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Estrogen receptor-dependent and independent roles of benzo[*a*]pyrene in Ishikawa cells

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Abstract

Polycyclic aromatic hydrocarbons (PAHs) are environmental pollutants generated from the incomplete combustion of organic material. PAHs have been studied as genotoxicants, but some also act via non-genotoxic mechanisms in estrogen-dependent malignancies, such as breast cancer. PAHs require metabolic activation to electrophilic metabolites to exert their genotoxicity but non-genotoxic properties may also contribute to their carcinogenicity. The role of PAHs in endometrial cancer, a cancer associated with unopposed estrogen action is unknown. We assessed the metabolism of the representative PAH, benzo[*a*]pyrene (B[*a*]P), to estrogenic compounds in Ishikawa human endometrial cells in the presence and absence of cytochrome P450 induction. Using stable-isotope dilution high-performance liquid chromatography and APCI tandem mass spectrometry in the selected reaction monitoring mode, we analyzed B[*a*]P metabolism in Ishikawa cells. Estrogenic activity of B[*a*]P metabolites was determined by the endogenous estrogen inducible alkaline phosphatase reporter gene and an exogenous estrogen response element (ERE) luciferase reporter gene construct. We also assessed whether PAHs can induce a proliferative phenotype via estrogen receptor (ER)- and non-ER-regulated pathways. We demonstrate that B[*a*]P can be metabolized in human endometrial cells into 3-OH-B[*a*]P and B[*a*]P-7,8-dione in sufficient amounts to activate ERs. We also show that only B[*a*]P-7,8-dione induces endometrial cell proliferation at concentrations lower than required to activate the ER; instead non-genomic signaling by the EGF receptor (EGFR) and activation of the mitogen-activated protein kinase (MAPK) pathway was responsible. This work indicates that human endometrial cells can metabolize PAHs into estrogenic metabolites, which may induce cell proliferation through non-ER-regulated pathways.

Key Words

- ▶ benzo[*a*]pyrene
- ▶ metabolism
- ▶ estrogen receptor
- ▶ Ishikawa
- ▶ endometrium

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Introduction

Polycyclic aromatic hydrocarbons (PAHs) are pervasive environmental toxicants and the representative compound, benzo[*a*]pyrene (B[*a*]P), is ranked as a Group 1 carcinogen by the International Agency for Research on Cancer (IARC) (Korsh *et al.* 2015). Ambient PAHs

are released primarily from the incomplete combustion of fossil fuels and motor vehicular traffic; however, oral uptake of PAHs may also occur via consumption of smoked and grilled meat and fish, while dermal exposure to PAHs may result from the use of consumer products that

contain black rubber (Armstrong *et al.* 2004, Korsh *et al.* 2015). PAHs have been implicated in divergent estrogen signaling mechanisms (Santodonato 1997, Hayakawa *et al.* 2011, Lam *et al.* 2018) in laboratory rodent models (Hirose *et al.* 2001, Kummer *et al.* 2008, Borek-Dohalska *et al.* 2016) and in in vitro human cell line models (Gozgit *et al.* 2004, Pliskova *et al.* 2005), suggesting that they can be added to the growing list of endocrine-disrupting chemicals.

PAHs have been implicated in estrogen receptor-dependent cancers. Women had a twofold increased risk of breast cancer associated with detectable levels of PAH relative to women with non-detectable levels, and this association increased with higher levels of PAHs rising to a level of absolute breast cancer risk (10-year risk $\geq 3.4\%$: OR=4.09, 95% CI=1.38–12.13) (Shen *et al.* 2017). PAHs have been extensively used to experimentally induce breast carcinogenesis (Morris & Seifter 1992). PAHs have been implicated in cancer initiation (including breast cancer) via the induction of DNA damage, through the formation of DNA adducts in target tissues (Kriek *et al.* 1993, Rundle *et al.* 2000, Gammon *et al.* 2002). However, PAH has also been linked to cancer promotion by cell proliferation via estrogen receptor (ER)-mediated signaling (Santodonato 1997). Two PAHs, benz[a]anthracene (B[a]A) and B[a]P, have been shown to stimulate the proliferation of human breast carcinoma MCF-7 cells via an ER-dependent mechanism, and this proliferation was blocked by the antiestrogen, ICI 182,780 (Plíšková *et al.* 2005). These data suggest that the proliferative effects of PAHs could contribute to tumor promotion in estrogen-sensitive tissues via the ER.

While there is ample evidence for genotoxic and non-genotoxic estrogenic signaling in breast cancer models, the role of PAHs in the development of other ER-dependent cancers such as endometrial cancer still needs clarification. Endometrial cancer is the most common malignancy within the female reproductive system (Siegel *et al.* 2019). Most endometrial malignancies are driven by aberrant hormone signaling, where estrogen signal transduction through estrogen receptor alpha (ER α) functions as an oncogenic signal (Rutledge 1976, Rodriguez *et al.* 2019).

PAHs have been marginally linked to endometrial cancer. Consumption of 101–200 g of red meat per day raises a woman's risk to endometrial cancer development by 2-fold, and this was attributed to the formation of heterocyclic amines and/or PAHs during the processing of meat at high temperatures (Plagens-Rotman *et al.* 2016). Additionally, a Taiwanese 30-year

population-based epidemiological study showed an increase in endometrial cancer with increasing air pollutants, such as B[a]P (Huang *et al.* 2012). In the immature rat uterotrophic assay in Wistar rats, PAHs including B[a]P, B[a]A or fluoranthene administered consecutively for 3 days at 10 mg/kg/day led to a significant increase of uterine weight and hypertrophy of the luminal epithelium, suggesting that they behaved as estrogen-like compounds (Kummer *et al.* 2008). These effects were likely facilitated by ER α , the predominant ER isoform in the uterus (Brandenberger *et al.* 1999), as they were abrogated by the ER antagonist, ICI 182,780. Non-ER-regulated pathways such as the EGF receptor (EGFR) (Tu *et al.* 2011) and/or mitogen-activated protein kinase (MAPK) (Zhou *et al.* 2011) pathways have also been implicated in estrogen signaling in endometrial cancer. PAHs have similarly been implicated in EGFR and MAPK pathways in their role in cancer development (Burdick *et al.* 2003, Rodríguez-Fragoso *et al.* 2009, Song *et al.* 2013). This evidence suggests that PAHs may play a role in endometrial cancer development via estrogen receptor and non-estrogen receptor pathways. However, there is a dearth of studies that assess PAH metabolism in endometrial cancer and the estrogenic properties of the metabolites that may form.

PAHs require metabolic activation to highly reactive electrophiles to exert their carcinogenicity (Shimada & Fujii-Kuriyama 2004). Cytochrome P450s (P450s) are implicated in the metabolism of PAHs and are inducible by PAHs and polyhalogenated hydrocarbons (such as 2,3,7,8-tetrachlorodibenzo[b,e][1,4]-dioxin (TCDD)), via the aryl hydrocarbon receptor (AhR) (Poland *et al.* 1976, Sanderson *et al.* 2001, Schaufler *et al.* 2002). It is thus important to conduct metabolism studies with and without TCDD induction.

In this study, we used Ishikawa endometrial cells to represent type 1 endometrioid adenocarcinoma cells, which respond to stimulation by unopposed estrogens. We tested the hypothesis that B[a]P metabolism is induced in these cells with TCDD to yield estrogenic compounds using stable-isotope dilution high-performance liquid chromatography and APCI mass spectrometry. We tested whether the metabolites formed induced an endogenous alkaline phosphatase reporter gene and an estrogen response element (ERE) luciferase reporter gene construct as read-outs of ER activation. We also investigated whether B[a]P metabolites can induce a proliferative phenotype via ER- and non-ER-regulated pathways.

Materials and methods

Hazardous agents used in this work were handled in accordance with the NIH Guidelines for the Laboratory Use of Chemical Carcinogens.

Reagents and chemicals

The unlabeled B[a]P metabolite standards were purchased from the NCI Chemical Carcinogen Standard Reference Repository (Midwest Research Institute, Kansas City, MO, USA). The [¹³C₄]-labeled B[a]P metabolite internal standards were synthesized as described (Ran *et al.* 2008). The identities and purity of all the standards were established by LC-MS and HPLC, as previously published (Lu *et al.* 2011). All solvents were of HPLC grade, and all other chemicals were of the highest purity available and were used without further purification. Cell culture media were obtained from Invitrogen Co. except for fetal bovine serum (FBS), which was from Hyclone Laboratories (Logan, UT, USA). *p*-Nitrophenyl phosphate (pNPP) was obtained from Sigma Aldrich, and the dual-luciferase reporter assay system was obtained from Promega.

Cell culture

Transformed human endometrial cells, Ishikawa cells, were obtained from Sigma Aldrich (99040201-1VL) and maintained in MEM+2 mM glutamine+1% non-essential amino acids (NEAA)+5% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 µg/mL streptomycin. All cells were maintained in 10² cm dishes and were incubated at 37°C in a humidified incubator maintained at 5% CO₂, and culture media were renewed every 2–3 days. The cells were subcultured every 4–5 days using 0.25% trypsin. Cultured cells with a passage number of 6–16 were used to reduce variability. Before each experiment, the cells were seeded in phenol red-free DMEM/F12 with 5% charcoal dextran stripped (CDS) FBS (estrogen depleted media) for 48 h.

Liquid chromatography tandem-mass spectrometric analysis

We reported a reversed-phase HPLC method, which separated all the major B[a]P metabolites over 60 min (Lu *et al.* 2011). To optimize shorter run times, we injected B[a]P metabolite standards individually as well as a mixture onto a Kinetex XB Biphenyl 100 x 2.1 mm 2.6 µm column (Phenomenex, Torrance, CA, USA).

Satisfactory chromatographic separation of B[a]P metabolites was obtained using 5 mM ammonium acetate in water plus 0.02% (v/v) formic acid as mobile phase A and 5 mM ammonium acetate in acetonitrile plus 0.02% (v/v) formic acid as mobile phase B. This mobile phase is compatible with LC-MS analysis due to the volatility of ammonium acetate. All B[a]P standards (200 pmol) of interest were well separated in a 25 min gradient.

A Finnigan TSQ Quantum Ultra spectrometer (Thermo Fisher) interfaced with a Waters Alliance 2690 HPLC system (Waters Corporation, Milford, MA, USA) was used for the LC-MS/MS analyses. Chromatographic separation for the LC-MS/MS analyses was achieved with the Kinetex XB Biphenyl column. The eluent from the HPLC column was directly introduced into a standard atmospheric pressure chemical ionization (APCI) source equipped with a corona discharge pin. Nitrogen was used as the nebulizing gas as well as the sheath gas and auxiliary gas. The vaporizer and capillary temperatures were set at 400 and 270°C, respectively. All the hydroxylated standards under positive APCI conditions were predominated by stable dehydrated precursors, expected from s-phase dehydration that results in loss of H₂O, which were chosen as parent ions in SRM analysis. B[a]P-7,8-*trans*-dihydrodiol formed a [M+H-H₂O]⁺ parent ion *m/z* 269 and a [M+H-2H₂O]⁺ product ion *m/z* 251; while 3-OH-B[a]P generated the [M+H]⁺ parent ion *m/z* 269 which lost a molecule of water [M+H-H₂O]⁺ to give a product ion *m/z* 251; B[a]P-7,8-dione formed a [M+H]⁺ parent ion *m/z* 283 and a product ion [M+H-CO]⁺ *m/z* 255; and B[a]P-tetraol gave a [M+H-2H₂O]⁺ as a parent ion of *m/z* 285 and a product ion of [M+H-3H₂O - CO]⁺ of *m/z* 239. By contrast B[a]P did not fragment and gave a parent ion of [M+H]⁺ *m/z* 253 only. The metabolites measured were therefore detected under the following SRM transitions: *m/z* 269 [M+H-H₂O]⁺ → *m/z* 251 [M+H-2H₂O]⁺ for B[a]P-7,8-*trans*-dihydrodiol; *m/z* 269 [M+H]⁺ → *m/z* 251 [M+H-H₂O]⁺ for 3-OH-B[a]P; *m/z* 283 [M+H]⁺ → *m/z* 255 [M+H-CO]⁺ for B[a]P-7,8-dione; *m/z* 285 [M+H-2H₂O]⁺ → *m/z* 239 [M+H-3H₂O - CO]⁺ for B[a]P-tetraol; and *m/z* 253 [M+H]⁺ for B[a]P.

To generate the calibration curves, ~100 pmol of [¹³C₄]-internal standards (B[a]P, B[a]P-7,8-*trans*-dihydrodiol, 3-OH-B[a]P and B[a]P-7,8-dione) were spiked with different nmol amounts of the unlabeled B[a]P and its metabolites (0, 0.3125, 0.625, 1.25, 2.5, 5 and 10) in a 150 µL volume. The HPLC method described previously was used, and the flow rate was set at 0.3 mL/min. The peak area ratios of the analyte/[¹³C₄]-internal standard were plotted against the concentrations of analytes.

The linearity of the calibration curves was considered satisfactory in the studied range of concentrations ($R^2 > 0.99$ for all B[a]P standards). The isotopically labeled B[a]P metabolite internal standards co-eluted with the unlabeled standards and gave m/z values that were 4 amu higher.

B[a]P metabolism in Ishikawa cells

The metabolism of B[a]P in Ishikawa cells was conducted according to published procedures (Lu *et al.* 2011). Briefly, the cells ($\sim 3 \times 10^6$) were induced with TCDD (10 nM) in 2 mL of charcoal dextran stripped FBS MEM medium for 24 h before B[a]P treatment (5 μ M, 0.1% DMSO) in 2 mL of HBSS (10 mM glucose). Control experiments were performed in a similar manner with the exception that DMSO was substituted for TCDD. Cells were sampled at 0, 3, 6, 12, and 24 h. The 2 mL media was siphoned into a 5 mL tube, after which 2 mL of fresh media was added to cells then harvested by a cell scraper into a 5 mL tube and the media and cells extracted twice with 3 mL of water-saturated ethyl acetate. Before extraction, a [$^{13}\text{C}_4$]-B[a]P metabolite standard cocktail (containing ~ 100 pmol analytes of each analyte) was added to each sample to normalize for losses in the sample workup and analysis. The organic extract was dried under vacuum, and the residue was dissolved in 150 μ L of 90% methanol and put into an autosampler vial for LC-MS/MS analysis. All incubations were performed in triplicate in three independent experiments.

Alkaline phosphatase activity assay

Ishikawa cells were seeded at a density of 5×10^4 cells in 96-well plates. Media was replaced with media containing 5% charcoal dextran stripped FBS and no phenol-red. They were treated with ethinyl estradiol (EE), or with the appropriate B[a]P metabolite in a dose-dependent manner, and after 48 h media was removed and lysis buffer (0.1 M Tris/HCL, 0.1% Triton X, 100 mM glycine, 1 nM MgCl, 1 mM ZnCl, pH 9.8) added and transferred to -80°C for 12 h before measuring alkaline phosphatase activity. Alkaline phosphatase activity was assayed by monitoring the hydrolysis of *p*-nitrophenyl phosphate (pNPP) to *p*-nitrophenol at pH 9.8 and measuring the formation of the *p*-nitrophenolate anion at 405 nm (molar extinction coefficient = $18,000/\text{M}^{-1}\text{cm}^{-1}$). Calibration curves were generated with *p*-nitrophenol under alkaline conditions, so that absorbance could be converted to pmoles (Littlefield *et al.* 1990).

Experiments were first conducted by increasing the concentration of either EE or B[a]P metabolite. The assays were repeated in the presence of 5 μ M B[a]P metabolites (predetermined) or 1 nM EE and a dose-dependent inhibition with fulvestrant (Sigma Aldrich), an estrogen receptor antagonist, was measured. All experiments were reproduced in three independent experiments. Curves were fitted to non-linear regression models in Prism version 7.00 for Windows (GraphPad Software).

Transient transfection and dual-luciferase activity assay

Ishikawa cells were seeded at a density of 2×10^4 cells in 96-well plates. The cells were transfected with the pERE-luciferase plasmid, which contains three copies of the *Xenopus laevis* vitellogenin A2 ERE upstream of the firefly luciferase reporter. To normalize for transfection efficiency a pRL-TK plasmid, which contains a cDNA encoding Renilla luciferase, was co-transfected. Co-transfection was conducted using 0.4 μ g of pERE-luciferase plasmid and 2.5 ng of pRL-TK plasmid with 0.5 μ L of lipofectamine 2000 reagent (Invitrogen). Transfection solutions were replaced after 24 h, and cells were treated with EE, or with the appropriate B[a]P metabolite in a dose-dependent manner. Cells were treated for 24 h prior to cell lysis and measurement of firefly- and Renilla luciferase luminescence using the Stop-Glo Reagent system (Promega). Experiments were repeated in the presence of 5 μ M of estrogenic B[a]P metabolites or 1 nM EE and dose-dependent inhibition with fulvestrant was measured. The magnitude of the Renilla luciferase signal showed that the variability introduced by transient transfection was less than 3%. All experiments were replicated in three independent experiments.

Cell proliferation and imaging

Cell proliferation assays were conducted through high-contrast brightfield counting via the Cytation 5 Cell Imaging Multi-Mode Reader (BioTek Instruments) every 24 h post-treatment. Images were captured at $4\times$ in the brightfield channel. Two high-contrast brightfield images were captured at each time point: an in-focus image used for reference, and a defocused image for cell counting. Image preprocessing was used to obtain the best possible enhancement of contrast, reducing each cell to a single bright spot. Gen5 software applied masks to identify each

cell for counting. All experiments were replicated in three independent experiments.

Western blot analysis

Ishikawa cells ($\sim 3.2 \times 10^6$) were treated with 10 nM or 100 nM B[a]P-7,8-dione in a time course (5, 10, 30, 60 and 120 min). Cells were washed in ice-cold PBS, and whole-cell extracts were prepared in lysis buffer containing radioimmunoprecipitation assay buffer (RIPA buffer) (ThermoFisher) and 1 tablet of protease inhibitor cocktail (Roche). Cells were collected, and debris was pelleted by centrifugation at 15,000 *g* for 10 min at 4°C. The supernatants were collected and stored at –20°C. Total protein (40 µg) was loaded onto 10% (v/v) SDS-PAGE gels, separated, and transferred to nitrocellulose. Membranes were incubated with monoclonal mouse phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (#9106) or polyclonal rabbit p44/42 MAPK (#9102) (Cell Signaling Technology). Bound antibodies were detected using horseradish peroxidase-conjugated secondary antibodies and chemiluminescent immunodetection with an ECL Detection Kit (GE Healthcare), and were visualized using the Bio-Rad ChemiDoc imaging system. In each case, three independent experiments were performed, followed by densitometric analysis and one-way ANOVA statistical analysis.

Results

Characterization of Ishikawa cells

Ishikawa cells were shown to express ER α , ER β and AhR by immunoblot analysis. We demonstrated the function of the AhR by measuring the induction of P450 activity using the AhR ligand TCDD to induce ethoxyresorufin *O*-deethylase activity and the function of the ER by measuring the induction of the estrogen-responsive alkaline phosphatase activity using EE as a ER ligand (Supplementary Figs 1, 2 and 3, see section on [supplementary materials](#) given at the end of this article).

Stable-isotope dilution LC-APCI-MS/MS detection of B[a]P metabolism in Ishikawa cells

To study B[a]P metabolism in Ishikawa cells, we implemented an APCI interface since it is more sensitive than electrospray ionization (ESI) in detecting B[a]P metabolites (Koeber *et al.* 1997). B[a]P and its metabolite

standards (B[a]P-7,8-*trans*-dihydrodiol, B[a]P-tetraols, 3-OH-B[a]P and B[a]P-7,8-dione) were directly infused to optimize ionization conditions for their identification. All the hydroxylated standards under positive APCI conditions were predominated by stable dehydrated precursors (see ‘Materials and methods’ section). Representative LC-SRM/MS chromatograms that demonstrate excellent separation and detection for all the analytes are shown in Fig. 1. Representative ion chromatograms of B[a]P metabolites and corresponding internal standards are shown in Supplementary Fig. 4. The peak area ratios of analyte and corresponding isotope-labeled internal standard were plotted against the added amount of analyte to generate calibration curves. For all standards analyzed, excellent linearity was found with $R^2 \geq 0.99$ (Supplementary Fig. 5).

With the method established, Ishikawa cells were then incubated with 5 µM B[a]P with or without prior P450 induction by TCDD, and metabolites were measured by SID-LC-APCI-MS/MS protocol over a 24 h time course. Representative ion chromatograms of B[a]P at 0 and 24 h are shown in Fig. 2. Over 3–24 h, B[a]P was depleted from the media and was taken up into the cells so that 2 µM of B[a]P was in the cellular component after 3–24 h in both the presence or absence of TCDD induction (Fig. 3A). 3-OH-B[a]P was the first metabolite to appear, and it was formed in greater amounts and at an earlier time point following TCDD induction. The formation of B[a]P-7,8-dione occurred later than 3-OH-B[a]P (Fig. 3B). Following TCDD induction B[a]P-7,8-dione was formed

Figure 1

HPLC separation and resulting ion chromatograms of B[a]P metabolites. Successful separation of B[a]P tetraols (1–4), B[a]P-7,8-*trans*-dihydrodiol, 3-OH-B[a]P, B[a]P-7,8-dione and B[a]P after injection of a mixture of 200 pM of each onto the column connected to an APCI-MS ion source. A representative ion chromatogram is shown. Retention times are indicated above the apex of each peak. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0579>.

Figure 2

Ion chromatograms of B[a]P metabolites formed in Ishikawa cells over 24 h. (A) At 0 h there are no B[a]P metabolites present in the media, but unmetabolized B[a]P was present. (B) At 24 h 3-OH-B[a]P and B[a]P-7,8-dione were present in the media while B[a]P-7,8-*trans*-dihydrodiol is absent and B[a]P is depleted. The ion chromatograms of the corresponding $[^{13}\text{C}_4]$ -internal standards are shown. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0579>.

in greater amounts and occurred at an earlier time point. With and without induction by TCDD, B[a]P-7,8-dione formation lagged behind 3-OH-B[a]P formation (Fig. 3C). Collectively, unmetabolized B[a]P, 3-OH-B[a]P and B[a]P-7,8-dione accounted for 70% of the B[a]P added. There was no evidence for the appearance of B[a]P-7,8-*trans*-dihydrodiol or B[a]P-tetraols in the organic soluble metabolites with or without TCDD induction of P4501A1/1B1 (data not shown).

Measurement of the estrogenic activity of B[a]P and its metabolites in Ishikawa cells

We took advantage of the endogenous reporter gene, alkaline phosphatase (AlkP), which is inducible by estrogens and other estrogenic compounds. The activity of the AlkP enzyme can be easily quantified *in situ* using a chromogenic substrate (Littlefield *et al.* 1990). Previous studies show that estradiol induces AlkP at concentrations as low as 10^{-12} M and antiestrogens completely block this effect. We confirmed this finding using EE to activate

AlkP and fulvestrant to inhibit it (Supplementary Fig. 3). The induction AlkP in Ishikawa is specific for estrogens and no other steroids tested, including androgens, progestins, mineralocorticoids or glucocorticoids induce AlkP; therefore these responses have been regarded to be solely mediated by ER (Littlefield *et al.* 1990).

Cells were treated with 3-OH-B[a]P, B[a]P-7,8-*trans*-dihydrodiol, B[a]P-9,10-*trans*-dihydrodiol, B[a]P-7,8-dione, B[a]P-4,5-dione, B[a]P-6,12-dione, B[a]P-1,6-dione, B[a]P-3,6-dione and B[a]P-r-7,t-8,t-9,c-10-tetrahydrodiol (B[a]P-tetraol-1) in a concentration dependent manner between 10^{-1} and 10^4 nM. Higher concentrations of these compounds were either insoluble or were cytotoxic in cell culture. B[a]P, 3-OH-B[a]P, B[a]P-9,10-*trans*-dihydrodiol and B[a]P-7,8-dione stimulated AlkP activity, while the rest of the metabolites tested yielded a less than 1-fold increase in AlkP activity (Supplementary Fig. 6). The induction of AlkP with 5 μM B[a]P, 3-OH-B[a]P, B[a]P-9,10-*trans*-dihydrodiol and B[a]P-7,8-dione was also inhibited by fulvestrant in a dose-dependent manner indicating that this activity was driven by ER (see representative Supplementary Fig. 7).

Figure 3

Quantification of B[a]P and metabolites formed in Ishikawa cells over 24 h. (A) B[a]P metabolism was measured over 24 h and the distribution of metabolites were measured in the media and cellular components. (B) Quantification of 3-OH-B[a]P in TCDD-induced and uninduced treated cells. (C) Quantification of B[a]P-7,8-dione in TCDD-induced and uninduced treated cells, see Materials and Methods for details. Mean values $\pm$ SD of $n = 3$ independent experiments are given. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$. One-way ANOVA was performed using GraphPad Prism. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0579>.

Induction of ERE luciferase activity by B[a]P and metabolites in Ishikawa cells

Our findings from the AlkP assays showed that B[a]P, 3-OH-B[a]P and B[a]P-7,8-dione metabolites were estrogenic using an endogenous reporter gene. We were concerned that the AlkP assay may not have been sensitive enough to detect the true potency of the estrogenic effects of B[a]P metabolites. To address this question, we used a more sensitive ERE luciferase activity assay and measured estrogenic activity using this exogenous reporter gene. Cells were transiently transfected with an ERE-luciferase reporter plasmid and luciferase activity was determined after 24 h treatment with test compounds as a measure of ER-mediated transcriptional activation. We found that the EC_{50} values obtained were similar to those observed in the AlkP assay but the fold induction with all ER ligands was greater (Fig. 4A). We also found that the luciferase activity induced by the B[a]P metabolites was inhibited by fulvestrant in a dose-dependent manner (Fig. 4B).

Figure 4

Induction of ERE luciferase activity by EE, B[a]P and its metabolites and its inhibition by fulvestrant. (A) B[a]P, B[a]P-7,8-dione, and 3-OH-B[a]P-induced ERE luciferase activity in a concentration-dependent manner with EC_{50} values in the low micromolar range, by comparison EE, induced ERE luciferase in the low nanomolar range. (B) Induction of ERE luciferase activity by 5 μM B[a]P, 5 μM B[a]P-7,8-dione, 5 μM 3-OH-B[a]P and 1 nM EE is inhibited by fulvestrant, an ER antagonist in a dose-dependent manner. EE2 = ethinyl estradiol. ERE luciferase activity was measured 24 h post-treatment, see 'Materials and methods' section. Mean values $\pm$ s.d. of $n = 3$ independent experiments are given. * $P \leq 0.05$; **** $P \leq 0.0001$. One-way ANOVA followed by Dunnett's multiple comparisons test was performed using GraphPad Prism. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0579>.

Induction of MCF-7 breast cancer cell proliferation by 17 α -ethinyl estradiol (EE) and B[a]P-7,8-dione and inhibition with fulvestrant

MCF-7 cells have an established estrogen-dependent proliferation phenotype that we sought to utilize as a positive control for imminent experiments in endometrial cells. We treated MCF-7 cells with EE and B[a]P-7,8-dione in a concentration-dependent manner, 10^{-1} and 10^4 pM for EE and between 10^{-1} and 10^4 nM for B[a]P-7,8-dione.

Figure 5

Inhibition of EE- and B[a]P-7,8-dione-induced MCF-7 cell and Ishikawa endometrial cell proliferation with fulvestrant. (A) A dose-dependent fulvestrant (ER antagonist) inhibition of 100 pM EE-induced MCF-7 cell proliferation over 192 h. (B) Fulvestrant inhibition of 1 μM B[a]P-7,8-dione-induced MCF-7 proliferation over 192 h. (C) A dose-dependent fulvestrant inhibition of 100 pM EE-induced Ishikawa cell proliferation over 96 h. (D) Lack of complete fulvestrant inhibition of 10 nM B[a]P-7,8-dione-induced Ishikawa proliferation over 96 h. Mean values $\pm$ s.d. of $n = 3$ independent experiments are given. $*P \leq 0.05$; $***P \leq 0.001$; $****P \leq 0.0001$. One-way ANOVA followed by Dunnett's multiple comparisons test was performed using GraphPad Prism. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0579>.

Cell proliferation was induced by both EE and B[a]P-7,8-dione (Supplementary Fig. 8) in a dose-dependent manner. The induction of cell proliferation by 100 pM EE (Fig. 5A) and 1 μM B[a]P-7,8-dione (Fig. 5B) in MCF-7 cells was inhibited by fulvestrant in a dose-dependent manner, suggesting that both EE- and B[a]P-7,8-dione-induced cell proliferation was driven by ERs in the MCF-7 cell line. The concentration at which we saw an effect of B[a]P-7,8-dione in MCF cells

was consistent with its EC_{50} value for ER activation in Ishikawa cells. These results were replicated in three independent experiments.

Induction of Ishikawa endometrial cancer cell proliferation by EE and estrogenic PAHs and inhibition with fulvestrant

Ishikawa endometrial cells were treated with EE (10^{-1} to 10^4 pM) and estrogenic PAHs – B[a]P, B[a]P-7,8-dione and 3-OH-B[a]P – in a concentration-dependent manner (10^{-1} to 10^4 nM). In total, 1 pM EE and 10 nM B[a]P-7,8-dione (Fig. 6) induced a significant increase ($p \leq 0.05$) in the proliferation of Ishikawa cells while B[a]P and 3-OH-B[a]P did not exhibit significant cell proliferation and were, in fact, cytotoxic at high concentrations (Supplementary Fig. 9). Notably, contrary to the trend observed in MCF-7 cells, B[a]P-7,8-dione-induced endometrial cell proliferation in the low nM range at concentrations insufficient to activate the ER in the alkaline phosphatase and ERE-luciferase reporter gene assays. Fulvestrant inhibited EE stimulated Ishikawa cell growth but did not inhibit B[a]P-7,8-dione-induced proliferation in Ishikawa cells (Fig. 5D). These data revealed that B[a]P-7,8-dione-induced endometrial cell proliferation in an ER-independent manner. In additional experiments, we also found that B[a]P-7,8-dione and EE induce the expression of the cell cycle gene, *CCND1* (Supplementary Fig. 10), and this induction was not inhibited by fulvestrant (Supplementary Fig. 11). We also found EE and B[a]P-7,8-dione to have no effect on apoptosis in the 0.1 nM to 1.0 μM concentration range (Supplementary Fig. 12). These supplemental experiments further reveal B[a]P-7,8-dione-induced endometrial cell proliferation to be a primary outcome of B[a]P-7,8-dione signaling in endometrial cells, as opposed to apoptosis.

Inhibition of B[a]P-7,8-dione-induced endometrial cell proliferation by EGFR-selective tyrosine kinase inhibitor-gefitinib

In an effort to decipher a mechanism for B[a]P-7,8-dione-induced endometrial cell proliferation that was independent of ER, we explored the role of EGFR since previous studies suggested that B[a]P quinones can induce MCF-7 cell proliferation by activation of the EGFR (Burdick *et al.* 2003). Cell proliferation was induced in Ishikawa cells using 10 nM B[a]P-7,8-dione in the presence and absence of the EGFR-selective tyrosine kinase inhibitor (gefitinib). We observed a dose-dependent inhibition of B[a]P-7,8-dione-induced proliferation by gefitinib in Ishikawa

Figure 6

Induction of Ishikawa cell proliferation with EE and B[a]P-7,8-dione. (A) EE (1 pM) and B[a]P-7,8-dione (1 nM) induce Ishikawa cell proliferation and (B) EE and B[a]P-7,8-dione induce Ishikawa cell proliferation in a dose-dependent manner. Mean values $\pm$ s.d. of $n = 3$ independent experiments are given. * $P \leq 0.05$; *** $P \leq 0.001$; **** $P \leq 0.0001$). One-way ANOVA was performed using GraphPad Prism. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0579>.

cells (Fig. 7A). These results suggest that EGFR activation is implicated in B[a]P-7,8-dione-induced endometrial cell proliferation. These results were replicated in three independent experiments.

Inhibition of B[a]P-7,8-dione-induced endometrial cell proliferation by MAPK inhibitor – PD98059

We wanted to determine whether the MAPK pathway was involved in B[a]P-7,8-dione-induced cell proliferation of Ishikawa cells. Cell proliferation was induced in Ishikawa cells using 10 nM B[a]P-7,8-dione in the presence and absence of the MAPK inhibitor, PD98059. We observed a dose-dependent inhibition of B[a]P-7,8-dione-induced proliferation by PD98059 in Ishikawa cells (Fig. 7B). These results suggest that B[a]P-7,8-dione-induced endometrial cell proliferation and implicated the MAPK pathway. These results were replicated in three independent experiments.

Phosphorylation of p44/42 MAPK by nM concentrations of B[a]P-7,8-dione in Ishikawa cells

We confirmed the activation of MAPK pathway by immunoblotting for phospho p44/42 MAPK (ERK1/ERK2)

after treating Ishikawa cells with nM concentrations of B[a]P-7,8-dione. In a time course spanning 5 min to 2 h we observed an increase in p44/42 MAPK phosphorylation that peaked at 30 min (Fig. 8) for both 10 nM and 100 nM B[a]P-7,8-dione treated cells. These results confirm our hypothesis that the MAPK pathway is indeed implicated in B[a]P-7,8-dione-induced endometrial cell proliferation. These results were replicated in three independent experiments.

Discussion

Endometrial cancer is the most common malignancy within the female reproductive system (Siegel *et al.* 2019). Exposure of the endometrium to unopposed estrogens from endogenous and exogenous sources is of concern as it has been shown to be the main driver of endometrial cancer. Polycyclic aromatic hydrocarbons (PAHs) have been extensively studied for their mutagenic and carcinogenic activities (Kummer *et al.* 2008). However, they also engender non-genotoxic effects associated with tumor promotion or endocrine disruption (Sjogren *et al.* 1996, Pliskova *et al.* 2005, Andrysik *et al.* 2007),

Figure 7

Inhibition of B[a]P-7,8-dione-induced endometrial cell proliferation with EGFR and MAPK inhibitors, gefitinib and PD98059, respectively. (A) The EGFR inhibitor (gefitinib) dose-dependent inhibition of Ishikawa cell proliferation induced by 10 nM B[a]P-7,8-dione over 96 h. (B) The MAPK inhibitor (PD98059) dose-dependent inhibition of Ishikawa cell proliferation induced by 10 nM B[a]P-7,8-dione over 96 h. Mean values $\pm$ s.d. of $n = 3$ independent experiments are given. ** $P \leq 0.01$; **** $P \leq 0.0001$; ns, not significant. One-way ANOVA was performed using GraphPad Prism.

A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0579>.

and others have shown PAHs and/or their metabolites to have xenoestrogenic activity in rodent and human breast cell line models (Arcaro *et al.* 1999, Charles *et al.* 2000, Fertuck *et al.* 2001, Gozgit *et al.* 2004, Abdelrahim *et al.* 2006). Activation of ER signaling in endocrine target tissues, such as the endometrium, may further contribute to the known carcinogenicity of PAHs (Santodonato 1997). While some work has been done to study reproductive toxicity associated with endometrial cell-mediated metabolism of PAHs (Iannaccone *et al.* 1984), insufficient information exists concerning the metabolic activation and estrogenic activity of PAH in human endometrial cells. The signaling mechanisms and biological outcomes of estrogenic and non-estrogenic signaling induced by PAH in endometrial cells also remain to be clarified.

We demonstrate that the Ishikawa endometrial cell line is appropriate for investigating the metabolic fate and estrogenic activity of B[a]P and its metabolites. This cell line contains the AhR and ERs (Supplementary Fig. 1) and is responsive to the induction of cytochrome P450s (P450s) and estrogenic activity by TCDD and EE, respectively (Supplementary Figs 2 and 3).

Utilizing stable-isotope dilution high performance liquid chromatography and APCI tandem mass spectrometry in the selected reaction monitoring mode (LC/APCI/MS/MS), we characterized and quantified B[a]P and its metabolites in the presence and absence of P450 induction by TCDD an AhR agonist. We found that B[a]P can be metabolized in Ishikawa cells to significant levels of 3-OH-B[a]P and B[a]P-7,8-dione, with the induction of P450s resulting in the formation of higher levels of both products. We tested the estrogenic activity of these metabolites using the inducible endogenous alkaline phosphatase reporter activity and the exogenous estrogen response element (ERE) driven luciferase reporter activity in Ishikawa cells. We demonstrated that B[a]P metabolites formed in Ishikawa cells can activate the ER in both assays and that the reporter gene activity was blocked with fulvestrant. We also found that B[a]P-7,8-dione promoted endometrial cell proliferation at nanomolar concentrations insufficient to activate the ER, and implicated EGFR and MAPK signaling in this response.

Figure 8

Concentration-dependent activation of MAPK by B[a]P-7,8-dione in Ishikawa cells. Top panel immunoblot showing 10 nM B[a]P-7,8-dione and 100 nM B[a]P-7,8-dione induction of p44/42 MAPK phosphorylation over at 5, 10, 30 min and 2 h. Tubulin is used as the loading control. Bottom panel densitometric quantitation of the immunoblot showing fold change of phosphor p44/42 over vehicle. Mean values $\pm$ s.d. of $n = 3$ independent experiments are given. * $P \leq 0.05$; *** $P \leq 0.001$; **** $P \leq 0.0001$. One-way ANOVA was performed using GraphPad Prism. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0579>.

Our study identified the main pathways of PAH metabolism in endometrial cells, and we identified the most important estrogenic PAH metabolites following PAH exposure that may act as endocrine-disrupting chemicals. 3-OH-B[a]P and B[a]P-7,8-dione were the main metabolites formed revealing evidence for P450-mediated mono-oxygenation and the *o*-quinone pathway. The latter requires the sequential action of P450, epoxide hydratase and aldo-keto reductases (Penning 2014). PAHs have previously been shown to engender estrogenic activity in rodent models and breast cancer cell lines but not in human endometrial cells; thus this work is significant because it illustrates the estrogenic character of PAHs and their metabolites in these cells. It is noteworthy that B[a]P-7,8-dione can redox cycle to the corresponding catechol, and B[a]P-7,8-catechol has structural similarity to catechol estrogens, which have genotoxic properties (Rempel *et al.* 2009, Wang *et al.* 2009, 2010, Fussell *et al.* 2011). Thus B[a]P-7,8-dione formed may play a significant role in endometrial cell genotoxicity in addition to its estrogenic action.

We assessed cell proliferation mediated by the estrogenic PAH metabolites because it is the primary phenotype attributed to estrogen signaling in female endocrine cancers. As a positive control, we observed a concentration-dependent increase in cell proliferation in MCF-7 breast cancer cells treated with EE, confirming previous results (Soto & Sonnenschein 1985, Furuya *et al.* 1989, Liao *et al.* 2014). B[a]P-7,8-dione exhibited a growth phenotype in MCF-7 cells at concentrations that elicit an estrogen response in our reporter gene assays which was inhibited by fulvestrant, suggesting that B[a]P-7,8-dione-induced cell proliferation was ER-dependent in this cell line. When we applied the same protocol to Ishikawa endometrial cells, we also observed an increase in proliferation after treatment with increasing concentrations of EE, and this response was attenuated by fulvestrant. Of the PAH metabolites tested only B[a]P-7,8-dione-induced Ishikawa cell proliferation but this effect was observed in the low nM range, at concentrations insufficient to activate the ER. Moreover, the growth phenotype in Ishikawa cells mediated by B[a]P-7,8-dione was not blocked by fulvestrant. We also investigated the effects of EE and B[a]P-7,8-dione on the induction of the cell cycle gene *CCND1* and found both to induce the expression of this gene in a dose-dependent manner. However, fulvestrant did not inhibit the induction of EE- and B[a]P-7,8-dione-induced *CCND1* expression. Additionally, we found both EE- and B[a]P-7,8-dione to not induce apoptosis in Ishikawa cells

in the 0.1 nM to 1 μ M range. These results suggest that B[a]P-7,8-dione-induced endometrial cell proliferation may not be ER-regulated.

Given that B[a]P quinones can induce EGFR signaling via ROS in breast epithelial cells (Burdick *et al.* 2003). We demonstrated that EGFR-selective receptor tyrosine kinase and MAPK inhibitors, gefitinib and PD98059, respectively, inhibit B[a]P-7,8-dione-induced cell proliferation in Ishikawa cells. Additionally, exposing Ishikawa cells to nM concentrations of B[a]P-7,8-dione resulted in the phosphorylation of p44/42 MAPK (ERK 1/2). These data suggest that the MAPK pathway is implicated in B[a]P-7,8-dione-induced endometrial cell proliferation.

Our work unveils two properties of PAHs and their metabolites in human endometrial cells. PAHs are estrogenic in human endometrial cells in the μ M concentration range but not the nM range. On the other hand, low concentrations (nM range) of B[a]P-7,8-dione elicit a growth phenotype in endometrial cells that do not seem to be modulated by ERs but implicate the MAPK pathway. Reactive oxygen species (ROS) are not only toxic to cells but can also modulate signaling during tumor progression. For instance, it has previously been demonstrated that the oxidation of protein cysteine (Cys) residues in EGFR and downstream phosphatases by ROS enhances EGFR-mediated signaling to promote tumor progression (Weng *et al.* 2018). The redox-cycling of B[a]P-7,8-dione, leading to ROS formation (Burczynski *et al.* 1999), could explain why it is the only B[a]P metabolite formed in Ishikawa cells to induce proliferation implicating the EGFR and MAPK pathways.

In conclusion, we have demonstrated that endometrial carcinoma cells can metabolize B[a]P to bioactive metabolites. Of these, both B[a]P-7,8-dione and 3-OH-B[a]P induce ER-dependent gene transcription, but this transcription does not result in cell proliferation. Importantly, low concentrations of B[a]P-7,8-dione affect endometrial carcinoma proliferation by an ER-independent pathway involving EGFR-MAP-kinase signaling.

Supplementary materials

Supplementary data has been deposited in the public data repository figshare:

Supplementary Figure 1: <https://doi.org/10.6084/m9.figshare.11284034.v2>
Supplementary Figure 2: <https://doi.org/10.6084/m9.figshare.11284052.v2>
Supplementary Figure 3: <https://doi.org/10.6084/m9.figshare.11284118.v2>
Supplementary Figure 4: <https://doi.org/10.6084/m9.figshare.12192531.v1>
Supplementary Figure 5: <https://doi.org/10.6084/m9.figshare.12192543.v1>
Supplementary Figure 6: <https://doi.org/10.6084/m9.figshare.12192579.v1>
Supplementary Figure 7: <https://doi.org/10.6084/m9.figshare.12192621.v1>
Supplementary Figure 8: <https://doi.org/10.6084/m9.figshare.11996658.v1>

Supplementary Figure 9: <https://doi.org/10.6084/m9.figshare.11996697.v2>
Supplementary Figure 10: <https://doi.org/10.6084/m9.figshare.11996727.v1>
Supplementary Figure 11: <https://doi.org/10.6084/m9.figshare.11996733.v1>
Supplementary Figure 12: <https://doi.org/10.6084/m9.figshare.11996742.v1>

Declaration of interest

T M P is the founder of Penzymes, LLC, receives a sponsored research agreement from Forendo and is on the expert panel of the Research Institute for Fragrance Materials. Our grant sponsor, National Institute of Environmental Health Sciences, is a non-commercial funding agency and was not involved in the acquisition and interpretation of the data or the decision to publish the work.

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