Jarvis, Ian WH; Bergvall, Christoffer; Bottai, Matteo;Westerholm, Roger; Stenius, Ulla; Dreij, Kristian.

Persistent activation of DNA damage signaling in response to complex mixtures of PAHs in air particulate matter.Toxicol Appl Pharmacol. 2013 Feb 1;266(3):408-18.

DOI: 10.1016/j.taap.2012.11.026

Toxicology and Applied Pharmacology 266 (2013) 408–418

Contents lists available at SciVerse ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

Persistent activation of DNA damage signaling in response to complex mixtures of PAHs in air particulate matter

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ARTICLE INFO

Article history:
Received 28 September 2012
Revised 21 November 2012
Accepted 25 November 2012
Available online 5 December 2012

Keywords:
PAHs
Air particulate matter
Complex mixtures
Risk assessment
Benzo[*a*]pyrene
Dibenzo[*a,l*]pyrene

ABSTRACT

Complex mixtures of polycyclic aromatic hydrocarbons (PAHs) are present in air particulate matter (PM) and have been associated with many adverse human health effects including cancer and respiratory disease. However, due to their complexity, the risk of exposure to mixtures is difficult to estimate. In the present study the effects of binary mixtures of benzo[*a*]pyrene (BP) and dibenzo[*a,l*]pyrene (DBP) and complex mixtures of PAHs in urban air PM extracts on DNA damage signaling was investigated. Applying a statistical model to the data we observed a more than additive response for binary mixtures of BP and DBP on activation of DNA damage signaling. Persistent activation of checkpoint kinase 1 (Chk1) was observed at significantly lower BP equivalent concentrations in air PM extracts than BP alone. Activation of DNA damage signaling was also more persistent in air PM fractions containing PAHs with more than four aromatic rings suggesting larger PAHs contribute a greater risk to human health. Altogether our data suggests that human health risk assessment based on additivity such as toxicity equivalency factor scales may significantly underestimate the risk of exposure to complex mixtures of PAHs. The data confirms our previous findings with PAH-contaminated soil (Niziolek-Kierecka et al., 2012) and suggests a possible role for Chk1 Ser317 phosphorylation as a biological marker for future analyses of complex mixtures of PAHs.

Introduction

Particulate matter (PM) in urban air is comprised of a complex mixture of chemicals. One group of chemicals associated with air PM are the polycyclic aromatic hydrocarbons (PAHs), ubiquitous environmental pollutants comprised of two or more fused aromatic rings that are formed and emitted into the atmosphere as a result of incomplete pyrolytic processes. Despite their structural similarities, PAHs vary greatly in their carcinogenic potency, with several classified as possible or probable human carcinogens (IARC, 2010). In order to exert their carcinogenic activity, PAHs require activation by the cytochrome P450 (CYP) enzymes, ultimately producing metabolites that can react with DNA to form mutagenic DNA adducts (IARC, 2010). Benzo[*a*]pyrene (BP) and dibenzo[*a,l*]pyrene (DBP) are two of the most mutagenic and carcinogenic PAHs identified to date. DBP is several orders of magnitude more potent in rodents than BP, most likely due to the formation of more persistent DNA adducts that can escape cellular repair processes (Dreij et al., 2005; Luch, 2009).

To date, few studies have investigated the effects of interactions between mixtures of PAHs either in binary or environmental samples, such as extracts from soil and air PM. Data from us as well as others clearly show interactions between PAHs leading to unexpected effects (Mattsson et al., 2009; Niziolek-Kierecka et al., 2012; Staal et al., 2007; Tarantini et al., 2009, 2011). We recently showed that exposing HepG2 cells to PAH-containing soil extracts results in prolonged activation of DNA damage signaling consistent with persistent DNA damage, whereas BP-induced DNA damage was rapidly repaired, suggesting a strong synergistic response between mixtures of PAHs (Mattsson et al., 2009; Niziolek-Kierecka et al., 2012). In addition, other studies have shown synergistic effects of interactions of PAHs on cellular DNA adduct levels in HepG2 cells exposed to binary or complex mixtures (Staal et al., 2007; Tarantini et al., 2009, 2011). Conversely, other studies have demonstrated antagonistic effects on the carcinogenic potency of PAHs in complex mixtures attributed to inhibitory effects on the metabolic activation by the CYP enzymes (Courter et al., 2008; Mahadevan et al., 2007; Marston et al., 2001).

Importantly, these results have serious implications for the risk assessment of mixtures of PAHs. The current approach which is used by the Swedish and US EPA and the WHO is based on the use of toxic equivalency factors (TEFs) or relative potency factors (RPFs) which

Abbreviations: PAH, polycyclic aromatic hydrocarbon; PM, particulate matter; BP, benzo[*a*]pyrene; DBP, dibenzo[*a,l*]pyrene; Chk1, checkpoint kinase 1; CYP, cytochrome P450; TEF, toxic equivalency factor; RPF, relative potency factor; SPE, solid phase extraction; DDR, DNA damage response; MAF, mixture assessment factor; TEQ, toxic equivalent quota; LOQ, limit of quantification.

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http://dx.doi.org/10.1016/j.taap.2012.11.026

express the carcinogenic potential relative to that of BP (Boström et al., 2002). This means that the TEF scale assumes additivity of cancer potency and thus does not take combination effects as a result of intra-mixture interactions into account. Furthermore, the TEF values that are quoted for different PAHs vary greatly depending on the source i.e. in vitro vs. in vivo data, different cancers and endpoints (California EPA, 2004; Collins et al., 1998; Larsen and Larsen, 1998; Muller, 1997; Nisbet and LaGoy, 1992). In addition to the results mentioned above which show that non-additive interactions occur between PAHs, animal studies have shown that BP as an indicator may markedly underestimate the cancer potency of PAH mixtures (Gaylor et al., 2000; Siddens et al., 2012). Together, this clearly shows that using the TEF approach could consequently lead to an underestimation of the risk.

In the present study we examined the effects of binary mixtures of BP and DBP and complex mixtures of PAHs in urban air PM extracts on activation of DNA damage signaling. We observed a more than additive response for binary mixtures of BP and DBP on activation of DNA damage signaling. Exposing cells to air PM extracts caused a persistent activation of DNA damage signaling at concentrations at least 100-fold lower than those for BP alone, which is not in accordance with results applying TEF scales. Furthermore, we demonstrate that the composition of PAHs contributed significantly to air PM extract potency, with stronger activation of DNA damage signaling and CYP1 induction observed in air PM fractions containing PAHs with more than 4 aromatic rings. Taken together, our results demonstrate that interactions between PAHs occur and that the use of BP as a marker in risk assessment seriously underestimates the risks to human health of exposure to complex mixtures of PAHs.

Materials and methods

Caution. PAHs are carcinogenic and experimental handling must be carried out under special safety conditions such as those outlined in the NCI guidelines.

Reagents and antibodies. Unless otherwise stated all chemicals, including BP, were of analytical grade and obtained from Sigma Aldrich. (Stockholm, Sweden). Hexane, toluene and methanol (HPLC grade) were obtained from Rathburn Ltd. (Walkerburn, UK). DBP was purchased from AccuStandard Inc. (New Haven, CT, USA). Detailed information on manufacturer and purity of the standards used for PAH analysis have been published previously (Sadiktsis et al., 2012). Gibco (Invitrogen, Paisley, UK) supplied all cell culture reagents. Electrophoresis reagents were obtained from Bio-Rad (Hercules, CA, USA). Cell Signaling Technology (Beverly, MA, USA) provided the following antibodies: Chk1 phosphorylated at Ser317, H2AX phosphorylated at Ser139 and p53 phosphorylated at Ser15. Antibodies against Cdk2 (M2) and secondary anti-rabbit and anti-mouse antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Air sampling and solvent extraction. Air PM was collected on a Teflon coated glass fiber filter (Ø149 mm, Pallflex Inc., Putnam, CT, USA) at the rooftop of the Arrhenius laboratory building situated on the main campus of Stockholm University. A total of 5205.768 m^3 of air were drawn through the filter with an average flow rate of 509 l min^{-1}. After sampling the filter was folded, wrapped in aluminum foil and stored in a freezer. The filter sample was extracted with pressurized fluid extraction using an ASE 200 accelerated solvent extraction system (Dionex Corporation, Sunnyvale, CA, USA) following a procedure developed and validated for analysis of PAHs in air PM using SRM 1649a urban dust (National Institute of Standards and Technology, Gaithersburg, MA, USA) (Bergvall and Westerholm, 2008). Toluene was used as an extraction solvent at 200 °C and 3000 psi for five consecutive 30 min static extraction cycles. An unsampled filter was extracted in the same manner as the filter sample to serve as a method blank.

Preparation and fractionation of the air raw extract. Two aliquots were removed from the crude air PM extract, gently evaporated to dryness under a stream of nitrogen gas and then reconstituted with dimethyl sulfoxide (DMSO). An aliquot of the crude extract was evaporated to approximately 0.5 ml under nitrogen gas and cleaned-up using a solid phase extraction (SPE) protocol with a silica cartridge as described in detail elsewhere (Bergvall and Westerholm, 2006; Christensen et al., 2005). The SPE eluate was reduced to dryness using nitrogen gas and re-dissolved with 130 μl of hexane. Fractionation of the extract was achieved using back flush high performance liquid chromatography (HPLC) on a nitrophenylpropylsilica column (4.0 mm i.d. × 125.0 mm, 5 μm particle-size) (Phenomenex, Torrance, CA, USA) with a hexane mobile phase. A Varian 9001 Solvent Delivery System (Varian Inc., Palo Alto, CA, USA) was used to deliver a mobile phase flow rate of 1.0 ml min^{-1}. PAH detection was enabled using a Varian 9050 Variable Wavelength UV−VIS detector (Varian Inc.) monitoring the HPLC column effluent at 254 nm. The flow direction of the HPLC column was reversed using an air actuated four port switching valve (Valco Instruments Inc., Houston, TX, USA). A 50 μl aliquot of the extract was loaded into the HPLC using a Rheodyne model 7125 syringe loading injector (Rheodyne Inc., Cotati, CA, USA) equipped with a 100 μl loop and a model 710 100 μl syringe (Hamilton, Bonaduz, Switzerland). During the HPLC run the flow direction of the column was reversed prior to elution of BP. The eluate was collected before and after the back flush yielding two fractions called F1 and F2, respectively. An aliquot of respective fraction was taken for PAH analysis as described in more detail in the next section. The fractions were then gently evaporated to dryness under nitrogen gas and reconstituted with DMSO. In the fraction where BP was present at blank concentrations, reconstitution was made to yield a similar proportion of the original raw extract per μl of DMSO as the fraction containing BP.

PAH analysis. Aliquots of crude and cleaned-up fractionated extract were fortified with internal standard solutions. The aliquots of the crude extract were cleaned-up using silica SPE cartridges in the same manner as described in the previous section. PAH analysis was carried out on a hyphenated HPLC–gas chromatography/mass spectrometry (HPLC–GC/MS) system. Detailed descriptions on the system set-up and the method used have been given elsewhere (Bergvall and Westerholm, 2006; Christensen et al., 2005; Sadiktsis et al., 2012) and will only be described in brief. The SPE extracts were further cleaned-up on the HPLC system using back flush on the same type of column as described in the previous section and with a mobile phase of hexane with 0.1% dodecane (v/v). The PAH enriched fraction from the HPLC was transferred to the GC where PAH separation was performed. Mass selective detection was carried out using a quadrupole mass spectrometer.

Cell culture and exposure. Human hepatocellular carcinoma cells (HepG2) were obtained from the American Type Culture Collection (Rockville, MD, USA). These cells were ideally suited to this study because of their metabolic competence and ability to activate PAHs and other mutagens (Knasmuller et al., 1998). Cells were cultured in minimal essential medium supplemented with 10% fetal bovine serum, sodium pyruvate (1 mM), non-essential amino acids (0.1 mM), penicillin (100 units/ml) and streptomycin (0.1 mg/ml), and maintained at 37 °C in 5% CO_2. For cell proliferation experiments, cells were seeded at 1×10^4 cells/ml in 96-well plates and cultured for 24 h, whereas for Western blot, qRT-PCR and Comet assay experiments, cells were seeded at 3×10^5 cells/ml in 6-well plates and cultured for 72 h. Cells were subsequently exposed to BP and DBP, solvent control (0.1% DMSO) or air PM extracts for up to 48 h. Cells were exposed

to air PM extracts using a concentration equivalent to a set concentration of BP.

Cell viability. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay as described previously (Mosmann, 1983). Briefly, HepG2 cells were exposed to PAHs for 48 h followed by incubation in serum- and phenol-red free medium containing 0.5 mg/ml MTT for 4 h, then washed and solubilized in DMSO. Optical density was measured at a wavelength of 590 nm. Data are expressed as percent of control.

Western blotting. Cells were washed with ice-cold PBS and scraped into IPB-7 buffer (20 mM triethanolamine-HCl pH 7.8, 0.7 M NaCl, 0.5% Igepal CA-630, 0.2% sodium deoxycholate) with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin, 1 mg/ml pepstatin, 1 mM sodium fluoride, 1 mM sodium orthovanadate, 0.1 mg/ml trypsin inhibitor and 1 mg/ml aprotinin). Protein content was measured and subjected to standard SDS–PAGE. Separated proteins were transferred to a PVDF membrane (Bio-Rad, Hercules, CA, USA) by wet electro-blotting. Non-specific antibody binding was reduced by incubating membranes in 5% non-fat dry milk. Signals were detected using enhanced chemiluminescence (Amersham GE Healthcare Bio-Sciences AB, Uppsala, Sweden). To confirm the signals were in the linear range of the detection system, blots were performed for all antibodies with increasing concentrations of protein lysate (0–30 μg) (data not shown). Experiments were performed at least in triplicate and analyzed separately. Densitometric analysis was performed using ImageJ software version 1.45 s (National Institute of Health, USA).

RNA purification and real-time RT-PCR. Total RNA was prepared using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and 1 μg used to generate cDNA with the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) according to protocol. Subsequently, quantification of gene expression was performed in duplicates using Maxima™ SYBR® Green qPCR Master Mix (Fermentas, St. Leon-Rot, Germany) with detection on an Applied Biosystems 7500 real-time PCR System (Applied Biosystems, Foster City, CA, USA). The reaction cycles used were 95 °C for 2 min, and then 40 cycles at 95 °C for 15 s and 60 °C for 1 min followed by melt curve analysis. Primer sequences were as follows: CYP1A1 forward CACCATCCCCCACAGCAC and reverse ACAAAGACACAACGCCCCTT, CYP1B1 forward AGTGCAGGCA GAATTGGATCA and reverse AGGACATAGGGCAGGTTGGG, and GAPDH forward CGAGATCCCTCCAAAATCAA and reverse TTCACACCCATGA CGAACAT. Relative gene expression quantification was based on the comparative threshold cycle method ($2^{-\Delta\Delta Ct}$).

Comet assay. The alkaline version of the comet assay was performed as described previously (Karlsson et al., 2005). In brief, slides pre-coated with agarose (0.3%) were covered with exposed cells resuspended in low melting point agarose (0.75% w/v). Following incubation in cold lysis buffer (1% Triton X-100, 2.5 M NaCl, 10 mM Tris, and 0.1 M EDTA, pH 10) for 1 h on ice, the slides were incubated in cold alkaline solution (0.3 M NaOH and 1 mM EDTA, pH > 13) for 40 min on ice. The comets were separated by electrophoresis using the alkaline buffer at 29 V (1.15 V/cm) for 30 min. Slides were neutralized in 0.4 M Tris–HCl (pH 7.5), dried overnight and fixed in methanol for 5 min. After staining with ethidium bromide, at least 50 comets were scored per treatment performed in triplicates using a Leica DMLB fluorescent microscope and Comet Assay 3 (Perceptive Instruments Ltd, Haverhill, UK).

Statistical analysis. All data presented are means ± SE. One-way ANOVA with Bonferroni's *t*-test correction was used to determine statistical significance in cell proliferation, Western blot, qRT-PCR and Comet assay experiments (significance rated as $p<0.05$). For the interaction analysis we estimated a generalized linear regression model with a log link. The potential within-batch correlation of the values for the outcome variable was taken into account by applying generalized estimating equations (Zeger and Liang, 1986). This method provides robust estimates for the standard errors of the regression coefficients and does not require making any assumptions about the distribution of the regression residual. We assumed an exchangeable working correlation structure. The models were estimated separately for each protein. The level of protein phosphorylation was the dependent variable. The independent variables for all the three proteins were BP, DBP, a natural cubic spline variable for BP (knots at 1, 2, and 3), and a natural cubic spline variable for DBP (knots at 0.5, 1, and 1.5). The interaction between BP and DBP and their respective spline variables was tested with the Wald test. Number and placement of the knots of the splines were chosen on a 0.5-point grid to maximize the goodness of fit.

Results

We have previously demonstrated that exposure of cells to PAH-contaminated soil extracts results in persistent DNA damage and a prolonged activation of DNA damage signaling that could not be attributed to nor explained by the concentration of BP in the samples (Mattsson et al., 2009; Niziolek-Kierecka et al., 2012). In these studies several key markers of DNA damage and signaling including phosphorylation of Chk1, Chk2, H2AX and p53 were investigated. In the present study the effects of BP and DBP in binary mixtures on DNA damage and DNA damage response (DDR) have been investigated, as well as PAH-containing urban air PM extracts using a dose response approach. We have also applied a statistical model to investigate potential effects due to interactions between BP and DBP in binary mixtures.

Binary mixtures of BP and DBP induce a stronger activation of DNA damage signaling than BP or DBP alone

To investigate potential interactions we studied the effect of single PAHs and binary mixtures on DNA damage signaling. Binary mixtures were prepared using a dose response approach to include increasing concentrations of BP with a constant DBP and vice versa. A single exposure time of 48 h was chosen based on previous results showing persistent DNA damage and prolonged DDR at this time following exposure to complex mixtures (Niziolek-Kierecka et al., 2012).

We have previously shown that the markers used in this study (Chk1, p53 and H2AX) are key signal transducers of DNA damage signaling in response to PAHs (Mattsson et al., 2009; Niziolek-Kierecka et al., 2012; Pääjärvi et al., 2008). The results of this study showed that both BP and DBP were significantly associated with increasing phosphorylation levels of Chk1, p53 and H2AX (Figs. 1 and 2). Statistically significant increases in Chk1 Ser317 (pChk1), p53 Ser15 (pp53) and H2AX Ser139 (γH2AX) phosphorylation from baseline levels were observed in cells exposed to 1000 nM ($p=0.003$), 1000 nM ($p=0.0029$) and 3000 nM ($p<0.001$) BP respectively, whereas for cells exposed to binary mixtures of BP and DBP, statistically significant increases were at 300 nM ($p=0.046$), 3000 nM ($p<0.001$) and 3000 nM ($p<0.001$) respectively (Fig. 1). For cells exposed to DBP alone, statistically significant increases from baseline levels were observed at 10 nM ($p=0.001$) for pChk1, 30 nM ($p<0.001$) for pp53 and 100 nM ($p<0.001$) for γH2AX, whereas for cells exposed to binary mixtures of DBP and BP, statistically significant increases were at 10 nM ($p=0.017$), 30 nM ($p=0.030$) and 30 nM ($p=0.010$) respectively (Fig. 2). Significant effects on cell viability was only observed in cells exposed to concentrations of BP and DBP greater than 300 and 30 nM respectively (Supplementary Figs. 1, A and B).

To relate the activation of DNA damage signaling to the levels of DNA damage, these were measured after 6 and 48 h by Comet assay. Comparable levels of damage were observed in cells exposed to BP or DBP alone (Fig. 3), and although both produced higher levels

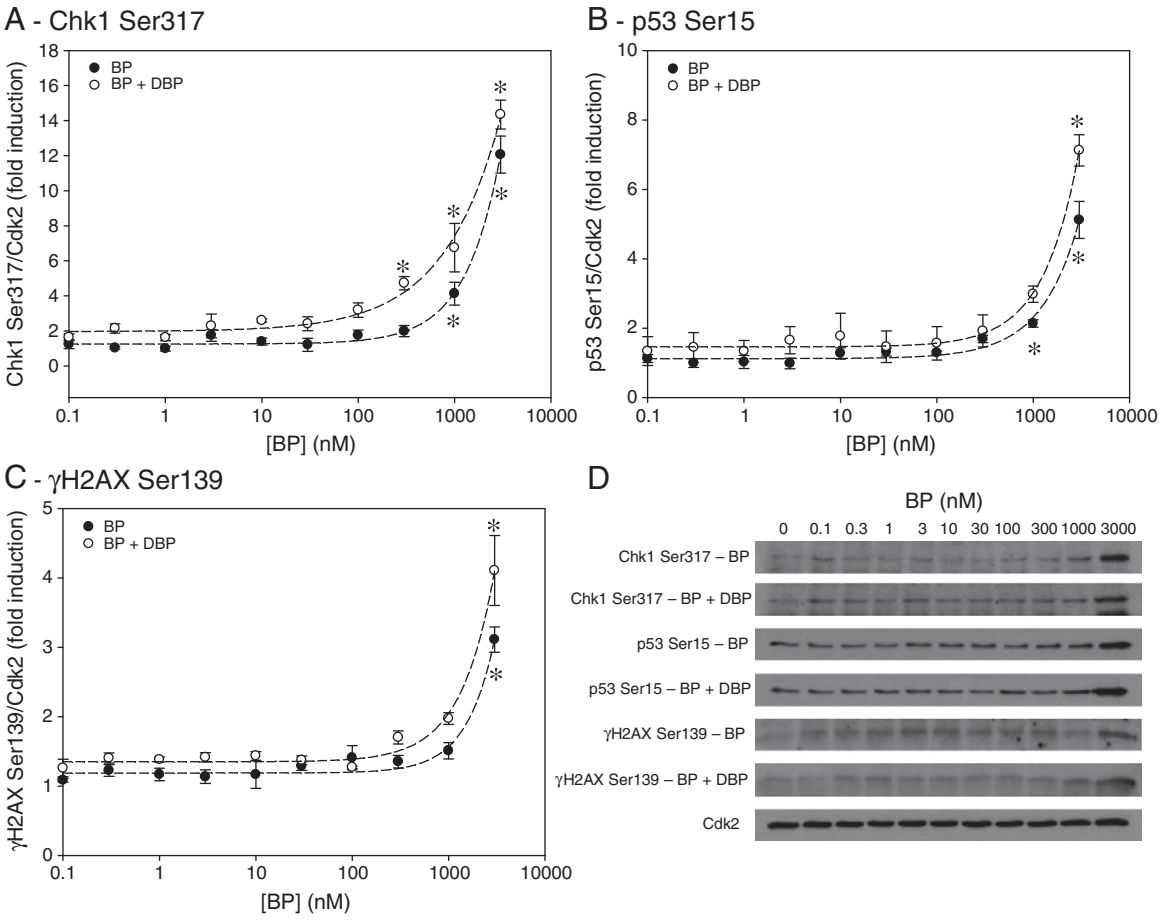

Fig. 1. Binary mixtures of BP and DBP are more potent at activation of DNA damage signaling than BP alone. Phosphorylation of Chk1 at Ser317 (A), p53 at Ser15 (B) and H2AX at Ser139 (C) was assessed by Western blot after 48 h exposure to 0–3000 nM BP alone (●) or with 1 nM DBP (○). Densitometric analysis for each protein is shown in (D). Cdk2 was used as a loading control. Experiments were performed in triplicate and data points represent means ± SE. Where not shown, error lies within the data points. Curves were fitted using the logistic 4 parameter equation in Sigmaplot 12. *$p<0.05$ as compared with control levels by one way ANOVA.

of damage than in the control, this was not statistically significant. Statistically significant levels of DNA damage were observed in cells exposed to binary mixtures at 6 and 48 h with higher levels at 48 h (Fig. 3). This increase could either be explained by formation of persistent DNA damage or by a continuous release of reactive oxygen species causing DNA damage. Together the results show that binary mixtures of BP and DBP induce a stronger DDR in HepG2 cells than BP or DBP alone. Furthermore, and in agreement with our previous data (Niziolek-Kierecka et al., 2012), this data identifies pChk1 as a potential marker for prolonged DNA damage signaling in response to PAHs.

In order to investigate if interactions between BP and DBP would lead to non-additive effects on DDR we applied a statistical model to the dose–response data shown in Figs. 1 and 2 as described in Materials and methods. The combination of a robust statistical model and all four data sets allowed for predicting level of protein phosphorylation at different concentrations of binary exposure. A statistically significant ($p<0.001$) interaction was observed between BP and DBP in binary mixtures compared to the individual chemicals on the phosphorylation of Chk1, with the effects of the binary mixture producing a more than an additive effect on the level of phosphorylation (Fig. 4). No statistically significant interactions (i.e. dose addition) were observed for the activation of pp53 ($p=0.9216$) or γH2AX ($p=0.1371$).

Analysis of PAH content in Stockholm air samples

The analytical setup allowed for identification of 42 unique PAHs present in air PM (Table 1), ranging from three to six aromatic rings. The raw air PM extract contained a total PAH concentration of 3392.6 pg/m^3 including 160 pg/m^3 of BP and 1.05 pg/m^3 of DBP. These values could be compared to concentrations of BP and DBP of 248 pg/m^3 and 4.8 pg/m^3, respectively, determined in PM_{10} collected in the street canyon of Hornsgatan (downtown Stockholm) during the same time period as the PM studied in the present work (Westerholm et al., 2012). For all fractions used in this study, PAH concentrations are presented in micromolar (Table 1). Fraction S-1 was prepared from the raw air PM extract to achieve a concentration of 1 μM BP (actual 0.91 μM). Fraction S-10 was prepared by concentrating an aliquot of fraction S-1, and contained a final concentration of 10.3 μM BP. Sub-fractions S^{F1} and S^{F2} were prepared by separating an aliquot of S-1 by size to yield a fraction containing 3- and 4-ring PAHs (S^{F1}) and a fraction containing PAHs with 5 rings and more (S^{F2}) with concentrations of BP of 0.004 and 1.14 μM respectively. A blank extract contained a total PAH content of 40 nM including 0.3 nM BP.

Exposure to air PM extract induces protein phosphorylation consistent with persistent activation of DNA damage signaling

To investigate DNA damage and DDR after exposure to air PM extracts, cells were exposed to extracts by using a dose equivalent to 0.1–10 nM BP (BP_{eq}) and levels of pChk1, pp53 and γH2AX were measured by Western blotting (Figs. 5, A and B). Prolonged activation of DNA damage signaling was observed in a dose-dependent manner. Statistically significant increases in pChk1, pp53 and γH2AX from baseline levels were observed in cells exposed to air fractions with 6 nM ($p=0.009$), 1 nM ($p=0.007$) and 10 nM ($p<0.001$)

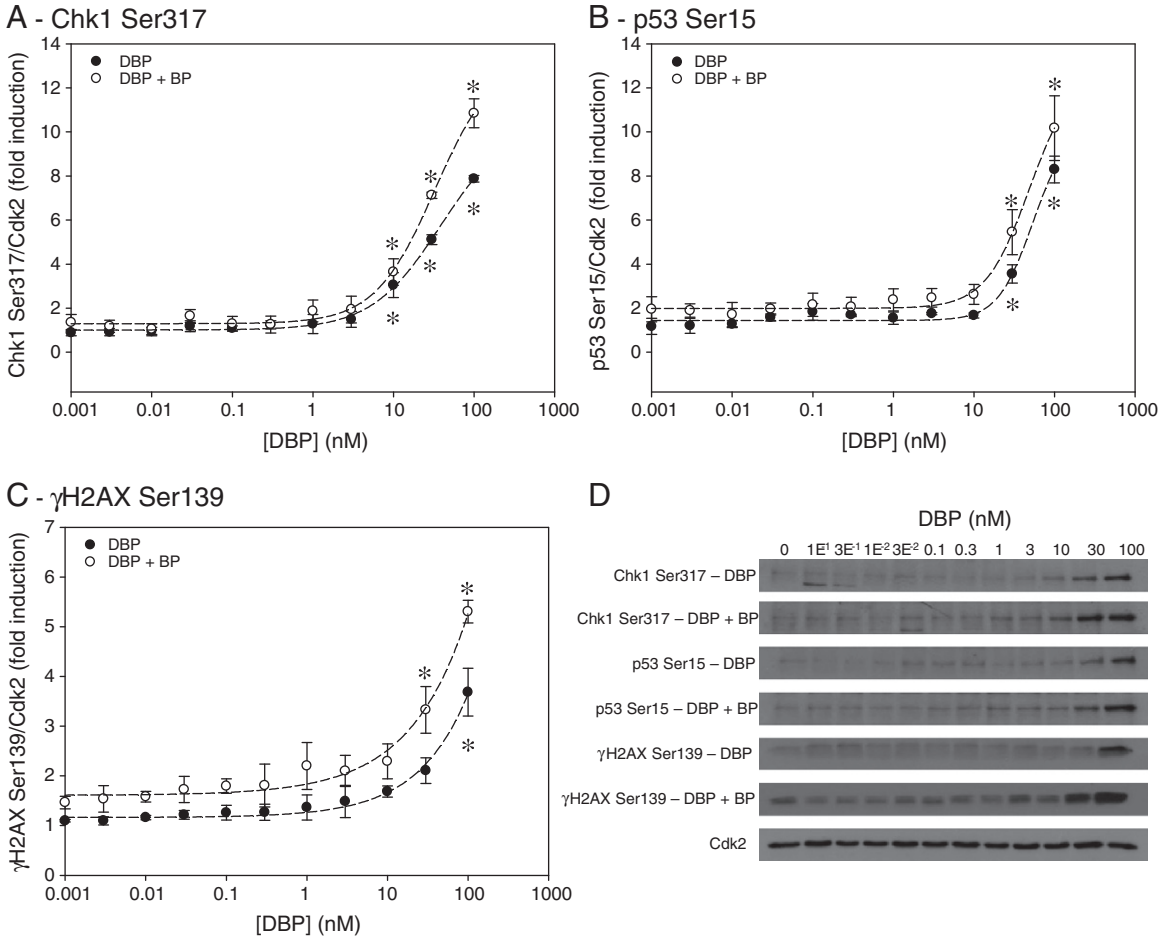

Fig. 2. Binary mixtures of DBP and BP are more potent at activation of DNA damage signaling than DBP alone. Phosphorylation of Chk1 at Ser317 (A), p53 at Ser15 (B) and H2AX at Ser139 (C) was assessed by Western blot after 48 h exposure to 0–100 nM DBP alone (●) or with 100 nM BP (○). Densitometric analysis for each protein is shown in (D). Cdk2 was used as a loading control. Experiments were performed in triplicate and data points represent means ± SE. Where not shown, error lies within the data points. Curves were fitted using the logistic 4 parameter equation in Sigmaplot 12. *$p<0.05$ as compared with control levels by one way ANOVA.

BP_{eq} respectively (Figs. 5, A and B). No statistically significant increase in DNA damage was observed in cells exposed to air PM compared to either control levels (Fig. 3). No response was observed in experiments with the blank sample (data not shown), and no significant effect on cell viability was observed with either the S-1 or S-10 fractions (Supplementary Fig. 1, C).

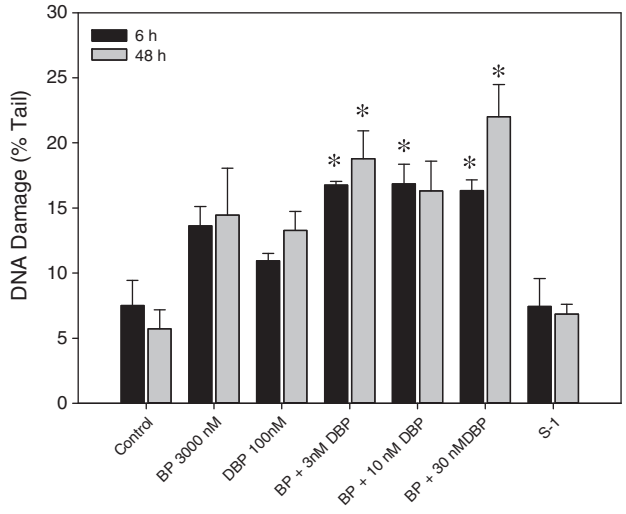

Fig. 3. Binary mixtures of BP and DBP result in persistent DNA damage. Levels of DNA damage in HepG2 cells exposed to BP or DBP alone, in binary mixtures of 3000 nM BP with DBP, and air PM extract S-1. DNA damage levels were determined after 6 and 48 h by Comet assay. Experiments were performed in triplicate and data points represent means ± SE. *$p<0.05$ as compared with control levels by one way ANOVA.

Many of the biological effects of PAHs, including oxidative stress and DNA damage, are believed to be mediated through activation of the aryl hydrocarbon receptor (AhR) and subsequent induction of cytochrome P450 enzymes (CYP) (Nebert et al., 2000). Activation of the AhR was assessed by measuring induction of gene expression of CYP1A1 and 1B1 using qRT-PCR following exposure to BP alone or the BP_{eq} dilutions described above (Fig. 5, C). Exposure to 1 and 10 nM BP alone did not induce either CYP1A1 or 1B1 (data not shown). In contrast, an 11- and 118-fold induction of CYP1A1 was observed following exposure to air PM fractions with 1 and 10 nM BP_{eq} respectively (Fig. 5, C). Statistically significant increases in CYP1A1 induction was observed from 6 nM ($p=0.008$). Induction of CYP1B1 was less strong compared to CYP1A1, with 3- and 18-fold induction observed following exposure to air PM fractions with 6 and 10 nM BP_{eq} respectively (Fig. 5, C). Statistically significant increases in CYP1B1 induction was observed from 10 nM ($p<0.001$).

Cellular responses to complex mixtures of PAHs cannot be predicted by applying TEF-based analyses

The data presented in Figs. 1–5 agree with our previous findings that PAHs in complex mixtures are more potent than BP alone at inducing DNA damage and subsequent DDR (Mattsson et al., 2009; Niziolek-Kierecka et al., 2012) and support the idea that BP alone as

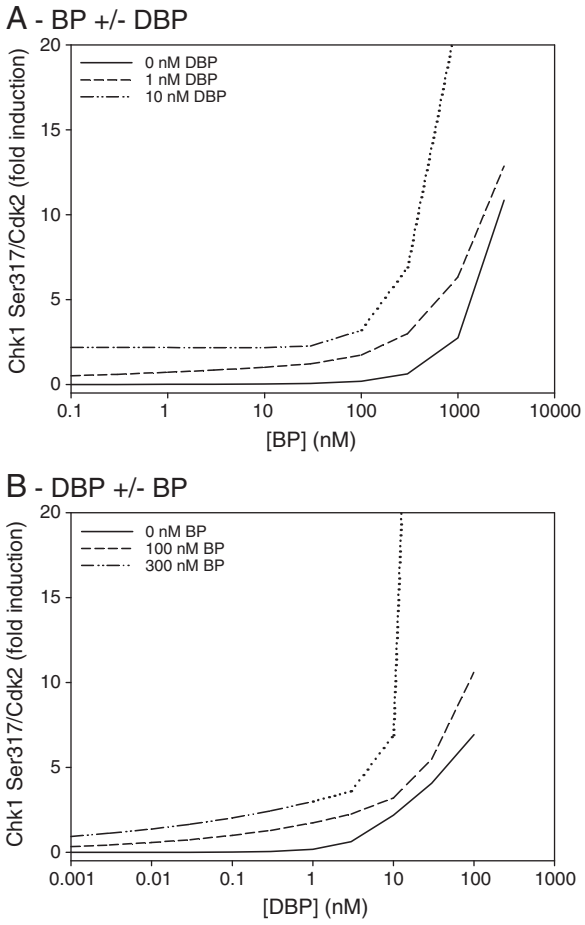

Fig. 4. Statistical analysis of binary BP and DBP mixtures reveal more than additive effects on Chk1 phosphorylation. Modeling of pChk1 in response to single and binary mixtures of BP (±DBP) and DBP (±BP) is shown in (A) respectively (B). The dotted part of the curve for the higher binary concentrations represents extrapolation outside the experimental range.

an indicator is not sufficient for assessing effects of exposure to mixtures of PAHs on human health. Comparing the dose-dependent phosphorylation of Chk1, p53 and H2AX in HepG2 cells exposed to BP, DBP or air PM extracts; we observed persistent phosphorylation of all three proteins at significantly lower concentrations in cells exposed to the air PM extracts (see Figs. 1, 2 and 5). The concentrations of BP, DBP and air PM extract (BP_{eq}) required to achieve a certain fold increase of phosphorylation for all three proteins are given in Table 2. As can be seen, DBP is up to 80-fold more potent in inducing the same levels of protein phosphorylation as BP. This number is in agreement with animal experiments where DBP has been shown to be about 100-fold more potent than BP as a carcinogen (Luch, 2009; Siddens et al., 2012). Comparing the air PM extract with BP shows an up to 250-fold higher potency in inducing DDR. This is significantly higher than the TEQ values, ranging from 1.74 to 4.71, calculated for the air PM extract using different available TEF scales (Table 3). This clearly demonstrates that complex mixtures are more potent than BP and that TEF scales are insufficient for predicting DDR in response to complex PAH mixtures.

PAH composition plays a significant role in complex mixture toxicity

Due to the vast array of chemicals in complex mixtures, it is plausible that the contributory potency of each individual chemical varies. In order to study the impact of mixture composition on the cellular DDR, time-dependent effects on level of pChk1, pp53 and γH2AX and CYP induction following exposure to the total air fraction (S-1) and subfractions S^{F1} and S^{F2} were assessed (Figs. 6, A–E). Exposure to the total and both subfractions induced a time-dependent increase in pChk1 up to 24 h, and then a decrease at 48 h (Fig. 6, A). Levels of p53 and γH2AX phosphorylation followed different time-dependent kinetics, increasing through 48 h (Figs. 6, B and C). Statistically significant increases were observed for all proteins following exposure to the S-1 fraction (Figs. 6, A–C) and for pChk1 and pp53 after exposure the subfraction containing the larger PAHs (S^{F2}, Figs. 5, A and B). No significant changes were detected for the subfraction with the smaller PAHs (S^{F1}). Significant increases in CYP1A1 mRNA expression were observed at all time-points following exposure to the S-1 fraction and at 6 and 24 h with the subfraction containing the larger PAHs (S^{F2}, Fig. 6, D), whereas the subfraction containing the smaller PAHs only induced significant expression at 6 h (S^{F1}, Fig. 6, D). Significant increases in CYP1B1 mRNA expression were only observed following exposure to the S-1 fraction (Fig. 6, E). Taken together, these data suggest that larger PAHs with 5 or more aromatic rings contribute more to the activation of DNA damage signaling and genotoxicity of PAHs in air PM.

Discussion

The aim of this study was to investigate the effects of low-levels of PAHs on DNA damage and DDR and to investigate how interactions in simple and complex mixtures can alter these responses. Persistent activation of DNA damage signaling was found for BP and DBP in a dose-dependent manner, both as individual chemicals and in binary mixtures. A more than additive effect was observed for binary mixtures on pChk1 activation compared to BP and DBP alone. Exposure to air PM extracts caused persistent activation of DNA damage signaling at significantly lower (BP_{eq}) concentrations than BP alone which could not be explained by TEF scale analysis, currently used in risk assessment of PAHs. The composition of PAHs in air PM extracts played a significant role in the effects on DNA damage signaling, with extracts containing PAHs with more than four aromatic rings demonstrating increased potency compared to smaller PAHs.

Our first approach to studying the potential interactions between PAHs was to identify the effects of binary mixtures of BP and DBP on activation of DNA damage signaling. DBP was chosen as it has demonstrated the highest carcinogenic potential of the PAHs analyzed so far (Luch, 2009), and hence, it is plausible that interactions between BP and DBP would lead to a higher carcinogenic potency than the individual chemicals. For all proteins analyzed in this study (Chk1, p53 and H2AX) the level of phosphorylation was higher in cells exposed to the binary mixtures than BP or DBP alone. The results were further supported by Comet assay which showed more persistent levels of DNA damage in response to binary BP and DBP exposure. We next applied a statistical model to the data to determine if any interactions were occurring in the binary mixtures. The applied model encompassed four unique data sets per protein (BP alone, DBP alone, BP with DBP and DBP with BP). A more than additive effect was observed for the activation of pChk1 with the binary mixtures compared to the individual compounds alone. This is in agreement with previous studies which have demonstrated synergistic or more than additive effects with mixtures of PAHs on early vertebrae development (reviewed in (Billiard et al., 2008)) and cellular DNA adduct levels (Staal et al., 2007; Tarantini et al., 2009, 2011). Together, these results confirm that interactions between PAHs can result in unexpected biological effects with important implications for human health.

The effect of complex mixtures of PAHs in air PM extracts was analyzed in comparison to BP alone using a dose response based approach similar to the experiments with binary mixtures. The presence of PAHs on air particles is considered to play an important role in the

Table 1
Concentration (μM) of PAHs in extracts of an urban air sample collected over 218 h on the rooftop of Stockholm University as described in Materials and methods. Sample S-1 is the raw Stockholm extract equivalent to 1 μM BP, which was evaporated to produce a ten-fold more concentrated sample (S-10) and from which fractions were prepared (S^{F1} and S^{F2}) based on the number of aromatic rings.

PAH	Air PM (pg/m^3)	S-1 (μM)	S^{F1} (μM)	S^{F2} (μM)	S-10 (μM)	Blank (μM)
Phenanthrene	260	2.10	1.47	0.00525	23.6	0.01
Anthracene	22.7	0.183	0.121	0.00377	2.06	0.008
3-Methylphenanthrene	47.4	0.355	0.186	0.00126	3.99	0.002
2-Methylphenanthrene	73.4	0.548	0.263	0.00193	6.17	0.002
2-Methylanthracene	8.38	0.0630	0.0328	0.000627	0.705	0.0004
9-Methylphenanthrene	36.4	0.272	0.137	0.00157	3.06	0.002
1-Methylphenanthrene	69.6	0.520	0.272	0.000763	5.85	0.001
4H-cyclopenta[*def*]phenanthrene	67.7	0.511	0.233	0.00300	5.76	0.0005
2-Phenylnaphthalene	34.4	0.242	0.225	0.000840	2.73	0.001
3,6-Dimethylphenanthrene	1.68	0.0117	0.0109	0.000524	0.132	0.0002
3,9-Dimethylphenanthrene	8.29	0.0577	0.0541	0.000451	0.650	0.001
Fluoranthene	357	2.54	2.69	0.00390	28.6	0.003
Pyrene	312	2.21	2.47	0.00539	24.9	0.003
1-Methylfluoranthene	47.3	0.314	0.350	0.00194	3.53	0.0004
Benz[*a*]fluorene	33.0	0.219	0.269	0.000600	2.47	0.0003
Benz[*b*]fluorene	17.3	0.115	0.144	0.000701	1.29	0.0001
2-Methylpyrene	18.9	0.125	0.149	0.000922	1.41	0.0004
4-Methylpyrene	23.9	0.159	0.197	0.000792	1.79	0.0003
1-Methylpyrene	21.1	0.140	0.174	0.00121	1.58	0.0004
Benzo[*ghi*]fluoranthene	144	0.917	1.04	0.00202	10.3	0.0004
Benzo[*c*]phenanthrene	58.7	0.370	0.359	0.000396	4.16	0.0006
Benzo[*b*]naphto[1,2-*d*]thiophene	2.42	0.0149	0.0142	0.000329	0.167	0.0002
Benz[*a*]anthracene	149	0.940	1.06	0.00374	10.6	0.0004
3-Methylchrysene	12.3	0.0731	0.0939	0.00109	0.823	0.0001
2-Methylchrysene	27.6	0.164	0.173	0.00156	1.84	0.0002
6-Methylchrysene	16.1	0.0955	0.114	0.000327	1.08	0.0001
1-Methylchrysene	30.4	0.180	0.194	0.00125	2.03	0.0002
Benzo[*b*]fluoranthene	335	1.91	0.00142	2.16	21.5	0.0003
Benzo[*k*]fluoranthene	137	0.780	0.000588	0.926	8.78	0.0002
Benzo[*e*]pyrene	225	1.28	0.00332	1.42	14.4	0.0003
Benzo[*a*]pyrene	160	0.910	0.00362	1.14	10.3	0.0003
Perylene	24.3	0.138	0.000726	0.162	1.56	0.0003
Indeno[1,2,3-*cd*]fluoranthene	27.3	0.142	0.000385	0.163	1.60	0.0002
Indeno[1,2,3-*cd*]pyrene	164	0.854	0.000800	1.04	9.62	0.0002
Dibenz[*a,h*]anthracene	26.1	0.135	0.000594	0.144	1.52	0.0002
Picene	31.1	0.161	<LOQ[a]	0.184	1.81	0.0002
Benzo[*ghi*]perylene	208	1.08	0.000928	1.40	12.2	0.0003
Dibenzo[*a,l*]pyrene	1.05	0.00497	<LOQ	0.00415	0.0559	0.0001
Dibenzo[*a,e*]pyrene	21.6	0.103	<LOQ	0.0953	1.16	0.0002
Coronene	126	0.604	0.00125	0.745	6.80	0.0003
Dibenzo[*a,i*]pyrene	4.39	0.0209	<LOQ	0.0219	0.235	0.0003
Dibenzo[*a,h*]pyrene	0.817	0.00388	<LOQ	0.00484	0.0437	0.0003
Total	3392.6	21.6	12.5	9.7	242.9	0.04

[a] LOQ = limit of quantification.

observed health effects related to exposure to air PM (Lewtas, 2007). We found that phosphorylation of Chk1, p53 and H2AX and CYP1 induction followed the same dose dependent increase but at significantly lower concentrations of air PM extracts (1 nM BP_{eq}) than BP alone. Assuming a typical human breathing volume of 1.2 m^3/h (ICRP, 1994), 1 nM BP_{eq} of air PM extract correlates to approximately 3.45 h of continuous breathing. These data suggest that the different PAHs in complex mixtures have a significantly more profound influence on potency than BP alone. This has important implications for the current approach of risk assessment of PAHs and is further discussed below. The data showed that pChk1 was the most sensitive marker with a 220-fold lower BP_{eq} dose of air PM extract inducing a 2-fold increase in pChk1 as compared to BP alone. This is in agreement with the analysis of the binary mixtures and our previous data (Niziolek-Kierecka et al., 2012) showing sustained levels of DNA damage in parallel to persistent activation of Chk1. Together these data suggests that Chk1 phosphorylation could be an important marker for future analyses of the effects of complex mixtures of PAHs on DNA damage signaling. This warrants further investigation and confirmation using other single and binary PAHs. Earlier studies have shown that deregulated Chk1 activity sensitizes cells to the BP diol epoxide metabolite (Chen et al., 2009; Guo et al., 2002). Activation of Chk1 by phosphorylation at Ser317 is mainly attributed to ATR signaling pathway in response to single strands breaks (Jazayeri et al., 2006) and bulky PAH-DNA adducts (Choi et al., 2007, 2009), suggesting that these are the more prominent types of DNA damage in cells exposed to mixtures of PAHs. This is in agreement with a recent study showing that a dose of 0.16 μM BP alone mainly formed DNA adducts in HepG2 cells while BP_{eq} complex mixtures of PAHs mainly caused strand breaks (Tarantini et al., 2009). Interestingly, they did not observe any strand breaks in response to a reconstituted PAH mixture suggesting the action of other compounds in the complex mixture sample. The methodology for preparation and fractionation of the air Pm extract used in the current study has previously been shown to exclude polar compounds such as nitro-PAHs and acridines (Christensen et al., 2005) but probably includes polychlorinated biphenyls (PCBs) and polychlorinated dibenzo-dioxins and -furans (PCDD/Fs) (Bandh et al., 1996; Piazza et al., 2012). Compounds such as PCBs and PCDD/Fs are known to induce the AhR pathway (Schmitz et al., 1995) possibly leading to further interaction effects contributing to the biological activity of the air PM extract.

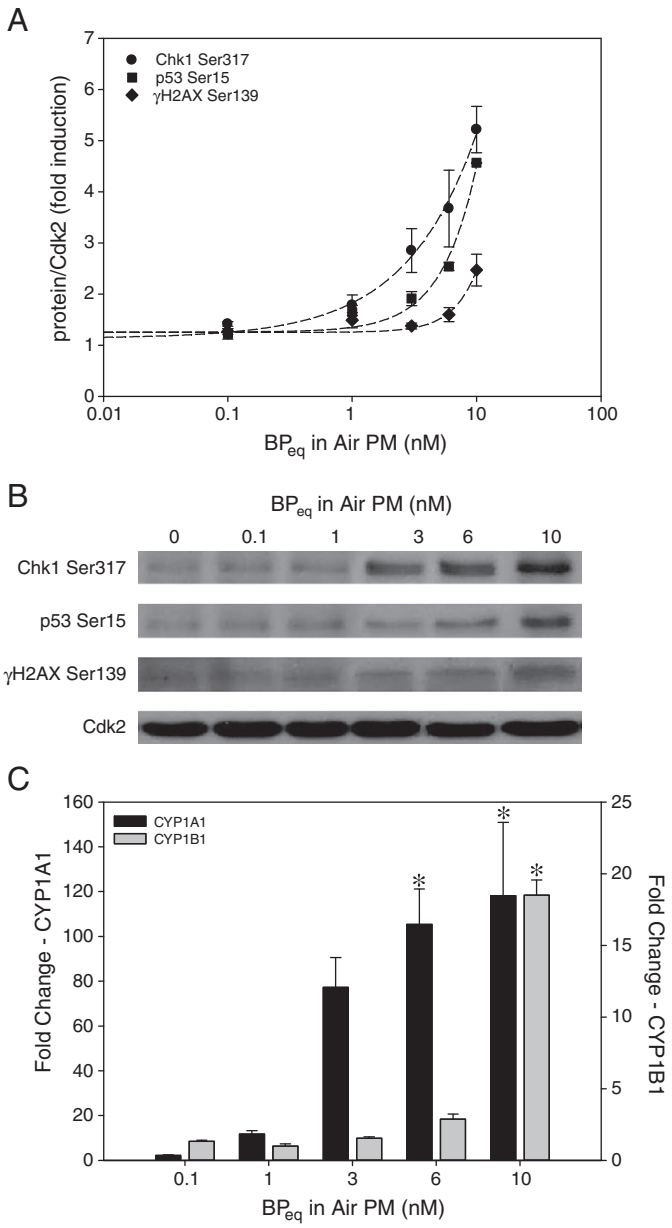

Fig. 5. Persistent activation of DNA damage signaling and cytochrome P450 induction in HepG2 cells in response to air PM extracts. Phosphorylation of Chk1 at Ser317, p53 at Ser15 and H2AX at Ser139 was assessed by Western blot after 48 h (A). Representative blots are shown in B. Cdk2 was used as a loading control. CYP1A1 and CYP1B1 mRNA levels were measured by qRT-PCR (C). Experiments were performed in triplicate and data points represent means ± SE. Where not shown, error lies within the data points. Curves were fitted using the logistic 4 parameter equation in Sigmaplot 12. *$p<0.05$ as compared with control levels by one way ANOVA.

In this study we found that fractions of air PM containing larger PAHs produced significant effects on DNA damage signaling, and in the case of Chk1 phosphorylation, comparable to levels seen in the total air fraction. No significant increase in protein phosphorylation was observed with the fraction containing smaller PAHs. In addition, induction of CYP1 was more pronounced with the subfraction containing larger PAHs. Earlier investigations on different effects on carcinogenicity between size-separated fractions of PAHs identified mixtures containing >3 aromatic rings as contributing to the majority of toxicity (reviewed in Jacob, 2008). More recently increased toxicity has been demonstrated in PAH fractions obtained from coal tar mixtures with 4 to 6 aromatic rings compared with fractions containing 2 and 3 rings (Reeves et al., 2001). Our data is also in line with the majority of PAHs classified by IARC as probably or carcinogenic to humans are "larger" PAHs (IARC, 2010). These findings support the hypothesis that different PAHs contribute to the toxicity of complex mixtures and that PAHs with ≥5 aromatic rings, including BP and DBP, are likely to provide a larger risk to human health.

Table 2
Concentrations of BP, DBP and air PM extract required to induce 2–5 fold inductions of Chk1 and p53 phosphorylation and 2–2.6 fold induction of γH2AX in HepG2 cells.

Protein	Increase in phosphorylation[a]	Concentration required (μM)			Fold difference	
		BP	DBP	Air PM	BP/DBP	BP/Air PM
Chk1	2 fold	0.31	0.0048	0.0014	64.6	221.4
	3 fold	0.65	0.0109	0.0037	59.6	175.7
	4 fold	0.97	0.0188	0.0065	51.6	149.2
	5 fold	1.24	0.0289	0.0096	42.9	129.2
p53	2 fold	0.76	0.0143	0.0039	53.1	194.9
	3 fold	1.58	0.0259	0.0067	61.0	235.8
	4 fold	2.26	0.0359	0.0090	63.0	251.1
	5 fold	2.93	0.0445	ND[b]	65.8	–
H2AX	2 fold	1.71	0.0209	0.0082	81.8	208.5
	2.2 fold	2.01	0.0289	0.0090	69.6	233.3
	2.4 fold	2.26	0.0378	0.0098	60.0	230.6
	2.6 fold	2.45	0.0470	ND	52.1	–

[a] Increase in phosphorylation is measured from fitted curves in Figs. 1, 2 and 5 and represents an increase from baseline levels.
[b] ND = No data available.

For risk assessment of PAHs, potency is often expressed relative to BP using toxic equivalency factors (TEFs) or relative potency factors (RPFs) (Boström et al., 2002). In addition, the use of genotoxic potency factors (GEFs) based on the activation of γH2AX in response to PAHs has recently been proposed (Audebert et al., 2012). A major problem with using the TEF/RPF approach is that the quantitative analysis of risk does not account for differences in mechanisms or endpoints of the chemicals in the mixtures, and cannot accurately reflect the different interactions that can occur. Furthermore, a significant inadequacy of using TEF values for assessment is that there are a number of different scales published and either the values for different PAHs are not available or the values in these scales differ significantly. For example, DBP is assigned a value of 1 in the TEF scale published by Larsen and Larsen (1998), whereas other scales have a value of 10 (Collins et al., 1998) and 100 (WHO/IPCS, 1998). Using TEF values from five different sources we calculated a TEQ value for the air PM extract ranging between 1.74 and 2.68. We also calculated a "maximum" TEQ value of 4.71 by taking the highest available value for each PAH from the five sources. However, from the experimental data obtained in this study we observed that the BP_{eq} concentrations of air PM extracts required achieving fold increases in phosphorylation of DNA damage signaling proteins was more than 100 times lower than BP alone. The lack of parity highlights that the TEF/RPF scheme is insufficient for predicting activation of DNA damage signaling in response to complex mixtures. This is in line with a recent study by Siddens et al (2012) which concluded that using the current RPFs led to a highly significant underestimation of the potency of coal tar extract as skin carcinogen. In addition, the high number of different compounds found in complex mixtures, which might affect the biological activity as discussed above, further adds to the uncertainty of using TEF scales for complex mixtures. These findings are

Table 3
Toxic equivalence (TEQ) values for PAHs present in the air PM extract according to different toxic equivalency factor (TEF) scales.

Source	Nisbet and LaGoy (1992)	Muller (1997)	Larsen and Larsen (1998)	Collins et al. (1998)	California EPA (2004)	Highest TEF value from all
TEQ	2.33	2.17	1.74	2.01	2.68	4.71

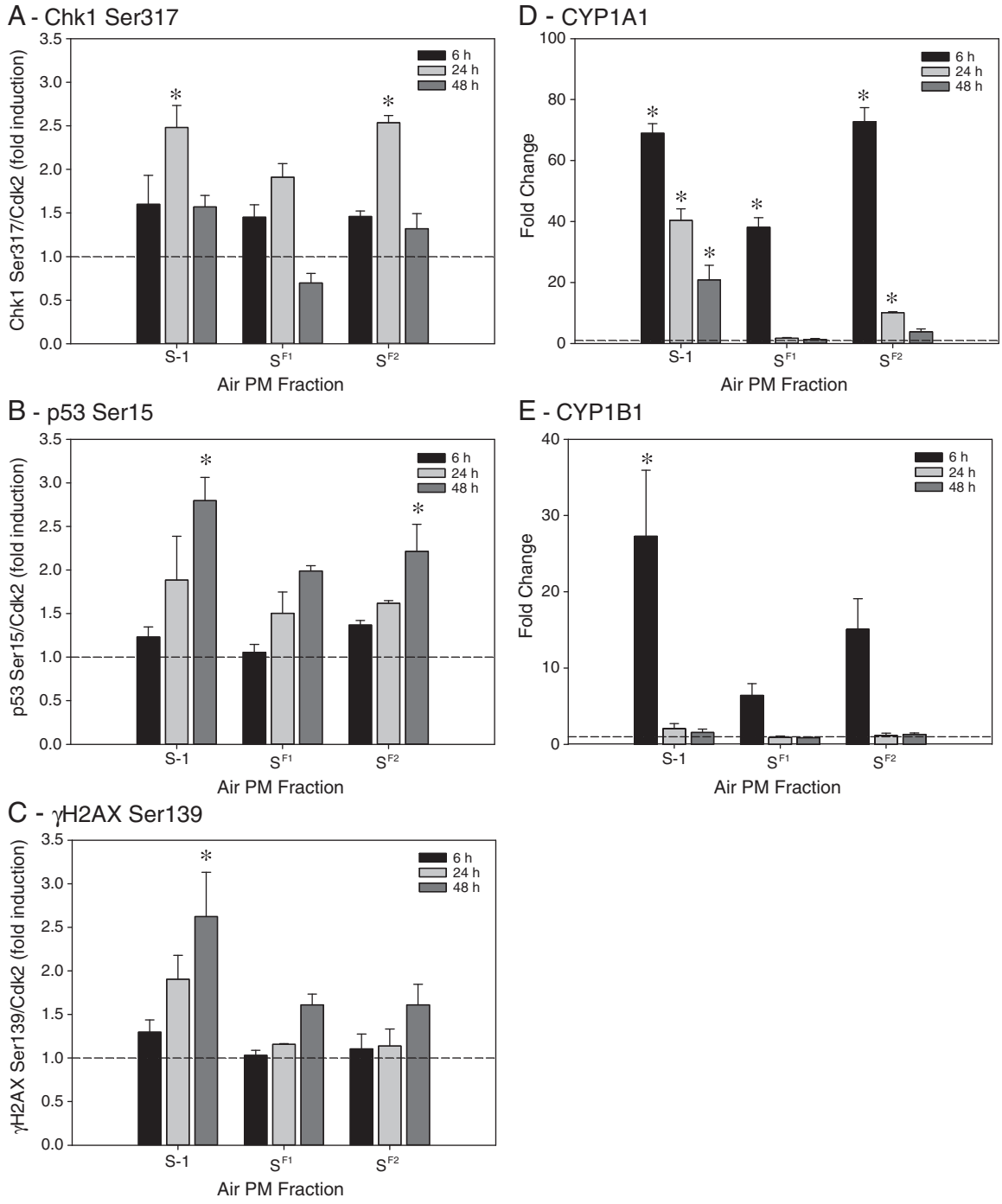

Fig. 6. PAH-size-dependent activation of DNA damage signaling and cytochrome P450 induction in HepG2 cells exposed to air PM extracts. Cells were exposed to air PM extract S-1 and subfractions prepared according to PAH size (S^{F1} containing 3- to 4-ring PAHs and S^{F2} containing PAHs with five or more rings). Phosphorylation of Chk1 at Ser317 (A), p53 at Ser15 (B) and H2AX at Ser139 (C) was assessed by Western blotting. CYP1A1 (D) and CYP1B1 (E) mRNA levels were measured by qRT-PCR. Experiments were performed in triplicate and data points represent means ± SE. Where not shown, error lies within the data points. The dashed line at $y = 1.0$ represents control levels. *$p < 0.05$ as compared with control levels by one way ANOVA.

in agreement with earlier published in vitro and in vivo studies by us and others (Gaylor et al., 2000; Mattsson et al., 2009; Niziolek-Kierecka et al., 2012; Siddens et al., 2012; Tarantini et al., 2009) and suggests that the current approach needs re-evaluation.

A potential alternative to component-based analyses such as the TEF and RPF scheme, would be to use "mixture assessment factors" (MAFs) similar to what has been discussed by both US EPA and within REACH under the EU commission (Backhaus et al., 2010; USEPA, 2000). MAFs do not rely on relative potency values assigned to the separate mixture components but instead compare the effects of whole mixtures assessed through a relevant biological endpoint thus including potential non-additive effects as a result of interactions. Based on the biological significance of DNA damage signaling in response to complex mixtures of PAHs in air PM extracts in this study and soil extracts (Niziolek-Kierecka et al., 2012), phosphorylation of Chk1 could be one candidate as a marker for future risk

assessment of complex mixtures using MAFs. The results presented here on Chk1 (see Table 2) showed sustained activation at BP_{eq} concentration 100–200-fold lower than BP alone. These results have important implications for the risk assessment of mixtures of PAHs and warrant further investigation using environmental PAH samples from different sources and in different experimental models.

In conclusion, this study addressed the effects of binary mixtures of PAHs on DNA damage and signaling and showed that interactions lead to more than additive effects. Effects on activation of DNA damage signaling were also observed at significantly lower concentrations of PAHs in complex mixtures compared with that of BP alone. Furthermore, these differences could not be correlated with methods currently used for risk assessment of PAHs. The increasing evidence highlighting the insufficiencies of using BP as an indicator for PAH toxicity highlights the need to develop more applicable methods for risk assessment of the effects of complex mixtures on human health.

Funding information

This work was supported by the Swedish Research Council Formas (U.S., C.B. and R.W.), Cancer- och Allergifonden (U.S.), Stockholm University (C.B. and R.W.) and an EU/FP7 Marie Curie IRG fellowship (K.D.). The authors declare no competing financial interests.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.taap.2012.11.026.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

The authors would like to thank Professor Bengt Jernström for valuable comments on the manuscript and helpful discussions throughout.

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