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Evidence of Estrogen- and TCDD-Like Activities in Crude and Fractionated Extracts of PM_{10} Air Particulate Material Using in Vitro Gene Expression Assays

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Polar and nonpolar fractions prepared from an organic extract of inhalable air particulate material collected from an urban location in downtown Toronto, Ontario, Canada, were examined for estrogen and Ah receptor-mediated activities using in vitro gene expression assays. The presence of estrogenic activity was determined using MCF-7 human breast cancer cells transiently transfected with a Gal4-human estrogen receptor chimera and a Gal4-regulated luciferase reporter gene. 2,3,7,8-Tetracholordibenzo-*p*-dioxin (TCDD)-like activity was detected using Hepa 1c1c7 cells transiently transfected with a CYP1A1-regulated reporter gene (pGudLuc 1.1). Significant estrogenic and TCDD-like activity was detected in the crude extract and in the nonpolar fractions. Results from the analyses of nine environmentally prevalent polyaromatic hydrocarbons (PAH) indicated that PAH might be significant contributors to the observed activity. Surprisingly, three PAH, namely benzo[*a*]pyrene, chrysene, and benz[*a*]anthracene, were found to substantially induce in vitro estrogenic and TCDD-like activities that were mediated by the estrogen and Ah receptors, respectively. Benzo[*k*]fluoranthene, dibenz[*a,h*]-anthracene, and anthracene also exhibited significant in vitro TCDD-like activity. These results demonstrate the utility of in vitro gene expression assays to identify the presence of potential endocrine disruptors within complex mixtures.

Introduction

There is increasing and widespread concern that man-made chemicals may interfere with the normal functioning of human and wildlife endocrine systems leading to adverse health effects such as increased rates of specific cancers, reproductive system abnormalities, learning and behavioral problems, and immune system deficiencies (*1*). These chemicals have been described as environmental endocrine disrupters by the United States Environmental Protection Agency and broadly defined as "exogenous agents that interfere with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development, and/or behavior". A further evolution of this definition that was endorsed during the Weybridge, United Kingdom, Endocrine Disrupter Meeting (*2*) describes "an endocrine disrupter as an exogenous substance that causes adverse health effects in an intact organism, or its progeny, secondary to changes in endocrine function". One premise behind endocrine disruption is that substances may mimic endogenous hormones by eliciting similar effects or by blocking or diminishing their function by competing for available receptor sites. However, some effects may not be directly mediated through mechanisms that involve nuclear steroid receptors. For example, the antiestrogenic effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) appear to be mediated by the aryl hydrocarbon receptor (AhR) (*3, 4*), while nongenomic events or membrane-bound steroid receptor-mediated events can occur in the pituitary (*5*) and other tissues (*6*).

Recent attention has focused on the endocrine-disrupting effects of estrogenic substances, often referred to as exo-estrogens, environmental estrogens, or xenoestrogens. The chemical structures of these compounds are diverse, extending across broad classes of chemicals including environmental pollutants, pharmaceuticals, and natural products, as well as industrial chemicals (*7, 8*). The agonist and antagonist activities of this range of chemical structures appear to be related to the observed promiscuity of the estrogen receptor to bind compounds with both planar features and polar properties (*9*−*11*).

Urban air particulate material (APM) is a complex mixture of chemicals released from vehicular and industrial sources, together with re-entrained particles from roads and agricultural sources. Of particular concern to human health are particles in the inhalable and respirable size ranges referred to as PM_{10} and $PM_{2.5}$, respectively. These particles readily enter the lungs and become trapped by the alveoli. Urban air particulates with mean aerodynamic diameters below 10 μ (i.e., PM_{10}) have been implicated as a causative agent in premature deaths among urban dwellers (*12, 13*). In addition, due to the increased sensitivity of children to the effects of particulate material, research performed to enhance the understanding of how urban APM effects children is high on the EPA's national agenda (*14*). Approximately 30% of the mass of respirable urban air particulate can be extracted using organic solvents such as dichloromethane to afford a complex mixture of aliphatic and aromatic chemicals, ranging from nonpolar to very polar in nature. Such extracts have been fractionated and analyzed using various chromatographic and bioassay protocols. Previous work has focused on multidimensional approaches for the identification of mutagenic polycyclic aromatic compounds (PACs) using the Ames *Salmonella typhimurium* microsome assay (*15*−*18*).

The presence of endocrine disrupters in APM is not unlikely. Nonchlorinated organics such as polycyclic aromatic hydrocarbons (PAHs) have been implicated in biological activity mediated through the aromatic hydrocarbon receptor (AhR) such as P4501A1 (*3, 19, 20*) loosely classifying

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them as TCDD-like compounds. Previous studies examining the in vivo and in vitro effects of manufactured gas plant residue, known to contain high levels of PACs, implicate this type of air particulate material in causing significant endocrine disrupting activities (*21*, *22*). However, there are no reports on the potential presence of estrogenic compounds in the complex mixture of urban air particulate material.

Previous studies have demonstrated the utility of in vitro reporter gene assays to screen for the presence of estrogenic and TCDD-like endocrine disrupters in complex mixtures. These assays have used a variety of endpoints including ligand binding (*23*−*25*), increased protein expression (*26*, *27*), enzyme induction (*26*, *28*) and cell differentiation or proliferation (*29*, *30*). In vitro assays are simple, cost-effective, and, consequently, capable of screening large numbers of samples. In addition, these assays are valuable because they are able to indicate a potential mechanism of action as well as account for synergistic, antagonistic, and additive interactions that may occur within a complex mixture. Although in vitro assays have been proposed as part of a tiered screening battery to identify and assess chemicals and complex mixtures that may elicit estrogenic activities (*8*, *31*, *32*), they cannot replace in vivo models. The pharmacokinetic and pharmacodynamic interactions that can occur in vivo are not taken into account; therefore, in vitro tests should be used to complement in vivo testing. In addition, in vitro assays may not take into consideration other mechanisms of action and/or cross-talk between mechanisms of action that may lead to adverse effects. Moreover, important factors such as critical lifecycle or sensitive developmental windows and the effects of bioconcentration and bioaccumulation cannot be accurately modeled using in vitro assay systems. However, results from in vitro assays may indicate potential mechanisms of action that could be used to refine in vivo studies. In this study, we examine the estrogenic and TCDD-like activity associated with the crude organic extract and chemical fractions derived from inhalable urban APM (PM_{10}) as well as determining the contributions of chemicals found within these extracts.

Materials and Methods

Air Particulate Collection and Fractionation. Air particulate material was collected with an Anderson PM_{10} hi-vol air sampler (General Metal Works, Village of Cleves, OH). The air sampling was carried out between September 1990 and January 1991 at the Ontario Ministry of Environment and Energy sampling site on Bay Street in downtown Toronto, Ontario, Canada. This site was considered to be representative of urban air particulate quality in Toronto. Inhalable particulate material was collected for 24 h on 8 × 10 in. Teflon-coated glass fiber filters (Pallflex TC40M120WW). A total of 15 filters from the Bay St. site were combined to afford a composite sample representing 0.595 g of respirable particulate material collected from 25 600 m^3 of air. The composite sample was batch extracted with dichloromethane for 24 h using a Soxhlet apparatus to afford a crude extract that was fractionated according to procedures previously reported (*18*).

A brief description of the fractionation process follows and is summarized in Figure 1. The crude extract of the combined filters was evaporated to dryness carefully onto 3.0 g of neutral alumina. This alumina was placed in a column atop 6.0 g of neutral alumina that had been activated at 170 °C for at least 48 h. The column (1.0 × 10 cm) was eluted with hexane (60 mL) to yield an aliphatic fraction (A1), followed by dichloromethane (60 mL) to afford a nonpolar aromatic fraction (A23), and finally by methanol (60 mL) then methanol:water (4:1, 60 mL) to produce a polar aromatic fraction A45. Additional aliphatic and simple aromatic compounds were removed from fraction A23 by chromatography on Sephadex LH20 using a mobile phase consisting of hexane:dichloromethane:methanol (6:4:3). Material eluting prior to the naphthalene standard was referred to as fraction A0, while material that eluted with and after naphthalene was referred to as A23/LH20.

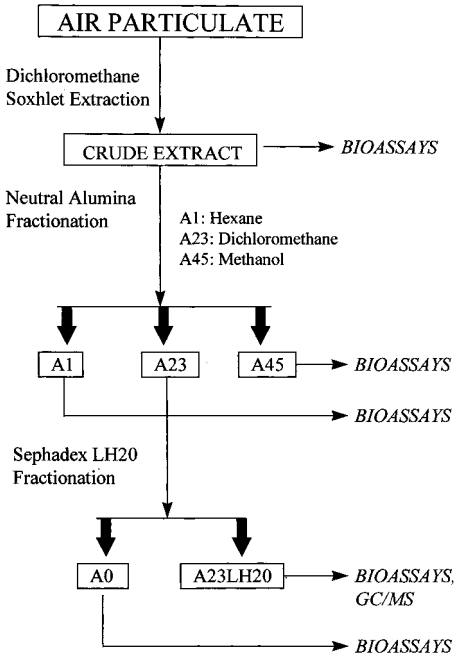

FIGURE 1. Schematic of the sample fractionation procedure for air particulate samples.

Chemical Analyses. Chemical analyses were performed by gas chromatography−mass spectrometry using a Hewlett-Packard 5890 Series II gas chromatograph and a Hewlett-Packard 5971 Mass Selective Detector with cool, on-column injection (1 mL toluene solution) using a Hewlett-Packard 7673A Automatic Injector. Helium was used as the carrier gas with a constant flow (1.00 mL/min) maintained using electronic pressure programming. A 60 m DB-5ms column (J&W Scientific, Folsom, CA, 0.25 i.d., 0.25 mm film thickness) was used with the following temperature program: initial temperature, 130 °C; program rate, 1.6 °C/min; final temperature, 300 °C; hold at 300 °C for 25 min. The transfer line temperature was 300 °C. Data were collected in selected-ion monitoring and full scan modes. Typical detection limits for PAHs under electron impact ionization conditions with selected ion monitoring ranged from 3 to 20 pg injected, which corresponded to a detection limit ranging from 0.15 to 1.0 pg/m^3 in air. Three deuterated PAHs (phenanthrene-d_{10}, benz[*a*]anthracene-d_{12}, and dibenz[*a*,*h*]anthracene-d_{14}) were added as surrogates to the filters in the Soxhlet apparatus prior to extraction; two deuterated internal standards (pyrene-d_{10} and perylene-d_{12}) were added to the A23/LH20 fraction for quantitation purposes.

Bioassay Chemicals. TCDD and ICI 164,384 were gifts from Dr. S. Safe (Texas A&M University) and Dr. A. Wakeling (ICI Pharmaceuticals, Cheshire, England), respectively. The PAHs were purchased from ICN (Mississauga, Ontario, Canada). All chemicals, TCDD, ICI 164,384, 17β-estradiol (E2), and the above air particulate extracts were prepared as solutions and serially diluted in dimethyl sulfoxide (DMSO). Phenol red-free Dulbecco's Modified Eagle's Medium (D-MEM) powder and media supplies were purchased from Gibco BRL (Burlington, Ontario, Canada). Fetal bovine serum (FBS), protease inhibitors as recommended by Sambrook et al. (*32*), and [γ^{32}P]dATP were purchased from ICN (Mississauga, Ontario, Canada). D-Luciferin was purchased from Molecular Probes (Eugene, OR). All other chemicals were of the highest quality available from commercial sources.

Cell Culture. The cell lines used in this study were (i) estrogen-responsive human breast cancer MCF-7 cells (kindly provided by Dr. L. Murphy, University of Manitoba) and (ii) wild-type mouse hepatoma-derived Hepa 1c1c7 cells and two Hepa 1c1c7 mutants, an AhR-deficient cell line and an AhR nuclear translocator (ARNT)-deplete cell line (all gratefully donated by Dr. O. Hankinson, UCLA, CA). Cells were maintained as per Zacharewski et al. (*33*). Briefly, the cells were grown in 10% FBS phenol red-free D-MEM supplemented with sodium bicarbonate (24 mM), glucose (11 mM), HEPES (20 mM), nonessential amino acids (10× solution), vitamin supplement solution (10× solution), sodium pyruvate (10 mM), lipoic acid (2 mg/mL), vitamin B_{12} (1.4 mg/mL), zinc sulfate (0.5 mM), and L-glutamine (2 mM). In addition, the antibiotics gentamycin (50 μg/mL) to prevent mycoplasma contamination, penicillin/streptomycin (100 IU/mL, 100 μg/mL) to control for both Gram negative and positive bacteria, and amphotericin B (2.5 μg/mL) to prevent fungal growth were added to the culture medium. All cells were kept at 37 °C in a 4% CO_2 humidified atmosphere.

Transfections and Reporter Gene Assays. Transfections and dosing protocols were carried out as per Zacharewski et al. (*32*) with a few exceptions. Cells were plated at approximately 4×10^6 cells per well in a 6-well tissue culture plate (50% confluence) in D-MEM supplemented with 10% FBS for the Hepa 1c1c7 cells and in D-MEM supplemented with 5% FBS stripped of serum-borne steroids by dextran-coated charcoal (DCC-FBS) treatment (*34*) for the MCF-7 cells. After 6 h, cells were transiently transfected by calcium phosphate coprecipitation (*34*). Plasmids transfected into each MCF-7 well were (i) 0.05 μg of pCMV, a β-galactosidas expression vector used to normalize the transfection efficiency between wells (*32*); (ii) 0.5 μg of Gal4-HEGO (provided by P. Chambon, IGBMC, Illkirch, France) which encodes for the Gal4-human estrogen receptor (ER) chimeric receptor (the yeast transcription factor Gal4 DNA binding domain linked to the ER ligand binding domain); and (iii) 1.5 μg of 17m5-G-Luc (provided by P. Chambon), the Gal4 regulated reporter construct consisting of five repeats of the Gal4 responsive elements (17mer) linked to the firefly luciferase cDNA reporter gene. The two plasmids transfected into the Hepa 1c1c7 cells were (i) 0.05 μg of pCMV (as above); and (ii) 2.5 μg of pGudLuc1.1 (provided by Dr. M. Denison, UC Davis, CA), a DNA construct containing the 482 bp (−1301 to −819) region of the murine-derived CYP1A1 upstream of the firefly luciferase cDNA reporter gene (*35*). Overnight transfections were followed by two rinses with sterile phosphate buffer saline replaced by the 10% FBS D-MEM for Hepa 1c1c7 cells and 5% DCC−FBS D-MEM for the MCF-7 cells.

For the agonist study, cells were dosed for 24 h with standards, TCDD (Hepa 1c1c7) or 17β-estradiol (MCF-7), serially diluted air particulate extracts, and PAHs at concentrations such that the DMSO concentration did not exceed 0.1% (2.5 μL in 2.5 mL medium/well). For the estrogen receptor antagonism studies, the cells were treated for 24 h with two doses of E2 (0.2×10^{-10} g/well, a submaximal dose, and 1×10^{-10} g/well, a maximal dose) and air particulate extract at three dose levels such that the DMSO concentration did not exceed 0.2% (0.005 mL in 2.5 mL medium/well). Each treatment was performed in duplicate, and two replicates were taken from each well affording, in total, four replicates per dose. The resultant reporter gene response was observed as relative light units on a 96-well plate luminometer (Luminoskan RS, Labsystems, Finland). The β-gal activity was quantified using standard protocols (*32*). The luciferase activity expressed in relative light units per β-galactosidase activity (RLU/β-gal) and is presented here as fold induction based on luciferase activity relative to the DMSO control. Dose−response data was analyzed by a sigmoidal curve-fitting method based on a logit function (GraphPad, San Diego, CA) for continuous response data (*36*). Means and standard deviations are given for the EC50 value.

Conformational assays were done to verify that the luciferase activity observed was (i) ER mediated in MCF-7 cell bioassay and (ii) AhR and ARNT dependent in the Hep1c1c7 cell assay. For the MCF-7 cells, transient transfections were performed in the absence of the Gal4-HEGO chimeric construct. In addition, the MCF-7 luciferase activity was antagonized by the addition of 10^{-7} M ICI 164,384 (*37*), the specific estrogen receptor antagonist (*38*). For the AhR bioassay, the Hep1c1c7 cell mutants substituted for the Hepa1c17 wild-type cells in that transient transfections were done as described above. An additional test, the gel retardation assay, was run to provide further evidence that the luciferase activity in the AhR bioassay was AhR dependent.

Gel Retardation Assay. The wild-type and mutant TCDD response element (DRE) oligonucleotides have been previously described (*33*, *4*). Briefly, the oligonucleotides were (i) the wild-type site D DRE (wtDRE), −998 to −973 of the CYP1A1 regulatory region [complementary strands of the wt DRE site D; 5′-gatctctTCTCACGCaActccgag-3′ and 5′-gatcctcggagTtGCGTGAGAaga-3′ (*39*)] and (ii) the point mutated DRE from the pS2 regulatory region (mutDRE) (5′-gggattacaGCGCGAccactgc-3′) (*4*). End labeling of the wild-type oligonucleotides (300 ng) was done with [γ^{32}P]dATP in the presence of T4 polynucleotide kinase (15 units) for 1 h at 37 °C. The strands were annealed in 100 mM NaCl and loaded on a 15% nondenaturing polyacrylamide gel, and electrophoresed to separate free [γ^{32}P]dATP from labeled oligonucleotides. The labeled, annealed strands were visualized by exposure to Kodak X-OMAT AR film and the resultant band was excised and extracted overnight in TE8 (Tris, 10 mM, and EDTA 1 mM, pH 8.0) buffer.

The gel retardation assay was followed as per Denison et al. (*40*). Guinea pig cytosol (20 mg/mL protein) was transformed (18 pg of TCDD, 2.3 mg of A23/LH20, 1 mg of crude extract) for 2 h at room temperature. The transformed cytosol was incubated for 15 min with 375 ng of poly(dIdC) (Boehringer Mannheim, Montreal, Canada) in HEGD (Hepes, 17 mM, EDTA, 4 mM, 10% glycerol and DTT, 5 mM) buffer containing 0.8 mM KCl. For competition assays, unlabeled annealed wt DRE (62.5 ng) and mutDRE (37.5 ng) oligonucleotides were added during the initial incubation. Labeled oligonucleotides (100 000 cpm, 2.5 ng) were then added and incubated for an additional 15 min. Samples were loaded onto a pre-electrophoresed 4% nondenaturing polyacrylamide gel run using 1× TBE running buffer (90 mM Tris, 120 mM boric acid, and 4 mM EDTA, pH 8). The gel was then dried, and specific DNA−protein interactions were localized following exposure to Kodak X-OMAT AR film.

Results and Discussion

APM from a downtown sampling site (Bay St., Toronto, Ontario, Canada) was extracted and chemically separated yielding three fractions: A1, nonpolar aliphatics, A23, nonpolar PACs, and A45, polar aromatic compounds (Figure 1). Fraction A23 was further refined using a Sephadex LH20 column to afford an aliphatic-free, nonpolar aromatic fraction (A23/LH20) and a residual fraction (A0) that contained some aliphatic compounds, simple aromatics, and phthalates. All four fractions (A0, A1, A23/LH20, and A45) and the original crude extract were assessed using reporter gene activities to investigate their estrogen and AhR-mediated activities. Selected PAHs, commonly found in APM using GC/MS analyses, were also tested for in vitro estrogenic and TCDD-like activity.

Detection of Estrogenic Activity. Transient cotransfection of MCF-7 cells with the Gal4-human ER (Gal4-HEG0) and a Gal4-responsive luciferase reporter (17m5-G-Luc) have

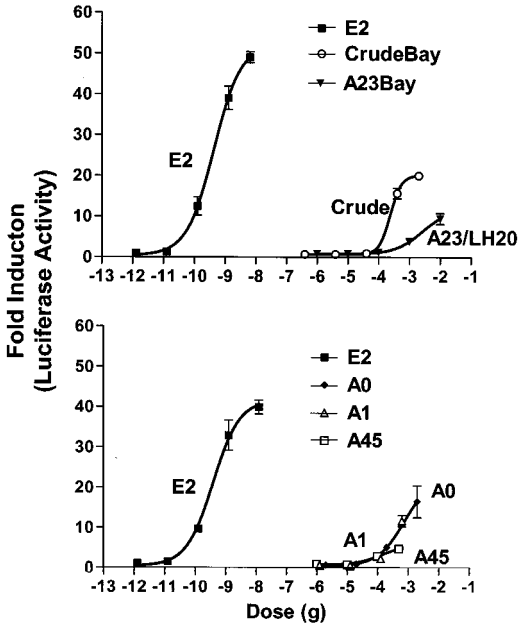

FIGURE 2. Gal4-HEG0-mediated induction of the Gal4-regulated reporter gene (17m5-G-Luc) by E2, crude extract, A23/LH20 fraction (top panel) and A0, A1, and A45 fractions (bottom panel). The data shown are representative of at least three independent experiments. Although the A45 fraction appears to have a very small amount of luciferase activity, this was not repeated in further experiments. The two graphs shown here represent two separate assays where each assay generates a standard E2 curve, against which the fractions and extract are compared.

been shown to be a responsive, reproducible, and efficient means of assessing the in vitro estrogenic activity of chemicals (*41*−*43*) and complex mixtures (*33*, *44*). The maximum luciferase activity ranged from 30- to 70-fold following treatment with 1.4 ng/plate (1 nM) E2, an exceptionally responsive system when compared to other in vitro assays assessing estrogenicity (*7*). The mean EC50 value for E2 was $(4.2 \pm 3.6) \times 10^{-10}$ g/plate [$(5.8 \pm 5.0) \times 10^{-10}$ M, $n = 18$]. The E2 EC50 values are reported in grams per plate to facilitate direct comparison to the APM EC50 values that are expressed in grams of respirable material dosed per plate. The crude fraction responded from 20 to 50% of the maximum E2 response suggesting the presence of partial ER agonists (Figure 2). On the basis of the luciferase activity observed with the four APM fractions, the nonpolar fractions (A1, A0, and A23/LH20) were the most potent possessing E2 equivalence ranging from 0.2 to 1.0 mg of E2/g respirable material and between 5 and 23 pg of E2/m^3 respired. The luciferase responses for the nonpolar fractions were within an order of magnitude of each other, while the A45 polar fraction failed to elicit any luciferase activity. There was only a small difference between A0, A1, and A23/LH20 fractions and the crude extract, consequently, it appeared that the crude extract activity represented the sum of the activity elicited in its fractions. Further support for the observed estrogenic activity in the Bay St. sample was seen upon comparison to a second urban APM sample. This latter sample, collected on Grosvenor St. in downtown Toronto, Ontario, Canada, was sampled and extracted in a manner similar to the Bay St. sample and had a PAH profile similar to that seen with the Bay St. sample.

No significant synergistic or antagonistic interactions were observed following co-treatment with E2 and either the crude extract or A23/LH20 fractions (data not shown). Yet, APM-induced luciferase activity was inhibited by greater than 90% when co-treated with the pure ER antagonist, ICI 164,384 (*37*, *38*). In addition, no induction of luciferase activity was

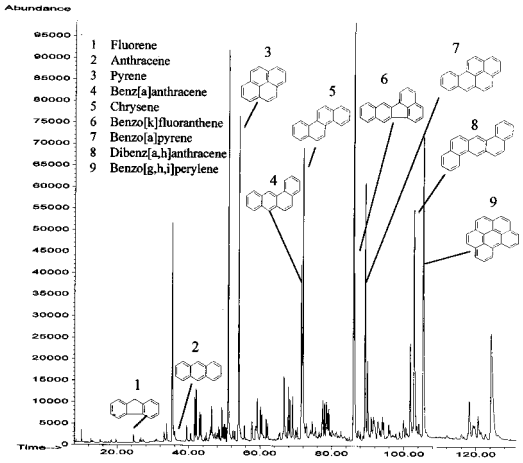

FIGURE 3. Total ion chromatogram of Bay St. air particulate fraction A23/LH20.

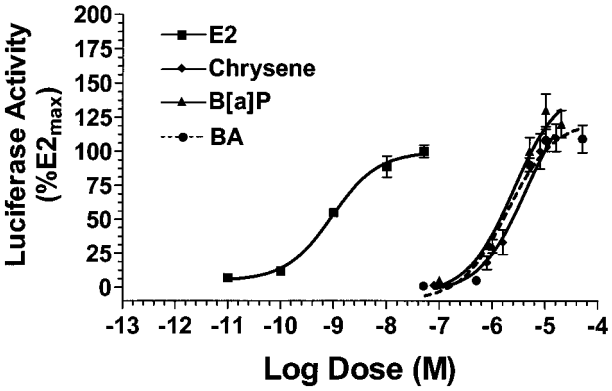

FIGURE 4. Gal4-HEG0-mediated induction of the Gal4-regulated luciferase reporter gene by E2 and 3 PAH, benzo[*a*]pyrene (B[a]P; ▲), chrysene (◆), and benz[*a*]anthracene (BA; ●). Luciferase activity for each PAH is expressed as the percent maximum activity as compared to the E2 maximum. The data shown are representative of at least three independent experiments. The EC50 values for each benzo[*a*]pyrene, chrysene, and benzanthracene ranged from 0.6 to 3 μM, 2 to 7 μM, and 2 to 7 μM, respectively.

observed when the 17m5-G-Luc reporter gene was transfected into the MCF7 cells without the Gal4HEG0 chimeric receptor construct (data not shown). Collectively, these results indicate that the chemicals present in the APM are (i) working through an ER-mediated pathway and (ii) not acting synergistically or antagonistically with E2.

As early as 1953, it was recognized that carcinogenic PAHs possess electronic and steric configurations similar to steroid hormones (*45*). It was subsequently hypothesized that PAHs may elicit their carcinogenic effects by acting at the same sites as steroid hormones and, therefore, interfere with normal steroid function (*46*). Several PAHs including 7- or 12-methylbenzanthracene, 7,12-dimethylbenz[*a*]anthracene, 2-aminophenanthrene, 2,4,7-trinitro-9-fluorenone, and benzo[*a*]pyrene have been shown to induce mammary cancer in female Sprague−Dawley rats following a single treatment (*47*). It is now known that the carcinogenicity of PAHs involves their bioactivation as a result of oxidative metabolism and subsequent adduction to DNA. However, the steroid-like activities of PAHs may also play a significant role in promoting the development of hormone-dependent carcinogenesis.

To test whether PAHs present in the APM (Figure 3) may be contributing to the estrogenic effect of APM, nine PAH (Figure 4) were tested with the E2 bioassay. The EC50 values were 1.3 ± 1.1, 4.0 ± 2.6, and 5.5 ± 2.7 μM for benzo[*a*]-

pyrene, benz[*a*]anthracene, and chrysene, respectively. Other PAHs, including fluorene, anthracene, dibenz[*a*,*h*]anthracene, benzo[*k*]fluoranthene, and benzo[*ghi*]perylene, did not exhibit agonist activity in the same assay (data not shown). Derivation of the estrogen equivalence for the three PAH agonists revealed that the PAHs were approximately 1000−10000 less potent than estrogen in the E2 bioassay.

Other PAHs that show competitive binding with [^{3}H]E2 for the ER include 3,9-dihydroxyanthracene (*48*) and 3,9-dihydroxy-7,12-dimethylbenz[*a*]anthracene (*49*). Another ER ligand binding study specifically examining the usefulness of selected tetrahydrochrysenes as fluorogenic ER ligands identified chrysene and alkyl-substituted chrysene as having binding affinity for the ER (*50*). Although no antiestrogenic activities have been observed using these mammalian gene expression assays, selected PAHs have been shown to antagonize E2-induced responses using gene expression assays in yeast (*51*) and MCF-7 foci development (*52*). These conflicting results may be due to the fact that the estrogenic activity of a chemical is species-, tissue-, cell-, and response-specific (*8, 53*). For example, tamoxifen, a widely used antiestrogen used for the treatment of breast cancer, blocks the activities of estrogen in breast tissue but behaves like an estrogen in the uterus. Moreover, epidemiological studies suggest that the weak estrogenic activity of tamoxifen in the uterus is believed to contribute to the development of endometriosis (*54, 55*). Collectively, these studies convincingly demonstrate that PAHs or their metabolites are capable of interacting in vitro with the ER and inducing an ER-mediated response. However, further studies are required to determine if PAHs are capable of eliciting in vivo ER-mediated responses. Recent in vivo studies conducted using ovariectimized DBA mice indicate that a coal tar distillate containing a complex mixture of PAHs was capable of inducing uterine wet weight gain as well as inducing reporter gene activity in a recombinant receptor/reporter gene assay (*56*).

PAHs, as well as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds, have also been shown to elicit antiestrogenic activity that is mediated by the AhR (*2, 57, 58*). Three mechanisms have been proposed to explain the antiestrogenic activities of AhR ligands (*3*): (i) induction of monooxygenases that metabolize estrogen, (ii) down-regulation of the ER, and (iii) inhibition of estrogen-induced genes. AhR-mediated antiestrogenic activities, like those of estrogen, are species-, tissue-, cell-, and response-specific. The antiestrogenic effects of TCDD and related compounds including PAHs may also be dependent on the expression of the high ligand affinity binding form of the AhR (*59*−*63*). Consequently, it may be inappropriate to assume that the AhR-mediated antiestrogenic activities of PAHs will negate the estrogenic effects of PAHs.

Detection and Verification of TCDD-like Activity. A TCDD-inducible reporter gene, pGudLuc1.1, was transiently transfected into Hepa 1c1c7 cells, to ascertain the presence of AhR ligands in the APM extract and its associated fractions. The maximum luciferase activity induced by 1.6×10^{-8} g of TCDD/dish (1×10^{-8} M) ranged from 20- to 40-fold. The mean TCDD EC50 value was $(2.2 \pm 2) \times 10^{-10}$ g/plate [$(1.4 \pm 1.3) \times 10^{-10}$ M, $n = 8$].

Significant luciferase-reporter gene induction was observed with the crude APM extract in the AhR bioassay when compared to the TCDD standard curve. In fact, the extract exhibits full efficacy as seen by the maximal luciferase activity achieved (Figure 5). From the 6 orders of magnitude difference in potency between TCDD and the crude extract (between 0.2×10^{-3} and 5.0×10^{-3} g/plate), TCDD equivalence (TEQ) can be derived. The equivalencies were in the range 0.2−15 mg of TCDD/g respirable material or 5−370 pg of TCDD/m^3 respired air. Typical urban background levels of polychlorinated dibenzo-*p*-dioxins (PCDD) in Hamilton, Ontario, a nearby industrial urban center, range from 0.013 to 0.19 pg of TCDD TEQ/m^3 (Ontario Ministry of Environment and Energy, unpublished data). Thus, background levels of PCDD cannot account for much, if any, of the response observed in these extracts. The potency of the nonpolar fractions, including A1 and A23/LH20, was noticeably stronger than the alipahatic nonpolar fraction, A0, inducing responses 2 orders of magnitude greater than the A0 fraction. This in not surprising in that it is unlikely that aliphatic compounds would be good ligands for the AhR. However, there were differences in potency between A1, A23/LH20, and A45 and the crude extract, where the sum of the fractions exhibited greater activity than the crude extract. This discrepancy may be due to antagonist interactions within the APM that are eliminated or minimized following fractionation of the crude mixture.

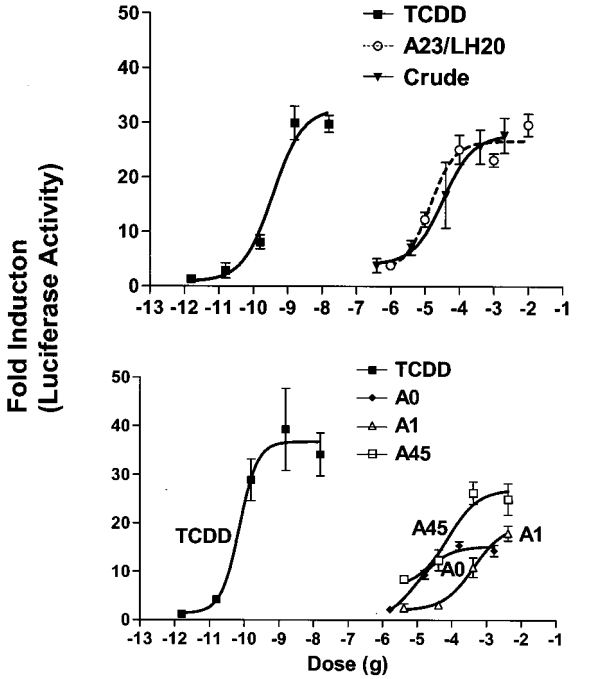

FIGURE 5. AhR-mediated induction of the DRE-regulated reporter gene (pGudLuc1.1) by TCDD, crude extract and A23/LH20 (top panel) and A0, A1, and A45 (bottom panel). The data shown are representative of at least three independent experiments. The two graphs shown here represent two separate assays where each assay generates a standard TCDD curve to which the fractions and extract are compared.

The CYP1A1 promoter from position −1309 to −819 (*35*) regulates expression of the pGudLuc1.1 reporter gene. Deletion analysis studies have identified five putative DREs in this region that are required but, in isolation, are not sufficient to mediate AhR-inducible gene expression. Consequently, this suggests that other mechanisms of induction may be possible especially when examining the activity of a complex mixture such as APM. To confirm that the TCDD-like activity observed in APM crude extracts and its fractions were AhR-mediated, the following two studies were conducted.

The role of the AhR and ARNT proteins in crude extract and A23/LH20 induction of pGudLuc1.1 was confirmed using two transiently transfected Hepa 1c1c7 mutant cell lines. These cell lines possess mutations within the structural genes encoding AhR and ARNT and, therefore, are nonresponsive to TCDD (*64*). Correspondingly, when these AhR-deficient and ARNT-deplete cells were dosed with the crude extract or A23/LH20, no luciferase activity was observed (data not shown).

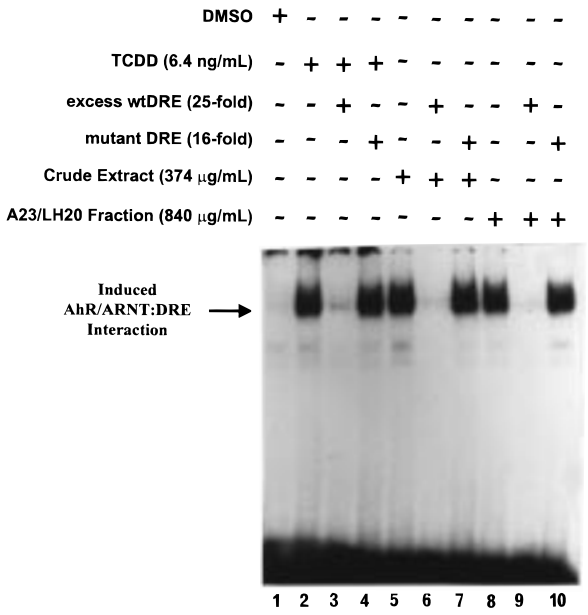

FIGURE 6. TCDD-, crude extract-, and A23/LH20 fraction-induced AhR/ARNT:DRE complexes (as indicated by arrow). The identity and specificity of the AhR/ARNT:DRE complex was confirmed by competing with a 25-fold excess of wt DRE (lanes 3, 6, and 9) and point-mutated DRE (lanes 4, 7, and 10).

Ligand-dependent transformation of the AhR is the initial step in AhR-mediated gene expression. In vitro gel retardation assays can be used to determine the ability of a pure compound or complex mixture to induce the formation of AhR/ARNT:DRE complexes. The strong induced band shown in Figure 6 represents the formation of an APM-inducible AhR−ARNT/DRE complex. These results are consistent with previous studies investigating the ability of complex mixtures to induce the formation of these complexes (*30*, *65*, *66*). The specificity of AhR/ARNT:DRE binding was demonstrated by the ability of (i) excess (25-fold) unlabeled DRE to outcompete the [γ^{32}P]-labeled DRE (lanes 3, 6, and 9) and (ii) the inability of the point-mutated DRE to compete with the [γ^{32}P]-labeled DRE (lanes 4, 7, and 10), thereby maintaining the strong AhR/ARNT:DRE induced band.

Further evidence of urban APM containing TCDD-like components in the crude extract and A23/LH20 fraction was found in the results in the second sampling site, located on Grosvenor St., which has a PAH profile similar to that seen in the Bay St. chemical analysis. Both the response level and potency of the Grosvenor St. sample were comparable to that seen with the Bay St. sample. Furthermore, the conformational studies done with the AhR-deficient and ARNT-deplete mutant cells line (data not shown) and with gel retardation assays corroborated the observed luciferase activity.

To determine the contribution that PAHs made to the observed TCDD-like activity, the same nine PAHs assayed for in vitro estrogenic activity were examined for their ability to induce AhR-mediated luciferase activity in the mouse hepatoma cells transiently transfected with pGudLuc1.1. Six of the nine PAHs tested for AhR-mediated gene expression were found to be agonists (Figure 7). The EC50 values for the PAHs ranged from 0.3 nM to 7 μM with TCDD toxic equivalent factors (TEFs) extending from 0.05 to 0.000 01 (Table 1). Dibenz[*a*,*h*]anthracene and benzo[*k*]fluoranthene were found to be the most potent followed by chrysene > anthracene > benz[*a*]pyrene = benz[*a*]anthracene (Table 1). This rank potency deviates from three recent studies using the ethoxyresorufin-*O*-deethylase (EROD) bioassay as the AhR-mediated endpoint. Two studies examined the

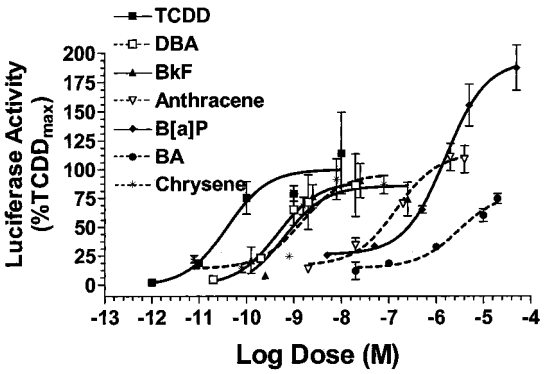

FIGURE 7. AhR-mediated induction of the DRE-regulated reporter gene (pGudLuc1.1) by TCDD and 6 PAH, benzo[*k*]fluoranthene (BkF; ▲), dibenzanthracene (DBA; □), chrysene (*), anthracene (▽), benzo[*a*]pyrene (B[a]P; ◆),and benz[*a*]anthracene (BA; ●). Luciferase activity for each PAH is expressed as the percent maximum activity as compared to the TCDD maximum. The data shown are representative of at least three independent experiments. The EC50 values for each BkF, DBA, Chrysene, anthracene, B[a]P and BA ranged from 0.6 to 4 nM, 0.3 to 3 nM, 0.6 to 2 nM, 42 to 150 nM, 0.5 to 2 μM, and 0.6 to 7 μM, respectively.

TABLE 1. Relative Potencies (RP)[a] for Selected PAH in MCF-7 and WT Cells Using 17β-Estradiol (E2) and 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), Respectively, as the Standard Compounds to Which PAHs Were compared

	MCF-7:ER		WT:AhR	
	RP	% max	RP	% max
standard	1.0	100	1.0	
fluorene	nd		nd	
anthracene	nd		0.000 1	89
pyrene	nd		nd	
chrysene	0.000 5	94	0.01	79
benz[*a*]anthracene	0.000 1	129	0.000 01	104
benzo[*a*]pyrene	0.001	158	0.000 01	208
benzo[*k*]fluoranthene	nd		0.05	53
benzo[*ghi*]perylene	nd		nd	
dibenz[*a*,*h*]anthracene	nd		0.05	107

[a] Equivalency factors were determined using the following formula: relative potency = standard (E2 or TCDD) EC50 PAH EC50. All values are derived from assays repeated at least three times. nd = not detected.

ability of PAHs to induce EROD in vitro in a rat hepatoma cell line, H4IIE, (*20*) or a fish liver cell line, RTL-W1 (*19*), while the third examined PAH EROD induction in chick embryos (*67*). All of these studies require that the PAHs transform the cytosolic AhR, heterodimerize with ARNT, and bind DRE to (i) upregulate the P4501A1 enzyme in the Willet et al. (*20*), Bols et al. (*19*), and Brunstrom et al. (*67*) studies or (ii) upregulate the luciferase enzyme in this study. In all of the these studies, benzo[*k*]fluoranthene was found to be the most potent PAH. The greatest discrepancy was with TEFs derived for chrysene, a PAH that is prevalent in many environmental matrices. Previous studies have demonstrated that TCDD and PCBs behave differently in different species (*68*−*71*) suggesting that rank potencies of PAH is also species dependent.

All PAHs exhibited high luciferase activity with maximum activities comparable to or greater than the response induced by 10 nM TCDD (32 ± 16-fold induction). However, despite the fact that benzo[*a*]pyrene was not as potent an AhR inducer as TCDD, it was consistently found to induce a response significantly greater than that seen with TCDD (Figure 7). This enhanced response of benzo[*a*]pyrene was not reported in H4IIE or RTL-W1 cells and may, therefore, be an additional

TABLE 2. List of Selected PAHs and Their Concentrations Found in a 16 Filter Composite Urban Air PM_{10} Sample from Bay St.

compd	concentration pg/m^3	concentration ug/g
fluorene	6.7	0.29
phenanthrene	113	4.9
anthracene	8.4	0.36
1-methylphenanthrene	15	0.63
anthraquinone	48	2.1
fluoranthene	186	8.0
pyrene	160	6.9
benzo[*a*]fluorene	17	0.75
benzo[*b*]fluorene	14	0.6
benz[*a*]anthracene	65	2.8
chrysene	153	6.6
benzo[*b*+*j*]fluoranthene	312	13
benzo[*k*]fluoranthene	89	3.8
benzo[*e*]pyrene	175	7.5
benzo[*a*]pyrene	58	2.5
perylene	18	0.77
indeno[1,2,3-*cd*]pyrene	248	15.0
dibenz[*ac*+*ah*]anthracene	35	1.5
benzo[*ghi*]perylene	148	8.93

species-specific effect or due to the presence of a novel metabolite or impurity.

Analysis of PAHs individually or in a complex matrix such as APM is complicated. Although they can be chemically detected through gas chromatography (Figure 3) and quantified with mass spectrometry (Table 2), analyzing the net biological activities is difficult. This may largely be ascribed to the biological reactivity of PAHs with AhR-mediated P450 enzymes. The most widely studied is the oxidation of hydrocarbons by P4501A1, an enzyme to which many PAHs act as autoinducers. Oxidation may be occurring in either cell line considering that P4501A1 activity is inducible in both MCF-7 (*72*, *73*) and Hepa 1c1c7 (64). However, other AhR-regulated P450 enzymes, such as P4501A2 (*73*, *74*) or P4501B1 (*75*), may also contribute to the generation of more potent receptor ligands. In addition, PAHs can be chemically modified in the atmosphere upon exposure to ultraviolet light. This photomodification can produce PAH-diol and quinone products (*76*, *77*) that can be more toxic to fish (*78*, *79*) and plants (*80*, *81*) than the parent PAH.

The metabolism and/or photooxidation of PAHs may also be involved in the formation of estrogenic compounds. The primary products of P450 oxidation are compounds that have an addition of hydroxyl groups at the lateral ends of the polyaromatic rings. This results in PAH products that may be structurally similar to E2 where the two hydroxylated rings of E2, A, and D contribute to (i) binding to the receptor and (ii) biological activity (*9*, *10*). In addition, upon examination of the E2 and TCDD equivalency factors derived for the reactive PAH (Table 1), the occurrence of three estrogenic compounds, benzo[*a*]pyrene, benz[*a*]anthracene, and chyrsene, also inducing AhR-mediated activity becomes apparent. Clearly, this further complicates assessing the role of PAHs in potentially causing receptor-mediated adverse effects in human and wildlife.

In conclusion, both estrogenic and TCDD-like activities are present in air particulate material. The health consequences of this observation is unknown, yet, the presence of these activities may contribute to and exacerbate adverse health effect evoked by APM. Lung tissue contains both ER proteins (*82*, *83*) and AhR (*84*, *85*). Consequently, these receptors may act as mediators to induce the production of more potent second messengers such as the arachidonic acid metabolites, cis-epoxyeicosatrienoic acids, and cis, trans-conjugated hydroxyeicosatetraenoic acids (*86*). In addition, there may be additional mediators of PAH activity such as ER β or other orphan receptors (*87*). However, further research looking at PACs in combination is required to determine if chemical composition is an important factor in the adverse effects elicited by air particulate material.

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