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Altered expression of histone deacetylases, inflammatory cytokines and contractile-associated factors in uterine myometrium of Long Evans rats gestationally exposed to benzo[a]pyrene

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Abstract

Etiology of preterm birth (PTB) is multifactorial; therefore, decreasing the incidence of PTB is a major challenge in the field of obstetrics. Epidemiological studies have reported an association between toxicants and PTB. However, there are no studies on the role of benzo[a]pyrene (BaP), an environmental toxicant, in the incidence of PTB. We first assessed the effects of BaP (150 and 300 $\mu\text{g kg}^{-1}$ body weight) dosed via gavage from day 14 to 17 of pregnancy on gestation length in Long Evans rats. We further assessed the histopathology of the uterus, expression of inflammatory cytokines, contractile-associated factors, histone deacetylases (HDACs) and NF κ B-p65 in myometrium collected on day 22 postpartum versus vehicle-treated controls. In our study, rats exposed to BaP delivered prematurely ($P < 0.05$) compared to control. Hematoxylin and eosin staining of uterus showed squamous metaplasia, glandular and stromal hyperplasia in BaP-exposed rats versus control. The concentrations of BaP metabolites measured by high-pressure liquid chromatography were higher in uterine myometrium of BaP-exposed rats while they were undetectable in controls. Quantitative real-time polymerase chain reaction showed significant increases in mRNA expression of interleukin-1 β and -8, tumor necrosis factor- α , connexin 43, cyclo-oxygenase-2 and prostaglandin F $_{2\alpha}$ receptor as compared to controls ($P < 0.05$). Western blot analysis revealed that BaP exposure caused decreases in class I HDACs 1 and 3 and increases in class II HDAC 5, cyclo-oxygenase-2 and nuclear translocation of NF κ B-p65 relative to controls. Our results suggest that gestational exposure to BaP increases incidence of PTB through

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Conflicts of interest

The authors did not report any conflict of interest.

epigenetic changes that causes increases in the expression of contractile-associated factors through the NF κ B pathway.

Keywords

Preterm birth; Benzo(a)pyrene; inflammatory cytokines; connexin 43; Cox2; Histone deacetylases; NF κ B; Environmental toxicant; Rats; Uterine myometrium

Introduction

Despite recent advances in medical sciences, the incidence of preterm birth (PTB) remains high (11.7%) costing US\$26 billion dollars annually. Epidemiological studies reported an association between environment pollution and PTB as well as environment-induced epigenetic changes and PTB (Bobak 2000; Hansen *et al.*, 2006; Huel *et al.*, 1993; Huynh *et al.*, 2006; Sagiv *et al.*, 2005; Wang *et al.*, 2000). Of the environmental pollutants, polycyclic aromatic hydrocarbons (PAHs) deserve special mention because of their release from automobile exhausts and combustion-related activities. As a good majority of combustion-derived particle sizes fall in the fine and ultrafine (particulate matter < 0.1) category, and the ability of PAHs to adsorb to these particles (Levy *et al.*, 2003) put people who are smokers and inhale contaminated air from occupational settings and vehicular emissions at a greater risk than others. Therefore, exposure to environmental pollutants, such as PAHs are high among populations who live near sources of anthropogenic sources of pollution such as hazardous waste sites, incineration plants and people working in industries such as petroleum handling, coke oven plants, aluminum manufacturing, graphite electrode manufacturing, etc. (Chuang *et al.*, 1999; Wernette and Nieves 1992). Particles with adsorbed PAHs deposit in the lungs, escape phagocytosis by alveolar macrophages and translocate to extrapulmonary organs through circulation (Janerich *et al.*, 1990; Martinez *et al.*, 1994; Schuster-Kolbe and Ludwig 1994). Studies in humans and animal models indicate that PAHs cross the placenta and reach fetal organs (Archibong *et al.*, 2002; Choi *et al.*, 2006; Huel *et al.*, 1993; Perera *et al.*, 2003). It has been suggested that these compounds interfere with placental development and disrupt function of cellular macromolecules such as DNA, RNA and proteins (Choi *et al.*, 2006; Wang *et al.*, 2000). For example, DNA-adducts formed by PAHs in cord blood leukocytes have been linked to decreased birth weights, lengths and head circumferences (Perera *et al.*, 2003). Studies using both animals and humans have shown that PAH exposure adversely affects growth, development and cognitive functions (Choi *et al.*, 2006; Etzel 1997; Weitzman *et al.*, 2002). Despite a large number of studies in this area, studies on disposition of PAH/metabolites in uterine tissue and effects of the metabolites on gestation is absent in the literature.

Exposure to PAHs have been reported to induce proinflammatory cytokines and cause histone modifications, which lead to functionally relevant changes in the genome but do not involve a change in the nucleotide sequence itself (Hou *et al.*, 2012; Veldhoen *et al.*, 2008). Histone acetylation was reported to increase while de-acetylation was reported to decrease the NF κ B activity (Greene and Chen 2004). Activation of NF κ B in turn was reported to increase inflammatory cytokines locally in the tissues (Karin and Greten 2005). Studies from

our laboratory and others have suggested that inflammation increases mRNA and protein content of oxytocin receptor, prostaglandin receptor, connexin 43 and cyclo-oxygenase-2 (COX-2) in the myometrium, that are essential in the parturition process (Romero *et al.*, 2006; Thota *et al.*, 2014). As proinflammatory cytokines have been reported to play a role in full-term as well as in spontaneous preterm deliveries (Gandley *et al.*, 2010; Romero *et al.*, 2008), we hypothesize that exposure to environmental pollutants during pregnancy potentiates epigenetic changes through histone modifications and enhance production of proinflammatory cytokines and expression of contractile-associated factors thereby increasing the risk for PTB.

Environmental tobacco smoke containing several PAHs, including BaP has been reported to alter vascular function (Gandley *et al.*, 2010). BaP has also been implicated in impaired fetal development (Martinez *et al.*, 1994; Weitzman *et al.*, 2002). *In utero* exposure to BaP has also been shown to impair fertility in F₁ mice and was shown to be positively associated with decreases in gonad size and altered folliculogenesis (Archibong *et al.*, 2012; MacKenzie and Angevine 1981). In spite of these findings that suggest a positive correlation between PAH exposure and increases in the incidence of PTB, there have been no studies on the effects of *in utero* BaP exposure on gestation and on morphological and molecular changes in myometrium postexposure. Therefore, in this study, we assessed the effects of *in utero* BaP exposure on (1) incidence of PTB, (2) histopathology of uterus, expression of inflammatory cytokines, (3) contractile-associated factors, (4) histone deacetylases (HDACs) and (5) NFκB-p65 in myometrial tissue in postnatal day 22 (PND22) rats.

Materials and methods

Chemicals and reagents

BaP (98% pure) and its metabolite standards (BaP diols, -phenols and -quinones) were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and the National Cancer Institute Chemical Carcinogen Repository (Midwest Research Institute, Kansas City, MO, USA) respectively. Solvents (high-pressure liquid chromatography [HPLC] grade) were obtained from Fisher Scientific Co. (Kennesaw, GA, USA). SYBR green was purchased from Bio-Rad (Hercules, CA, USA).

Animal exposure

Timed-pregnant Long-Evans rats were obtained from Harlan Sprague–Dawley (St. Louis, MO, USA) on gestation day (GD) 11 and were housed in the animal care facility in a climate-controlled room with a 12/12 h light/dark cycle schedule. They were fed standard rat chow and water ad libitum. All the animal care and use protocols were approved by the Institutional Animal Care and Use Committee of Meharry Medical College. Pregnant rats were randomly assigned to control and experimental groups. Dams were given peanut oil alone (control), or 150 and 300 µg kg⁻¹ BaP (Sigma Chemical Co., St. Louis, MO, USA) in a total volume of 0.875 ml peanut oil by oral gavage once daily from GD14 to 17 (Brown *et al.*, 2007). Pregnant rats were observed for labor and delivery from day 17 once every 4 h during the day. The time and day of delivery of the first pup were recorded in control as well as in exposure groups. On an average, deliveries occurring 12–24 h previous term deliveries

in controls were considered as pre-term deliveries (Roizen *et al.*, 2008; Wang *et al.*, 2008). Pups were weaned on postpartum day 21. Both the control and BaP-exposed rats were killed on PND22 and uterine tissues were dissected. Part of the uterus was fixed in buffered 10% formalin. Endometrial tissue was separated and discarded and the remaining uterine myometrial tissue was snap frozen in liquid nitrogen and stored at -80°C until analysis.

Extraction and analyses of myometrial tissue for benzo[a]pyrene metabolites

We subjected myometrium obtained from both control and BaP-exposed rats to liquid–liquid extraction with methanol, chloroform and water. The extracts after further processing and cleanup were analyzed by a reverse phase HPLC method to measure the concentrations of BaP metabolites as described earlier (Ramesh *et al.*, 2001). Six different metabolites, BaP 4,5 diol, BaP 7,8 diol, BaP 9,10 diol, BaP 3,6 dione, 3-hydroxy BaP and 9-hydroxy BaP, were measured and the values are represented as ng g^{-1} tissue.

Histopathology

To assess the effect of BaP exposure on the histopathology of the uterus we performed hematoxylin and eosin staining. Uterine tissue sections were briefly deparaffinized using xylene and rehydrated. They were first subjected to hematoxylin followed by eosin staining. Slides were then dehydrated using xylene and mounted using Permount. Uterine sections from control and experimental groups were observed under a confocal microscope in a bright field (TE2000-E; Nikon, Tochigi, Japan). All images were captured at $\times 20$ magnification under similar conditions.

Total RNA isolation and reverse transcription

Total RNA was extracted from uterine myometrium obtained from the vehicle- and BaP-treated rats using RNeasy kit as per the supplier's instructions (Qiagen, Gaithersburg, MD, USA). After removing genomic DNA contamination using DNaseI (Qiagen), we used $1\text{ }\mu\text{g}$ total RNA to prepare complementary DNA. RNA was mixed briefly with 3.0 nmol of oligodeoxythymine, $200\text{ }\mu\text{mol l}^{-1}$ deoxyribonucleotide triphosphate, 10 U of avian myeloblastosis virus reverse transcriptase, 5 U of RNase inhibitor in a total volume of $20\text{ }\mu\text{l}$ and subjected to reverse transcription in a thermal cycler set at 25°C for 10 min and at 42°C for 60 min for one cycle.

Quantitative polymerase chain reaction

To assess the expression of cytokine, inflammatory genes and contractile-associated proteins, we measured mRNA expression levels of IL-1 β , IL-8, TNF α , connexin 43, cox-2 and prostaglandin receptor in the myometrium of rats exposed to BaP ($0, 150$ and $300\text{ }\mu\text{g kg}^{-1}$ body weight) by quantitative polymerase chain reaction (PCR). PCR primers used were obtained from published literature (Table 1). All primer sets used for quantitative PCR generated a single amplicon. Briefly, 50 ng of complementary DNA were added to the master mix containing appropriate primers and SYBR green. Quantitative PCR was performed in a Bio-Rad MyiQ5. After an initial denaturation step, cDNA was amplified at 95°C for 15 s and 60°C for 1 min for 40 cycles using gene-specific primers. The GAPDH values of the respective samples were used to normalize the data.

Whole cell lysate preparation

We prepared whole cell lysates from myometrial tissue of rats, as described earlier (Thota *et al.*, 2014). Briefly, 25 mg of myometrium tissue was ground to powder in liquid nitrogen using pestle and mortar. An ice-cold RIPA buffer containing $\times 1$ protease inhibitor cocktail, 1 mM sodium vanadate and 1 mM sodium fluoride (Sigma) was added to the homogenized tissue and triturated using a p1000 micropipette. The whole cell lysate was stored at -80°C until further use.

Cytosol and nuclear protein preparation

Cytosol and nuclear fractions of myometrium were prepared using NE-PER nuclear and cytoplasmic extraction reagent (Thermo Scientific, Rockford, IL, USA) according to the suppliers' instructions. Myometrial tissue was cut into pieces weighing about 20–100 mg. After a brief wash with phosphate-buffered saline, tissue pieces were ground in CER I reagent using pestle and mortar. The ground tissue was vortexed for 15 s at high speed (16 000 *g*) on a bench top centrifuge followed by a 10 min incubation on ice. Next CER II reagent was added to the tubes, vortexed and incubated again for 1 min on ice. Tubes were then centrifuged at 16 000 *g* for 5 min and the supernatant (cytoplasmic fraction) was transferred immediately into a clean prechilled tube and stored at -80°C until use. Nuclear pellets were suspended in ice-cold NER reagent and vortexed for 15 s every 10 min for a total of 40 min. Tubes were then centrifuged at high speed (16 000 *g*) for 10 min and the supernatant (nuclear fraction) was stored immediately at -80°C until further use.

Western blot analysis

Western blot analysis was performed using equal amounts of protein. Whole cell lysates, cytosolic and nuclear fractions were resolved on a 10% Tris-Bis gels and transferred on to PVDF membranes. Western blot analysis was performed using primary antibodies against cox-2 (1 : 500; Cayman Chemicals, Ann Arbor, MI), HDACs 1, 3, 4 and 5 (1 : 1000; Cell Signaling, Beverly, MA) and NF κ B-p65 (2 ng ml $^{-1}$; Abcam, Cambridge, MA). Intensity of protein signals observed in autoradiographs was quantified using image documentation system (ProteinSimple, Santa Clara, CA, USA) and normalized with the corresponding β -actin (1 : 5000; Sigma) values.

Statistical analysis

Statistical analysis of quantitative PCR data was performed using the Bio-Rad iQ5 Optical System Software Version 2.0. Two-way ANOVA was used to evaluate differences in metabolite concentrations between the controls and BaP treatments. One-way ANOVA was used to evaluate differences between treatments and control. The data are represented as the mean $\pm$ SEM of five separate experiments.

Results

Rats treated with benzo[a]pyrene delivered preterm

Rats exposed to 150 μg of BaP from embryonic day 14 to 17 during pregnancy delivered at 21.20 ± 0.35 and those exposed to 300 μg kg $^{-1}$ delivered at 20.90 ± 0.35 days as compared

to rats in the control (vehicle) group that delivered at 22.25 ± 0.35 days of gestation ($P < 0.05$).

Concentrations of benzo[a]pyrene metabolites in myometrial tissue of benzo[a]pyrene-exposed rats are significantly higher

The BaP metabolites detected in myometrium samples were BaP 4,5-diol, BaP 7,8-diol, BaP 9,10-diol, BaP 3,6-dione, 3-hydroxy BaP and 9-hydroxy BaP. The concentrations of the above BaP metabolites measured showed a dose-dependent increase with the $300 \mu\text{g kg}^{-1}$ BaP treatment group registering greater concentrations than its $150 \mu\text{g kg}^{-1}$ counterpart (Fig. 1).

Immunohistochemical analysis of rat uterine tissues exposed *in utero* to benzo[a]pyrene showed structural abnormalities

We performed hematoxylin and eosin staining to assess the effects of BaP exposure on histopathology of uterus. On examination under a microscope after staining, we observed hyperplasia in the glandular region of the uterus in rats treated with $150 \mu\text{g}$ and stromal hyperplasia and squamous metaplasia in rats treated with $300 \mu\text{g}$ of BaP compared to rats in the control (vehicle) group (Fig. 2).

Messenger RNA expression of proinflammatory markers was significantly higher in uterine myometrial tissue of benzo[a]pyrene-exposed rats

To assess if changes observed in the morphology of the uterus are because of increased expression of inflammatory markers and contractile-associated factors we measured their mRNA expression using the PCR technique. Real-time PCR analysis of cDNA obtained from myometrium showed a significant ($P < 0.05$) increase in mRNA expression of IL-1 β , IL-8 and TNF α (Fig. 3) and connexin 43, cox-2 and prostaglandin receptor 2 α (Fig. 4) in BaP-treated rats compared to the vehicle-treated controls.

Upregulation of contractile-associated factor protein expression in benzo[a]pyrene-exposed rats

To confirm the changes observed in cox-2 mRNA expression we performed Western analysis.

Results showed a significant increase ($P < 0.05$) in cox-2 expression in rats treated with $300 \mu\text{g}$ of BaP-versus vehicle-treated controls (Fig. 5).

Histone deacetylase expression is differentially regulated in benzo[a]pyrene-exposed rats

Because epigenetic changes have been reported to play a role in increased inflammation, we measured protein expression of HDACs in the myometrium of rats treated with BaP. Western analysis of whole cell lysate obtained from rat myometrium exposed to BaP showed a significant decrease ($P < 0.05$) in HDACs 1 and 3, while HDAC 5 showed a significant increase ($P < 0.05$) compared to the control. No significant increases were observed in HDAC 4 (Fig. 6).

Benzo[a]pyrene-treatment enhanced nuclear translocation of NF κ B-p65 in rats

Furthermore, to assess if changes in HDACs affect the NF κ B pathway, we measured NF κ B-p65 in cytosolic and nuclear fractions in myometrium obtained from both control and BaP-exposed rats. The expression of NF κ B-p65 was significantly higher ($P < 0.05$) in the nuclear fraction and lower ($P < 0.05$) in the cytosolic fraction of myometrium obtained from BaP-exposed versus controls (Fig. 7).

Discussion

The BaP doses used in this study were chosen based on published reports and allowing for scenarios of exposure to episodic release of high levels of BaP into the general or occupational environment in some circumstances. Diet-related BaP intake by humans have been reported to range from 8.4 $\mu\text{g person}^{-1} \text{ day}^{-1}$ (Falco *et al.*, 2003) to 17 $\mu\text{g person}^{-1} \text{ day}^{-1}$ (de Vos *et al.*, 1990). An additional intake of 0.1 $\mu\text{g day}^{-1}$ is expected for smokers, who use a pack of cigarettes per day, as mainstream smoke yield of BaP per cigarette amounts to 10 ng per cigarette (Grimmer *et al.*, 1987). In this context, a 3 h stay in pubs/taverns exposes patrons to 6.3 ng m^{-3} BaP released from environmental tobacco smoke (Besaratnia *et al.*, 2002; Bolte *et al.*, 2008) with a greater propensity of repeated exposure of waitresses and bartenders to BaP in unrestricted smoking areas in these establishments. Coke oven workers are exposed to 42 $\mu\text{g m}^{-3}$ BaP (Lewtas *et al.*, 1997). Mean concentrations of BaP in the ambient air of highly polluted cities have been estimated to peak at about 20 $\mu\text{g m}^{-3}$ (Chorazy *et al.*, 1994). Furthermore, indoor exposure to BaP from cooking oil fumes range between 19 and 23 $\mu\text{g m}^{-3}$ (Chiang *et al.*, 1999). In rural areas of poor countries, where women use firewood for cooking and home heating, exposes them to 100 $\mu\text{g BaP m}^{-3}$ (Viau *et al.*, 2000). Therefore, either individual or cumulative exposure of women to all the above-mentioned BaP sources in some settings cannot be ignored, which may poses a hazard to their successful child-bearing abilities.

Our results demonstrate that *in utero* exposure to BaP results in preterm delivery of offspring. HPLC analysis of myometrial tissue samples obtained from BaP-exposed rats showed higher concentrations of BaP metabolites. We documented increases in inflammatory cytokines IL-1 β , IL-8 and TNF α , inflammatory markers cox-2, contractile-associated protein connexin 43 and prostaglandin receptor PGF2 α in BaP-exposed rats. Our studies revealed a robust downregulation in the expression of HDACs 1 and 3, and upregulation in HDAC 5 protein expression in BaP-exposed rats. Additionally, BaP-exposed rats demonstrated a propensity for upregulation of NF κ B protein expression in the nuclear fraction with an apparent corresponding decrease in the cytosolic fraction in myometrial tissue, as compared to the control rats. Results from this study suggest that BaP exposure during pregnancy increase the incidence of PTB by precipitating epigenetic changes that probably enhance expression of inflammatory cytokines and contractile-associated factors via the NF κ B pathway.

BaP-exposed rats in our study showed a significant reduction in gestation length as compared to controls. To our knowledge, this is the first report on the length of gestation in rats treated with BaP. Other studies reported decreases in fetal survival and fetal growth in F-344 rats exposed to BaP through inhalation (Archibong *et al.*, 2002, 2012). BaP was

reported to metabolize in uterine tissues (Ramesh *et al.*, 2001). Increase observed in BaP metabolites in uterine myometrium of rats pre-exposed to BaP in our study indicates that these BaP metabolites are probably involved in the disruption of myometrial function leading to increased incidence in PTB. Increases in BaP metabolites have been reported to affect fetal survival and growth negatively (Bobak 2000; Choi *et al.*, 2008; Geer *et al.*, 2012; Martinez *et al.*, 1994; Perera *et al.*, 2003). Within this context, it is of interest to note that, exposure to tobacco smoke- and traffic-related air toxicants has been reported to cause preterm deliveries (Fantuzzi *et al.*, 2007; Machado *et al.*, 2014; Padula *et al.*, 2014; Wilhelm *et al.*, 2011). Findings from this study support the contention that disposition of BaP metabolites in myometrium contribute to the incidence of PTB. Based on our current findings, we believe that increased incidence of PTB in a minority population is at least partially due to higher exposure to PAHs in this population (Chuang *et al.*, 1999; Wernette and Nieves 1992).

We observed glandular hyperplasia in rats treated with 150 µg and stromal hyperplasia in the uterus of rats exposed to 300 µg of BaP kg body weight. Similar changes in the glandular cells of the airway tract were reported in mice exposed to tobacco smoke suggesting that exposure to environmental pollutants produces systemic effects (Jones and Reid 1978). Furthermore, increases observed in the expression of inflammatory cytokines IL-1β, IL-8 and TNFα in rats exposed to BaP in the present study and PAHs in other studies (N'Diaye *et al.*, 2006; Veldhoen *et al.*, 2008), suggests that environmental pollutants have the potential to increase the inflammatory response. We assessed if an increase in inflammation observed in dams treated with BaP lead to increase in contractile-associated factors. Results from our study showed an increase in connexin 43 and cox-2 an enzyme involved in the synthesis of prostaglandin F2α receptor in BaP-exposed rats. This along with the increases observed in aortic smooth muscle cell contractions subsequent to BaP treatment (Yan *et al.*, 2000), suggests that BaP causes contractions through increases in contractile-associated factors. To our knowledge, this is the first such study reporting increases in biochemical factors involved in parturition subsequent to BaP exposure. These results suggest that BaP enhances the expression of inflammatory cytokines, which in turn increase contractile-associated factors. However, additional studies are needed to understand the molecular level mechanism involved in BaP-induced increases in contractile-associated factors in the uterus during the process of parturition.

Because exposure to environmental pollutants cause epigenetic changes that can negatively impact the normal development and function of the uterus, we assessed the expression of HDACs in the myometrium of PND22 rats exposed to BaP during pregnancy. The results showed an overall modulation of protein expression with downregulation of class I HDAC 1 and 3 and upregulation in class II HDAC 5 in BaP-exposed rats. Despite the limitations to the interpretation of our findings, we support the contention that selective acetylation and deacetylation of genes and selective transcription leads to increases in inflammatory and contractile-associated factors (Teneng *et al.*, 2011). Supporting this contention, increases in HDAC 3 have been reported to repress transcriptional activity of NFκB, while decreases in HDAC 3 were reported to cause increases in inflammatory response (Chen *et al.*, 2001; Greene and Chen 2004; Rahman and Adcock 2006). Long-term exposures to HDAC inhibitors have been reported to decrease NFκB DNA binding and decrease in

proinflammatory markers (Lindstrom *et al.*, 2008). Even though HDAC 5 is reported to play a role in skeletal myogenesis (McKinsey *et al.*, 2000), there are no reports to date on its role in myometrial contractions in the pregnant uterus. Therefore, further investigation is needed to assess its role. Taken together, these reports are supportive of the findings in the present study towards *in utero* BaP exposure being a risk factor for PTB. Decrease in HDACs 1 and 3 and a consequent increase in cytokines and contractile factors in BaP-exposed rats in our study further confirms the role of HDACs in the repression of transcription. Additional studies are needed to elucidate the mechanism involved in HDACs 1- and 3-induced nuclear translocation of NF κ B-p65 in BaP-exposed rats. However, this finding is strongly suggestive of our contention that the inflammatory response was mediated by NF κ B. While increases in HDAC 3 have been reported to repress transcriptional activity of NF κ B, long-term exposures to HDAC inhibitors have been reported to decrease NF κ B DNA binding and thus, decrease proinflammatory markers suggesting a controversial role for HDACs in mediating inflammation responses (Chen *et al.*, 2001; Lindstrom *et al.*, 2008).

From a human exposure standpoint, our studies have serious implications. Exposure of women to BaP and other PAHs emanated during cooking and home heating with biomass or fossil fuels rich in PAHs (Zhang and Smith 1996) place them at a greater risk concerning their fertility and childbearing. The same analogy could be applied to women who smoke and work in unregulated occupational settings. As epigenetic changes imposed by BaP exposure from the above-mentioned exposure scenarios adversely affect the uterine function, additional studies are warranted to characterize fully the effects of *in utero* exposure to BaP on the role of individual HDACs.

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Figure 1.

Metabolite composition of BaP in myometrial tissue obtained from Long Evans rats pre-exposed to BaP. Six different metabolites: BaP 4,5 diol, BaP 7,8 diol, BaP 9,10 diol, BaP 3,6 diol, 3-hydroxy BaP and 9-hydroxy BaP were measured in myometrium in rats gavaged with 150 and 300 µg kg⁻¹ body weight of BaP from gestation day 14 to 17 and killed on day 22 postpartum. The values are represented as ng g⁻¹ tissue. Sum of individual concentrations of BaP metabolites formed ($n = 5$ for each treatment category) is represented as total. Groups with asterisks (** $P < 0.01$) are significantly different from the vehicle-treated control. BaP, benzo[a]pyrene.

Figure 2.

H&E staining of uterus obtained from Long Evans rats pre-exposed benzo(a)Pyrene [B(a)P]. Pregnant rats were gavaged with 150, and 300ug/kg body wt. of B(a)P once every day from D14–17 of pregnancy. Dams were sacrificed on day 22 and uterus collected was subjected to H&E staining. Uterus from rats treated with 150ug of B(a)P showed hyperplasia in stromal region compared to control while those treated with 300ug of B(a)P showed hyperplasia in glandular region (lower left) and metaplasia in squamous epithelium (lower right).

Figure 3.

Effect of BaP on the mRNA expression of chemokine and cytokine genes in myometrial tissue obtained from Long Evans rats. Pregnant rats were gavaged with 150 and 300 $\mu\text{g kg}^{-1}$ body weight of BaP once every day from gestation day 14 to 17. Myometrium tissue was collected on day 22 postpartum and the effects on IL-8, IL-1 β and TNF α were assessed using reverse transcription and qPCR analysis. Data were normalized to respective GAPDH values. The bars represent the mean $\pm$ SEM from five replicates in each group. Groups with asterisks (* $P < 0.05$; ** $P < 0.01$) are significantly different from control. BaP,

benzo[*a*]pyrene; IL, interleukin; TNF, tumor necrosis factor. [Correction added on 16 October 2015, after first online publication: “BaP (μg)” corrected to “BaP (μg kg⁻¹)” in the x-axis legend.]

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Figure 4.

Effect of BaP on the mRNA expression of contractile-associated genes in myometrial tissue obtained from Long Evans rats. Pregnant rats were gavaged with 150 and 300 µg kg⁻¹ body weight of BaP from gestation days 14 to 17. Myometrium tissue was collected on day 22 postpartum and the effects on the prostaglandin F_{2α} receptor, cox-2 and connexin-43 were assessed using reverse transcription and qPCR analysis. The data were normalized to respective GAPDH values. The bars represent the mean ± SEM from five replicates in each group. Groups with asterisks (**P* < 0.05; ***P* < 0.01) are significantly different from control. BaP, benzo[*a*]pyrene; Cont, control.

Figure 5.

Effect of BaP on the protein expression of cox-2 in myometrial tissue obtained from Long Evans rats. Pregnant rats were gavaged with 150 and 300 $\mu\text{g kg}^{-1}$ body weight of BaP from gestation day 14 to 17. Myometrium tissue was collected on day 22 postpartum and the effect on cox-2 expression was assessed using Western analysis. The data were normalized to respective β -actin values. The bars represent the mean $\pm$ SEM from five replicates in each group. Groups with asterisks (** $P < 0.01$) are significantly different from control. BaP, benzo[*a*]pyrene; Cont, control.

Figure 6.

Effect of BaP on the protein expression of HDACs in myometrial tissue obtained from Long Evans rats. Pregnant rats were gavaged with 150 and 300 $\mu\text{g kg}^{-1}$ body weight of BaP from gestation day 14 to 17. Myometrium tissue was collected day 22 postpartum and the effects on class 1 HDACs 1 and 3 and class 2 HDACs 4, 5 and 6 were assessed using Western analysis. The data were normalized to respective β -actin values. The bars represent the mean $\pm$ SEM from five replicates in each group. Groups with asterisks (* $P < 0.05$; ** $P < 0.01$) are significantly different from control. BaP, benzo[*a*]pyrene; HDACs, histone deacetylases.

Figure 7.

Effect of BaP on the expression of NFκB-p65 in myometrial tissue obtained from Long Evans rats. Pregnant rats were gavaged with BaP from gestation day 14 to 17. Cytosolic and nuclear fractions prepared from myometrium tissue collected on day 22 postpartum were subjected to Western analysis using NFκB-p65 antibodies. The data were normalized to respective β-actin values. The bars represent the mean $\pm$ SEM from five replicates in each group. Groups with asterisks (* P < 0.05; ** P < 0.01) are significantly different from control. BaP, benzo[*a*]pyrene.

Table 1

Primers for chemokine, cytokines and contractile-associated factors

Primers	Accession no.	Forward primer	Reverse primer
TNF α	NM_012675.3	5'-AAATGGGCTCCCTCTCATCAGTTC-3' (339–362)	5' TCTGCTTGGTGGTTTGCTACGAC 3' (446–427)
IL-1 β	NM_031512.2	5'-CACCTCTCAAGCAGACACAG-3' (773–793)	5' GGGTTCCATGGTGAAGTCAAC3' (851–831)
IL-8	NM_011339.2	5'-AATTTCACCGCAATGAAGC-3' (339–359)	5' AGGTCTCCCGAATTGGAAAGG 3' (452–432)
Connexin 43	NM_012567.2	5'-TGTAACACTCAACAACCTGGC-3' (356–376)	5'-GGTTTTCTCCGTGGGACGTGA-3' (817–797)
Prostaglandin receptor F _{2α}	NM_013115.1	5'-TCAGCAGCACAGGCAAGGCA-3' (835–854)	5'-TGGCCATCGTCACCAGAAAGGGA-3' (944–922)
Cox-2	NM_017232.3	5'-ATGACGAGCGACTGTTCCAA-3' (929–948)	5'-TGAAGTGGTAACCGCTCAGG-3' (1030–1011)
GAPDH	NG_007904.2	5'-GTATTGGGCGCCTGGTCACC-3' (146–165)	5'-CGC TCC TGG AAG ATG GTG ATG G-3' (353–332)

Cox-2, cyclo-oxygenase 2; IL, interleukin; TNF, tumor necrosis factor.