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Nanomolar levels of PAHs in extracts from urban air induce MAPK signaling in HepG2 cells

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HIGHLIGHTS

- Benzo[*a*]pyrene (BP) and air PM extracts from Sweden and Brazil were compared.
- Air PM extracts induced a stronger activation of MAPK signaling and downstream stress response compared to BP.
- MAPK signaling was shown to be mediated through the MEK4/JNK/AP-1 axis.
- ATF2 was identified as a sensitive marker for cellular stress following exposure to nanomolar levels of PAHs in extracts from urban air.

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are common environmental pollutants that occur naturally in complex mixtures. Many of the adverse health effects of PAHs including cancer are linked to the activation of intracellular stress response signaling. This study has investigated intracellular MAPK signaling in response to PAHs in extracts from urban air collected in Stockholm, Sweden and Limeira, Brazil, in comparison to BP in HepG2 cells. Nanomolar concentrations of PAHs in the extracts induced activation of MEK4 signaling with down-stream increased gene expression of several important stress response mediators. Involvement of the MEK4/JNK pathway was confirmed using siRNA and an inhibitor of JNK signaling resulting in significantly reduced MAPK signaling transactivated by the AP-1 transcription factors ATF2 and c-Jun. ATF2 was also identified as a sensitive stress responsive protein with activation observed at extract concentrations equivalent to 0.1 nM BP. We show that exposure to low levels of environmental PAH mixtures more strongly activates these signaling pathways compared to BP alone suggesting effects due to interactions. Taken together, this is the first study showing the involvement of MEK4/JNK/AP-1 pathway in regulating the intracellular stress response after exposure to nanomolar levels of PAHs in environmental mixtures.

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1. Introduction

Human exposure to environmental pollutants in air particulate matter (PM) has been identified to cause a number of adverse health effects including cancer and various cardiovascular and respiratory diseases (Pope et al., 2002; Brunekreef and Holgate, 2002). One important group of environmental pollutants that are associated with PM and play an important role in the reported detrimental health effects are the polycyclic aromatic hydrocarbons (PAHs) (Lewtas, 2007). PAHs are ubiquitous environmental pollutants that are naturally present as mixtures and

Abbreviations: AP-1, activator protein 1; BP, benzo[a]pyrene; ERK, extracellular regulated kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEK4, MAPK kinase 4; PAH, polycyclic aromatic hydrocarbon; PM, particulate matter; TEF, toxic equivalency factor; TNF, tumor necrosis factor.

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are formed during combustion of carbon-containing fuels. Both individual and mixtures of PAHs are classified as carcinogens or probable carcinogens by the International Agency for Research on Cancer (IARC Working Group, 2010). Recent data from us and others have suggested synergistic effects due to interactions between PAHs in complex mixtures on the genotoxic and carcinogenic properties of PAHs (Niziolek-Kierecka et al., 2012; Jarvis et al., 2013; Tarantini et al., 2009; Siddens et al., 2012), though conversely, strong antagonistic effects have also been observed in human cells, probably resulting from competitive inhibition of metabolizing enzymes (Libalova et al., 2012, 2014; Tarantini et al., 2011). However, the role of interactions between PAHs in complex mixtures in relation to the adverse health effects of PAHs is poorly understood (Jarvis et al., 2014).

The activator protein-1 (AP-1) transcription factor is a dimeric complex comprising members of the ATF, FOS, JUN and MAF protein families that has functions in many areas of cellular homeostasis (Eferl and Wagner, 2003; Lopez-Bergami et al., 2010). In response to cellular stress stimuli AP-1 proteins are activated by the mitogen-activated protein kinase (MAPK) family of proteins, including c-Jun N-terminal kinase (JNK), p38 and extracellular signal-regulated kinase (ERK) (Karin, 1995; Whitmarsh and Davis, 1996). The protein MAPK kinase 4 (MEK4/MKK4) specifically activates JNK and p38 (Derijard et al., 1995) and has been identified as an important metastasis suppressor in several organs (Whitmarsh and Davis, 2007) and a possible target for small molecule inhibition in therapy for tumor necrosis factor alpha (TNF) mediated diseases (Kim et al., 2009). The present study focuses on activation of MAPK signaling in response to environmental mixtures of PAHs compared to single benzo[*a*]pyrene (BP) exposure in human-derived hepatoma (HepG2) cells. It has previously been shown that in vitro exposure to BP leads to activation of MAPK signaling associated with apoptotic cell death via p53 (Kim et al., 2005; Lin et al., 2008). However, the role of MAPK signaling in the stress response after exposure to nanomolar concentrations of PAHs in air PM remains unclear.

Using PAH-containing air PM extracts from Stockholm, Sweden and Limeira, Brazil at nanomolar concentrations we studied the time-dependent activation of MAPK signaling and downstream gene expression in HepG2 cells. Our results show that nanomolar concentrations of PAH extracts more strongly activate MAPK signaling and proteins of the AP-1 transcription factor than BP alone suggesting effects due to interactions and that this activation is mediated via MEK4 and JNK. Furthermore, the transactivation of cellular stress mediators including interleukin 8 in response to PAH extract was shown to be mediated through a MAPK. To the authors knowledge this is the first study to demonstrate activation of MEK4/JNK/AP-1 with downstream increased gene expression in the cellular stress response after exposure to nanomolar levels of PAHs found in air PM.

2. Materials and methods

2.1. Reagents and antibodies

Unless otherwise stated all chemicals, including BP, were of analytical grade and obtained from Sigma–Aldrich (Stockholm, Sweden). Detailed information on manufacturer and purity of the standards and solvents used for PAH analysis have been published previously (Sadiktsis et al., 2012; Ahmed et al., 2013). Gibco (Invitrogen, Paisley, UK) supplied all cell culture reagents. Cell Signaling Technology (Beverly, MA) provided the following antibodies: phospho-ATF2 Thr71, phospho-c-Jun Ser63, JNK, phospho-JNK Thr183/Tyr185, MEK4, phospho-MEK4 Thr261. Santa Cruz Biotechnology (Santa Cruz, CA, USA) provided the Cdk2, phospho-Erk Tyr204 and phospho-p38 Thr180/Tyr182 antibodies, secondary anti-rabbit, anti-mouse and siRNA against MEK4 and control siRNA-A. Calbiochem (Gibbstown, NJ, USA) provided the JNK inhibitor VIII.

2.2. Air sampling, sample preparation and PAH analysis

Air PM was collected at two sites: the campus of Stockholm University, Stockholm, Sweden and the campus of the Faculty Technology at UNICAMP, Limeira, Brazil. At both collection sites total PM was collected. The air PM sample from Stockholm was collected at roof-top level on a Teflon-coated glass fiber filter (Ø149 mm, Pallflex Inc., Putnam, CT, USA) with an average flow rate of 509 $l\,min^{-1}$ for 170 h. The total PM concentration for this sample was not determined, but PM concentration from other similar urban sites in Stockholm during the same season ranged from 10.4–19.4 $\mu g/m^3$ (unpublished data). The air PM sample from Limeira was collected at street level on a glass-fiber filter (254 mm × 233 mm, 0.33 mm pore size, Energética Ind. Com. LTDA, Rio de Janeiro, RJ, Brazil) using a high-volume sampler (Energética Ind. Com. LTDA, Rio de Janeiro, RJ, Brazil) operated at an average flow rate of 1130l min^{-1} for 24 h. The total PM concentration in the Limeira sample was 95.8 $\mu g/m^3$ (Umbuzeiro et al., 2014). Extraction was performed using an ASE 200 accelerated solvent extraction system (Dionex Corporation, Sunnyvale, CA, USA). Toluene was used as an extraction solvent at 200 °C and 3000 psi for five consecutive 30 min static extraction cycles as described previously (Jarvis et al., 2013). PAH content in the extracts was determined by HPLC–GC/MS as described previously (Jarvis et al., 2013; Sadiktsis et al., 2012).

2.3. Cell culture and exposure

Human-derived hepatocellular carcinoma cells (HepG2) were obtained from the American Type Culture Collection (Rockville, MD, USA). The motivation for the use of this cell line in this study is the metabolic competence for PAHs (Knasmuller et al., 1998) and a previously demonstrated response to low levels of PAHs (in mixtures) extracted from environmental samples by ourselves and others (Niziolek-Kierecka et al., 2012; Jarvis et al., 2013; Tarantini et al., 2009). HepG2 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. Prior to exposure cells were seeded at 3×0^5 cells/ml in 6-well plates and cultured for 72 h unless otherwise stated. Cells were exposed to solvent control (0.1% DMSO), BP, or PAH extracts for up to 24 h.

2.4. RNA interference

Transfection of cells was performed using Lipofectamine 2000 reagent (Invitrogen, Paisley, UK). Briefly, cells were seeded into 35 mm culture dishes and after 24 h transfected with 50 nM siRNA. siRNA sequences are shown in Supplementary Table 1. After 48 h of incubation cells were exposed to PAHs or solvent control, and then harvested for Western blot analysis.

2.5. Western blotting

Western blotting was performed as described previously (Jarvis et al., 2013). Briefly, whole cell lysates were subjected to standard SDS-PAGE and separated proteins 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). Experiments were performed at least in triplicate and

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