transcription (Nilsson et al., 2001, Kuiper et al., 1998, Sheeler et al., 2000, Wetherill et al., 2007).

Increasing studies found possible connections between health issues and exposure to BPA during pregnancy and development. A 2007 study investigated the interaction between BPA and estrogen-related receptor γ (ERR- γ) (Matsushima et al., 2007). This orphan receptor (endogenous ligand unknown) behaves as a constitutive activator of transcription. BPA seems to bind strongly to ERR- γ (dissociation constant = 5.5 nM), but not to the ERs. BPA binding to ERR- γ preserves its basal constitutive activity. It can also

protect it from deactivation by the selective ER modulator 4-hydroxytamoxifen. This may be one of the mechanisms by which BPA acting as a xenoestrogen (Matsushima et al., 2007). Differential expression of ERR- γ in different parts of the body may account for variations in BPA effects. For instance, ERR- γ has been found in high concentrations in the placenta, explaining reports of high BPA accumulation in this tissue (Taketa et al., 2009).

1.7.2 G protein coupled receptor 30 (GPER-30)

More recent studies suggested that BPA can also act through ER-independent mechanisms, which are defined as mechanisms involved in direct impact on intracellular signal transduction pathways in which activation of various signaling molecules such as G protein-coupled receptors and receptor tyrosine kinases (Revankar et al., 2005). Nongenomic estrogen actions mediated through GPER may be susceptible to disruption by xenoestrogens because several of them including bisphenol A (BPA), 4-nonylphenol and chlordecone (Kepona) show relatively high binding affinities for human GPER (IC₅₀ of BPA for zebrafish GPER (53 nM)) and have GPER agonist activities, mimicking the stimulation of cAMP production by E₂ (Thomas et al., 2005). Bouskine et al. (2009) reported that BPA activated both cAMP-dependent protein kinase and cGMP-dependent protein kinase pathways and triggered a rapid (15 min) phosphorylation of the transcription factor cAMP response-element-binding protein (CREB) and the cell cycle regulator retinoblastoma protein (Rb). This non-genomic activation did not involve classical ERs because it could not be reversed by ICI 182780 (an ER antagonist) or

reproduced either by E2 or by diethylstilbestrol (a potent synthetic estrogen), which instead triggered a suppressive effect. This activation was reproduced only by E2 coupled to bovine serum albumin (BSA), which is unable to enter the cell. As with E2-BSA, BPA at very low concentrations (10^{-9} to 10^{-12} M) similar to those found in human fluids promoted JKT-1 cell proliferation through a G-protein-coupled non-classical membrane ER (GPER). Low environmentally-realistic concentrations (10 nM) of BPA and related alkylphenols (4-nonylphenol, tetrabromobisphenol A and tetrachlorobisphenol A) mimic the inhibitory actions of E2 and GPR 30 on the resumption of meiosis, delaying it for several hours. This inhibitory action of BPA on spontaneous maturation of denuded oocytes is not blocked by co-treatment with actinomycin D and by incubation with control rabbit IgG, but is blocked by incubation of denuded oocytes with the specific GPER antibody, indicating BPA's action is nongenomic and mediated through GPER (Fitzgerald et al., 2015).

1.8 BPA and Cell Cycle Modulators

Cell division consists of two consecutive processes, mainly characterized by DNA replication and segregation of replicated chromosomes into two separate cells. Originally, cell division was divided into two stages: mitosis (M), i.e. the process of nuclear division; and interphase, the interlude between two M phases (Vermeulen et al., 2003). Replication of DNA occurs in a specific part of the interphase called S phase. S phase is preceded by a gap called G1 during which the cell is preparing for DNA synthesis and is followed by a gap called G2 during which the cell prepares for mitosis. Cells in G1 can, before commitment to DNA replication, enter a resting state called G0. Cells in G0 account for

the major part of the non-growing, non-proliferating cells in the human body (Vermeulen et al., 2003).

1.8.1 Cyclin-dependent kinase (CDK) regulation

Key regulatory proteins are the cyclin-dependent kinases (CDK), a family of serine/threonine protein kinases that are activated at specific points of the cell cycle. Until now, nine CDK have been identified and, of these, five are active during the cell cycle, i.e. during G1 (CDK4, CDK6 and CDK2), S (CDK2), G2 and M (CDK1). When activated, CDK induce downstream processes by phosphorylating selected proteins (Morgan, 1995; Pines, 1995). CDK protein levels remain stable during the cell cycle, in contrast to their activating proteins, the cyclins. Cyclin protein levels rise and fall during the cell cycle and in this way they periodically activate CDK (Evans et al., 1983; Pines, 1991). The D types cyclins bind to CDKs are essential for entry in G1 (Sherr, 1994). Another G1 cyclin is cyclin E which associates with CDK2 to regulate progression from G1 into S phase (Ohtsubo et al., 1995). Cyclin A binds with CDK2 and this complex is required during S phase (Girard et al., 1991; Walker & Maller, 1991). In addition to cyclin binding, CDK activity is also regulated by phosphorylation on conserved threonine and tyrosine residues (Jeffrey et al., 1995).

1.8.2 CDK substrates

When CDK is active, target proteins become phosphorylated on CDK consensus sites, resulting in changes that are physiologically relevant for cell cycle progression. The most frequently studied target is the substrate of CDK4/6-cyclin D, which is the product of the retinoblastoma tumour suppressor gene (pRb) (Kato et al., 1993). DNA damage

checkpoints are positioned before the cell enters S phase (G1-S checkpoint) or after DNA replication (G2-M checkpoint) and there appears to be DNA damage checkpoints during S and M phases. At the G1/S checkpoint, cell cycle arrest induced by DNA damage is p53-dependent. Usually, the cellular level of p53 is low but DNA damage can lead to rapid induction of p53 activity (Levine, 1997). Different protein kinases ‘recognize’ DNA damage, e.g. ataxia-telangiectasia-mutated (ATM), ataxia and rad3 related (ATR). These kinases phosphorylate p53 in response to DNA damage (Siliciano et al., 1997).

1.8.3 BPA and cell cycle

Previous studies have established that BPA modulates cell cycle- and apoptosis-related proteins and genes in MCF7 cells (Mlynarcikova et al., 2013; Lee et al., 2012). BPA also shown to has negative impact on noncarcinogenic cells. Human normal breast cells HBL-100 at 1 μ M promoted highest cell proliferation rate, at 1 μ M, 0.1 μ M and 0.001 μ M dose, BPA promoted more cells to enter into G2/M phase and caused an increase in the expression of cyclinD1 and CDK4 (Wu et al., 2012). Liu et al (2013) found that rat midbrain (MB) cells treated with 10⁻¹²–10⁻⁴ M BPA and only 10⁻⁴ M BPA inhibited proliferation and differentiation and caused an explicit S phase and G2/M phase arrest in the cell cycle through the JNK, CREB and p53 signaling pathways. BPA at 100 μ g/ml but not at 1 μ g/ml and 10 μ g/ml significantly decreased follicle growth, alters cell cycle regulators and induces atresia in antral follicles, inhibiting follicle growth independently of genomic pathway by through up-regulating *Cdk4*, Cyclin D2 (*Ccne1*), and *p53* expression, and down-regulating Cyclin *E1* (*Ccnd2*) expression (Peretz et al., 2012).

The aryl hydrocarbon receptor (AhR) is a cell cycle mediator and a toxin sensor known to regulate cellular responses to oxidative stress, inflammation, and growth and development (Gonzalez and Fernandez- Salguero, 1998; Gu et al., 2000) by regulating cell-cycle genes such as nuclear factor-thyroid 2-related factor-2 (Nrf2) (Lu et al., 2011), p53 (TRP53; Reyes-Hernandez et al., 2010), retinoblastoma (RB1; Ge and Elferink, 1998), and NFκB (Vogel et al., 2007). Several studies reports a role for the AhR in cell cycle control (Ge and Elferink, 1998; Kolluri et al., 1999; Elizondo et al., 2000; Tohkin et al., 2000; Elferink et al., 2001). AhR can also activate genes involved in xenobiotic metabolism, such as CYP1A1, CYP1A2 and CYP1B1 (Gonzalez et al., 1984). Exposure *in utero* of murine embryo to BPA at 0.02, 2, 200, and 20,000 µg/kg/day increased AhR mRNA expression in the cerebra, cerebella, and gonads (testes and ovaries) of male and female mid- and late-developmental stage (14.5- and 18.5-days post coitum or dpc, respectively) embryos (Nishizawa et al. 2005).

1.9 BPA and DNA damage

BPA has been shown to induce DNA adduct formation both *in vitro* and *in vivo* due to formation of bisphenol-o-quinone in the presence of cytochrome P450 (Atkinson and Roy, 1995a, 1995b). Brieño-Enríquez et al. (2012) characterized the gene expression of human fetal oocytes in culture, as well as to evaluate the effect of BPA in cultured human oocytes. BPA up-regulated expressions of Spo11 (Initiator of meiotic double stranded breaks), H2AX, Blm (Bloom syndrome protein), Rpa /9replication protein A), ER-α, ER-ββ and ERR-γ genes, in addition to genes involved in double strand break

generation, signaling and repair. De Flora et al. (2011) examined the effects of BPA in male Sprague-Dawley rats and found that BPA up-regulated clusterin expression in atrophic prostate epithelial cells and induced lipid peroxidation and DNA fragmentation in spermatozoa. Significant levels of DNA adducts were formed in prostate cell lines (PNT1a nontumorigenic epithelial cells and PC3 metastatic carcinoma cells) treated with high 200 and 250 μ M BPA for 24 h. However, low dose 1nM BPA for 2 months treatment in PNT1a nontumorigenic epithelial cells, increased the DNA adducts formation was borderline to statistical significance ($p=0.08$).

Tiwari et al. (2012) determined genotoxic and mutagenic effects of BPA in male and female rats and observed that BPA exposure caused a significant increase in the frequency of micronucleus (MN) in polychromatic erythrocytes (PCEs), an increase in structural chromosome aberrations in bone marrow cells and DNA damage in blood lymphocytes, an increase in levels of 8-hydroxydeoxyguanosine in the plasma, an increase in lipid peroxidation in liver, and decreased glutathione levels in liver. Dobrzyńska and Radzikowska (2013) observed DNA strand breaks in spleen cells, whereas BPA induced DNA strand breaks in lymphocytes and in cells from spleen, kidneys, and lung and in germ cells of Pzh:SFIS male mice. Recent research has shown that BPA at 50 μ M and 100 μ M dose induced ROS production and DNA double strand breaks in insulinoma INS-1 cells and significantly increased DNA damage associated protein p53, p-Chk2 (T68) expression (Xin et al., 2014).

BPA has been investigated for its genotoxic and carcinogenic properties, but the results have been controversial. BPA does not exhibit a mutagenic activity at 10, 50 and 100 mg/kg body weight in single oral exposure and 10 mg/kg in repeated oral exposure

(for 5 days) in the bone marrow cells, the chromosomal aberration test, micronucleus assay results were negative, but its genotoxic potential was manifested in the form of achromatic lesion and c-mitotic effects (Naik and Vijayalaxmi, 2009). Izzotti et al (2009) reported that the formation of DNA adducts in both liver and mammary cells of female CD-1 mice receiving BPA in their drinking water (200 mg/kg body weight) for eight consecutive days. It can also be speculated from the results that the threshold concentration of BPA required for the formation of MN is much higher than that for the induction of c-mitotic effects. BPA induced DNA strand breaks in L5178Y mouse lymphoma cells (Lee et al., 2003) and induced aneuploidy and structural chromosomal aberrations in ER-positive MCF-7 and in CHO-K1 cells (Tayama et al., 2008). Similarly, Allard and Colaiacovo (2010) showed that exposure of nematode *Caenorhabditis elegans* to BPA, at concentrations similar to those used in mammalian models, causes chromosome damage and impaired meiotic double-strand break repair. The genotoxic potential of BPA was also presented by Lee et al. (2013b) who observed chromosomal aberrations and double-strand breaks in mutant chicken DT40 cell lines deficient in DNA repair pathways. Similarly, it was shown that BPA, by generating ROS, was capable of inducing oxidative modification of DNA bases (Kovacic, 2010). Opposite data were obtained by Audebert et al. (2011) who, based on the detection of histone H2A phosphorylation, evaluated genotoxicity of BPA on the human intestinal cell line LS174T, hepatoma cell line HepG2 and renal cell line ACHN. As a result, the authors did not observe any genotoxic potential of BPA. Some studies showed that damage to DNA may be caused by BPA metabolites. Atkinson and Roy (1995) showed that the BPA metabolite 3,4-quinone-BPA (BPA-quinone) was able to form covalent adducts with

DNA and Kolsek et al. (2012) showed that BPA-quinone behaved as a potential DNA mutagen because it formed adducts with deoxyguanosine.

1.10 BPA and Steroid Synthesis and Metabolism

Arase et al. (2011) examined effects of fetal exposure to low-dose BPA on the development of the prostate in mice. In the BPA treated mice, the urogenital sinus (UGS), E2 levels and CYP19A1 (cytochrome P450 aromatase) activity were significantly increased. The mRNAs of steroidogenic enzymes, Cyp19a1 and Cyp11a1, and the sex-determining gene, Nr5a1 (nuclear receptor subfamily 5 group a member 1), were up regulated specifically in the BPA-treated group. The up-regulation of mRNAs was observed in the mesenchymal component of the UGS as well as in the cerebellum, heart, kidney, and ovary but not in the testis.

1.11 Hypothesis and objectives of the study

As reviewed above, there has been an increased report on the developmental effects of BPA. Because BPA is an estrogen analog capable of interrupting signaling pathways of estrogen receptors (ERs), it is plausible that BPA may have similar effects to those estrogens. ER- α or ER- β are expressed in the lung in response to exposure to estrogens (Mollerup et al., 2002; Ciana et al., 2001; Marino et al., 2006). Estrogens also affect pulmonary alveolar development (Massaro and et al., 2007). Lung pathology often has signs of altered ER expression (Logginidu et al., 2000). In addition, BPA may also act through the membrane receptor G protein-coupled receptor (GPR30) and affect down-

stream messengers in the lung, which has been implicated in other lung pathologies (Zhang et al., 2014). Low environmentally-realistic concentrations (10 nM) of BPA and related alkylphenols (4-nonylphenol, tetrabromobisphenol A and tetrachlorobisphenol A) mimic the inhibitory actions of E2 and GPR 30 on the resumption of meiosis, delaying it for several hours. This inhibitory action of BPA on spontaneous maturation of denuded oocytes is not blocked by co-treatment with actinomycin D and by incubation with control rabbit IgG, but is blocked by incubation of denuded oocytes with the specific GPER antibody, indicating BPA's action is nongenomic and mediated through GPER (Fitzgerald et al., 2015).

BPA has been shown to induce DNA adduct formation both *in vitro* and *in vivo* due to formation of bisphenol-o-quinone in the presence of cytochrome P450 (Atkinson and Roy, 1995a, 1995b). Kolsek et al. (2012) showed that BPA-quinone behaved as a potential DNA mutagen because it formed adducts with deoxyguanosine. Dobrzyńska and Radzikowska (2013) observed DNA strand breaks in spleen cells, whereas BPA induced DNA strand breaks in lymphocytes and in cells from spleen, kidneys, and lung and in germ cells of Pzh:SFIS male mice. Recent research has shown that BPA at 50 μ M and 100 μ M concentration induced ROS production and DNA double strand breaks in insulinoma INS-1 cells and significantly increased DNA damage associated protein p53, p-Chk2 (T68) expression (Xin et al., 2014).

AhR is a soluble cytosolic protein in a complex with the chaperone proteins HSP90 (Perdew, 1988) and HSP23 (Kazlauskas et al, 1999). Upon ligand activation, the AhR translocates into the nucleus, dissociates from the hsp proteins, and binds to DNA response elements (known as a xenobiotic responsive element or XRE) with the ARNT

protein, a heterodimerization partner (Lees and Whitelaw, 1999), and activates the expression of AhR target genes, such as CYP1A1 and CYP1B1 (Kumar et al., 1999; Elferink et al., 2001; Beischlag et al., 2002; Wang and Hankinson, 2002). There is evidence showing that crosstalk between the ER and AhR leads to inhibition of estrogenic signaling both *in vitro* and in experimental animals (Safe et al., 2000). The signals that recruit ER α to the activated AhR complex are unknown, and may include the activation of other signaling pathways, such as kinases, since the ER α AF1 is needed for recruitment to and interaction with the AhR complex (Ohtake et al., 2003). Reports also show that AhR–ARNT complexes competitively inhibit the binding of ER-alpha to imperfect estrogen response element (ERE) sites adjacent to or overlapping with XRE sites (Klinge et al., 1999). AhR recruits unliganded or liganded ER α away from ER regulated genes to AhR regulated genes, leading to inhibition of estrogen targeted gene transcription (Matthews et al., 2005). Interestingly, it was shown that ARNT acted more potently on ER- β than ER- α (Ruegg et al., 2008, Brunnberg et al., 2003).

However, the effects and underlying mechanisms of action of BPA in fetal lung development have not been reported. Therefore, the thesis project aims to investigate the molecular mechanisms involving in the early developmental effects of BPA using HFLF as an *in vitro* model.

Based on the above information, it can be hypothesized that 1) BPA may act as a ligand to ER- α or ER- β or GPR30 that affect the fetal lung maturation in HFLF, 2) BPA may act as a ligand to the AhR, 3) BPA may be oxidized to quinones by CYP1B1 and/or other CYPP450 enzymes in HFLF cells which increases ROS production, causing DNA

damage/repair, cell cycle arrest and senescence, which may lead to immunosuppression and delay in fetal lung maturation.

1.12 The rational for using the HFLF as *in vitro* model

Estrogen is known to play an important role in fetal lung development and maturation (Seaborn et al., 2010). The estrogens also accelerated depletion of fetal lung glycogen. 17β -Estradiol also increased the rate of choline incorporation into phosphatidylcholine as well as the activity of cholinephosphate cytidyltransferase in fetal rabbit lung explants *in vitro* (Khosla et al., 1983). As an environmental estrogen, BPA–estrogen receptor interactions have been proposed as possible explanation underlying some of the epidemiological association between estrogen activity and asthma (Bonds et al., 2013). This study showed that a rise in rates and severity in asthma as women enter puberty and into adulthood. Recent study by Van Winkle et al. (2013) found that BPA exposure of the dam during late term significantly accelerated secretory cell maturation in the proximal airways of the rhesus macaque's fetus, suggeststhe fetus at the third trimester of gestation is more susceptible to BPA's effects. Exogenous estrogen accelerated female rat fetal lung maturation by increasing surfactant synthesis through enhanced uptake of estrogen by fibroblasts and subsequent release of maturation factors from fibroblasts to epithelium (Adamson et al., 1990), implying a role of fibroblasts in regulating fetal lung maturation. As well as, fibroblasts respond to and also produce inflamntory mediators (Alkhouri et al., 2014), and they secrete extracellular matrix for tissue remodeling that may become aberrant in lung diseases, including asthma (Fixman et al., 2007; Dijkstra et al., 2006).

HFLF is one of the few available and culturable normal human embryonic cell lines, it is non-cancerous and non-transformed and can potentially differentiate into many cell types. Therefore, characterizing the effects of BPA in HFLF will assist in understanding the reported effects of prenatal exposure to BPA on fetal lung development.

1.13 Various BPA concentrations used for the cell culture

In vitro low dose studies are important to understand the mechanisms of BPA. For *in vivo* toxicological studies, levels of BPA below the current (NOAEL) of 50 µg/kg.bw/d established by United States Environmental Protection Agency (EPA) were considered as low-dose, while low-dose concentration for *in vitro* cell culture studies on BPA is generally defined as less than the NOAEL cut-off value of 50 ng/mL (0.219 µM) (Welshons et al., 2006; Wetherill et al., 2007). LOAEL for BPA in laboratory animals is 5 mg/kg/d. The low BPA doses used in the published *in vitro* studies varied considerably, and doses far below the 10^{-7} M (1nM) level have been used in some studies (Wanatabe et al., 2003; Hugo et al., 2008; LaPense et al., 2009). However, the background BPA levels in experimental materials must be considered when deciding on the lowest dose BPA to be used. The background BPA levels in disposable pipettes and pipette tips were <0.2 ng/ml (0.88 nM); in commercial buffer solutions were 0.02 ng/ml (0.088 nM) and in all cell culture media obtained from various sources were 0.08 to 4.26 ng/ml (0.35 to 19 nM) with average of 0.83 ng/ml (3.5 nM) (Cao et al., 2010) that we can't go lower than 3.5 nM. Therefore, our lowest BPA concentration was 10 nM. However, most of published *in vitro* cell culture studies showed that BPA dose effects have seen only at higher concentration (1µM) or high concentration ($\geq 10\mu\text{M}$) level (Pennie et al., 1998; Lee et al.,

2003; Yoneda et al., 2003). Some studies showed that similar and/or different effects are produced in the same cell lines by BPA at low and high concentrations (Inadera et al., 2000; Smith et al., 2003; Miyatake et al., 2006). Based on this information, the concentrations of BPA used were 10 nM and 100 nM as low concentrations and 1 μ M, 10, 20, 50 and 100 μ M as high concentrations in this study.

Furthermore, cells were treated with BPA for 24 h in this study. The overall cell attachment to the culture dishes and to finish one cycle of cell replication needs 16 to 24 h depends on cell type or line that using. The 16, 48 and 72 h culture time points were also tested. However, we observed that the cells were not completely attached at 16h and too confluent by 48 and 72 h of culture. The cells are completely attached and reached 75% confluency (which is the best stage for a connective tissue like fibroblast cells) at 24h and this culture was used in this study.

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

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 (hFLF) 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 α , ER β and G-protein coupled estrogen receptor 30 (GPR30), and effects of ERs antagonists on BPA-induced changes in endithelin-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. hFLF expresses all three ERs. BPA had no effects on either cellular distribution or protein expression of ER α , however, at 100 $\mu\text{mol/L}$ (or 23 $\mu\text{mol/L}$ intracellular BPA) increased ER β 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, interleukine-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 release of immune and developmental modulators in hFLF, 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.

2.1 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 α 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 remodelling 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 (Alkhoury 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- κ B p65) in cultured normal human fetal lung fibroblasts (hFLF) and the potential role of ERs in the action of BPA in this cell line.

2.2 Materials and methods

2.2.1 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 μ mol/L BPA as working stock solutions. We used 0, 0.01, 0.1, 1, 10, 20, 50 and 100 μ 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 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.

2.2.2 Cell culture

Normal human fetal lung fibroblasts (hFLF) (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 Eagle's Minimal Essential Medium (EMEM) with 10% fetal bovine serum at 37°C and 5% CO₂.

2.2.3 Cellular BPA content and localization

The hFLF (4×10^4 cells/mL or 4×10^5 cells/dish) were seeded in 10 cm culture dishes overnight, then treated with 0, 0.01, 0.1, 1, 10, 20, 50, or 100 $\mu\text{mol/L}$ 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 analysed 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 hFLF were seeded at 4×10^4 cells/mL (4×10^5 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 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 minutes 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-CME-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× objective and photographed with a SPOT RT3 digital camera and SPOT basic software (Diagnostic Instrument Inc., Sterling Heights, MI, USA).

2.2.4 Cell viability, proliferation, and membrane integrity

The hFLF were seeded at 4×10^4 cells/mL (4×10^5 cells/dish) in 96 well culture plates overnight, then treated with 0, 0.01, 0.1, 1, 10, or 100 $\mu\text{mol/L}$ 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 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).

2.2.5 Phosphorylation of NF-κB and release of GDF-15, IL-6, and IP-10

For measuring NF-κB p65 phosphorylation, hFLF were seeded at 4×10^4 cells/mL (4×10^5 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-κB p65 antibody and rabbit polyclonal anti-NF-κB 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 μg/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× 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 were counted for each dose group (0 and 100 µmol/L BPA) using Image J software, and NF-κB 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, hFLF were seeded at 4×10^4 cells/mL (4×10^5 cells/dish) in 10 cm culture dishes overnight and dosed with BPA at 0, 0.01, 0.1, 1, and 100 µmol/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 ELISA kit (Abcam Inc, Toronto, ON, Canada) according to instruction provided by the manufacturer.

2.2.6 Cellular localization of ERs

The hFLF were seeded at 4×10^4 cells/mL (4×10^5 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 α (Cell Signaling Technology Inc., Danvers, MA, USA), mouse monoclonal anti-ER β (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 μ 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). 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).

2.2.7 Protein expressions of ERs

Protein expression levels of ER α , ER β 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 μ 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 nuclear and cytoplasmic extraction reagents (Thermo Scientific, Pierce Biotechnology, Rockford, USA). A

volume of 200 μ 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 μ L cytoplasmic extraction buffer II (CERII) was added, vortexed and centrifuged at 4°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 μ 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 α , ER β , 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 μ 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 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°C with a rabbit polyclonal anti-human ER α (Epitomics, CA, USA), Rabbit polyclonal anti-human ER β (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®WestFemto reagent (Thermo Scientific, ON, Canada), protein bands were

visualized under a digital phosphorimager (FluorChem HD2, Alpha Innotech/ProteinSimple, CA, USA).

2.2.8 Effect of BPA and ERs antagonists on ET-1 release

The hFLF were seeded at 4×10^4 cells/mL (4×10^5 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 ERs antagonists on ET-1 release, hFLF were cultured as described above and pre-treated with ERs antagonists including MPP dihydrochloride (1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinyloxy)phenol]-1H-pyrazole dihydrochloride, ER- α selective), PHTPP (4-[2-Phenyl-5,7-bis(trifluoromethyl) pyrazolo[1,5-a]pyrimidin-3-yl]phenol, ER- β 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°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.

2.2.9 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 HSD test were used to compare differences among treatment groups. Differences were considered significant when $p < 0.05$.

2.3 Results

2.3.1 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 hFLF treated with or without BPA for 24 hr. In this study, BPA was directly added to the cell culture media. Although many in vitro studies on BPA have been published, very few, if any, have correlated effects of BPA with intracellular BPA concentrations. The cellular uptake and cytotoxicity of BPA were measured in HFLF using various doses (0, 0.01, 0.1, 1, 10, 20, 50 and 100 μM). The lower doses (0, 0.01, 0.1, 1 $\mu\text{mol/L}$) were uptaken by BPA. However, the high doses (10, 20, 50 and 100 $\mu\text{mol/L}$) uptakes were decreased with increase of BPA concentration applied to culture. The cellular content of BPA increased with BPA concentration applied to culture (Fig.2.1). 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 HFLF (Fig.2.2). 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 HFLF 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 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.

2.3.2 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 HFLF using multiple techniques. BPA treatment showed no significant effects on the live cell protease activity (Fig.2.3a), and the total, viable and dead cell numbers (Fig.2.3b), suggesting a lack of influence of BPA on cell viability and proliferation of HFLF under the conditions used. Interestingly, BPA at 100 $\mu\text{mol/L}$ decreased extracellular protease activity, a measurement of protease leakage caused by cell death and membrane damage (Fig.2.3a). The intracellular and extracellular protease assays measure two different proteases. It remains to be clarified if BPA at 100 $\mu\text{mol/L}$ decreased expression levels or activity of the type of protease that is measured in the extracellular protease assay.

2.3.3 NF- κ B p65 phosphorylation and release of GDF-15, IL-6, and IP-10

NF- κ B 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- κ B p65 activation. We examined effects of BPA treatment on NF- κ B p65 phosphorylation at S536 in hFLF by immunocytochemical staining. NF- κ B p65 p S536 positive staining was located mainly in the nuclei of both control and 100 μ M BPA-treated cells (Fig.2.4a). Quantitative analysis suggested that 24 hr BPA treatment significantly decreased the number of NF- κ B p65 p S536 positively stained nuclei (Fig. 4a). We also stained total NF- κ B protein and found no difference between control and BPA-treated cells (data not shown). GDF-15 is known to suppress NF- κ B activation and thus has anti-inflammatory function. Thus we examined the effects of BPA on GDF-15 secretion in hFLF. Treatment with BPA at 0.01 and 1 μ mol/L had no effects on the release of GDF-15 from hFLF after 24 hr of exposure (Fig.2.4b). However, at 100 μ mol/L, BPA increased GDF15 release after 24 hr of exposure (Fig.2.4b). Both IL-6 and IP-10 are NF- κ 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 hFLF treated with or without BPA. Treatment with 0.01 and 1 μ mol/L BPA for 24 hr showed no effects on IL-6 and IP-10 release from hFLF (Fig. 4b). However, treatment with 100 μ mol/L BPA significantly decreased IL-6 and IP-10 release (Fig.2.4b).

2.3.4 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 hFLF. ER- α and ER- β positive staining was localized in both nuclei and cytoplasm (Fig.2.5). GPR30 was found in the plasma membrane and nuclei of HFLF. 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- α , but slightly increased ER- β positive staining in the cytoplasm and had no influence on ER- β positive staining in the nuclei. The same concentration of BPA also increased GPR30 positive staining mainly in the nuclei. These results suggest that HFLF, a cell line traditionally viewed as non-estrogen responsive, also expresses all three estrogen receptors, although their roles in cellular signalling and metabolic responses remain to be established.

2.3.5 Protein expression of ERs

ELISA quantification of ERs protein revealed that 100 $\mu\text{mol/L}$ BPA did not affect ER- α protein levels in either cytoplasmic or nuclear fractions (Fig.2.6a). However, it increased ER- β protein levels in the cytoplasmic fractions, with a trend of decrease in the nuclear fractions (Fig.2.6b). 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.2.6c). Western blot analysis of ERs protein expression confirmed the results of ELISA analysis (Fig.2.6d). All these results are consistent with the findings from immunocytochemical staining.

2.3.6 Effects of BPA and ERs 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.2.7a). Antagonists MPP, PHTPP and G15, which are specific for ER- α (Fig. 7b), ER- β (Fig.2.7c), and GPR30 (Fig.2.7d), respectively, had little or no effect on BPA-induced decrease in ET-1 release.

2.4 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 hFLF. 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 hFLF, 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 μ mol/L dose level on cell viability, proliferation, and membrane integrity after 24 hr of exposure. Rather, we found a decreased extracellular protease activity in hFLF exposed to 100 μ mol/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 μ mol/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- κ B activation, the release of GDF-15, IL-6, IP-10, and ET-1, and protein expression of ERs in hFLF, which are all known to have

regulatory impacts on cellular processes involved in fetal lung development and maturation, as well as development and progression of asthma.

NF- κ B is a transcription factor regulating many genes involved in inflammation, immunity, cell proliferation, apoptosis, and stress response. Persistent activation of NF- κ B has been implicated in inflammatory airway diseases including asthma (Pantano et al., 2008, Schuliga, 2014). Site specific phosphorylation of p65 serves as a code to target NF- κ B transcriptional activity to distinct gene subsets (Hochrainer et al., 2013). In this study we observed a decreased phosphorylation of NF- κ B p65 at S536, and decreased secretion of downstream genes IL-6 and IP-10, suggesting an anti-inflammatory effect of BPA treatment in hFLF.

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- κ B activity via delaying I κ B degradation (Vanhara et al. 2009). In this study, BPA treatment increased GDF-15 secretion from hFLF, resulting in decreased NF- κ 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 hFLF, although its physiological implication in childhood asthma warrants more investigation.

IL-6 plays an important role in lung angiogenesis and morphogenesis (Parera et al., 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 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 hFLF, 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 hFLF, 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 ER α and ER β 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 hFLF. BPA treatment had no effects on either distribution or protein level of ER α , but increased cytoplasmic expression level of ER β and nuclear expression level of GPR30. Antagonist specific for ER α , ER β , or GPR30 had little effect on BPA-

induced decrease in ET-1 release. These data indicate that although ERs are expressed in hFLF, 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 β -dependent fashion in mouse myoblasts (Robb and Stuart, 2014). The increased cytoplasmic expression of ER- β by BPA in HFLF may also contributed to BPA-induced stress resistance observed in hFLF.

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 μ 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 HFLF 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 hFLF 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.

Figure and legends of Chapter 2

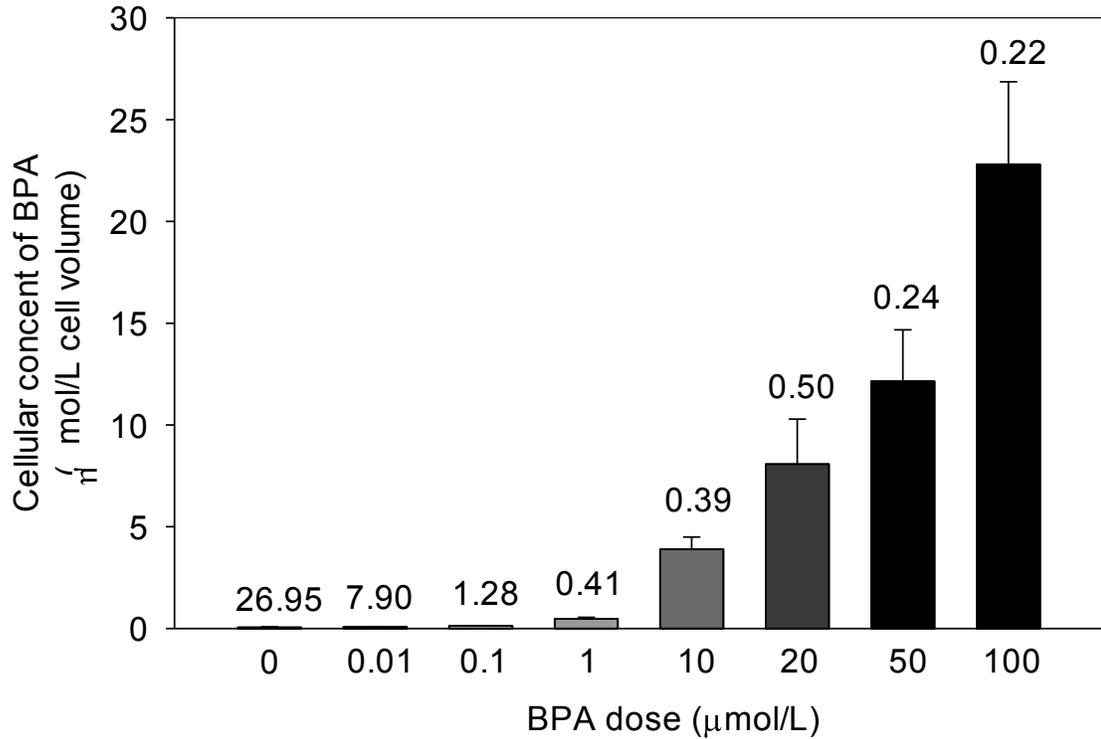


Fig.2.1. Cellular contents of BPA. The HFLF were exposed to different concentrations of BPA for 24 hr. Cellular contents of BPA were measured using GC-MS. Values were normalized against cell volume and expressed as means of four independent experiments. Error bars are the standard errors of the means. The values written on the top of vertical bars are the ratios of cellular BPA contents to BPA doses applied in the culture. The value for 0 $\mu\text{mol/L}$ 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.

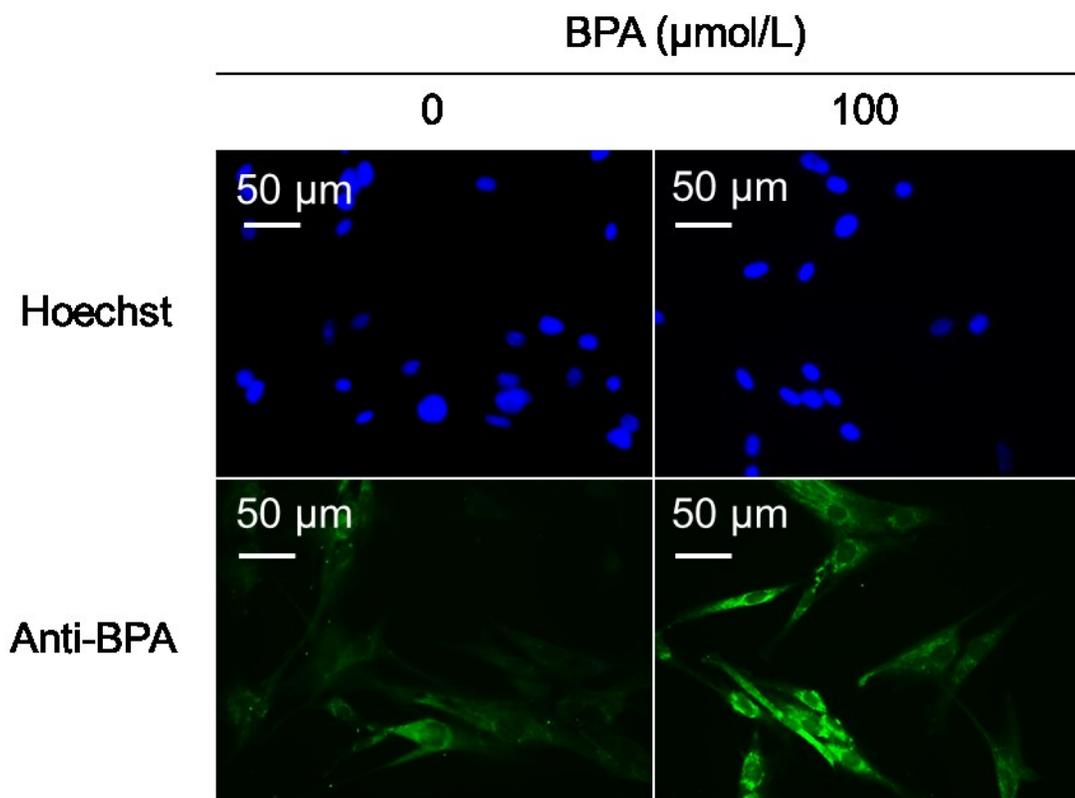


Fig.2.2. Cellular localization of BPA. The HFLF were fixed in 4% paraformaldehyde after 24 hr of exposure to 0 and 100 $\mu\text{mol/L}$ BPA. Fixed cells were incubated with anti-bisphenol A-CME antibody or antibody dilution buffer only (as negative control) for 24 hr, followed by incubation with Hoechst and Alexa Fluor goat anti-rabbit IgG (H+L). Images were obtained at 40 \times objective lens under fluorescence microscopy

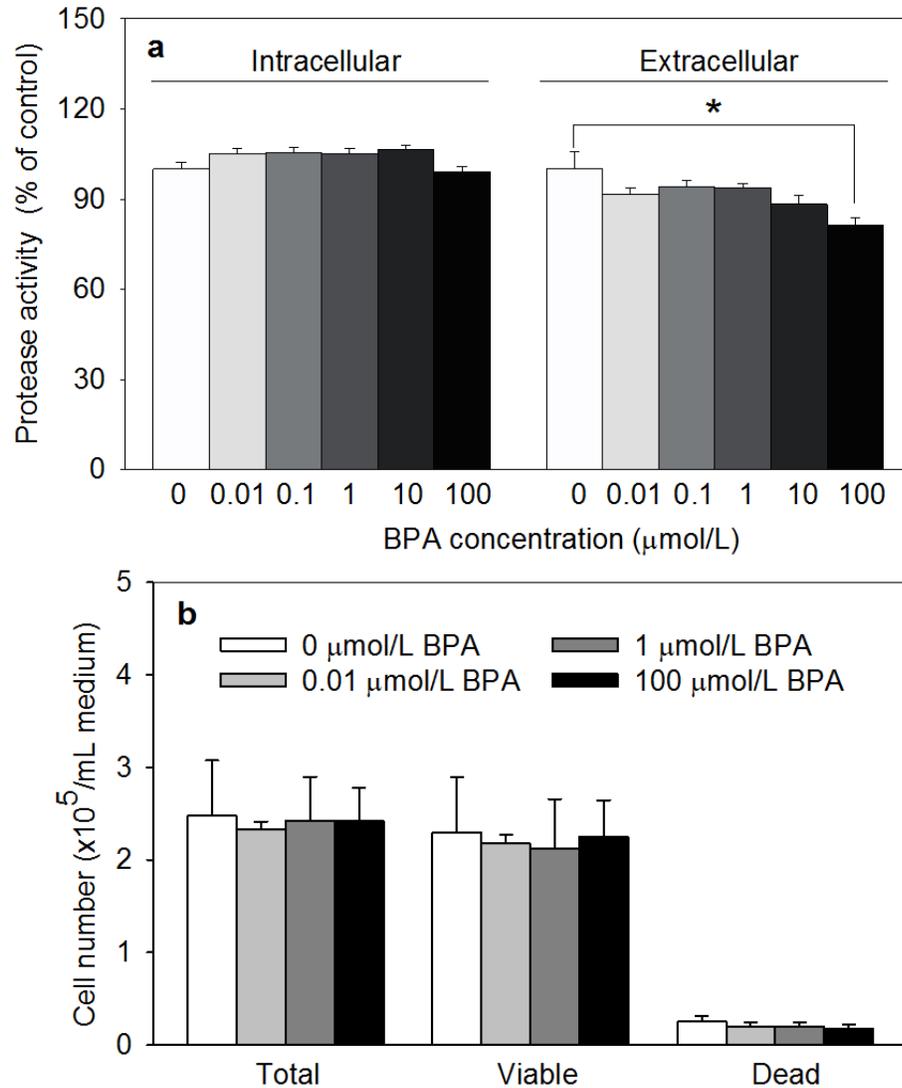


Fig.2.3. Effects of BPA on cell viability, membrane integrity and proliferation. The HFLF were exposed to BPA at 0-100 μmol/L for 24 hr. Cell viability and membrane integrity was measured by intracellular and extracellular protease activity using Multi-Tox assay (a); The total, viable and dead cell numbers 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 $p < 0.05$.

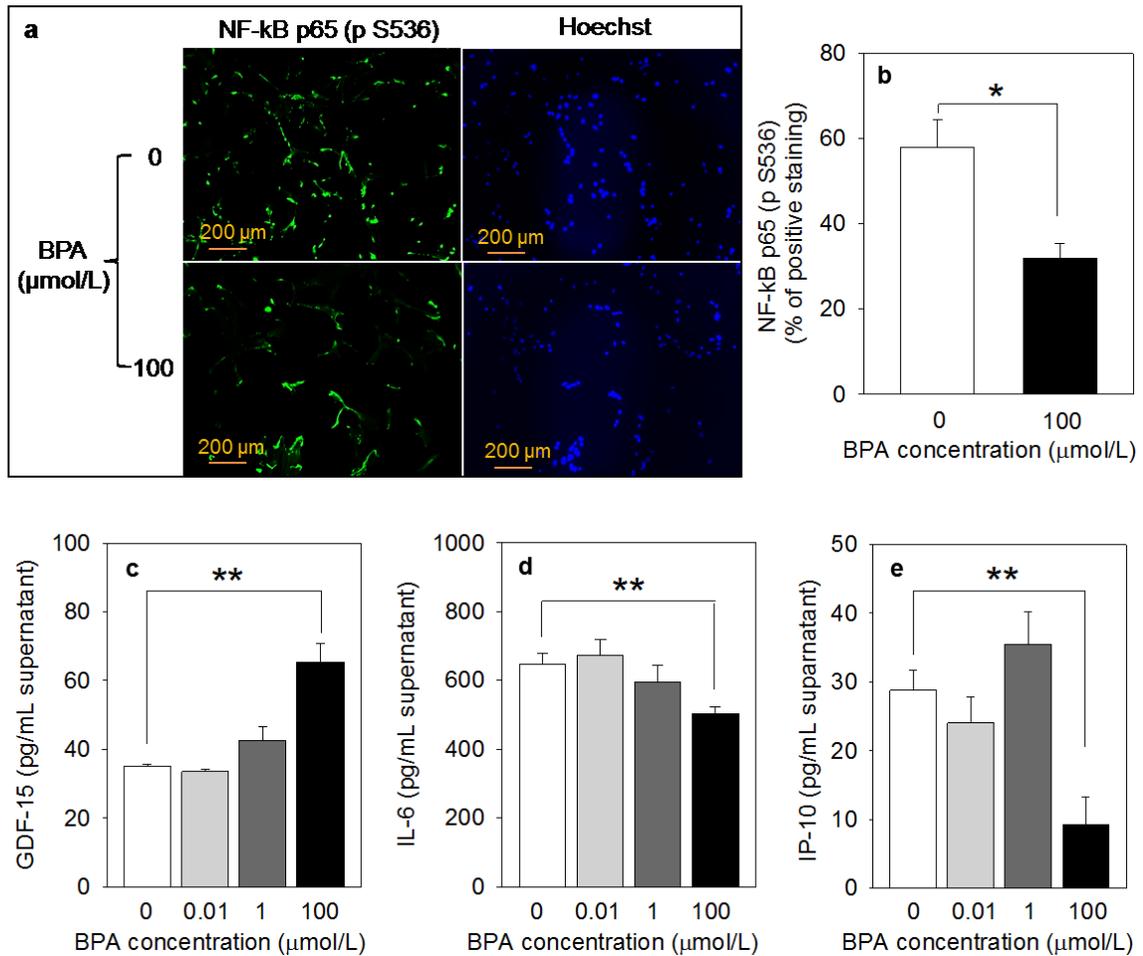


Fig.2.4. Effects of BPA on GDF-15, IL-6, and IP-10 release and NF-κB phosphorylation. The HFLF were treated with 0 or 100 μmol/L BPA for 24 hr and fixed in 4% paraformaldehyde. Fixed cells were stained with rabbit polyclonal anti-NF-κB p65 (p S536) and Alexa Fluor 488 goat anti-rabbit IgG. Images were 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 HFLF culture supernatants were collected after 24 hr exposure to 0, 0.01, 1 or 100 μmol/L BPA. PDG-

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.

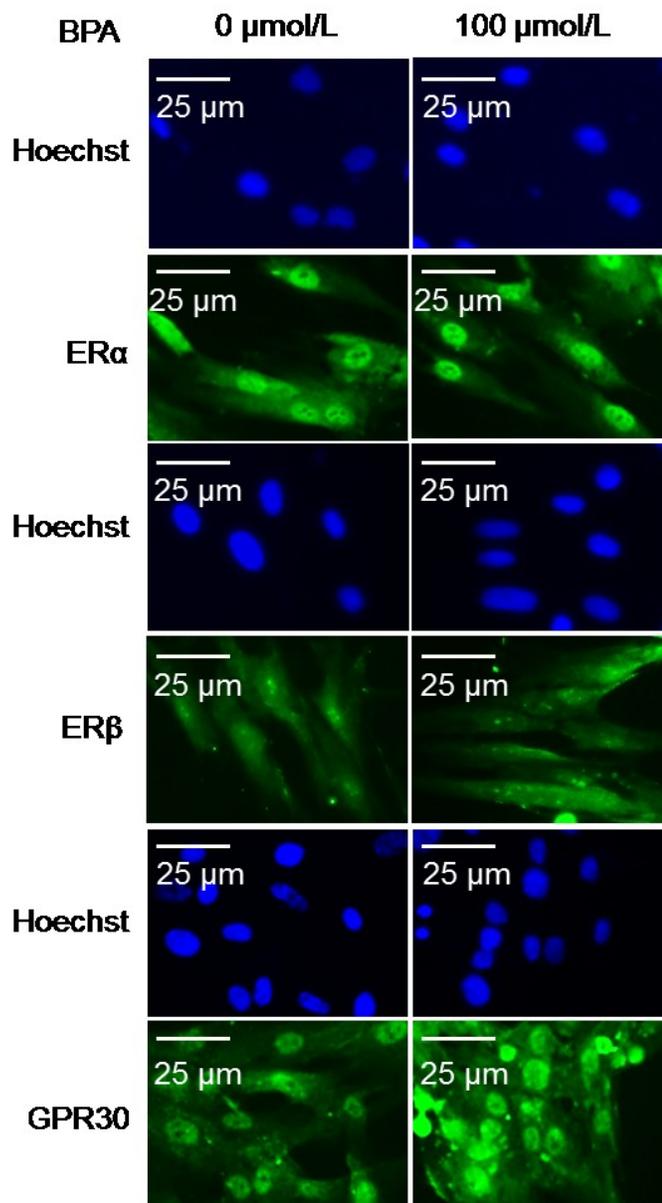


Fig.2.5. Subcellular localization of ER α , ER β , and GPR30 in HFLF. The hFLF were fixed after 24 hr exposure to 0 or 100 $\mu\text{mol/L}$ BPA and incubated with antibodies against human ER α , ER β , or GPR30, respectively, or antibody dilution buffer only (as negative control) followed by incubation 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.

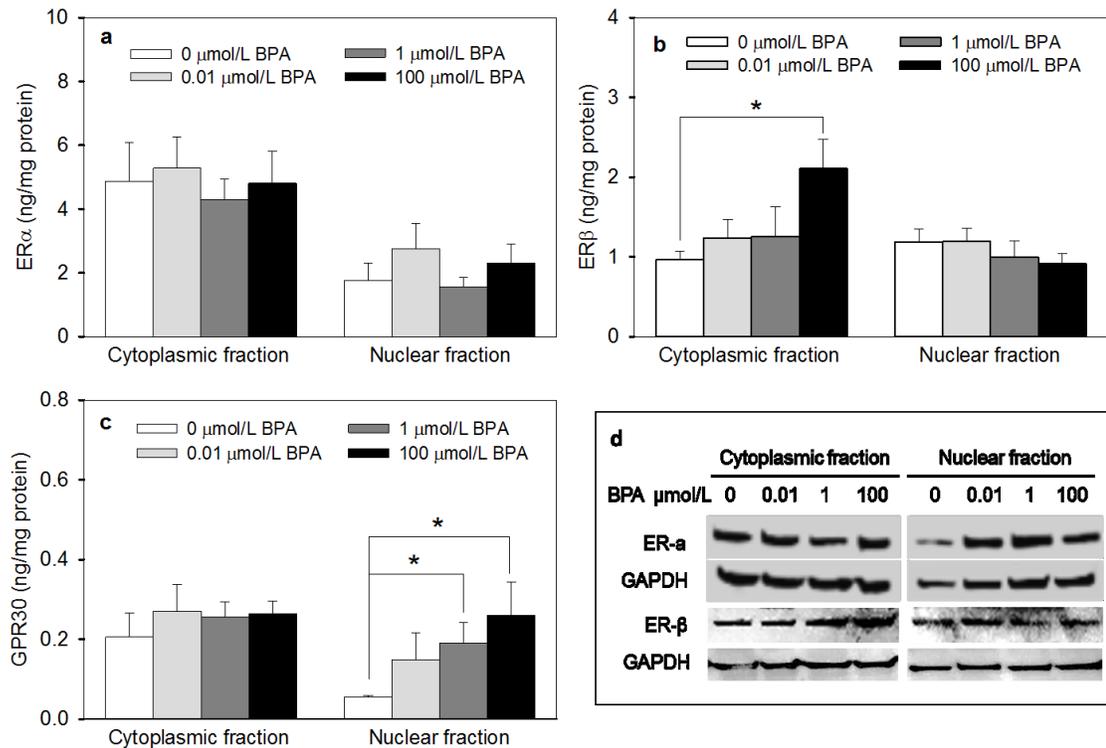


Fig.2.6. Protein expression of ERs. HFLF were exposed to 0, 0.01, 1, and 100 $\mu\text{mol/L}$ BPA for 24 hr. Cellular nuclear and cytoplasmic fractions were obtained from cell lysates using NE-PER nuclear and cytoplasmic extraction buffers. ER α (a), ER β (b) and GPR30 (c) protein levels in cytoplasmic 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 $p < 0.05$.

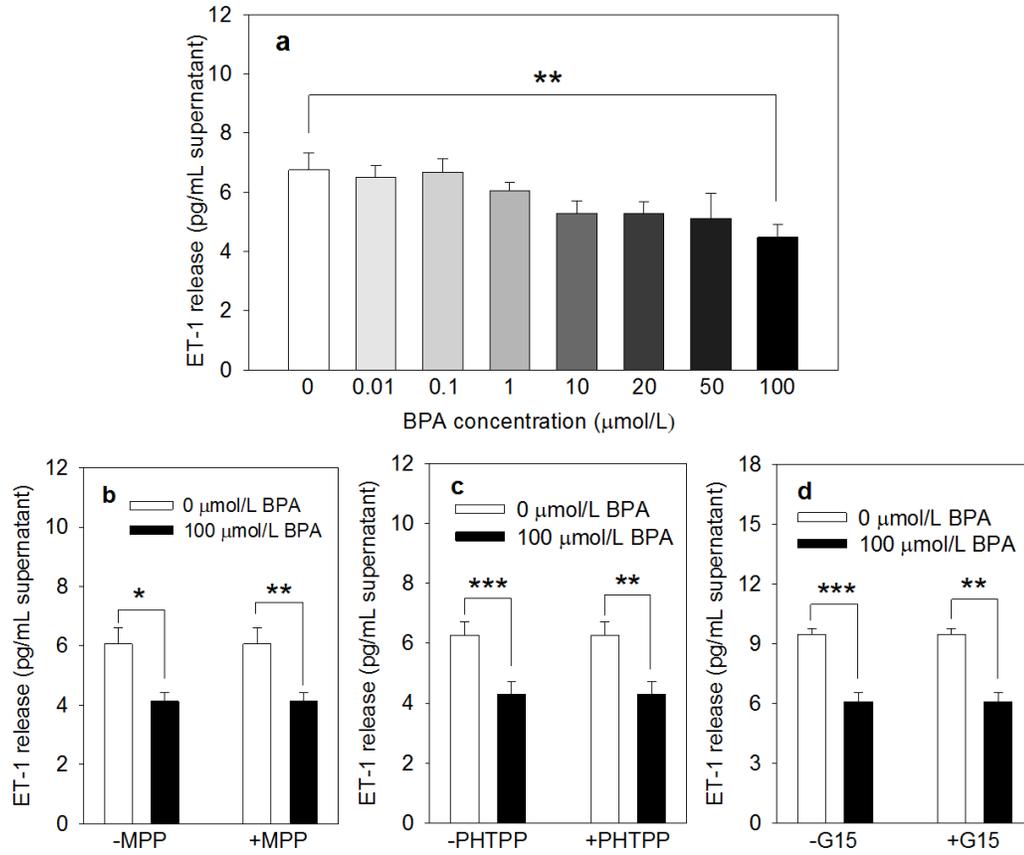


Fig.2.7. Effects of BPA and ERs antagonists on ET-1 release. The HFLF were dosed with 0, 0.01, 0.1, 1, 10, 20, 50 and 100 μmol/L BPA for 24 hr. Supernatants were collected and analyzed for ET-1 release (a) using ELISA kits. Effects of ERα selective antagonist, MPP (b), ERβ selective antagonist, PHTPP (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.

3 Chapter: Bisphenol A induces DSB-ATM-p53 signalling leading to cell cycle arrest, senescence, autophagy, stress response, and estrogen release in human fetal lung fibroblasts

Abstract

Experimental and/or epidemiological studies suggest that prenatal exposure to bisphenol A (BPA) may delay fetal lung development and maturation and increase the susceptibility to childhood respiratory disease. However, the underlying mechanisms remain to be elucidated. In our previous study with cultured human fetal lung fibroblasts (HFLF), we demonstrated that 24 h exposure to 1 and 100 $\mu\text{M/L}$ BPA increased GPR30 protein in the nuclear fraction. Exposure to 100 μM BPA had no effects on cell viability, but increased cytoplasmic expression of ER β and release of GDF-15, as well as decreased release of IL-6, ET-1, and IP-10 through suppression of NF κ B phosphorylation. By performing global gene expression and pathway analysis in this study, we identified molecular pathways, gene networks, and key molecules that were affected by 100, but not 0.01 and 1 $\mu\text{M/L}$ BPA in HFLF. Using multiple genomic and proteomic tools, we confirmed these changes at both gene and protein levels. Our data suggest that 100 $\mu\text{M/L}$ BPA increased CYP1B1 and HSD17B14 gene and protein expression and release of endogenous estradiol, which was associated with increased ROS production and DNA double strand breaks, upregulation of genes and/or proteins in steroid synthesis and metabolism, and activation of Nrf2-regulated stress response pathways. In addition, BPA also activated ATM-p53 signaling pathway, resulting in increased cell cycle arrest at G1 phase, senescence and autophagy in HFLF. Fetal lung development and maturation requires paracrine interaction between fetal lung alveolar type II epithelial cells and fibroblasts.

The results from our studies suggest that prenatal exposure to BPA at high enough concentrations may affect fetal lung development and maturation by altering the release of developmental, immune, and hormonal modulators from fetal lung fibroblasts, and thereby possibly affecting susceptibility to childhood respiratory diseases.

3.1 Introduction

Bisphenol A (BPA) is widely used in the manufacture of products containing polycarbonate and epoxy resin including food and drink containers. Human exposure to BPA occurs mainly through ingestion of contaminated food and drink (Gao et al., 2016, Cao et al., 2015, Kang et al., 2006.), although thermal paper, dust, cosmetics and BPA manufacturing are other potential exposure sources (Russo et al., 2017, Gao et al., 2016, Hines et al., 2017). More than 90% of the U.S. population has detectable levels of BPA in urine samples, with the highest concentration found in children, followed by adolescents (Calafat et al., 2005, Hines et al., 2017).

In both humans and animals, BPA is metabolized mainly by uridine 5'-diphospho-glucuronosyltransferases (UGTs) into glucuronide (Hanioka et al., 2008, Yokota et al., 1999). However, maternal exposure to BPA can lead to fetal exposure to BPA via placental transfer of maternal BPA-glucuronide conjugate that can be deconjugated to form free BPA by β -glucuronidase in the fetus (Nishikawa et al., 2010; Takahashi and Oishi, 2000). In fact, BPA has been detected in human placenta, fetal blood, amniotic fluid, and fetal liver at concentrations ranging from undetectable to hundreds of ng/g (Vandenberg et al., 2012; Cao et al., 2012; Edlow et al., 2012; Ikezuki et al., 2002, Nahar et al., 2015). With much lower expression of metabolic enzymes, the

fetus has limited capacity to detoxify xenobiotics comparing with the adult. Fetal lung has much lower expression of UGTs (2B7/2B15 in human, 2B1 in rat), but higher expression of β -glucuronidase (Lucier et al., 1977), than fetal liver. It also has higher surface to volume ratio, and exposure through both cord blood and amniotic fluid, which renders it more susceptible to BPA toxicity (Ekström et al., 2013). Recent studies revealed that fetal exposure to BPA may delay lung maturation and increase childhood susceptibility to respiratory disease in both animal model and human subjects (Spanier et al., 2012, Hijazi et al., 2015). However, the underlying mechanisms remain to be elucidated.

In a previous study with human fetal lung fibroblasts (HFLF), a cell type important in regulating fetal lung differentiation and development, we found that BPA at the concentration and duration of exposure used caused no change in cell viability, but decreased release of two important regulators of embryo development and immune response, endothelin-1 (ET-1) and interleukin-6 (IL-6), suppressed phosphorylation of nuclear factor kappa b (NF κ B), while also increased cytoplasmic expression of ER β and nuclear expression of G-protein coupled estrogen receptor 30 (GPR30). However, ER α , ER β and GPR30 antagonists could not block the effect of BPA on ET-1 release. This suggested that BPA suppressed ET-1 release through an ER-independent metabolism, although the exact molecular pathways leading to the effects of BPA remain to be investigated.

In addition to glucuronidation, sulfonation and oxidation of BPA also occur in vivo, especially when high dose of BPA is used (Ye et al., 2011, Atkinson and Roy, 1995). Bisphenol ortho-quinone, potentially formed via oxidation of BPA by cytochrome

P450 (CYP) enzymes, can bind DNA and form DNA adducts (Atkinson and Roy, 1995). CYP1B1 is a major CYP450 enzyme expressed in human extrahepatic tissues including lung (Bièche et al., 2007). CYP1B1 is capable of oxidizing catechol estrogens to their respective quinones that can further react with DNA (Zhang et al., 2007). However, there is a lack of information as to the effect of BPA on CYP1B1 expression in fetal lung cells. It is reasonable to speculate that BPA is oxidized to quinones by CYP1B1 in human fetal lung cells where CYP1B1 is constitutively expressed or induced by BPA exposure.

As an endocrine disruptor, BPA has been studied repeatedly for its effects on steroidogenesis (Bloom et al., 2016, Williams et al., 2014). Fetal exposure to low dose BPA was found to increase in situ estrogen level and CYP19A1 and CYP11A1 mRNA levels in postnatal day 1 urogenital sinus of both male and female mice (Arase et al., 2011). However the effects of BPA on steroidogenesis in fetal lung cells have not been reported. Estrogen is known to play an important role in fetal lung development and maturation (Seaborn et al., 2010). Exogenous estrogen accelerated female rat fetal lung maturation by increasing surfactant synthesis through enhanced uptake of estrogen by fibroblasts and subsequent release of maturation factors from fibroblasts to epithelium (Adamson et al., 1990), implying a role of fibroblasts in regulating fetal lung maturation. Therefore, characterizing the effects of BPA in HFLF will assist in understanding the reported effects of prenatal exposure to BPA on fetal lung development (Hijazi et al., 2015).

To reveal the key molecular pathways involved in the developmental effects of BPA in human fetal lung and their potential implications in the reported link between prenatal exposure to BPA and increased sensitivity to childhood respiratory diseases, we

examined the gene expression profiles in HFLF treated with non-cytotoxic concentrations of BPA. Using Ingenuity Pathway Analysis (IPA), we identified molecular pathways, gene networks, and key molecules that were affected by BPA in HFLF. Using multiple genomic and proteomic tools, we confirmed these changes at both gene and protein levels. Our data suggest that BPA exposure increases CYP1B1 and HSD17B14 gene and protein expression and release of endogenous estradiol, which is associated with increased ROS production, upregulation of genes and/or proteins involved in steroidogenesis, and activation of Nrf2-regulated stress response pathway. BPA exposure also caused DNA double strand breaks (DSBs), leading to activation of ATM-p53 signaling pathway, resulting in increased cell cycle arrest at G1 phase, senescence, and autophagy in HFLF.

3.2 Materials and Methods

3.2.1 Chemicals

BPA powder (99%) was purchased from Sigma-Aldrich (Oakville, Ontario, Canada). A 1.0 mM stock solution of BPA was prepared and diluted in distilled and deionized water (DDW) to obtain 0.1, 1, 10, 100 and 1000 μM BPA as working stock solutions. We used 0, 0.01, 0.1, 1, 10 and 100 μM BPA doses in our experiments. Our previous analysis of BPA in distilled and deionized water, media, buffer, and serum demonstrated that BPA is present at nM or sub-nM concentrations in all tested materials used in culture studies (ref. 51). The lowest concentration used in this study (0.01 μM) is a few folds above the background BPA concentration.

3.2.2 Cell culture and BPA treatment

Normal human fetal lung fibroblasts (HFLF) (WI-38, CCL-75) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cell culture medium and fetal bovine serum were obtained from Invitrogen-Gibco (Carlsbad, CA, USA). Cells were maintained in phenol red-free Eagle's Minimal Essential Medium (EMEM) with 10% fetal bovine serum at 37°C and 5% CO₂. For BPA treatment, cells were seeded at 4x10⁴ cells/mL in EMEM overnight and then treated with 0 (water), 0.01, 1, 10 and 100 µM BPA in the same medium at 37°C and 5% CO₂ for 24 h. These BPA doses were selected based on our previous study.

3.2.3 Total RNA isolation

HFLF cells (4x10⁴ cells/mL) were seeded in 10 cm culture dishes containing supplemented media overnight. After 24 h exposure to BPA at 0, 0.01, 1, or 100 µM, the cells were washed twice with ice-cold PBS buffer, scraped off, immediately frozen in liquid nitrogen, and then stored at -80°C until RNA isolation. Total RNA from each frozen cell pellet was extracted using the RNeasy Mini Kit as described by the manufacturer (Qiagen, Mississauga, ON, Canada). Briefly, cells were lysed by adding RLT Buffer and subsequently homogenized on QIA shredder spin columns. Total RNA was extracted, purified and DNase I treated on RNeasy mini columns. The concentration of the total RNA was determined using a NanoDrop ND-1000 UV/Vis Spectrophotometer (NanoDrop Technologies, Inc. Wilmington, DE, USA). The quality of the purified RNA was assessed by a Bioanalyzer (Agilent 2100 Bioanalyzer, Agilent

Technology, Waldbronn, Germany). High quality RNA samples (RNA integrity number above 9.0) were selected for subsequent microarray analysis and cDNA synthesis.

3.2.4 Microarray hybridization and image acquisition

Twenty-four RNA samples (100 ng/ μ L) were labeled and hybridized to Affymetrix 1.0 ST human gene arrays for microarray analysis at the Ontario Genomics Innovation Centre StemCore Microarray Facility (Ottawa, ON, Canada). These samples consisted of six replicates from six independent experiments treated with 0, 0.01, 1, or 100 μ M BPA. The microarray data were analyzed as previously described (Chen et al., 2011). Briefly, the CEL file for each array was read using the Bioconductor package Affy in R (R Development Core Team, 2005). Expression summary values were generated using the Robust Multi-Array Average method (Irizarry et al., 2003). Box plots, MA plots and heat maps were used to evaluate the quality of the microarray data.

3.2.5 Bioinformatics analysis

To detect differentially expressed genes between the control and treated groups, an analysis of variance (ANOVA) model was applied using the microarray ANOVA library in R (Cui et al., 2005). The model included the date of hybridization as a block effect and fixed effect of treatment. The F_s statistic (Wu et al., 2003), a shrinkage estimator for the gene-specific variance components, was used to test the treatment effect. The p values for all statistical tests were estimated by the permutation method using

residual shuffling followed by adjustment for multiple comparisons by using the Benjamini-Hochberg false-discovery rate approach (ref. 56). Significantly affected genes were identified as having an adjusted p value ≤ 0.05 . Estimated marginal means, also known as the least squares means were estimated for each group. These means are a function of the model parameters and are adjusted for the other factors in the model. The least squares means were then used to estimate the fold change for each contrast that was tested. Genes with an adjusted p value ≤ 0.05 were considered as profoundly differentially expressed genes and subjected to network, functional, and canonical pathway analyses using the “Core Analysis” of Ingenuity Pathways Analysis (IPA) software released in June, 2017 (Ingenuity® Systems, Redwood City, CA, USA). Datasets containing the Affymetrix gene identifiers and their corresponding p values and fold-change values were uploaded as tab-delimited text files. Each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base. These differentially expressed genes, called Network Eligible Molecules, were overlaid onto a global molecular network developed from information contained in Ingenuity’s Knowledge Base. Networks of Network Eligible Molecules were then algorithmically generated based on their connectivity and assigned a score. Scores of two or above have at least a 95% likelihood of not being generated by chance. The Functional Analysis was conducted to identify the Molecular and Cellular Functions or Physiological System Development and Functions that were most significant to the input differentially expressed gene set. A right-tailed Fisher’s exact test was used to calculate a p -value determining the probability that each biological function for that data set is due to chance alone.

Canonical pathway analysis was conducted to identify the up- or down-regulated pathways from the IPA library of canonical pathways that were most significant to the input data set. The significance of the association between the data set and the canonical pathway was measured in two ways: 1) A ratio of the number of genes from the data set that map to the pathway divided by the total number of genes that make up the canonical pathway provided an estimation of the extent of pathway involvement and 2) Fisher's exact test was used to determine the probability that the association between the genes in the data set and the canonical pathway is due to chance alone.

3.2.6 mRNA expression by RT-qPCR.

Procedures for cDNA synthesis and RT-qPCR have been described previously (ref. 52). Briefly, 2 µg of total RNA was reverse-transcribed to synthesize cDNA with Retroscript Kit (Ambion, Austin, TX, USA). RT-qPCR was performed on a MX4000 Multiplex Quantitative PCR System (Stratagene, La Jolla, CA, USA) using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA). The gene expression value were normalized by housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

3.2.7 DNA damage response

ATM and histone H2A phosphorylation

HFLF cells (4×10^4 cells/mL) were seeded in 4-well glass chamber culture slides (BD Biosciences, California, USA) and then dosed with 0 and 100 μ M BPA for 24 h overnight. After removing media, each chamber was washed twice with ice-cold PBS. Cells were then fixed in 4% paraformaldehyde (Santa-Cruz Biotechnology, Inc. California, USA) for 20 min at room temperature. The cells were washed three times with PBS, and then permeabilized in 0.1% Triton X-100 for 15 minutes at room temperature. After being rinsed three times with PBS, cells were incubated with rabbit monoclonal anti-ATM (phospho S 1981) (Abcam Inc, Cambridge, USA) (diluted 1:100) in IHC-Tek antibody diluent (IHC World, LLC, Ellicott City, Maryland, USA) at 4 °C overnight. After being washed, cells were incubated with Alexa Fluor 488 goat anti-rabbit IgG (Life Technologies, Carlsbad, CA, USA) (1:500 in IHC-Tek antibody diluent) for 2 h at room temperature. After being rinsed in PBS three times for 5 min, the cells were incubated with 2 μ g/mL Hoechst 33342 (Life Technologies Inc., Burlington, ON, Canada) diluted in deionized and distilled water in the dark for 10 min. Slides were mounted with mounting media (Dako, Burlington, Canada). The positive staining was assessed under a Zeiss Axiovert 40CFL inverted microscope (Carl Zeiss Microscopy, LLC) using 20X objective and photographed with a SPOT RT3 digital camera and SPOT basic software (Diagnostic Instrument Inc., Sterling Heights, MI, USA). Six hundred (600) cells were counted per experiment from 3 independent experiments using ImageJ software (imagej.nih.gov/ij). Cells having bright spots were considered as ATM positive cells.

To determine localization of γ -H2AX, immunocytochemistry assay was conducted as describe above using rabbit monoclonal anti-phospho histone H2AX (Ser139) (Millipore Canada Ltd, Etobicoke, ON, Canada). To quantify its protein

expression, cell lysates were collected and were measured for levels of phosphorylated H2AX (γ -H2AX) using γ -H2AX Pharmacodynamic Assay ELISA Kit (Trevigen Inc, Gaithersburg, MD, USA) according to the manufacturer's instructions.

3.2.8 Cell cycle, senescence, and autophagy

Total and phospho p53 expression

HFLF were cultured as describe above. Cells were harvested and lysed in Pierce RIPA buffer (Thermo Scientific, Rockford, IL, USA) containing HaltTM protease and phosphatase inhibitors (Pierce Biotechnology, Rockford, USA). Protein expression of total and phospho p53 at S15 (p53 pS15) was measured in cell lysates using ELISA kits (Abcam Inc, Toronto, ON Canada) according to manufacturer's instructions. Total protein concentrations of cell lysates were determined by DC Protein Assay (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada). The p53 pS15 protein levels in samples were normalized against total protein concentrations.

Phosphorylation of Cdk2 at Try15 and H3 at Ser10

Cells were seeded in 24-well glass bottom plate (Greiner Bio-One) and grown in EMEM overnight as described above, and treated with 0 and 100 μ M BPA for 24 h. The level of phosphorylated Cdk2 (CdK2 pTry15) and histone H3 (H3 pSer10) were measured using Cell Cycle In-Cell ELISA Kit (Abcam Inc, Toronto, Canada) according to the manufacturer's instructions. Briefly, cells were fixed with 4% paraformaldehyde for 10 mins and permeabilized with 1% Triton X-100. Cells were blocked for 2 hrs, and

then incubated with primary antibody overnight at 4 °C. Next day, cells were washed and incubated with secondary antibody for 2 hrs and the fluorescent intensity of Cdk2 pTry15 were measured at excitation 360 nm and emission 460 nm, the histone H3 pSer10 were measured at excitation 530 nm and emission 590 nm using a microplate reader (POLARstar OPTIMA, BMG LABTECH, Germany). To determine the fluorescence signal (RFU) in wells, the mean of background signal (with no primary antibody) was subtracted from all other experimental signals. The fluorescence intensity of different antibodies was normalized by dividing the Ganus Green staining recorded at OD 595nm.

β-Galactosidase expression

The β-Galactosidase Staining kit (Cell Signaling, Danvers, MA, USA) was used to detect β-galactosidase in 0 and 100 μM BPA treated cells. After 24 h exposure to BPA in the 6 well plates, cells were washed by PBS and fixed by 1X fixative solution (2% formaldehyde, 0.2% glutaraldehyde in 1X PBS). β-Galactosidase staining solution (pH 5.9-6.1) were prepared for each 35 mm well with mixture of 930 μL staining solution A (500 nM potassium ferrocyanide), 10 μL of staining solution B (500 nM potassium ferricyanide) and 50 μL of X-gal (20 mg 5-bromo-4-chloro-3-indolyl-βD-galactopyranoside powder in 1 mL of N-N-dimethylformamide). Cells were incubated 12-13 hrs in a 37 °C dry incubator. Then, cells were washed 3X with PBS. The β-galactosidase blue staining was assessed under a Zeiss Axiovert 40 CFL inverted microscope (Carl Zeiss Microscopy, LLC) and photographed with a SPOT RT3 digital camera and SPOT basic software (Diagnostic Instrument Inc., Sterling Heights, MI, USA). The staining was quantified by counting of the positively stained flat cells using

ImageJ software (imagej.nih.gov/ij). Proportion of big flat senescent cells with positive staining was calculated as percentage of total number of cells.

LC3B expression and autophagic activity

HFLF (4×10^4 cells/mL) were seeded in 4-well glass chamber culture slides (BD Biosciences, California, USA) overnight and then dosed with 0 and 100 μ M BPA for 24 h. Autophagy Detection Kit (Abcam Inc, Cambridge, USA) was used to detect the autophagic activity in dosed HFLF. Briefly, each chamber was washed twice with assay buffer. Then, cells were incubated with Microscopy Dual Detection Reagent at 37 °C in the dark. After 30 min incubation, cells were washed with assay buffer and slides were mounted with mounting media (Dako, Burlington, Canada). The staining was assessed under a Zeiss Axiovert 40 CFL inverted microscope (Carl Zeiss Microscopy, LLC) and the positive staining was quantified using ImageJ software (imagej.nih.gov/ij). The number of positively stained cells was calculated as percentage of total number of cell. Western blot analysis was conducted as described above using rabbit polyclonal anti-LC3B antibody (Novus Biologicals Canada, Oakville, ON, Canada) and protein bands were analyzed by ImageJ software.

3.2.9 ROS production and Nrf2-regulated oxidative stress and antioxidant defense response

ROS detection

The level of intracellular ROS was measured with the Cell ROX CellROX Deep Red Reagent. Briefly, 4×10^4 cells/mL were seeded in 12-well glass bottom culture plates (In Vitro Scientific, Sunnyvale, CA 94086, USA) and grown in EMEM overnight before being dosed with BPA as described above. ROS production was measured using a fluorescent dye, CellROX Deep Red Reagent (Life Technologies Inc, Burlington, ON, Canada) which was added to the culture dishes to a final concentration of 10 μ M, 24 h after dosing with BPA. After 30 min incubation at room temperature, the fluorescent intensity was measured at excitation 640 nm and emission 665 nm using a microplate reader (POLARstar Optima, BMG Labtech, Germany). ROS values were normalized against Hoechst (2 μ g/mL) staining measured at excitation 360 nm and emission 475 nm. Meanwhile the positive staining was assessed under a Zeiss Axiovert 40CFL inverted microscope (Carl Zeiss Microscopy, LLC, Thornwood, NY, United States) using 20X objective and photographed with a SPOT RT3 digital camera and SPOT basic software (Diagnostic Instrument Inc., Sterling Heights, MI, USA).

Nrf1/2 and HO-1 protein expression

HFLF (4×10^4 cells/mL) were seeded in 10 cm culture dishes containing EMEM and incubated overnight at 37°C and 5% CO₂, and then dosed with 0, 0.01, 1, or 100 μ M BPA for 24 h. At the end of BPA exposure, medium was removed, and cells were washed three times with ice-cold PBS buffer. A volume of 50 μ L ice-cold RIPA lysis buffer (Millipore, ON, Canada) containing protease inhibitors (Complete Mini, Roche, QC, Canada) was added to each dish. Cells were scraped, sonicated, and centrifuged at 13,000 rpm and 4°C for 10 min. The supernatant was transferred into clean Eppendorf vials and protein concentration was determined using the DC protein assay kit (Bio-Rad

Laboratories, CA, USA). Thirty μg of cell lysate protein was resolved by electrophoresis on 4-20% Precise Tris-HEPES Gel (Thermo Fisher Scientific, ON, Canada). Separated proteins were transferred to PVDF membranes, which was then blocked with 5%-skimmed milk dissolved in TBST (10 mM Tris base, 150 mM NaCl, and 0.1% Tween 20, pH 7.5), and probed overnight at 4°C with a mouse monoclonal anti-human Nrf1 (H-4, NFE2L1) antibody, a rabbit polyclonal anti-human Nrf2 (H-300, NFE2L2) antibody, or a mouse monoclonal anti-human HO-1 (HMOX-1) antibody, all from Santa Cruz Biotechnology (Santa Cruz, CA, USA). After four washes in the TBST, the membrane was probed with a horse radish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Bio-Rad, Mississauga, ON, Canada) or anti-mouse IgG (Bio-Rad, Hercules, CA, USA). The membrane was also probed with monoclonal anti-actin antibody (Sigma-Aldrich Canada Ltd, Oakville, ON, Canada) or rabbit polyclonal anti-GAPDH antibody (Sigma-Aldrich Canada Ltd, Oakville, ON, Canada). Beta actin or GAPDH bands were used as loading controls. After incubation of the membrane with SuperSignal®WestFemto reagent (Thermo Fisher Scientific, ON, Canada), protein bands were visualized under a digital phosphorimager (FluorChem HD2, Alpha Innotech/ProteinSimple, Santa Clara, CA, USA). Densitometry analysis was performed on images of protein bands using ImageJ software (imagej.nih.gov/ij).

3.2.10 Endogenous estradiol production and metabolism

HFLF were cultured as described above. After exposure to 0, 0.01, 1, 10 and 100 μM BPA, estradiol levels in supernatants were measured using Estradiol E2 Human

ELISA kit from Abcam (Abcam Inc, Toronto, Canada). The HSD17 β 14 protein level was measured in cell lysates using commercial ELISA kits from USCN Life Science Inc. (Wuhan, P.R. China) according to manufacturer's instructions. CYP1B1 protein level in cell lysates was measured using Western blot analysis. Rabbit polyclonal anti-CYP1B1 antibody IgG (Abcam Inc, Toronto, Canada) and the HRP conjugated secondary antibody (Dako, Denmark) were used to identify CYP1B1 protein bands. The protein band intensity was measured by ImageJ software (imagej.nih.gov/ij). Cellular localization of CYP1B1 was examined using the same antibody by immunocytochemical staining as described above.

3.3 Results

3.3.1 BPA exposure alters expression of over two thousand genes involved in multiple canonical pathways and cellular processes in the absence of cytotoxicity

Our previous study demonstrated that BPA at $\leq 100 \mu\text{M}$ did not affect HFLF cell viability after 24 h of exposure (²⁰ Mahemuti et al. 2016). However, the molecular targets and pathways affected by BPA remained unclear. To answer this question, we examined global gene expression in HFLF cells treated with 0, 0.01, 1 or 100 μM BPA for 24 h using mRNA microarray analysis. Clustering analysis of gene expression data revealed a dramatic differential gene expression pattern in the 100 μM BPA dose group as compared to other dose groups (Fig.3.1A). These differences represent significant ($p \leq 0.05$) changes in the expression of 2527, 1923, and 2220 genes in 100 μM BPA dose group as compared to 0, 0.01, and 1 μM dose groups, respectively (Fig.3.1B). Some of

the differentially ($p \leq 0.05$) expressed genes between 100 and 0 μM BPA dose groups with a fold of change ≥ 1.5 were validated by RT-qPCR (Fig.3.1C).

To determine the gene functions and pathways affected by 100 μM BPA, we conducted a Core Analysis using the most current version of IPA (IPA, June 2017, <http://www.ingenuity.com>). The results of the IPA analysis suggests that the genes affected by BPA treatment are involved in many canonical pathways including Cell Cycle Control of Chromosomal Replication (CCCCR), Role of BRCA1 in DNA Damage Response, ATM Signaling, Nrf2-mediated Oxidative Stress Response and Cholesterol Synthesis (Fig.3.1D). IPA also revealed that expression of 23 genes associated with CCCCR canonical pathway was significantly decreased by 100 μM BPA, implying a suppression of G1 to S phase transition (Fig.3.2A). Expression of 15 genes was altered by 100 μM BPA in the direction consistent with an increased breakage of chromosome (Fig.3.2B). Expression of 22 genes were altered by 100 μM BPA in the direction consistent with a decrease in homologous recombination (HR) (Fig.3.2C). These gene expression changes indicated an impact of BPA exposure on DNA integrity and cell cycle.

3.3.2 BPA increases DNA double strand breaks (DSB) leading to H2AX and ATM phosphorylation, and ATM signaling

As revealed by IPA analysis, ATM Signaling is one of the pathways affected by 100 μM BPA treatment (Fig.3.3A). ATM, known as ataxia-telangiectasia mutated, is a serine/threonine protein kinase that is recruited and activated by DSB and phosphorylates histone H2A at serine 139, initiating DNA DSB repair pathway. Further examination into

gene functions, we noticed that expression of 18 genes was altered by BPA treatment in the direction consistent with an increase in the formation of γ -H2AX (Fig.3.3A). To confirm the gene expression data, we determined phosphorylation of H2AX (γ -H2AX) and ATM (p s1981) using immunocytochemical staining and ELISA (Fig.3.3B). We detected a significant increase in γ -H2AX protein level (Fig.3.3C) and percentage of ATM-ps1981 positive cells (Fig.3.3D) in BPA-treated HFLF, supporting the notion that 100 μ M BPA treatment induced DNA DSB, leading to the activation of the ATM signaling pathway. Interestingly, we also found increased expression of ATM target genes including RAD50 and 53BP1, two important proteins involved in DSB recognition and repair, as well as tumor suppressor protein p53, a key regulator of cell cycle control (Fig.3.3A). Increased expression of 53BP1 may imply an activation of non-homologous end joint (NHEJ) repair of DSB, although more experiments are warranted to confirm this.

3.3.3 BPA increases p53 phosphorylation but decreases Cdk2 phosphorylation, leading to increased senescence

As shown in Fig.3.3A, the expression of p53 gene was upregulated by 100 μ M BPA. It is known that ATM phosphorylates several key proteins including p53 that initiate activation of the DNA damage checkpoint, leading to cell cycle arrest and senescence. To further understand the effects of BPA on cellular response downstream of ATM signaling, we measured total protein level of p53 and phosphorylation of p53 at S15 (p53pS15) (Fig.3.4A). We found a significant increase in p53 pS15 in HFLF after 24 h of exposure to 100 μ M BPA, suggesting an activation of p53. To determine if BPA-

induced activation of ATM and p53 affected cell cycle in HFLF, we determined phosphorylation of CDK2 at Tyr15 (CDK2 pTyr15), which is known to be elevated in G1/S phase and essential for G1/S transition, and phosphorylation of H3 at S10 (H3 pS10), which is known to be elevated in G2/M phase. Not surprisingly, we detected a significant decrease in CDK2 pTyr15, but no changes in H3 pS10, by BPA treatment, implying a suppression of the G1/S phase transition (Fig.3.4B). The gene function analysis also showed that expression of 19 genes was altered by BPA treatment in the direction consistent with a decrease in the G1/S phase transition (Fig.3.4C), suggesting that BPA at 100 μ M caused cell cycle arrest at the G1 phase. Knowing that p53 is also an important mediator of cellular senescence, we measured expression of β -galactosidase known to accumulate in senescent cells using immunocytochemical staining. Our results indicated a significant increase in percentage of β -galactosidase-positive cells (Fig.3.4D). This finding is in full agreement with the result of gene function analysis showing that expression of 41 genes were changed by BPA treatment in the direction consistent with an increase in cellular senescence (Fig.3.4E).

3.3.4 BPA increases autophagy

In response to DNA damage and/or other forms of stress, p53 can be activated and acts through both transcription-dependent and -independent mechanisms to coordinate cellular responses, which either prevent or repair genomic damage or eliminate potentially oncogenic cells. In addition to causing cell-cycle arrest and senescence, p53 is also known to regulate autophagy, a lysosomal pathway of cellular self-digestion. With the activation of p53 in BPA treated HFLF, we predicted that BPA treatment may also

induce autophagy, and thus using both immunocytochemical and Western blot methods we measured protein expression of microtubule-associated proteins 1A/1B light chain 3B (MAP1LC3B or LC3B) (Fig.3.5A), which is a central protein in the autophagy pathways. Our results demonstrated that 24 h exposure to 100 μ M BPA increased protein expression of LC3B, especially in the perinuclear region, and more specifically LC3BII (Fig.3.5A). We also observed a significant increase in percentage of cells containing autophagic vacuoles (Fig.3.5B). This is consistent with the result of microarray analysis, which showed that many genes including MAP1LC3B2 that are known to be involved in autophagy or regulation of autophagy were upregulated (Fig.3.5C).

3.3.5 BPA increases cellular ROS level and activates Nrf2-regulated stress response and xenobiotic detoxification pathways

Microarray and IPA revealed that BPA at 100 μ M increased gene expression of nuclear factor (erythroid-derived 2) factor 2 (Nrf2), a transcription factor known to regulate oxidative stress/antioxidant defense response pathways (Fig.3.1D). In this pathway, we found increased expression of Nrf2 (or NFE2L2) gene and its downstream target genes which are known to be involved in antioxidant defense, xenobiotic transport and metabolism, and protein degradation (Fig.3.6A). To confirm this finding, we measured protein levels of Nrf1 and Nrf2 and its downstream target, HMOX1 using Western blot. Our results showed that BPA treatment significantly increased Nrf2 and HO-1 protein expression after 24 h of exposure (Fig.3.6B). It is well established that Nrf2-regulated oxidative stress/antioxidant response pathways are activated by an increased production of reactive oxygen species (ROS). To confirm this, we measured

ROS production in HFLF after 24 h of exposure to 100 μ M BPA using CellRox deep red dye, fluorescent imaging and fluorescent plate reader. We detected a significant increase in ROS level in BPA- treated cells (Fig.3.6C).

3.3.6 BPA increases endogenous estradiol release and HSD17B14 and CYP1B1 gene and protein expression

Our microarray analysis showed that a number of genes in the cholesterol and estrogen synthesis and metabolism pathways including 17-beta-hydroxysteroid dehydrogenases 14 (HSD17B14) and CYP1B1 were upregulated in HFLF after 24 h of exposure to 100 μ M BPA. To confirm the gene analysis data, we measured protein expression of CYP1B1 and HSD17B14 using Western blot, ELISA and immunocytochemical staining. Western blot of CYP1B1 revealed three protein bands at 52, 70, and 110 kDa. BPA at both 1 and 100 μ M significantly increased the 110 kDa protein expression (Fig3.7A). CYP1B1 was located at the perinuclear region of the cell and BPA treatment increased overall staining intensity and nuclear localization of CYP1B1 (Fig.3.7B). Protein expression of HSD17B14 was also significantly increased by both 1 and 100 μ M BPA after 24 h of exposure (Fig.3.7C), suggesting a potential increase in estradiol synthesis and/or metabolism. To confirm this, we measured estradiol release into the culture media, and found that BPA at 100 μ M significantly increased release of estradiol from HFLF (Fig.3.7D), which is consistent with the effects of BPA on expression and regulation of genes involved in steroid synthesis and metabolism (Fig.3.7E). Since BPA also increased mRNA level of aryl hydrocarbon

receptor (AhR), it is likely that the increased CYP1B1 expression was a result of AhR gene upregulation by BPA, although more experiments are needed to confirm this.

3.4 Discussion

In our previous and present studies, using HFLF and various proteomic and genomic tools, we investigated the molecular mechanisms underlying the reported effects of prenatal exposure to BPA on fetal lung development and maturation in animal models and humans. Our results revealed that cell cycle control, stress response, autophagy, xenobiotic detoxification, and steroid synthesis and metabolism are the major molecular pathways and cellular processes affected by 100 μ M BPA after 24 h of exposure. BPA at 1 μ M significantly increased protein expression of ER β , GPR30, HSD17B14 and CYP1B1, but did not induce changes in global gene expression, suggesting that protein translation, modification and/or translocation of estrogen receptors (ER β and GPR30) and enzymes involved in steroid synthesis and metabolism are more sensitive targets of BPA in HFLF.

DSBs are the most deleterious DNA lesions, which, if unrepaired, may lead to chromosome aberrations, genomic instability, and/or cell death (Jackson et al., 2002). RAD50 is a DSB repair protein. Upon occurrence of DSBs, it forms complex with Mre11 and NBS1, which plays a central role in DSB repair, DNA recombination, maintenance of telomere integrity and meiosis. BPA treatment increases RAD50 gene expression, suggesting a need of RAD50 at DSB sites. In response to ROS-induced DSBs, ATM is recruited by MRE11- RAD50-NBS1 complex to the site of DSBs and

activated by autophosphorylation that allows inactive dimer of ATM to dissociate into active monomers. Phosphorylated ATM (pATM) acts on downstream target genes including H2AX, 53BP1 and p53 which regulate DSB repair and cell cycle control processes. Increased pATM, γ -H2AX, and p53 pS15 by 100 μ M BPA further supports the notion that BPA exposure increases DNA DSBs in HFLF after 24 h of exposure.

It is well established that oxidative stress-induced DNA damage induces ATM signaling pathway subsequently leading to p53 activation (Ben-Porath and Weiberg, 2005). It is known that p53 activation inhibits cyclin E and its partner CDK2, a complex required for progression of the cell cycle from G1 to S phase (Sancar et al., 2004). The p53 is a key mediator of cell cycle arrest and senescence in response to stress (Tonnessen-Murray et al., 2017). Senescent cells are viable and metabolically active, increased size, flat morphology, but unable to replicate their DNA (Hayflick and Moorhead, 1961). In addition to DNA DSBs and cell cycle arrest at G1 phase, BPA exposure also increases senescence in HFLF as shown by increased β -galactosidase and changes in expression of 41 genes in the direction consistent with increased cellular senescence. In our study, BPA treatment resulted in cells that are large and flat, the typical morphology of senescent cells. BPA-induced activation of ROS-DSB-pATM-p53S15-pCDK2-G1/S transition-senescence axis in HFLF further confirms the role of ATM, p53, and CDK2 in regulating cellular response to chemical induced DNA damage.

Autophagy can be induced by DNA damage to degrade aggregated proteins, damaged mitochondria and even ribosome (Abedin et al., 2007). When DNA damage is mild and repairable, cytoprotective autophagy will be activated to promote cellular survival via enhanced turnover of DNA repair proteins (Zhang et al., 2015). LC3 plays

multiple roles in autophagy including membrane fusion, cargo selection and autophagosome transport. It is regarded as the most promising autophagosomal marker in mammals (Kabeya et al., 2000). p62/SQSTM1 delivers ubiquitinated cargoes for autophagic degradation. In some cell lines, overexpressed p62 enhances protein aggregation and has a protective effect on cell survival (Paine et al., 2005). p53 is known to promote autophagy through upregulation of DDIT4 (DNA damage-inducible transcript 4) (Mathiassen et al., 2017). It has been shown that DDIT4 mediates methamphetamine-induced autophagy and apoptosis through mTOR signaling pathway in cardiomyocytes (Chen et al., 2016). FIP200 (focal adhesion kinase family interacting protein of 200 kD) also known as RB1CC1, is required for the formation of ULK1-ATG13-FIP200 complex which acts as a node for integrating incoming autophagy signals into autophagosome biogenesis (Ganley et al., 2009). In this study, BPA at 100 μ M significantly increased expression of a number of genes known to be involved in autophagy. BPA also increased LC3B2 protein expression and the formation of autophagic vacuoles in HFLF after 24 h of exposure. It is possible that ROS-DSB-ATM-p53-DDIT4-LC3B2 pathway acts in the BPA-induced autophagy in HFLF, which implies an adaptation and survival response to BPA-induced DNA damage and stress.

By regulating the expression of genes coding for antioxidant, anti-inflammatory and detoxifying proteins, Nrf-1 and Nrf-2 transcription factors play a pivotal role in the cellular defense against the toxic effects of ROS (Loboda et al., 2016). HO-1, a known Nrf2-dependent gene (Alam et al., 1999), is often upregulated by oxidative stress, and has been implicated in cytoprotective defense response against oxidative injury (Otterbein et al., 2000). In this study, BPA induced ROS production resulted in activation of Nrf2-

regulated oxidative stress/antioxidant defense response pathway, leading to increased expression of downstream genes and/or proteins including HO-1, NQO1, EPHX1, and ABCCs. These genes play important roles in the antioxidant and anti-inflammation processes, as well as metabolism and transport of BPA in HFLF. This is in concert with our previous observation that BPA at 100 μ M decreased the release of cytokines and caused no change in cell viability.

It is unexpected that BPA increased release of estradiol at 100 μ M. This increase in estradiol release by BPA correlated with an increase in HSD17B14 (or DHRS10) and CYP1B1 gene and protein expression, suggesting that steroidogenesis in human fetal lung is a target of BPA exposure. This finding is consistent with the report that prenatal exposure to low dose BPA increased estradiol level and aromatase activity in both female and male mouse urogenital sinus measured on postnatal day 1 (Arase et al., 2011). The fact that BPA at 100 μ M, but not 1 μ M, caused significant changes in gene expression compels us to speculate that the much greater degree of increase in estradiol release by 100 μ M BPA may be responsible for most of the effects of BPA we observed at this dose level.

CYP1B1 is known to catalyze hydroxylation of estradiol at the C-2 and C-4 positions, resulting in formation of catechol estrogens 2-hydroxyestradiol and 4-hydroxyestradiol, which can be oxidized to quinones and quinone derivatives that can increase ROS production and form adducts with DNA, resulting genotoxicity (Yager 2000). Although the source of BPA-induced ROS in HFLF remains to be identified, hydroxylation and oxidation of estradiol by CYP1B1 is highly possible. Hydroxylation (Knaak and Sullivan, 1966, Jaeg et al., 2004) and formation of the semiquinone and

quinone have been reported for BPA (Roy et al., 1997). In this study, BPA increased gene and/or protein expression of CYP1B1, which may also contribute to hydroxylation and oxidation of BPA in HFLF, although more investigation is required to confirm this.

Based on the data generated from this study and our previous studies, we proposed a mechanistic model for the action of BPA in HFLF as shown in Fig. 8. BPA at 1 μ M induces a small degree of GPR30 nuclear translocation, a small increase in HSD17B14 and CYP1B1 protein expression, which does not influence global gene expression, resulting in no changes in ROS production and cell viability and growth. BPA at 100 μ M induces a large increase in nuclear GPR30 and cytoplasmic ER β protein expression leading to unclear downstream changes, which still need to be characterized. Meanwhile, BPA at 100 μ M increases gene expression of AhR, CYP1B1, POR, HSD17B14, and CYP51A1, leading to increased synthesis and release of estradiol.

Part of the estradiol and BPA may be hydrolyzed by EPHX1 and then oxidized by CYP1B1, leading to increased ROS production, which results in increased DNA DSBs, increased Nrf2 gene and protein expression and activation of Nrf2-regulated oxidative stress/antioxidant defense response pathways. This action leads to increased expression of antioxidant enzymes such as HO-1 and NQO1 and transporters such as ABCC1/4 (MRP1/2), which protect cells from ROS induced injury, while also suppressing NF κ B signaling, resulting in decreased release of cytokines (IL-6, IP-10, ET-1). The increased DNA DSBs activates ATM autophosphorylation, which leads to phosphorylation and stabilization of p53 for regulating transcription activity, which in turn increases expression and release of GDF-15. GDF-15 and p53 act to downregulate genes involved in cell cycle such as CDK1/2, CCCND1, CCNB1, CCNA2, CDC25, and FANCD2, and

decreases CDK2 phosphorylation, resulting in decreased G1/S transition and increased cell cycle arrest and senescence. GDF-15 can also decrease NFκB regulated cytokines release leading to immune suppression. p53 upregulates expression of LC3B2, RB1CC1 (FIP200), CTSs, and AGT2B, along with Nrf2-regulated SQSTM1, causing increased autophagy.

Through paracrine mechanisms, fetal lung fibroblasts secrete various growth factors, cytokines and hormones into extracellular matrix to regulate surfactant production, differentiation, and replication of alveolar type II cells during fetal lung development and maturation (Post et al., 1986). For example, upon induction by parathyroid hormone-related peptide, fetal lung fibroblasts secrete IL-6 to stimulate surfactant synthesis in pulmonary epithelial cells (Torday et al., 1998). BPA-induced decrease in IL-6 secretion from HFLF may cause delayed lung maturation, if it occurs *in vivo*.

The effects of BPA on the secretome, growth, and metabolic activation of HFLF observed in our studies mainly occurred at 100 μM dose level corresponding to 23 μM intracellular BPA concentration, which is markedly higher than what was detected in human fetal liver and amniotic fluid. However, it is still unclear what concentrations of BPA the human fetal lung is exposed to. With lower detoxification capability, higher surface to volume ratio, and multiple exposure sources, the fetal lung may accumulate BPA to much higher concentrations than other fetal tissues, and thus may be more vulnerable to BPA-induced toxicity. Our studies revealed possible mechanisms underlying the reported effects of prenatal exposure to BPA on fetal lung development

and maturation, which may contribute to increased sensitivity to respiratory disease in postnatal life.

Figure and Legends of Chapter 3

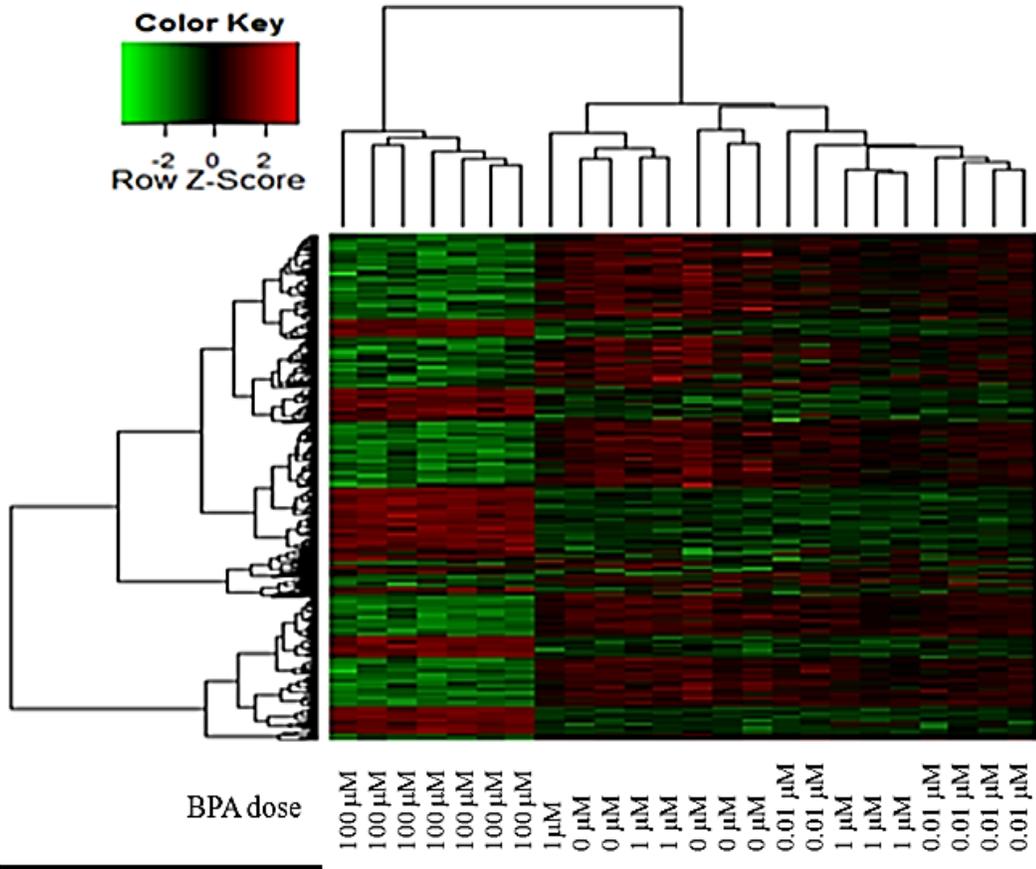


Fig.3.1A Heatmap representing hierarchical clustering of differentially expressed genes with $p \leq 0.05$. HFLF cultured as described in Methods. And exposed to 0, 0.01, 1, or 100 μM BPA for 24 h. mRNA was isolated and subjected to microarray analysis using Affymetrix 1.0ST human gene arrays. Clustering analysis of gene expression data revealed differential gene expression pattern in the 100 μM BPA dose group as compared to other dose groups Red and green colours represent expression levels greater than or less than the mean, respectively.

Number of genes among 26242 probes shown significant differential expression between treatment groups	
Comparison of treatment groups	<i>p</i><0.05
0.01 versus 0 μM BPA	0
1.00 versus 0 μM BPA	0
100 versus 0 μM BPA	2527
1.00 versus 0.01 μM BPA	0
100 versus 0.01 μM BPA	1923
100 versus 1.00 μM BPA	2220

Fig.3.1B The number of differentially expressed genes ($p \leq 0.05$) in HFLF.

These differences represent significant ($p \leq 0.05$) changes in the expression of 2527, 1923, and 2220 genes in 100 μ M BPA dose group as compared to 0, 0.01, and 1 μ M dose groups, respectively.

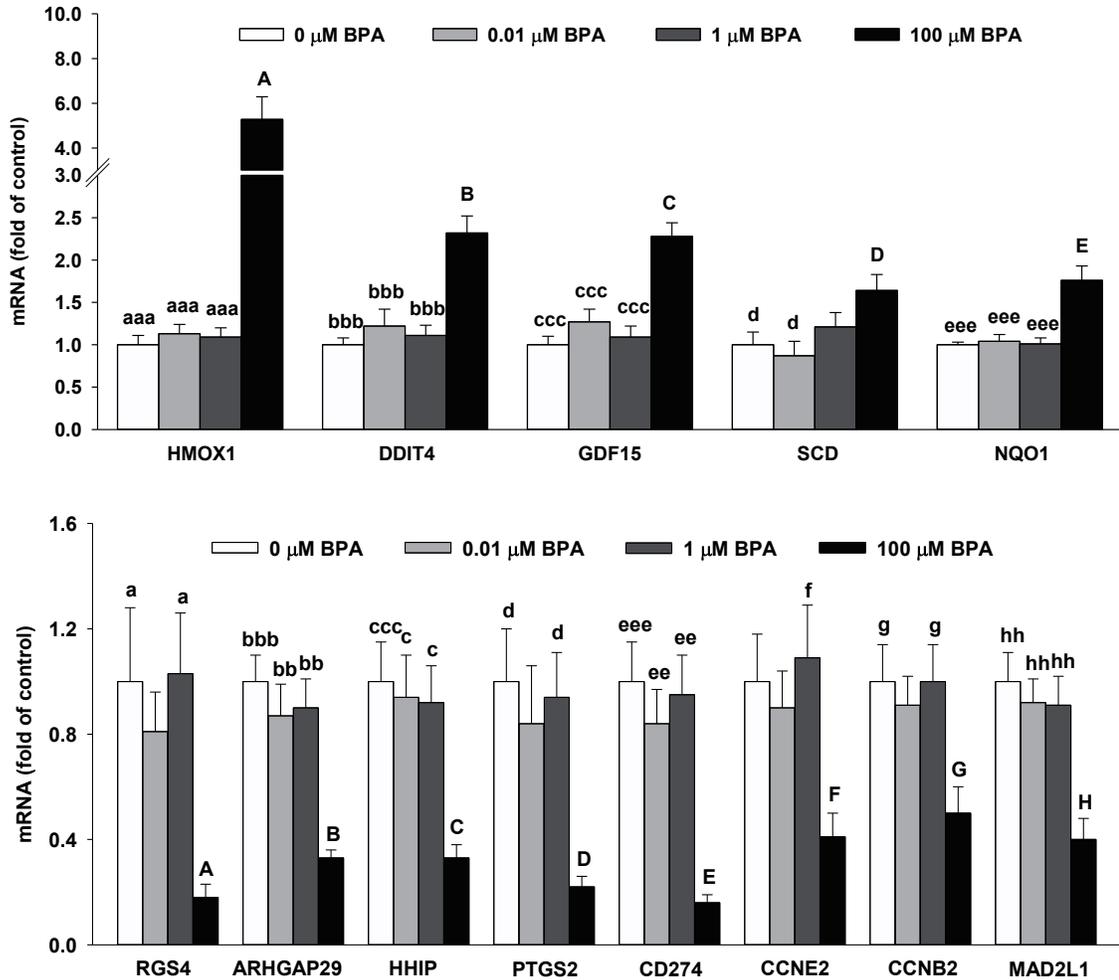


Fig.3.1C Validation of selected genes using RT-qPCR. HFLF cells cultured as described in Methods exposed to 0, 0.01, 1, or 100 μM BPA for 24 h. mRNA was isolated and subjected to microarray analysis using Affymetrix 1.0ST human gene arrays. Some of the differentially ($p \leq 0.05$) expressed genes between 100 and 0 μM BPA dose groups with a fold of change ≥ 1.5 were validated by RT-qPCR. Vertical bars represent means of 5-7 independent experiments. Error bars are the standard errors of the means.. “A”, “B”, “C”, and “E” is significantly different from “aaa”, “bbb”, “ccc” and “eee” at $p < 0.001$, respectively. “B”, “D”, “E”, and “H” is significantly different from “bb”, “dd”, “ee”, and “hh” at $p < 0.01$, respectively. “A”, “C”, “D”, “E”, “F” and “G” are significantly different from “a”, “c”, “d”, “e”, “f”, and “g” at $p < 0.05$, respectively.

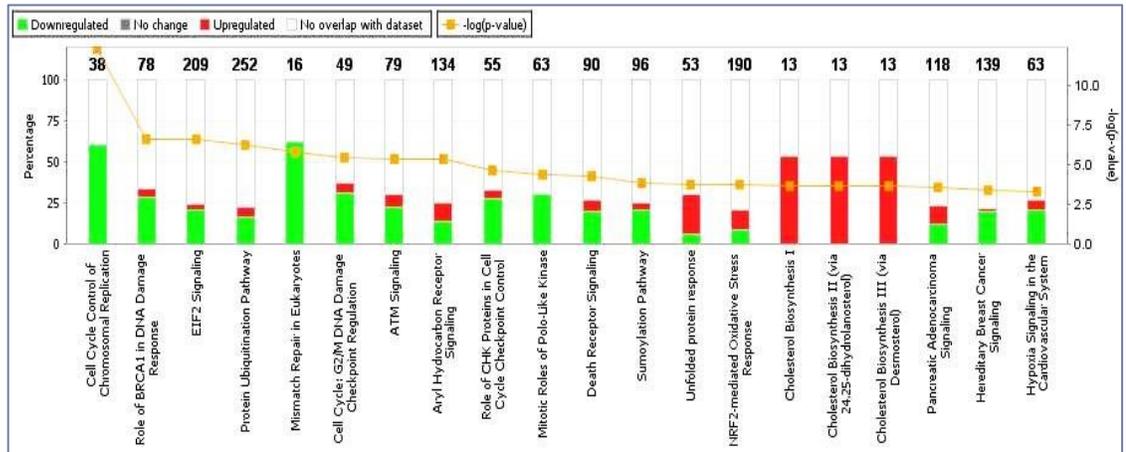


Fig.3.1D Top canonical pathways identified by IPA analysis in HFLF. HFLF cultured as described in Methods. And exposed to 0, 0.01, 1, or 100 μ M BPA for 24 h. mRNA was isolated and subjected to microarray analysis using Affymetrix 1.0ST human gene arrays. All genes with $p \leq 0.05$ were subjected to IPA. The top canonical pathways affected by BPA treatment are Cell Cycle Control of Chromosomal Replication (CCCCR), Role of BRCA1 in DNA Damage Response, ATM Signaling, Nrf2-mediated Oxidative Stress Response and Cholesterol Synthesis.

Gene Function Annotation	p-value	Activation z-score	# of Molecules
Viral Infection	2.68E-05	-4.369	312
infection of embryonic cell lines	5.37E-05	-3.727	61
homologous recombination	7.39E-07	-3.416	36
cytokinesis	6.7E-12	-3.387	59
M phase	2.2E-21	-3.218	90
cell survival	1.91E-10	-2.646	308
cell proliferation of fibroblasts	2.89E-09	-2.511	92
association of chromosome components	3.8E-06	-2.488	13
association of chromatin	3.32E-06	-2.455	10
metabolism of DNA	2.14E-13	-2.44	112
DNA replication	4.87E-14	-2.402	80
G1/S phase transition	1.49E-05	-2.37	52
chromosomal congression of chromosomes	2.6E-09	-2.345	11
proliferation of fibroblast cell lines	3.15E-05	-2.332	94
repair of DNA	1.83E-12	-2.259	91
repair of cells	1.47E-05	-2.254	28
cell viability	1.45E-10	-2.253	292
segregation of chromosomes	1.57E-20	-2.113	59
chromosomal aberration	0.000049	1.963	42
formation of mitotic spindle	2.86E-09	1.982	29
ploidy of fibroblasts	1.92E-05	1.982	5
apoptosis	5.82E-15	2.28	550
damage of chromosomes	1.72E-05	2.549	19
formation of nuclear foci	1.79E-08	2.739	32
breakage of chromosomes	1.67E-05	2.819	18
senescence of cells	9.16E-10	2.973	78
necrosis	1.14E-21	3.221	589
cell death	1.85E-19	3.227	707
formation of gamma H2AX nuclear focus	1.49E-06	3.341	22

Table 3.1 Gene functions significantly affected by 100 versus 0 μ M BPA exposure with $-2.0 \geq z\text{-score} \geq 2.0$.

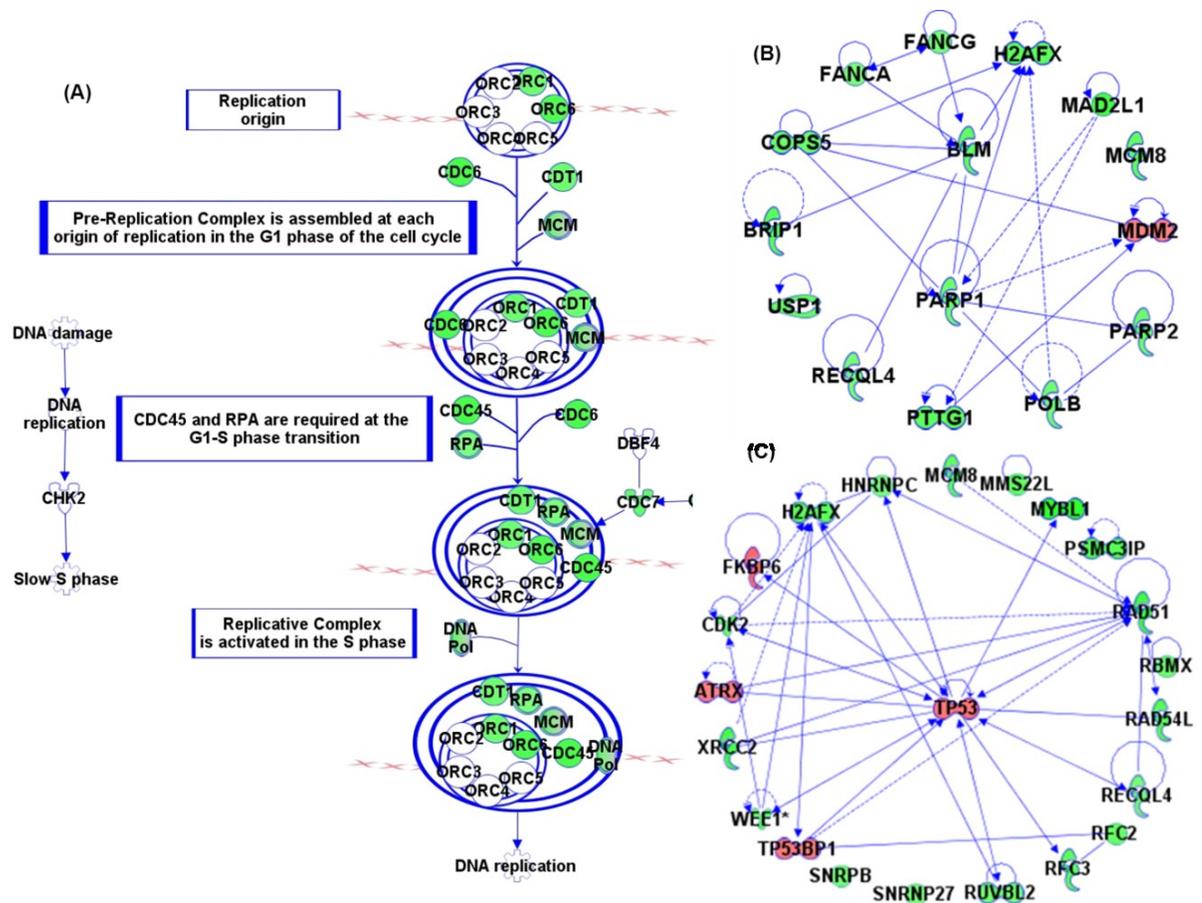


Fig.3.2 Expression and interaction of genes in cell cycle control of chromosomal replication (A), breakage of chromosome (B) and homolog recombination (C) identified by IPA in HFLF exposed to 0 and 100 μ M BPA for 24 h. HFLF were treated with BPA as described in Methods. mRNA was isolated from 6 independent experiments and subjected to microarray analysis using Affymetrix 1.0ST human gene arrays. All genes with $p \leq 0.05$ were subjected to IPA. Genes are represented as nodes with various shapes for different types. Red and green nodes indicate up-regulated and down-regulated genes induced by BPA, respectively. Connective edges represent various types of biological relationships among these genes. Expression of 23 genes associated with cell cycle control of chromosomal replication was altered by BPA treatment. $p = 5.14E-$

13 (A). Expression of 15 genes altered by BPA treatment in the direction consistent with increased breakage of chromosome with $z=2.819$, overlap $p=1.67E05$ (B). Expression of 22 genes altered by BPA treatment in the direction consistent with decrease in homologous recombination with $z\text{-score}=3.416$, overlap $p =7.39E-07$ (C).

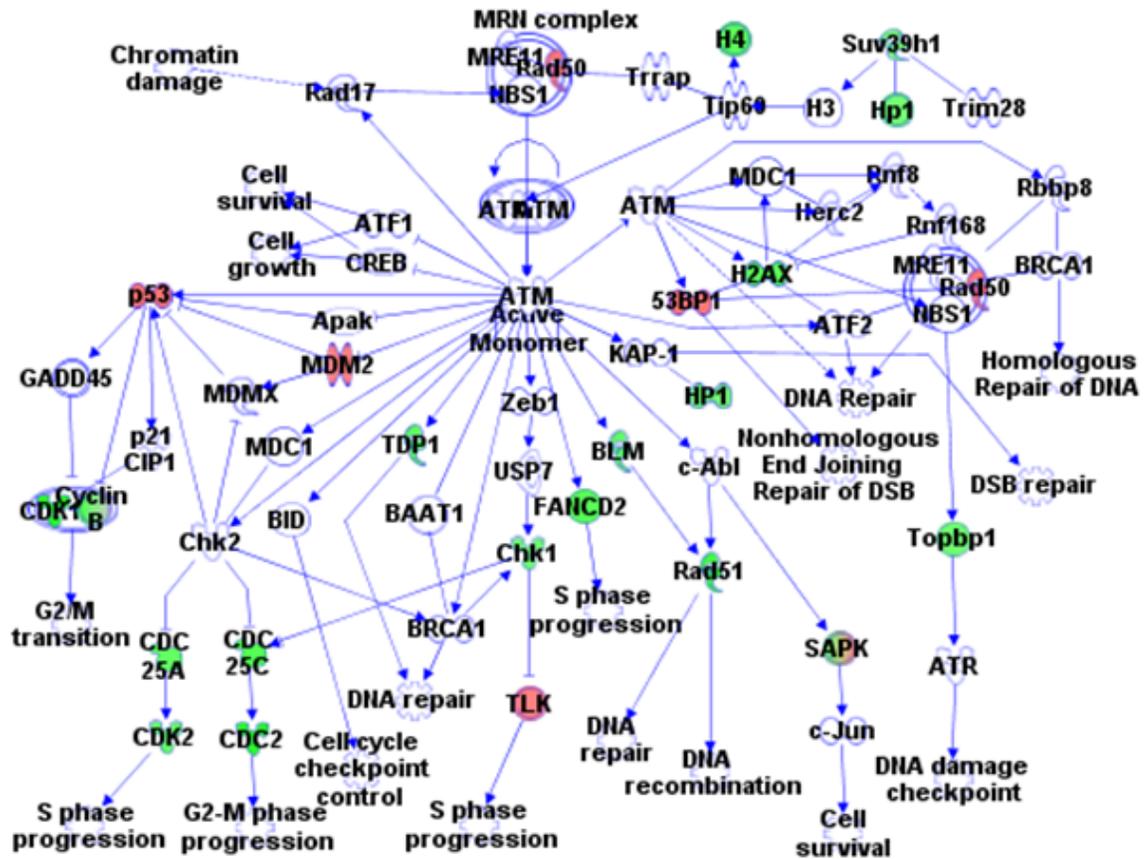


Fig.3.3A Expression of genes and interaction networks in ATM Signaling pathway identified by IPA in HFLF exposed to 0 and 100 μM BPA for 24 h. HFLF were treated with BPA as described in Methods. mRNA was isolated from 6 independent experiments and subjected to microarray analysis using Affymetrix 1.0ST human gene arrays. All genes with $p \leq 0.05$ were subjected to IPA. Genes are represented as nodes with various shapes for different types. Red and green nodes indicate up-regulated and down-regulated genes induced by BPA, respectively. Connective edges represent various types of biological relationships among these genes. Expression of 18 genes were altered by BPA treatment in the direction consistent with increase in formation of $\gamma\text{-H2AX}$ with z-score=3.341 overlap $p=1.49\text{E-}06$.

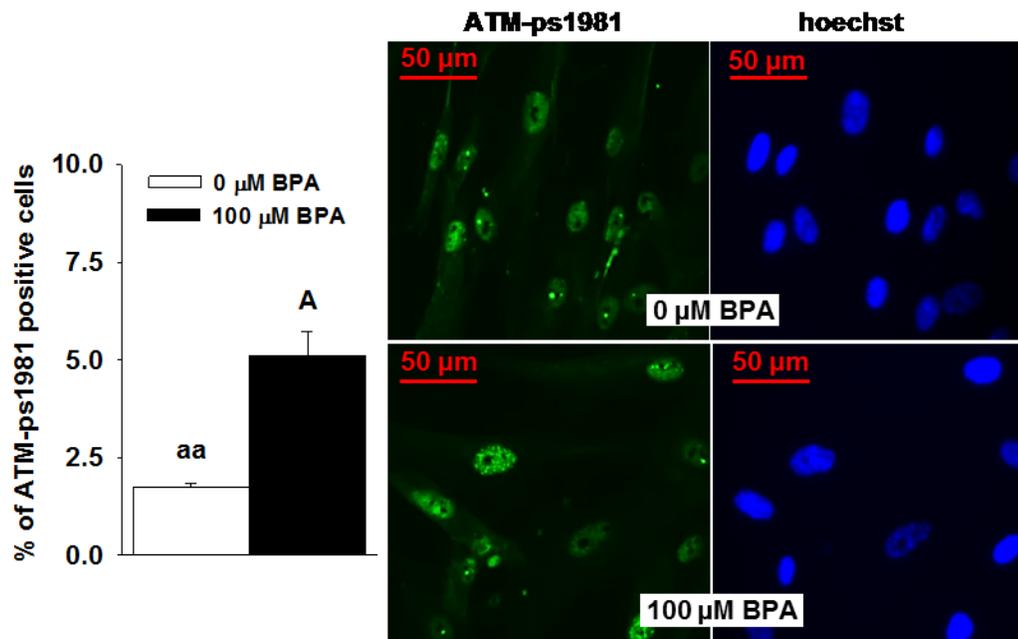


Fig.3.3B Immunocytochemical staining of Phosphorylated ATM (p s1981) in HFLF exposed to 0 and 100 μM BPA for 24 h. HFLF were treated with BPA as described in Methods. Cells were incubated with rabbit monoclonal anti-ATM (phospho S 1981) (Abcam Inc, Cambridge, USA) and Alexa Fluor 488 goat anti-rabbit IgG (Life Technologies, Carlsbad, CA, USA). Slides were mounted with mounting media (Dako, Burlington, Canada). The positive staining was assessed under a Zeiss Axiovert 40CFL inverted microscope (Carl Zeiss Microscopy, LLC) using 20X objective and photographed with a SPOT RT3 digital camera and SPOT basic software (Diagnostic Instrument Inc., Sterling Heights, MI, USA). Six hundred (600) cells were counted per experiment from 3 independent experiments using ImageJ software (imagej.nih.gov/ij). Cells having bright spots were considered as ATM positive cells. Values represent the means of 3 independent experiments. Error bars are the standard errors of the means. “aa” is significantly different from “A” at $p < 0.01$, respectively.

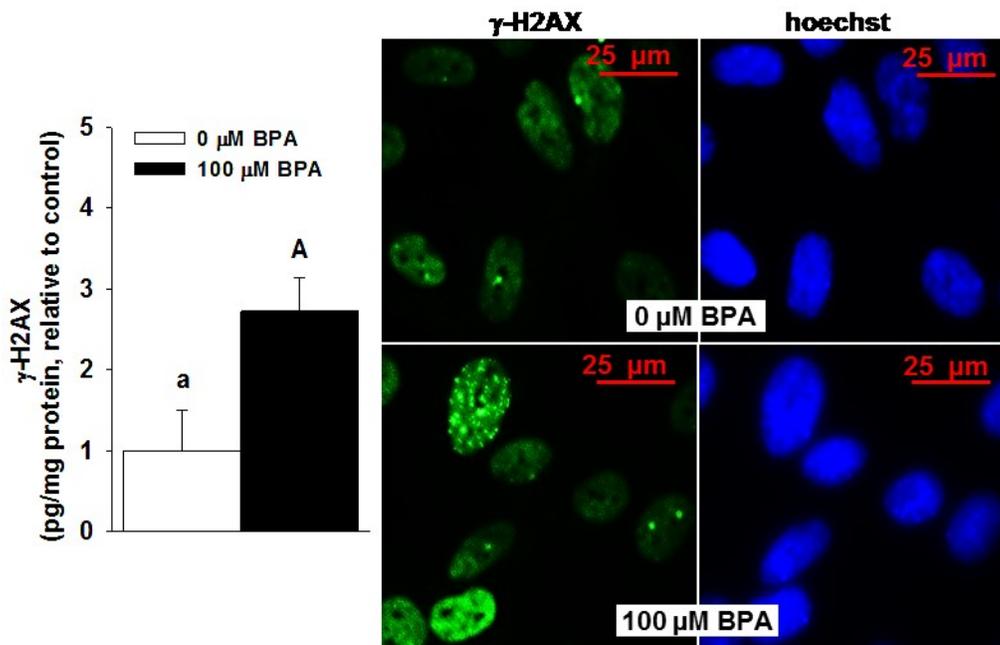


Fig.3.3C Phosphorylated H2AX (γ -H2AX) protein expression in HFLF exposed to 0 and 100 μ M BPA for 24 h. Localization of γ -H2AX, using immunocytochemistry assay was conducted as describe in methods using rabbit monoclonal anti-phospho histone H2AX (Ser139) (Millipore Canada Ltd, Etobicoke, ON, Canada). To quantify its protein expression, cell lysates were collected and were measured for levels of phosphorylated H2AX (γ -H2AX) using γ -H2AX Pharmacodynamic Assay ELISA Kit (Trevigen Inc, Gaithersburg, MD, USA) according to the manufacturer's instructions. Values represent the means of 3 independent experiments. Error bars are the standard errors of the means. "a" and is significantly different from "A" at $p < 0.05$, respectively.

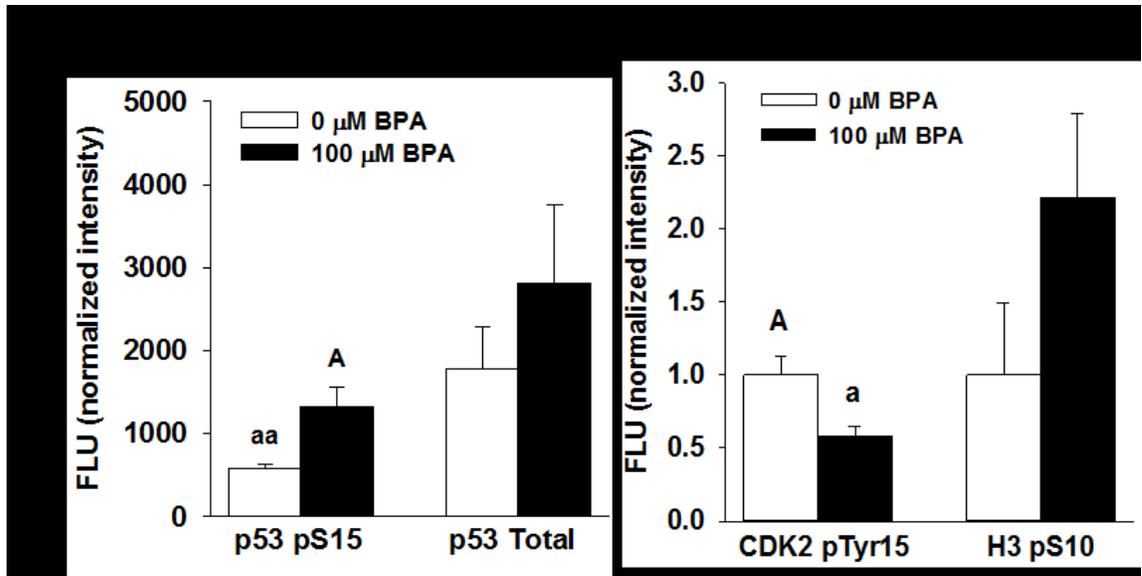


Fig.3.3 (D, E) Total and phosphorylated p53 (pS15); phosphorylated CDK2 (pTyr15) and Histone H3 (pS10) protein expression in HFLF exposed to 0 and 100 μM BPA for 24 h. HFLF were cultured and cell lysates were harvested as describe in Methods section. Protein expression of total and phospho p53 at S15 (p53 pS15) was measured in cell lysates using ELISA kits (Abcam Inc, Toronto, ON Canada) according to manufacturer’s instructions (A). The p53 pS15 protein levels in samples were normalized against total protein concentrations. The level of phosphorylated Cdk2 (CdK2 pTry15) and histone H3 (H3 pSer10) were measured using Cell Cycle In-Cell ELISA Kit (Abcam Inc, Toronto, Canada) according to the manufacturer’s instructions (B). The fluorescence signal (RFU) intensity was normalized by subtracting the mean of background signal (with no primary antibody) from all other experimental signals. The fluorescence intensity of different antibodies was normalized by dividing the Ganus Green staining recorded at OD 595nm. Vertical bars represent means of 3 independent experiments. Error bars are the standard errors of the means. “A” is significantly different from “a” and “aa” at $p < 0.05$ and 0.01 , respectively.

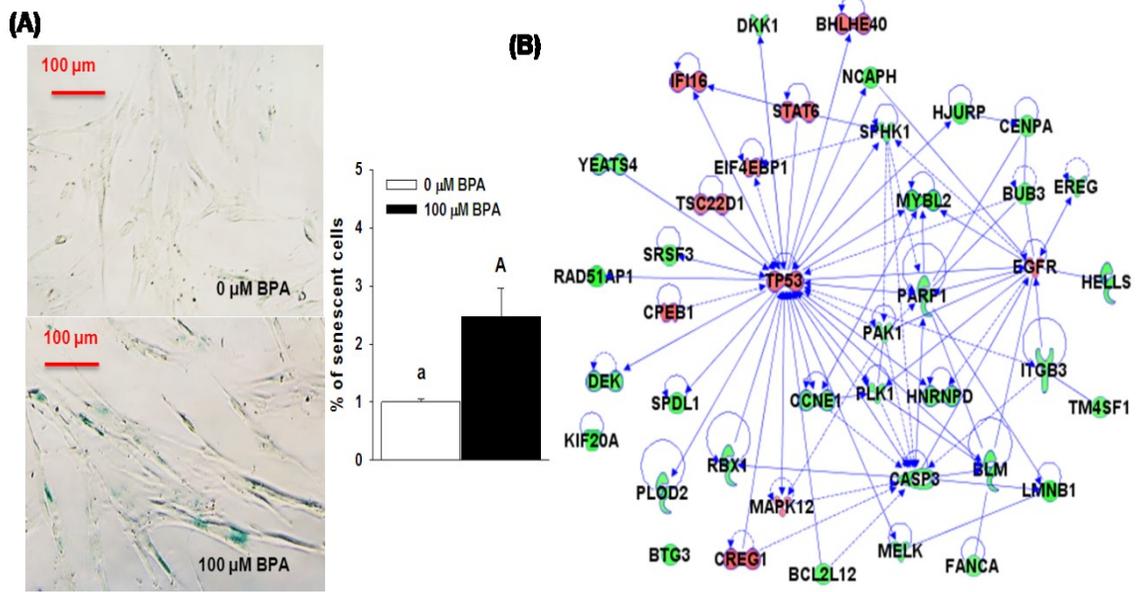


Fig.3.4 β -galactosidase staining of senescent cells and expression networks of senescence associated genes in HFLF cells exposed to 0 and 100 μ M BPA for 24 h.

HFLF cells after 24 h exposure to 0 and 100 μ M BPA in the 6 well plates, the β -galactosidase detected using β -Galactosidase Staining kit (Cell Signaling, Danvers, MA, USA) as described in Methods. The β -galactosidase blue staining was assessed under a Zeiss Axiovert 40 CFL inverted microscope (Carl Zeiss Microscopy, LLC) and photographed with a SPOT RT3 digital camera and SPOT basic software (Diagnostic Instrument Inc., Sterling Heights, MI, USA). The staining was quantified by counting of the positively stained flat cells using ImageJ software (imagej.nih.gov/ij). Proportion of big flat senescent cells with positive staining was calculated as percentage of total number of cells (A). Vertical bars represent means of 3 independent experiments. Error bars are the standard errors of the means. “A” is significantly different from “a” at $p < 0.05$ and 0.01, respectively. Gene expression networks were analysed by IPA (B). Expression of 41 genes was altered by BPA treatment in the direction consistent with increase in senescence of cells. z -score=2.973, overlap $p = 9.16E-10$.

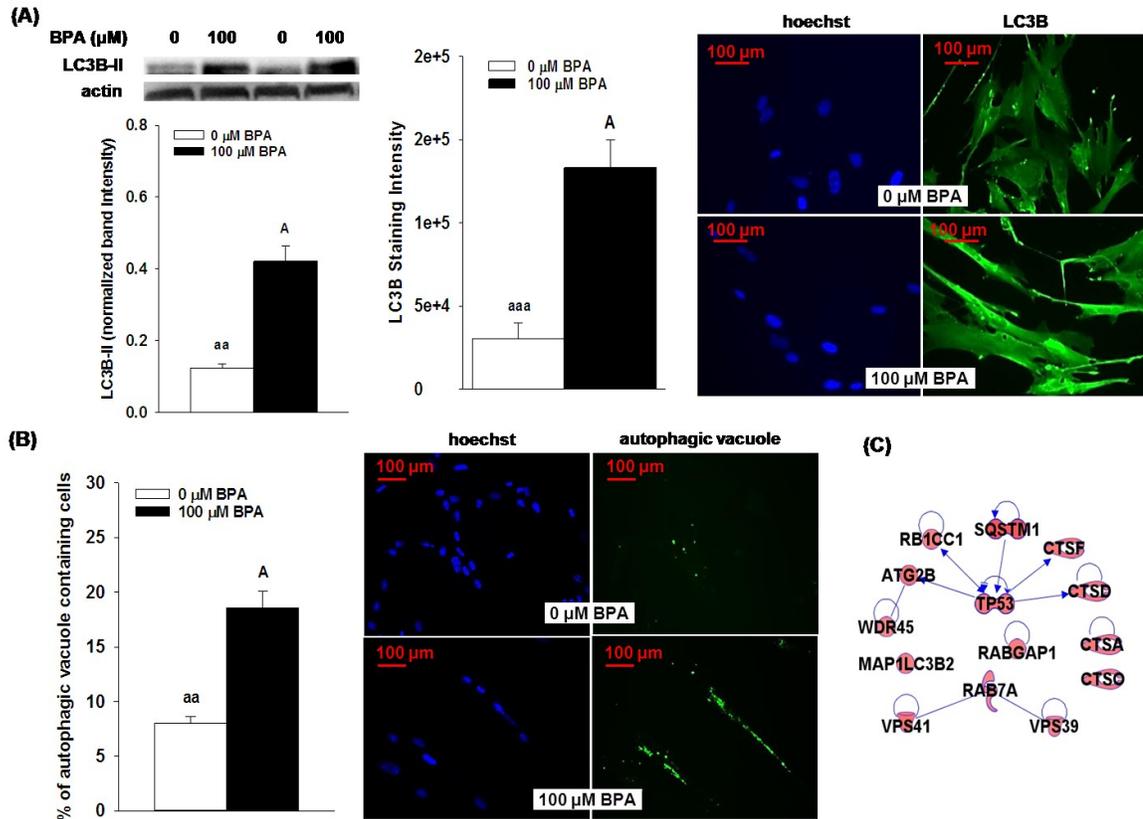


Fig.3.5 LC3B protein expression and cellular localization (A); autophagic vacuole staining (B); and upregulated expression of genes involved in autophagy identified by IPA (C) in HFLF exposed to 0 and 100 μM BPA for 24h. HFLF were treated with BPA as described in Methods. mRNA was isolated from 6 independent experiments and subjected to microarray analysis using Affymetrix 1.0ST human gene arrays. All genes with $p \leq 0.05$ were subjected to IPA. Genes are represented as nodes with various shapes for different types. Red nodes indicate up-regulated genes induced by BPA. LC3B protein expression and cellular localization were measured by Western blot and immunocytochemical staining as described in Methods. Autophagic activity was measured using a commercial kit. Vertical bars represent means of 3-4 independent experiments. Error bars are the standard errors of the means. “A” is significantly different from “aa” and “aaa” at $p < 0.01$ and 0.001 , respectively.

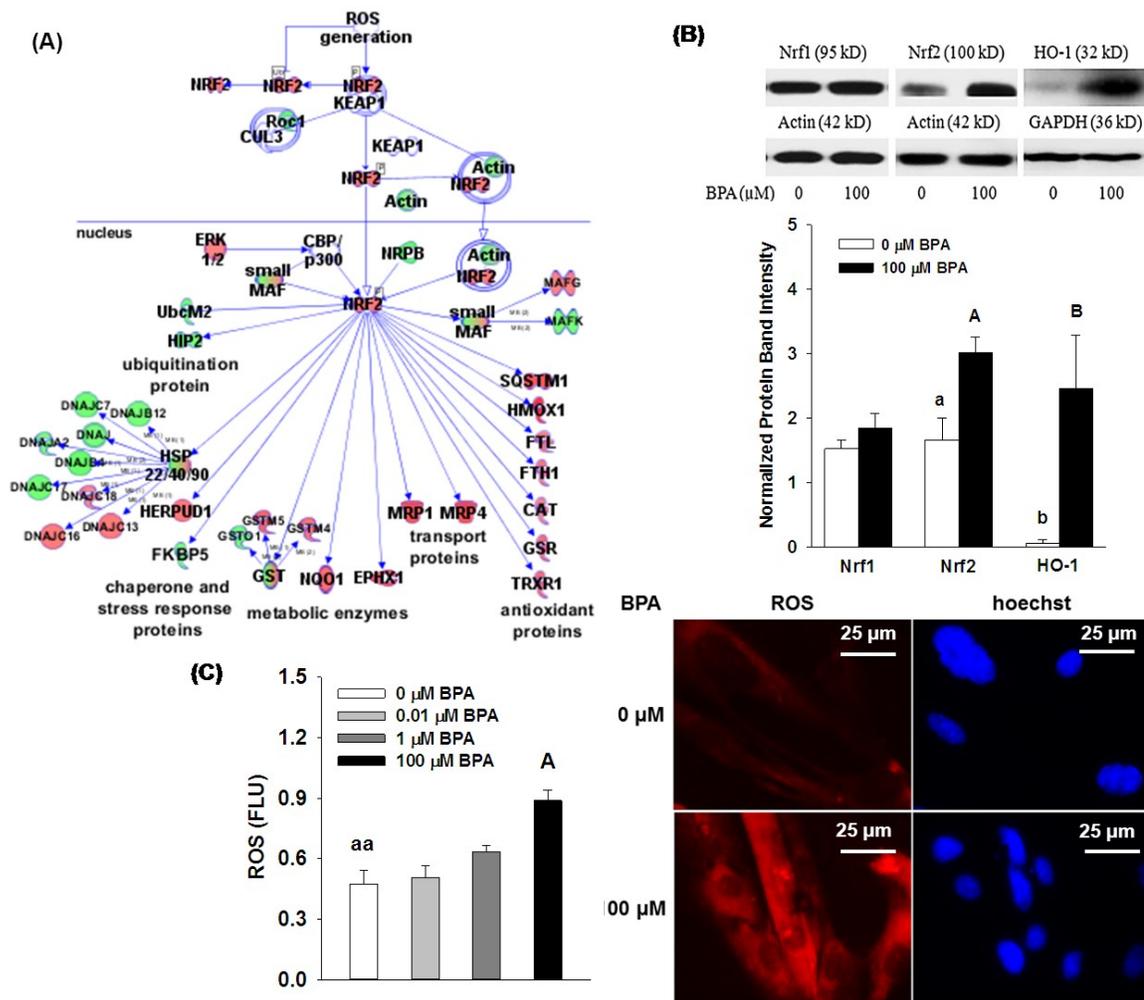


Fig.3.6 The gene expression and interaction in Nrf-2-mediated oxidative stress/antioxidant defense response pathway identified by IPA (A), Nrf1 and Nrf2, and HO-1 protein expression (B), and ROS production (C) in HFLF exposed to 0, 0.01, 1, or 100 μ M BPA for 24 h. HFLF were treated with BPA as described in Methods. mRNA was isolated from 6 independent experiments and subjected to microarray analysis using Affymetrix 1.0ST human gene arrays. All genes with $p \leq 0.05$ were subjected to IPA. Genes are represented as nodes with various shapes for different types. Red and green nodes indicate up-regulated and down-regulated genes induced by BPA, respectively. Connective edges represent various types of biological relationships

among these genes. Nrf1 and Nrf2, and HO-1 protein expression levels were measured by Western blot as described in the Methods. Band intensity was quantified and normalized against actin or GAPDH band intensity using ImageJ software. Values represent the means of four independent experiments. Error bars are the standard errors of the means. ROS was measured using CellRox dye. Vertical bars represent means of 3-4 independent experiments. Error bars are the standard errors of the means. “A” is significantly different from “a” and “aa” at $p < 0.05$ and 0.01 , respectively. “B” is significantly different from “b” at $p < 0.05$.

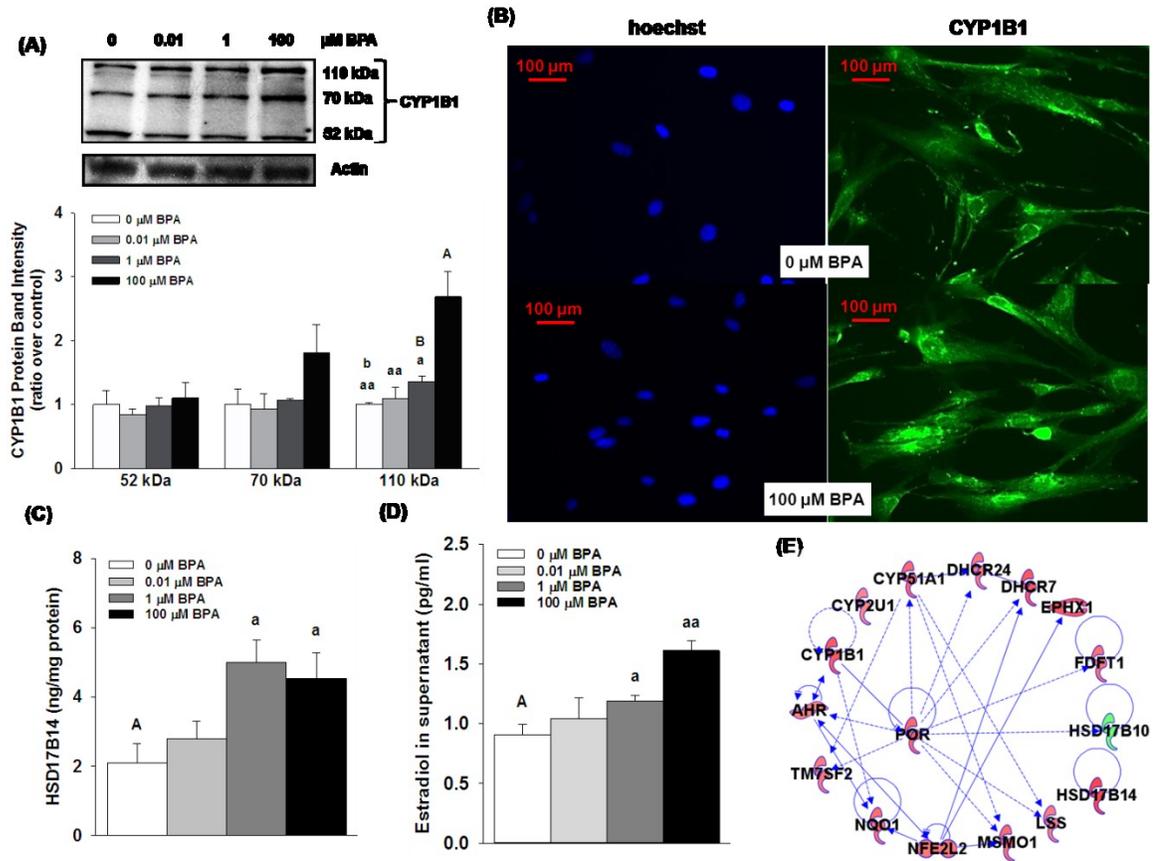


Fig.3.7 CYP1B1 protein expression (A) and cellular localization (B); HSD17B14 protein (C); release of estradiol (D); and upregulated genes involved in steroid synthesis and metabolism identified by IPA (E) in HFLF exposed to 0, 0.01, 1, and/or 100 μ M BPA for 24 h. HFLF were treated with BPA as described in Methods. mRNA was isolated from 6 independent experiments and subjected to microarray analysis using Affymetrix 1.0ST human gene arrays. All genes with $p \leq 0.05$ were subjected to IPA. Genes are represented as nodes with various shapes for different types. Red and green nodes indicate up-regulated and down-regulated genes induced by BPA, respectively. Connective edges represent various types of biological relationships among these genes. Protein expression of CYP1B1 was determined using Western blot. The HSD17B14 protein level was measured using commercial ELISA kits. (C). Estradiol in

supernatants was measured using ELISA kit. The steroids synthesis and metabolism genes were identified by IPA. Vertical bars represent means of 3-4 independent experiments. Error bars are the standard errors of the means. . “A” is significantly different from “a” and “aa” at $p < 0.05$ and 0.01 , respectively. “B” is significantly different from “b” at $p < 0.05$.

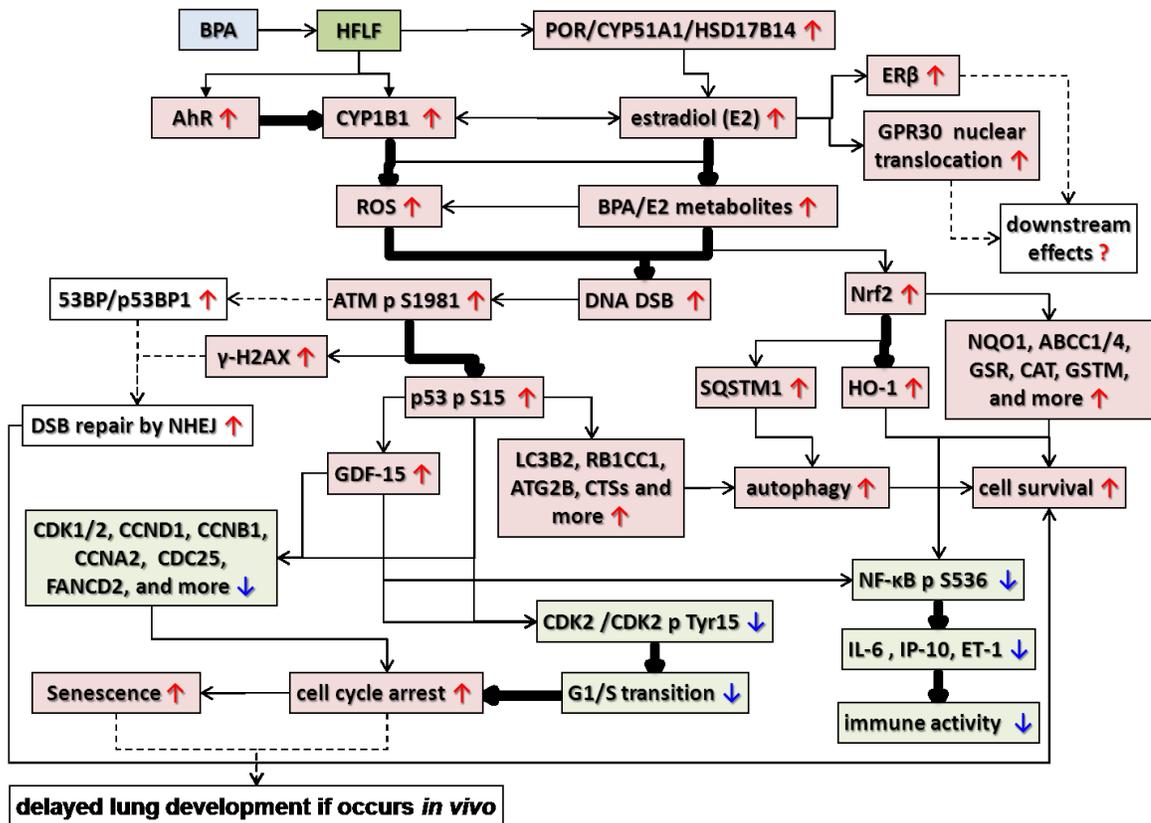


Fig.3.8 Proposed working model for the action of BPA in HFLF. BPA exposure induces a large increase in nuclear GPR30 and cytoplasmic ER β protein expression leading to unclear downstream changes. Meanwhile, BPA exposure increases gene expression of AhR, CYP1B1, POR, HSD17B14, and CYP51A1, leading to increased synthesis and release of estradiol. Part of the estradiol and BPA may be hydrolyzed by EPHA1 and then oxidized by CYP1B1, leading to increased ROS production, which results in increased DNA DSB, increased Nrf2 gene and protein expression and activation of Nrf2-regulated oxidative stress and antioxidant defense response pathway, which in turn leads to increased expression of antioxidant enzymes such as HO-1 and NQO1 and transporters such as ABCC1/4 (MRP1/2). These enzymes protect cells from ROS induced injury, and suppress NF κ B signaling, resulting in decreased release of cytokines (IL-6, IP-10, ET-1). The increased DNA DSB activates ATM autophosphorylation, which leads

to phosphorylation and stabilization of p53 for regulating transcription activity, which in turn increases expression and release of GDF-15. GDF-15 and p53 act to downregulate genes involved in cell cycle such as CDK1/2, CCCND1, CCNB1, CCNA2, CDC25, and FANCD2, and decrease CDK2 phosphorylation, resulting in decreased G1/S transition and increased cell cycle arrest and senescence. GDF-15 can also decrease NFκB regulated cytokines release leading to immune suppression. p53 upregulates expression of LC3B2, RB1CC1 (FIP200), CTSs, and AGT2B, along with Nrf2-regulated SQSTM1, causing increased autophagy. In addition, activated ATM increases phosphorylation of H2AX, possibly also 53BP1, which initiates and participates in DSB repair, likely through NHEJ.

4 Chapter: Discussion, conclusions and future directions

Many studies have focused on the effects of BPA, including its possible links to diabetes, obesity, reproductive disorders, cardiovascular diseases, birth defects, kidney diseases, and cancer (Wetherill et al., 2007; Rezg et al., 2014; Seachrist et al., 2016). However, conflicting findings on BPA toxicity and the reported differences between low-dose and high dose effects have led to continuing debate over the effects of BPA and its mechanisms of action (Vanderberg et al., 2009; Valentine et al., 2016). As a result, several legislations have deemed BPA irrelevant to human health and still permit the wide use of BPA for the production of plastic materials. An active topic of recent research focuses on the role of BPA in the development of lung related disease (Robinson and Miller, 2015). Most experiments on this topic have been performed on the pups, and these experiments demonstrated the adverse effects of BPA on normal fetal lung and airway development and these effects have been seen at high concentration of BPA exposure (Hijazi et al., 2015; Nakajima et al., 2012; Midoro-horiuti et al., 2010).

As a feeder cell, fibroblasts secrete various growth factors, cytokines and hormones into extracellular matrix to regulate growth and differentiation of other cell types, and thus play an important role in embryo development. Maternal and fetal BPA concentrations in the serum and placenta and amniotic fluid is well documented where the fetus will be exposed to BPA. On average, the concentration of BPA in the placenta during pregnancy ranges from 1.0 to 104.9 ng/g and the average fetal plasma concentrations range from 0.2 to 9.2 ng/ml (Schonfelder et al., 2002), and 8.3 ng/ml in amniotic fluids (Ikezuki et al., 2002). However, the observable response was 3-5

magnitudes higher than the 0.87-87 nM/L (0.2-20 ng/ml) of BPA found in maternal and fetal body fluids (Vanderberg et al., 2007).

The low dose effects didn't affect the HFLF probably due to the culture media with 10 to 15% serum which is rich in nutrients and growth factors was too protective that response that was seen in the high dose. Also, because of cell type specific gene expression patterns and the role of varied specific co-regulatory factors (AP-1, Sp-1, TIF-2, etc.), the effects of BPA can vary depending on intrinsic and extrinsic influences (Chariot et al., 1998; Klinge, 2000; Singleton et al., 2006).

There is no reported study that shows what concentration of BPA the human fetal lung is exposed to. The main effects are observed at 23 μ M intracellular BPA, which had no effect on cell viability and BPA at the chosen short-term 24 h exposure (while the timing of exposure is critical in effects of BPA) may only stimulate acute stress-resistance by increasing the Nrf-HO-1 regulated oxidative stress-antioxidant response pathway which was revealed in our IPA. Meanwhile, 23 μ M intracellular BPA also induced genes involved in cell cycle arrest, autophagy as well as DNA damage repair genes suggesting an adaptive response of HFLF to oxidative stresses induced by BPA. However, if the cell cycle arrest continuously occurs *in vivo* in HFLF, it may cause delay in fetal lung development and maturation.

IPA analysis revealed that cell death and apoptosis pathways are upregulated, as shown in Table 3.1. Based on our cell viability and cell count assay, there was little cell death observed. There were no apoptotic cells observed when cells were stained with DAPI. IPA analysis shows the main apoptotic gene, Caspase-3, is downregulated. To confirm if there is cell death or apoptosis, the protein expression of cell death markers

CD274 (Programmed cell death 1 ligand 1) and PDCDLG2 (Programmed cell death 1 ligand 2) were measured using Western blot and ELISA in 4 batches of cells treated with BPA. There are no significant differences observed in protein expression of CD274 and PDCDLG2 in BPA-treated cells versus controls. This suggests that BPA is not cytotoxic to the HFLF cells at the concentrations are used.

As a prototypical non-steroidal estrogen, BPA interferes with the activity of endogenous estrogens (e.g. 17 β -estradiol) by disrupting the proper activity of the estrogen nuclear hormone receptors in a diverse set of target tissues (Matthews et al., 2001; Vivacqua et al., 2003; Recchia et al 2004). There is additional evidence for secondary metabolic and pharmacokinetic actions of BPA that impacts its bioavailability and bioavailabilities of endogenous steroid hormones have also been described. Those secondary effects include modification of cytochrome P450 enzyme expression and activity (Cannon et al., 2000; Hanioka et al., 1998, 2000). Recent studies have also begun to highlight the potential mode of action via epigenetic mechanisms such as methylation-mediated promoter silencing (Anway et al., 2005, 2006; Ho et al., 2006). However, because of the pleiotropic mechanisms of BPA action, narrowly defining BPA as a selective estrogen receptor modulator (SERM) is inaccurate (Wetherill et al., 2007).

We observed all three estrogen receptors are expressed in HFLF cells with increased expression of ER- β in the cytoplasmic fraction and GPR-30 in the nuclear fractions only at 23 μ M intracellular BPA dose. This is consistent with findings that BPA affects the ER- α and ER- β expression only at high concentrations (Routledge et al., 2000; Singleton et al., 2006). This study explains that the activation of ERs depend on the effects of BPA on ERs co-activator recruitment. This was not the case in GPR-30

activation. Sheng et al (2013) found that BPA at low concentration boosts mouse spermatogonial cell proliferation by inducing the GPR-30 expression. This discrepancy with our findings is probably due to the different cell-specific effects of BPA and also because of spermatogonial cells, as a reproductive cell line, is more sensitive to the BPA effects.

ERa, ERb and GPR30 gene expressions were not revealed in microarray gene profiling. We consider that the microarray analysis would not be able to detect the low expression possibilities of ERs in this cell line. Therefore, we pooled three batches of cell culture plates to extract the ER proteins and were able to see the changes induced by BPA in protein expression level, as revealed in our Chapter 2 Results section. This is probably a reason for why the BPA low dose effects were not observed in this study.

ET-1 is known for its mitogenic and functional role in developing airways (Goldie et al., 1995). It is also known that estradiol inhibits ET-1 synthesis and that it is ER-mediated (Morey et al., 1998; Akishita et al., 1998). The effects of BPA on ET-1 expression were measured in HFLF and the ER-specific antagonists used to block the effects of BPA on ET-1 secretion. However, the ER-specific antagonists were not able to abolish the effects of BPA on the ET-1 release, suggesting that the BPA-ERs interaction may not be the main mechanism of BPA action in HFLF. The BPA's effects on ER- β expression in cytoplasmic fraction and GPR-30 in nuclear fraction need a more in-depth study to elucidate.

As mentioned earlier, there is additional evidence for secondary metabolic and pharmacokinetic actions of BPA that impact its bioavailability and bioavailabilities of endogenous steroid hormones through secondary effects include modification of

cytochrome P450 enzyme expression and activity. The microarray gene analysis data revealed that, BPA is up-regulated expression of AhR and its downstream genes including CYP1B1 and CYP51A1. Both BPA and its metabolites may act as a ligand of AhR. Given this hypothesis, the protein expression of AhR was measured using immunocytochemical and Western blot analysis in HFLF cells treated with various concentrations of BPA. The expression of AhR was very low, even the AhR specific ligand TCDD was not able to induce the fold changes of AhR in HFLF. The AhR receptor specific antagonist also used to measure the down stream CYP1B1 protein expression and the antagonist was not able to abolish the effects of BPA on CYP1B1 expression (data not shown). This suggests that BPA may not be a ligand for AhR in HFLF cells. The role of AhR in the mode-of-action of BPA remains to be confirmed in further study.

BPA activated CYP1B1 expression and increased estradiol synthesis in HFLF. It is also plausible to say BPA or estradiol is metabolized by CYP1B1, generating quinones and ROS, latter cause DNA damage and cell cycle arrest, which might be the main mechanisms of action of BPA in fetal lung development and maturation. Given this hypothesis, we measured the protein expression of key cell cycle control markers revealed in IPA, such as ATM, p53, CDK2, H3. ATM and p53 were upregulated and CDK2 (which elevates in G1/S phase of cell cycle) downregulated by BPA and no changes have seen in H3 (which elevates in G2 phase of cell cycle) suggesting, BPA contributed to the cell cycle arrest in HFLF. However, this effect only has seen at 23 μ M intracellular BPA concentration. The longer time exposure to BPA might be needed to see the similar effect in lower doses.

The effects of BPA in HFLF observed in our studies mainly occurred at 100 μM corresponding to 23 μM intracellular BPA concentration, which is markedly higher than what was reported for human fetal tissues and amniotic fluid. However, the reference dose (50 $\mu\text{g}/\text{kg}/\text{d}$) represents a daily limit of exposure for the human population, and in the case of BPA was built on the assumption that the predominant exposure pathways in humans is through the ingestion of contaminated dietary sources, followed by gastrointestinal (GI) absorption in the small intestine (Hemgstler et al., 2011).

There are fewer studies assessing the potential for BPA exposure from non-dietary source through dermal absorption, inhalation, sublingual absorption from other sources such as toys, feeding utensils, pacifiers, teething rings, electronics, chopping boards, cooking utensils, plastic food wrap, mobile phones, computers, sunglasses, CDs, DVDs, electronic equipments, automobiles, sports equipment and bicycle helmets, dental composites and sealants, thermal print paper for receipts, hair care products, magazines and books and dusts.

The residual BPA monomers in polycarbonate plastics are generally reported in the range of 1-140 mg/kg, also higher concentrations up to the maximum recommended migration level set by the European Food Safety Authority (EFSA) of 600 mg/kg have occasionally been reported (Hoeskstra et al., 2013). The interpretation of the laboratory animal use of ultrapure water may underestimate actual BPA migration in real-world scenarios as micronutrients and enzymes contained in human and animal body may facilitate increased release of BPA from polycarbonates. The fetus with lower detoxification capability, higher surface to volume ratio, and multiple exposure sources,

the fetal lung may accumulate BPA to much higher concentrations than found in the biological fluids. Therefore, fetuses are more vulnerable to BPA toxicity than adults.

The observed responses of HFLF to BPA is a first study in human lung cells which laid the groundwork for future *in vitro* studies to determine potential mechanisms underlying the contribution of BPA to fetal lung development. This study may provide some insight for consideration of a need to improve the fetal and early childhood specific environmental health risk assessment models. We didn't observe adverse effects at human relevant BPA concentrations, probably HFLF cells are not enough sensitive to the BPA. Further, to develop more sensitive cell model system in lung to study the BPA effects and mechanisms are needed. In addition, the 24h exposure time is probably too short to explain the chronic effects of BPA. Also, because of limited BPA project fundings, we are not able to repeat the microarray analysis with longer exposure time and with more BPA concentration ranges.

Further, explore the human relevant BPA exposure levels on the immune system and elucidate the molecular mechanism of acute versus chronic exposure to BPA need to be distinguished and deciphered. Define and consider the tissue and cell-type specific sensitivity and windows of susceptibility to BPA across the life span of experimental animals and humans should be pursued in future research.

Several future directions could be considered. For example, use the longer exposure times of days to weeks may help elucidate low-dose effects of BPA. Most importantly, use the Benchmark Dose Modeling approach to construct a set of dose response curves and see genes along with these curves to extrapolate the high dose effects of BPA observed in this study to the human relevant exposure dose.

Appendix

List of Publications

The following publications, manuscripts and conference presentations were produced during this project.

Research Papers

Mahemuti L, Chen Q, Coughlan MC, Florian M, Mailloux RJ, Cao XL, Scoggan K, Willmore WG, Jin X, 2016. Bisphenol A exposure alters release of immune and developmental modulators and expression of estrogen receptors in human fetal lung fibroblasts. *J Environ Sci.* 48, 11-23.

Mahemuti L, Chen Q, Coughlan MC, Qiao CY, Woodworth R, Dong D, Florian M, Yan J, Cao XL, Scoggan K, Jin X, Willmore WG. Bisphenol A induces DNA double strand break, activates the ATM-p53 signalling pathway leading to cell cycle arrest and increase in autophagy and senescence, while also increases stress response and estradiol release, in the human fetal lung fibroblast (HFLF), 2017. Submitted to *Cell death and Differentiation*; Submission number: CDD-17-0792.

Mailloux RJ, Fu A, Florian M, Petrov I, Chen Q, Coughlan MC, **Mahemuti L**, Yan J, Caldwell D, Patry D, Lalande M, Wang GS, Willmore WG, Jin X, 2015. A Northern contaminant mixture impairs pancreas function in obese and lean JCR rats and inhibits insulin secretion in MIN6 cells. *Toxicol.* 334, 81-93.

Mailloux RJ, Coughlan MC , Gagnon C, Florian M, **Mahemuti L**, Lalande M, Caldwell D, Willmore WG, Ratnayake N, Jin X, 2014. Effects of a Northern

Contaminant Mixture (NCM) on Hepatic Fatty Acid Profile in Lean and Obese JCR Rats Fed a Normal or High Fat/Sugar Diet. PLoS ONE 9(9): e106832.

Florian M, Yan J, Ulhaq S, Coughlan MC, **Mahemuti L**, Willmore WG, Jin X, 2013. Northern contaminant mixtures induced morphological and functional changes in human coronary artery endothelial cells under culture conditions typifying high fat/sugar diet and ethanol exposure. *Toxicol.* 313 (2–3), 103-112.

Conference Proceedings

Mahemuti L, Chen Q, Coughlan MC, Florian M, Scoggan K, Willmore WG, Jin X, 2016. Bisphenol A Induces DNA Double Strand Break and Activates the ATM-P53-GDF15 Signaling Pathway Leading to Cell Cycle Arrest and Senescence in Human Fetal Lung Fibroblasts (HFLF). Oral presentation. Health Canada Science Forum, Ottawa, Canada.

Mahemuti L, Willmore WG, Jin X, 2015. Bisphenol A Induces DNA Double Strand Break and Activates the ATM-P53-GDF15 Signaling Pathway Leading to Cell Cycle Arrest and Senescence in Human Fetal Lung Fibroblasts (HFLF). *Free Radical Biology and Medicine.* 87 (S1), S102-S103. Abstract/Poster, 21th Annual meeting, SFRBM, Boston, USA.

Mahemuti L, Chen Q, Coughlan MC, Zhang M, Florian M, RJ. Mailloux RJ, Cao X, Scoggan K, Willmore WG, Jin X, 2014. Bisphenol A (BPA) exposure altered release of immuno and developmental modulators and expression of estrogen receptors (ERs) in human fetal lung fibroblasts (HFLF). Abstract/Poster, Chemical Management Program Workshop, Health Canada, Ottawa.

Mahemuti L, Coughlan MC, Florian M, Willmore WG, Jin X, 2013. Effects of Bisphenol A (BPA) on Protein Expression of Estrogen Receptors (ERs) in Human

Fetal Lung Fibroblasts (HFLF). Abstract/Poster, 19th Annual Meeting, SFRBM, San Diego, USA.

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