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

Jarvis IWH[a], Bergvall C[b], Morales DA[c], Kummrow F[d], Umbuzeiro GA[c], Westerholm R[b], Stenius U[a] and Dreij K[a]*

[a]Institute of Environmental Medicine, Karolinska Institutet, Box 210, SE-171 77 Stockholm, Sweden.

[b]Department of Analytical Chemistry, Stockholm University, Svante Arrhenius väg 16, SE-106 91 Stockholm, Sweden.

[c]Faculty of Technology, State University of Campinas (UNICAMP), Limeira, SP, 13484-332, Brazil.

[d]Institute of Environmental, Chemical and Pharmaceutical Sciences, Federal University of São Paulo (UNIFESP), Diadema, SP, 09972-270, Brazil.

***Corresponding Author**

Kristian Dreij, PhD

Address: Institute of Environmental Medicine, Karolinska Institutet, Box 210, SE-171 77

Stockholm, Sweden. Email: Kristian.Dreij@ki.se Tel: +46 8 524 87566

Running Title

Environmental PAH mixtures activate MAPK signaling

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;

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 cJun. 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.

Key Words

PAHs, Air particulate matter, Complex mixtures, Benzo[*a*]pyrene, MAPK

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 [1, 2]. 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) [3]. PAHs are ubiquitous environmental pollutants that are naturally present as mixtures and 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 [4]. 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 [5-8], though conversely, strong antagonistic effects have also been observed in human cells, probably resulting from competitive inhibition of metabolizing enzymes [9-11]. However, the role of interactions between PAHs in complex mixtures in relation to the adverse health effects of PAHs is poorly understood [12].

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 [13, 14]. 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) [15, 16]. The protein MAPK kinase 4 (MEK4/MKK4) specifically activates JNK and p38 [17] and has been identified as an important metastasis suppressor in several organs [18] and a possible target for small molecule inhibition in therapy for tumor necrosis factor alpha (TNF) mediated diseases [19]. 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 [20, 21]. 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 [22, 23]. Gibco (Invitrogen, Paisley, UK) supplied all cell culture reagents. Cell Signaling Technology (Beverly, MA) provided the following antibodies: phospho-ATF2 Thr71, phospho-cJun 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.

105 **2.2. Air sampling, sample preparation and PAH analysis**

106 Air PM was collected at two sites: the campus of Stockholm University, Stockholm, Sweden
107 and the campus of the Faculty Technology at UNICAMP, Limeira, Brazil. At both collection
108 sites total PM was collected. The air PM sample from Stockholm was collected at roof-top
109 level on a Teflon-coated glass fiber filter (Ø149 mm, Pallflex Inc., Putnam, CT, USA) with an
110 average flow rate of 509 l min^{-1} for 170 h. The total PM concentration for this sample was not
111 determined, but PM concentration from other similar urban sites in Stockholm during the
112 same season ranged from 10.4 – 19.4 µg/m^3 (unpublished data). The air PM sample from
113 Limeira was collected at street level on a glass-fiber filter (254 × 233 mm, 0.33 mm pore size,
114 Energética Ind. Com. LTDA, Rio de Janeiro, RJ, Brazil) using a high-volume sampler
115 (Energética Ind. Com. LTDA, Rio de Janeiro, RJ, Brazil) operated at an average flow rate of
116 1130 l min^{-1} for 24 h. The total PM concentration in the Limeira sample was 95.8 µg/m^3 [24].
117 Extraction was performed using an ASE 200 accelerated solvent extraction system (Dionex
118 Corporation, Sunnyvale, CA, USA). Toluene was used as an extraction solvent at 200 °C and
119 3000 psi for five consecutive 30 min static extraction cycles as described previously [6]. PAH
120 content in the extracts was determined by HPLC-GC/MS as described previously [6, 22].
121

122 **2.3. Cell culture and exposure**

123 Human-derived hepatocellular carcinoma cells (HepG2) were obtained from the American
124 Type Culture Collection (Rockville, MD, USA). The motivation for the use of this cell line in
125 this study is the metabolic competence for PAHs [25] and a previously demonstrated response
126 to low levels of PAHs (in mixtures) extracted from environmental samples by ourselves and
127 others [5-7]. HepG2 cells were cultured in minimal essential medium supplemented with 10
128 % fetal bovine serum, sodium pyruvate (1 mM), non-essential amino acids (0.1 mM),
129 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 3×10^5 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 [6]. 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 analyzed separately. Densitometric analysis was performed using ImageJ software version 1.45s (National Institute of Health, USA).

2.6. 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. 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 using GAPDH as housekeeping gene. Primer sequences are shown in Supplementary Table 1. Relative gene expression quantification was analyzed with the mathematical model described in [26].

2.7. Statistical analysis

All data presented are means ± SE. One-way ANOVA with Bonferroni's t-test correction was used to determine statistical significance ($p < 0.05$).

3. Results and discussion

3.1. Analysis of PAH content in air PM extracts

The PAH content in the two air PM extracts was determined using our recently developed methodology that is capable of quantifying 42 unique PAHs of between 3 and 6 rings [6, 24] (Supplementary Table 2). PAHs at the LIMEIRA sampling site mainly result from heavy traffic (including cars and trucks), industrial emissions and biomass burning, whereas those at the STHLM site can mainly be attributed to heavy traffic emissions. The results showed that the air PM extract from Limeira (LIMEIRA) had approximately 3-fold higher total PAH content than the extract from Stockholm (STHLM) (10169.2 and 3392.6 pg/m^3, respectively), in agreement with recent data [24]. The level of BP was approximately 5-fold higher in the LIMEIRA sample (807 pg/m^3) compared to the STHLM sample (160 pg/m^3). The level of the highly potent dibenzo[*a*,*l*]pyrene was approximately 9-fold higher in the LIMEIRA sample (9.07 pg/m^3) compared to the STHLM sample (1.05 pg/m^3).

In order to compare the cellular response to the two PAH extracts and with BP alone, the extracts were prepared in DMSO to contain a concentration equivalent of 1 µM BP (BP_{eq}) (actual 0.91 and 1.10 µM for STHLM and LIMEIRA respectively, Supplementary Table 2). Cells were exposed to the PAH extracts with a final concentration of 1 nM BP_{eq}, and hence, cells were also exposed to 1 nM BP alone for comparative purposes. The final total PAH concentration the cells were exposed to was 21.6 and 14.4 nM for the STHLM and LIMEIRA extracts respectively. When assessing human exposure to PAHs in ambient air BP is often used as a surrogate indicator [27, 28] and thus, normalizing the PAH extracts to a set concentration of BP allows us to evaluate how well the BP content can predict the observed effects. Neither the Stockholm [6] nor the Limeira extract (data not shown) demonstrated cytotoxicity in the HepG2 cell line.

3.2. Nanomolar levels of PAH extracts activate JNK signaling

Activation of DNA damage signaling in cells after exposure to nanomolar concentrations of the STHLM extract has been demonstrated previously [6]. To identify regulatory pathways involved in different cellular responses including inflammation, apoptosis and cell cycle control, we assessed activation of MAPK proteins that have previously been shown to be activated in response to PAHs [29-32]. The results showed a significant increase in levels of phosphorylated JNK (pJNK) whilst no significant effect was observed on total JNK protein or on phosphorylation of Erk (pERK) or p38 (pp38) (Figure 1). Phosphorylation of JNK in response to the two PAH extracts followed different kinetics with the STHLM extract inducing a persistent increase in activation throughout the 12 hours whereas the LIMEIRA extract induced the highest levels of pJNK after 3 h which decreased thereafter. It is probable that this difference in trends is a result of different extract composition though further

investigation is required to confirm this. Both extracts induced a stronger activation of JNK than exposure to 1 nM BP alone (Figure 1).

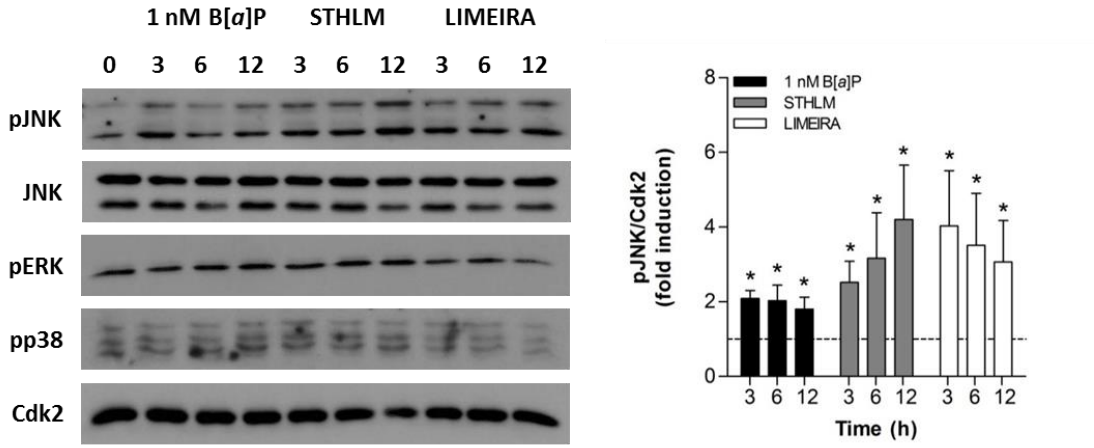

Figure 1. Nanomolar concentrations of PAH extracts activate JNK signaling. Cells were exposed to the PAH extracts (1 nM BP_{eq}) or 1 nM BP alone. Total and phosphorylated levels of the indicated proteins were determined by Western blot at 3, 6 and 12 h. Cdk2 was used as a loading control. Densitometric analysis is shown to the right. *$p<0.05$ compared with DMSO control levels.

3.3. AP-1 transcription factors are activated in response to nanomolar levels of PAH extracts

ATF2 and cJun are important proteins that form part of AP-1 and that are activated downstream of JNK [33, 34]. In agreement with the increased levels of pJNK, we observed increased phosphorylation of ATF2 and cJun (Figure 2A). Levels of phosphorylated ATF2 (pATF2) showed a significant increase in response to both BP_{eq} extracts, with the STHLM extract inducing higher and more sustained levels of pATF2 than the LIMEIRA extract, displaying >8-fold higher levels than control up to 12 h post-exposure. Levels of phosphorylated cJun (pcJun) displayed the same pattern as pATF2 with the highest levels in response to the STHLM extract (Figure 2A). Similar to the effect on pJNK, both extracts

induced higher levels of phosphorylation of ATF2 and cJun compared to 1 nM BP alone (Figure 2A). As shown in Figure 2B, significant increase in level of pATF2 could be detected in response to the STHLM extract at BP_{eq} concentrations down to 0.1 nM (1.8-fold, $p = 0.03$, total PAH concentration = 2.2 nM). These results suggest that pATF2 could be a sensitive biological marker for activation of MAPK signaling in response to PAHs. ATM-mediated activation of ATF2 by phosphorylation at Ser490/498 is implicated in the DNA damage response [35] and the possible role of ATF2 connecting MAPK and DNA damage signaling in response to PAHs is interesting.

The results presented here show clear differences in the observed responses between the PAH extracts. The signaling was also in general much stronger in response to the PAH extracts compared to BP alone, similar to what we have reported earlier for DNA damage signaling [6]. Since the total PAH content was quite similar for the 1 nM BP_{eq} STHLM and LIMEIRA extracts this implies that the observed differences more likely are due to sample composition. An explanation for this observation could be that there are different PAHs present in the STHLM extract that are more potent MAPK inducers than BP. Alternatively, substituted PAHs such as nitro-PAHs, which are known to induce stress signaling [36], might also contribute to the effects of the extracts. In addition, non-PAH compounds could affect the response of the extracts. The level of polychlorinated biphenyls were approximately 0.5 % of the PAH level (pg/m^3) in the STHLM extract (unpublished data) and thus are predicted to have minimal effects. Other polychlorinated compounds such as dibenzo-dioxins and furans may also contribute to the overall effect of the extracts [37, 38]. Metals are also known to be present in environmental air PM, and although the levels of metals in our extracts is unknown, we speculate that due to the sampling and processing methods the levels are likely to be low [11].

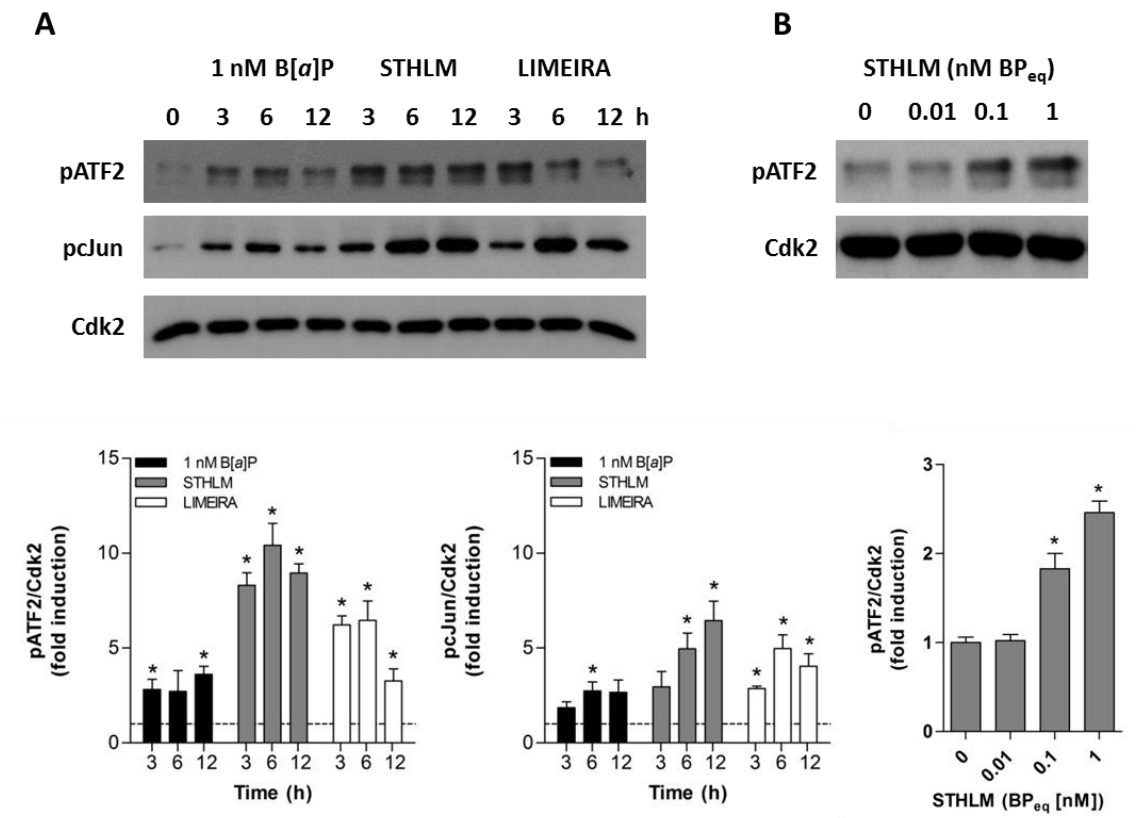

Figure 2. AP-1 transcription factors are activated in response to nanomolar levels of PAH extracts. Cells were exposed to the PAH extracts (1 nM BP_{eq}) or 1 nM BP alone. Total and phosphorylated levels of the indicated proteins were determined by Western blot at 3, 6 and 12 h (A). Cells were exposed to decreasing concentrations of the STHLM extract and level of pATF2 was assessed at 12 h (B). Cdk2 was used as a loading control. Densitometric analysis is shown below. *p<0.05 compared with DMSO control levels.

3.4. Activation of JNK/AP-1 signaling is mediated through MEK4

MEK4 has previously been shown to be involved in regulating pro-apoptotic signaling in human 293T and HeLa cells and rat F258 cells in response to BP treatment, albeit at higher concentrations than used here, leading to downstream caspase activation [39, 40]. However, it has not been established if MEK4 is activated in response to levels of PAHs found in urban air. The results showed significantly increased levels of phosphorylated MEK4 (pMEK4)

after exposure to both extracts and 1 nM BP, though highest levels were observed after 12 h exposure to the STHLM extract (4.5-fold, $p = 0.028$, Figure 3A). No changes were observed on levels of total MEK4. These results clearly show that MEK4 is activated in response to nanomolar levels of PAHs in mixtures and that the levels are higher compared to BP alone.

To determine if JNK/AP-1 activation was dependent on activation of MEK4 HepG2 cells were exposed to the STHLM extract for 12 hours following pre-treatment with siRNA against MEK4 (Figure 3B). Based on the effects in the time-response experiments shown in Figure 3A, only exposure to the STHLM extract was investigated with siRNA. Transfecting the cells with siRNA against MEK4 significantly reduced the levels of total MEK4 protein resulting in significantly reduced signaling through JNK, ATF2 and cJun (Figure 3B). No effect was observed with control siRNA confirming that the observed response resulted from interference of MEK4 and was not a by-product of the transfection procedure. Taken together, these data show that activation of JNK/AP-1 signaling in response to environmental PAH extracts results from activation of the upstream MAPK kinase MEK4. To our knowledge this is the first time MEK4 is identified as a mediator of stress signaling in response to concentrations of PAHs found in urban air.

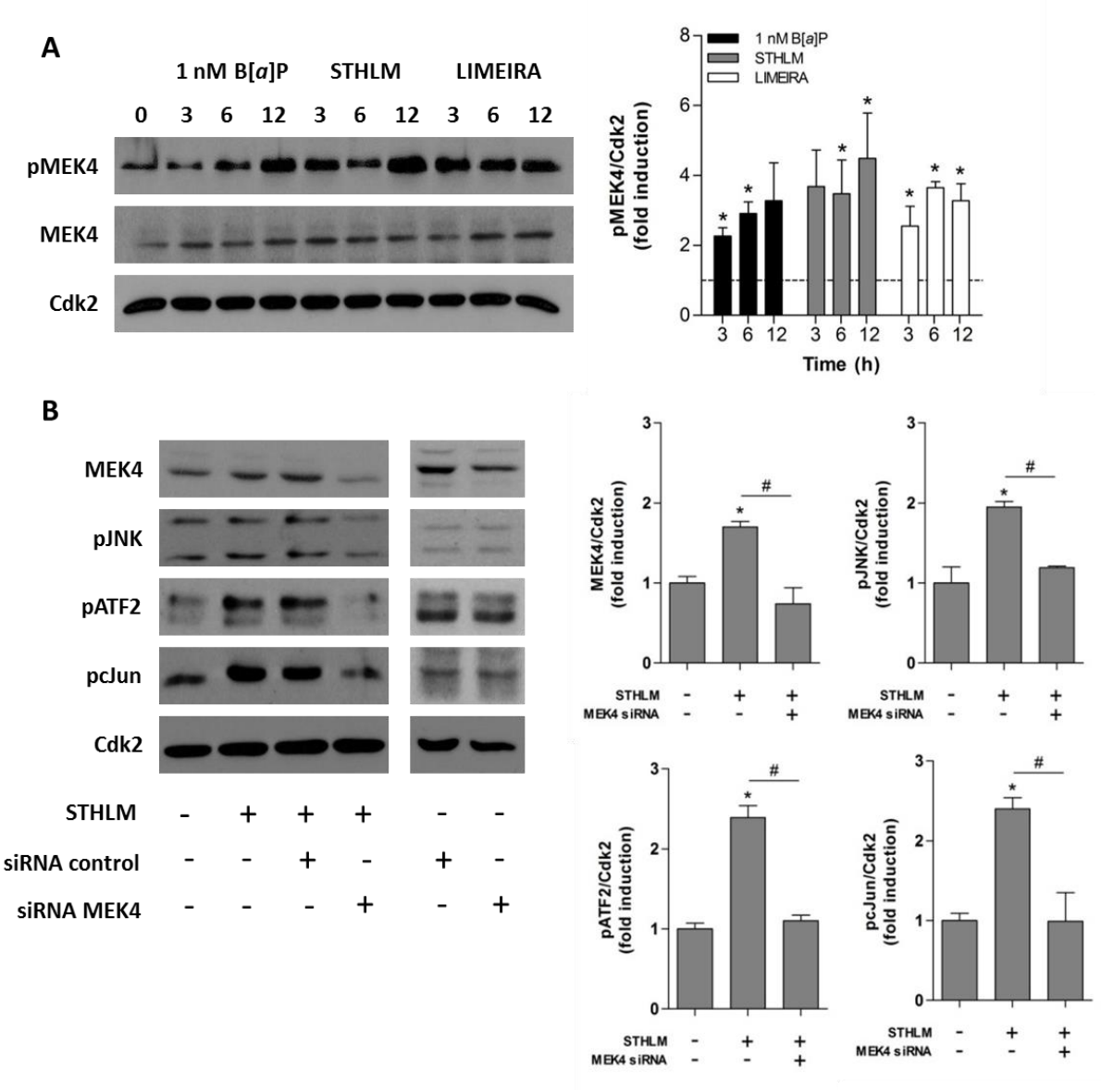

Figure 3. MEK4 mediates signaling through MAPK leading to activation of AP-1 transcription factors. Cells were exposed to the PAH extracts (1 nM BP_{eq}) or 1 nM BP alone and total and phosphorylated levels of MEk4 were determined by Western blot at 3, 6 and 12 h (A). Cells transfected with either mock siRNA or siRNA against MEK4 were exposed to the STHLM extract (1 nM BP_{eq}) for 12 h and effects on phosphorylation status of JNK, ATF2 and cJun were assessed by Western blot (B). Cdk2 was used as a loading control. Densitometric analysis is shown to the right. *$p<0.05$ compared with DMSO control levels. $^{\#}p<0.05$ compared with STHLM treatment.

3.5. JNK mediates transactivation of cellular stress mediators through AP-1

To confirm the role of activation of JNK signaling in activation of AP-1 proteins in response to PAH extracts, HepG2 cells were pre-treated for 1 h with a JNK inhibitor (JNKi, 20 µM) [41] followed by exposure to the STHLM extract for 12 h (Figure 4). Significantly reduced levels of both pATF2 and pcJun were observed confirming the involvement of JNK in activation of ATF2 and cJun in this study. JNK and cJun have previously been reported to be mediators of cellular stress responses to PAHs [42, 43]. However, this is the first time that activation of ATF2 can be linked to cellular stress signaling in response to PAHs.

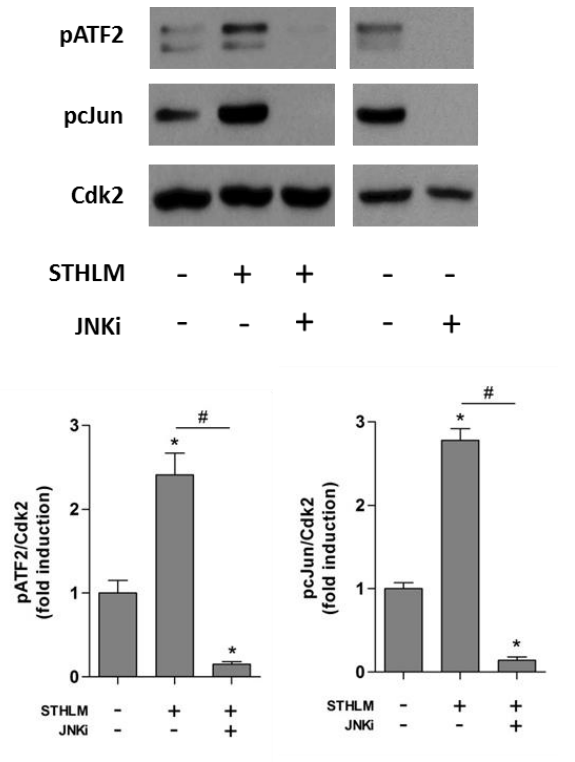

Figure 4. Activation of AP-1 transcription factors is mediated through JNK. Cells pretreated for 1 h with JNKi (20 µM in DMSO) were exposed to the STHLM extract (1 nM BP_{eq}) for 12 h and effects on phosphorylation status of ATF2 and cJun was assessed by Western blot. Densitometric analysis is shown below. *$p<0.05$ compared with DMSO control levels. $^{\#}p<0.05$ compared with STHLM treatment.

To investigate if activation of MAPK signaling in response to nanomolar levels of PAH extracts would result in induction of cellular stress mediators, effects on expression of MAPK regulated genes were examined by qRT-PCR (Figure 5). The results showed significant effects on the mRNA levels of tumor necrosis factor alpha (TNF), interleukin 8 (IL-8), tumor necrosis factor receptor superfamily member 6 (TNFR6 or FAS) and, epidermal growth factor receptor (EGFR). Expression levels of TNF and IL-8 displayed the strongest response with 13.3- and 5.7-fold up-regulation at 12 h, respectively. These genes are transactivated via MAPK signaling and involved in stimulating inflammation, proliferation or apoptosis and have all been shown to be play important roles in regulation of human diseases such as cancer (reviewed in [13, 14]. Moreover, in the presence of JNKi, mRNA levels of TNF, IL-8 and FAS were significantly reduced 12 h post treatment to the level of the DMSO control. Although the mRNA levels of EGFR were reduced in response to blocked JNK signaling 24 h post treatment, no significant effects were observed. These results confirm the involvement of MAPK signaling in transactivation of cellular stress mediators in response to nanomolar levels of PAH mixtures.

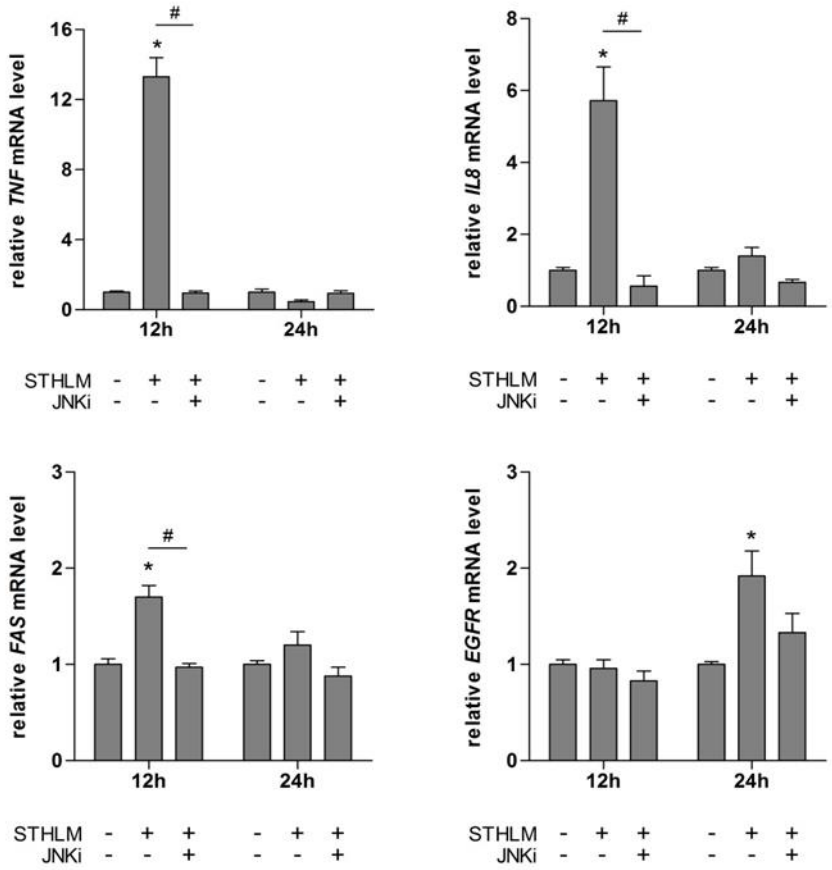

Figure 5. Induction of gene expression of stress response mediators is mediated through a MEK4/JNK/AP-1 signaling pathway. Cells were exposed to the STHLM extract (1 nM BP_{eq}) for 12 h with or without JNKi pretreatment and effects on gene expression were determined by qRT-PCR. *p<0.05 compared with DMSO control levels. #p<0.05 compared with STHLM treatment.

3.6. Comparison with toxic equivalency factors

In order to compare the extracts with BP they were prepared to have a final exposure concentration equivalent to 1 nM BP. An alternative approach is to derive BP equivalent concentrations using toxic equivalency factor (TEF) scales. Using previously published TEF values [44] we calculated BP_{TEQ} concentrations of 1.74 and 1.46 for the STHLM and LIMEIRA samples. Data for fold difference for pATF2, pcJun, pJNK and pMEK4 are given in Supplementary Table 3 and are presented as fold differences compared to BP. As can be

seen, the fold differences for pATF2 are higher than predicted based on BP_{TEQ} concentrations of the PAH extracts, emphasizing pATF2 as a sensitive biological marker for activation of MAPK signaling following PAH exposure. An explanation for this observation is that there could be different PAHs present in the extracts that are more potent inducers of the stress response than BP. Fold differences were similar to what would be predicted for pcJun, pJNK and pMEK4 based on BP_{TEQ} concentrations. However, it should be noted that there are a number of important considerations and requirements that make the TEF-based approach insufficient for mixture assessment as discussed recently [12].

4. Conclusion

Here we report that nanomolar concentrations of environmental PAH mixtures, extracted from urban air PM collected in Stockholm, Sweden and Limeira, Brazil, induce a time-dependent activation of MAPK signaling in HepG2 cells. We show that exposure to low levels of PAH extracts more strongly activates signaling pathways compared to BP alone suggesting possible effects from interactions. Exposure to the PAH extracts induces activation of MEK4 signaling with down-stream increased expression of several important stress response mediators. Abrogation of the MEK4-JNK pathway using siRNA and a specific inhibitor against JNK significantly reduces the transactivation mediated through AP-1 transcription factors ATF2 and cJun. This is the first study showing activation of MEK4/JNK/AP-1 pathway in in response to nanomolar levels of PAHs in environmental mixtures.

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

Jarvis IWH[a], Bergvall C[b], Morales DA[c], Kummrow F[d], Umbuzeiro GA[c], Westerholm R[b], Stenius U[a] and Dreij K[a]*

[a]Institute of Environmental Medicine, Karolinska Institutet, Box 210, SE-171 77 Stockholm, Sweden.

[b]Department of Analytical Chemistry, Stockholm University, Svante Arrhenius väg 16, SE-106 91 Stockholm, Sweden.

[c]Faculty of Technology, State University of Campinas (UNICAMP), Limeira, SP, 13484-332, Brazil.

[d]Institute of Environmental, Chemical and Pharmaceutical Sciences, Federal University of São Paulo (UNIFESP), Diadema, SP, 09972-270, Brazil.

***Corresponding Author**

Kristian Dreij, PhD

Address: Institute of Environmental Medicine, Karolinska Institutet, Box 210, SE-171 77 Stockholm, Sweden. Email: Kristian.Dreij@ki.se Tel: +46 8 524 87566

Running Title

Environmental PAH mixtures activate MAPK signaling

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;

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 cJun. 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.

Key Words

PAHs, Air particulate matter, Complex mixtures, Benzo[*a*]pyrene, MAPK

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 [1, 2]. 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) [3]. PAHs are ubiquitous environmental pollutants that are naturally present as mixtures and 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 [4]. 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 [5-8], though conversely, strong antagonistic effects have also been observed in human cells, probably resulting from competitive inhibition of metabolizing enzymes [9-11]. However, the role of interactions between PAHs in complex mixtures in relation to the adverse health effects of PAHs is poorly understood [12].

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 [13, 14]. 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) [15, 16]. The protein MAPK kinase 4 (MEK4/MKK4) specifically activates JNK and p38 [17] and has been identified as an important metastasis suppressor in several organs [18] and a possible target for small molecule inhibition in therapy for tumor necrosis factor alpha (TNF) mediated diseases [19]. 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 [20, 21]. 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 [22, 23]. Gibco (Invitrogen, Paisley, UK) supplied all cell culture reagents. Cell Signaling Technology (Beverly, MA) provided the following antibodies: phospho-ATF2 Thr71, phospho-cJun 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 µg/m^3 (unpublished data). The air PM sample from Limeira was collected at street level on a glass-fiber filter (254 × 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 1130 l min^{-1} for 24 h. The total PM concentration in the Limeira sample was 95.8 µg/m^3 [24]. 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 [6]. PAH content in the extracts was determined by HPLC-GC/MS as described previously [6, 22].

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 [25] and a previously demonstrated response to low levels of PAHs (in mixtures) extracted from environmental samples by ourselves and others [5-7]. 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 3×10^5 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 [6]. 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 analyzed separately. Densitometric analysis was performed using ImageJ software version 1.45s (National Institute of Health, USA).

2.6. 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

644 Mix (Fermentas, St. Leon-Rot, Germany) with detection on an Applied Biosystems 7500
645 Real-Time PCR System. The reaction cycles used were 95 °C for 2 min, and then 40 cycles at
646 95 °C for 15 s and 60 °C for 1 min followed by melt curve analysis using GAPDH as
647 housekeeping gene. Primer sequences are shown in Supplementary Table 1. Relative gene
648 expression quantification was analyzed with the mathematical model described in [26].
649
650 **2.7. Statistical analysis**
651 All data presented are means ± SE. One-way ANOVA with Bonferroni's t-test correction was
652 used to determine statistical significance ($p < 0.05$).
653
654 **3. Results and discussion**
655
656 **3.1. Analysis of PAH content in air PM extracts**
657 The PAH content in the two air PM extracts was determined using our recently developed
658 methodology that is capable of quantifying 42 unique PAHs of between 3 and 6 rings [6, 24]
659 (Supplementary Table 2). PAHs at the LIMEIRA sampling site mainly result from heavy
660 traffic (including cars and trucks), industrial emissions and biomass burning, whereas those at
661 the STHLM site can mainly be attributed to heavy traffic emissions. The results showed that
662 the air PM extract from Limeira (LIMEIRA) had approximately 3-fold higher total PAH
663 content than the extract from Stockholm (STHLM) (10169.2 and 3392.6 pg/m^3, respectively),
664 in agreement with recent data [24]. The level of BP was approximately 5-fold higher in the
665 LIMEIRA sample (807 pg/m^3) compared to the STHLM sample (160 pg/m^3). The level of the
666 highly potent dibenzo[*a*,*l*]pyrene was approximately 9-fold higher in the LIMEIRA sample
667 (9.07 pg/m^3) compared to the STHLM sample (1.05 pg/m^3).

In order to compare the cellular response to the two PAH extracts and with BP alone, the extracts were prepared in DMSO to contain a concentration equivalent of 1 µM BP (BP_{eq}) (actual 0.91 and 1.10 µM for STHLM and LIMEIRA respectively, Supplementary Table 2). Cells were exposed to the PAH extracts with a final concentration of 1 nM BP_{eq}, and hence, cells were also exposed to 1 nM BP alone for comparative purposes. The final total PAH concentration the cells were exposed to was 21.6 and 14.4 nM for the STHLM and LIMEIRA extracts respectively. When assessing human exposure to PAHs in ambient air BP is often used as a surrogate indicator [27, 28] and thus, normalizing the PAH extracts to a set concentration of BP allows us to evaluate how well the BP content can predict the observed effects. Neither the Stockholm [6] nor the Limeira extract (data not shown) demonstrated cytotoxicity in the HepG2 cell line.

3.2. Nanomolar levels of PAH extracts activate JNK signaling

Activation of DNA damage signaling in cells after exposure to nanomolar concentrations of the STHLM extract has been demonstrated previously [6]. To identify regulatory pathways involved in different cellular responses including inflammation, apoptosis and cell cycle control, we assessed activation of MAPK proteins that have previously been shown to be activated in response to PAHs [29-32]. The results showed a significant increase in levels of phosphorylated JNK (pJNK) whilst no significant effect was observed on total JNK protein or on phosphorylation of Erk (pERK) or p38 (pp38) (Figure 1). Phosphorylation of JNK in response to the two PAH extracts followed different kinetics with the STHLM extract inducing a persistent increase in activation throughout the 12 hours whereas the LIMEIRA extract induced the highest levels of pJNK after 3 h which decreased thereafter. It is probable that this difference in trends is a result of different extract composition though further

investigation is required to confirm this. Both extracts induced a stronger activation of JNK than exposure to 1 nM BP alone (Figure 1).

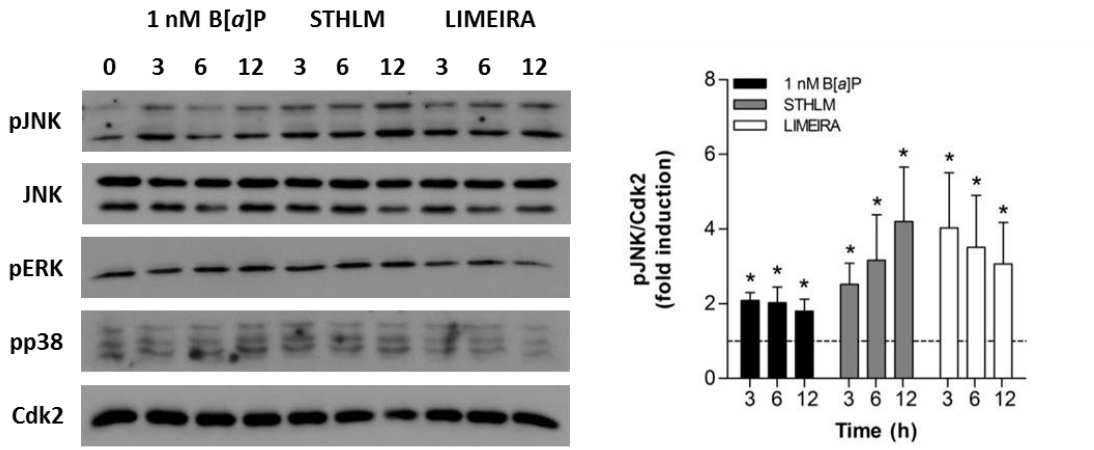

Figure 1. Nanomolar concentrations of PAH extracts activate JNK signaling. Cells were exposed to the PAH extracts (1 nM BP_{eq}) or 1 nM BP alone. Total and phosphorylated levels of the indicated proteins were determined by Western blot at 3, 6 and 12 h. Cdk2 was used as a loading control. Densitometric analysis is shown to the right. *$p<0.05$ compared with DMSO control levels.

3.3. AP-1 transcription factors are activated in response to nanomolar levels of PAH extracts

ATF2 and cJun are important proteins that form part of AP-1 and that are activated downstream of JNK [33, 34]. In agreement with the increased levels of pJNK, we observed increased phosphorylation of ATF2 and cJun (Figure 2A). Levels of phosphorylated ATF2 (pATF2) showed a significant increase in response to both BP_{eq} extracts, with the STHLM extract inducing higher and more sustained levels of pATF2 than the LIMEIRA extract, displaying >8-fold higher levels than control up to 12 h post-exposure. Levels of phosphorylated cJun (pcJun) displayed the same pattern as pATF2 with the highest levels in response to the STHLM extract (Figure 2A). Similar to the effect on pJNK, both extracts

induced higher levels of phosphorylation of ATF2 and cJun compared to 1 nM BP alone (Figure 2A). As shown in Figure 2B, significant increase in level of pATF2 could be detected in response to the STHLM extract at BP_{eq} concentrations down to 0.1 nM (1.8-fold, $p = 0.03$, total PAH concentration = 2.2 nM). These results suggest that pATF2 could be a sensitive biological marker for activation of MAPK signaling in response to PAHs. ATM-mediated activation of ATF2 by phosphorylation at Ser490/498 is implicated in the DNA damage response [35] and the possible role of ATF2 connecting MAPK and DNA damage signaling in response to PAHs is interesting.

The results presented here show clear differences in the observed responses between the PAH extracts. The signaling was also in general much stronger in response to the PAH extracts compared to BP alone, similar to what we have reported earlier for DNA damage signaling [6]. Since the total PAH content was quite similar for the 1 nM BP_{eq} STHLM and LIMEIRA extracts this implies that the observed differences more likely are due to sample composition. An explanation for this observation could be that there are different PAHs present in the STHLM extract that are more potent MAPK inducers than BP. Alternatively, substituted PAHs such as nitro-PAHs, which are known to induce stress signaling [36], might also contribute to the effects of the extracts. In addition, non-PAH compounds could affect the response of the extracts. The level of polychlorinated biphenyls were approximately 0.5 % of the PAH level (pg/m^3) in the STHLM extract (unpublished data) and thus are predicted to have minimal effects. Other polychlorinated compounds such as dibenzo-dioxins and furans may also contribute to the overall effect of the extracts [37, 38]. Metals are also known to be present in environmental air PM, and although the levels of metals in our extracts is unknown, we speculate that due to the sampling and processing methods the levels are likely to be low [11].

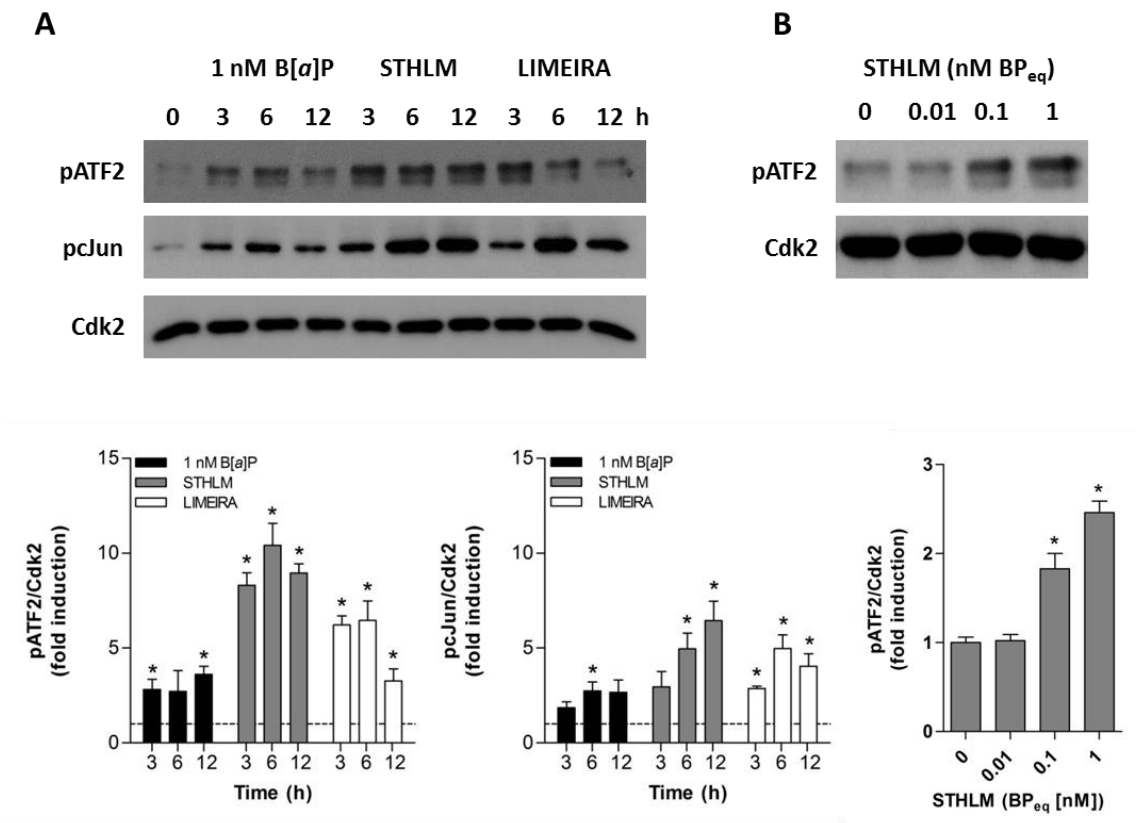

Figure 2. AP-1 transcription factors are activated in response to nanomolar levels of PAH extracts. Cells were exposed to the PAH extracts (1 nM BP_{eq}) or 1 nM BP alone. Total and phosphorylated levels of the indicated proteins were determined by Western blot at 3, 6 and 12 h (A). Cells were exposed to decreasing concentrations of the STHLM extract and level of pATF2 was assessed at 12 h (B). Cdk2 was used as a loading control. Densitometric analysis is shown below. *$p<0.05$ compared with DMSO control levels.

3.4. Activation of JNK/AP-1 signaling is mediated through MEK4

MEK4 has previously been shown to be involved in regulating pro-apoptotic signaling in human 293T and HeLa cells and rat F258 cells in response to BP treatment, albeit at higher concentrations than used here, leading to downstream caspase activation [39, 40]. However, it has not been established if MEK4 is activated in response to levels of PAHs found in urban air. The results showed significantly increased levels of phosphorylated MEK4 (pMEK4)

751 after exposure to both extracts and 1 nM BP, though highest levels were observed after 12 h
752 exposure to the STHLM extract (4.5-fold, $p = 0.028$, Figure 3A). No changes were observed
753 on levels of total MEK4. These results clearly show that MEK4 is activated in response to
754 nanomolar levels of PAHs in mixtures and that the levels are higher compared to BP alone.

755 To determine if JNK/AP-1 activation was dependent on activation of MEK4 HepG2
756 cells were exposed to the STHLM extract for 12 hours following pre-treatment with siRNA
757 against MEK4 (Figure 3B). Based on the effects in the time-response experiments shown in
758 Figure 3A, only exposure to the STHLM extract was investigated with siRNA. Transfecting
759 the cells with siRNA against MEK4 significantly reduced the levels of total MEK4 protein
760 resulting in significantly reduced signaling through JNK, ATF2 and cJun (Figure 3B). No
761 effect was observed with control siRNA confirming that the observed response resulted from
762 interference of MEK4 and was not a by-product of the transfection procedure. Taken together,
763 these data show that activation of JNK/AP-1 signaling in response to environmental PAH
764 extracts results from activation of the upstream MAPK kinase MEK4. To our knowledge this
765 is the first time MEK4 is identified as a mediator of stress signaling in response to
766 concentrations of PAHs found in urban air.

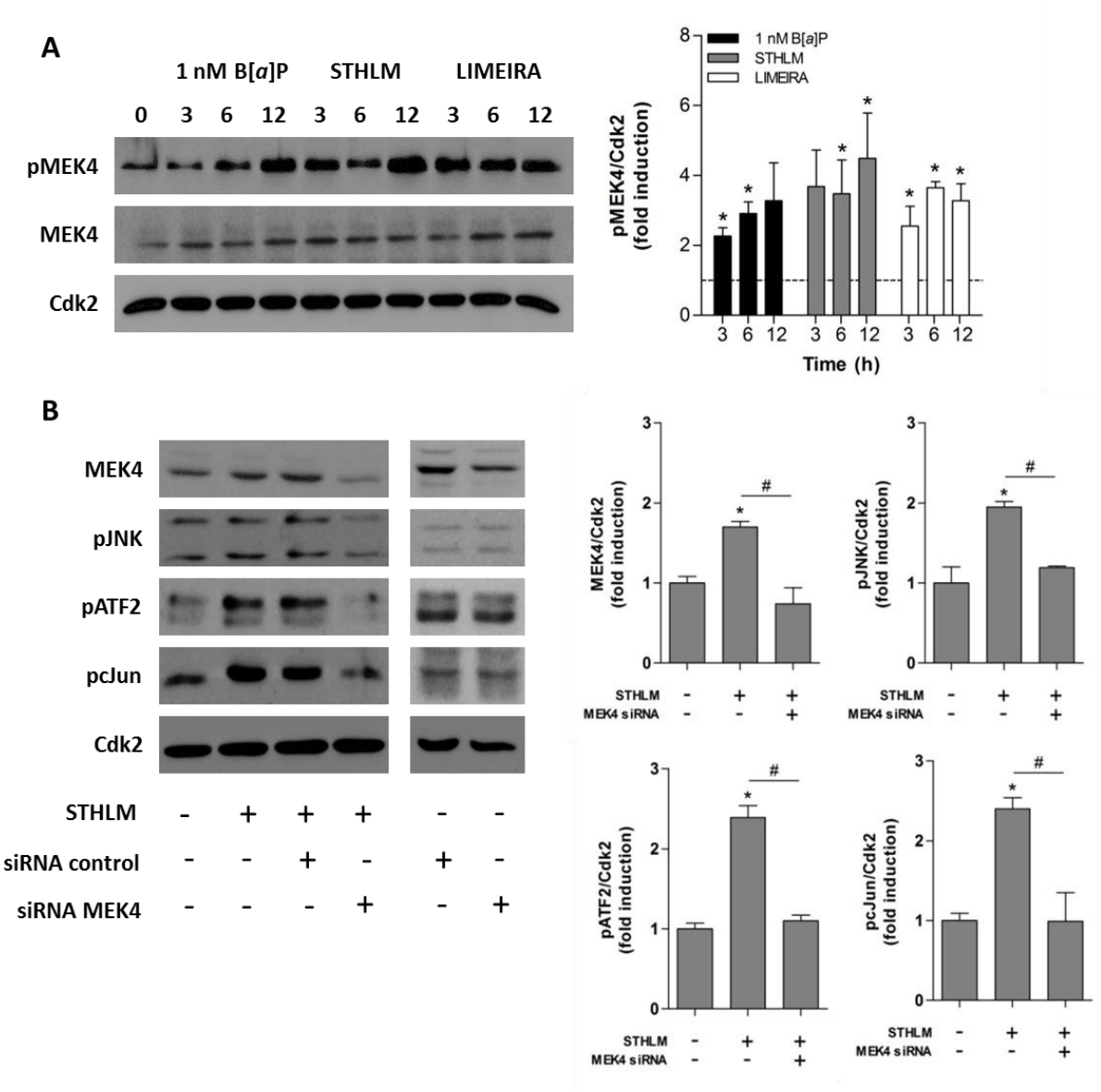

Figure 3. MEK4 mediates signaling through MAPK leading to activation of AP-1 transcription factors. Cells were exposed to the PAH extracts (1 nM BP_{eq}) or 1 nM BP alone and total and phosphorylated levels of MEk4 were determined by Western blot at 3, 6 and 12 h (A). Cells transfected with either mock siRNA or siRNA against MEK4 were exposed to the STHLM extract (1 nM BP_{eq}) for 12 h and effects on phosphorylation status of JNK, ATF2 and cJun were assessed by Western blot (B). Cdk2 was used as a loading control. Densitometric analysis is shown to the right. *$p<0.05$ compared with DMSO control levels. $^{\#}p<0.05$ compared with STHLM treatment.

3.5. JNK mediates transactivation of cellular stress mediators through AP-1

To confirm the role of activation of JNK signaling in activation of AP-1 proteins in response to PAH extracts, HepG2 cells were pre-treated for 1 h with a JNK inhibitor (JNKi, 20 µM) [41] followed by exposure to the STHLM extract for 12 h (Figure 4). Significantly reduced levels of both pATF2 and pcJun were observed confirming the involvement of JNK in activation of ATF2 and cJun in this study. JNK and cJun have previously been reported to be mediators of cellular stress responses to PAHs [42, 43]. However, this is the first time that activation of ATF2 can be linked to cellular stress signaling in response to PAHs.

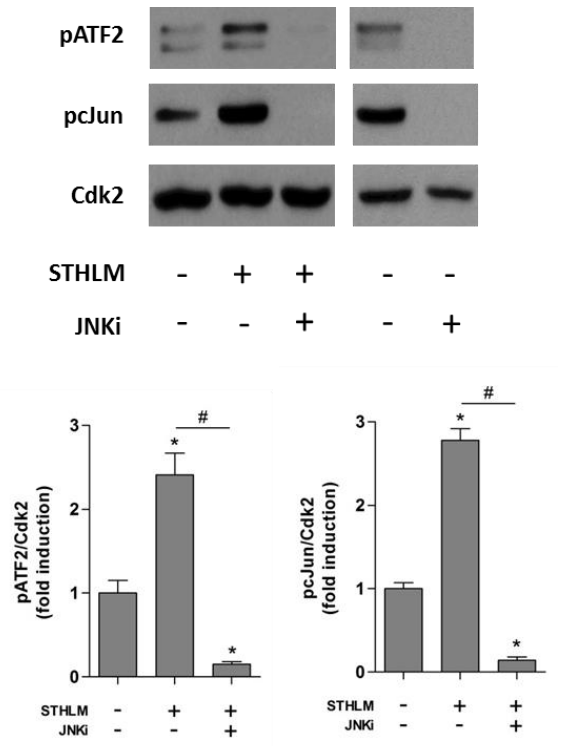

Figure 4. Activation of AP-1 transcription factors is mediated through JNK. Cells pretreated for 1 h with JNKi (20 µM in DMSO) were exposed to the STHLM extract (1 nM BP_{eq}) for 12 h and effects on phosphorylation status of ATF2 and cJun was assessed by Western blot. Densitometric analysis is shown below. *$p<0.05$ compared with DMSO control levels. $^{\#}p<0.05$ compared with STHLM treatment.

To investigate if activation of MAPK signaling in response to nanomolar levels of PAH extracts would result in induction of cellular stress mediators, effects on expression of MAPK regulated genes were examined by qRT-PCR (Figure 5). The results showed significant effects on the mRNA levels of tumor necrosis factor alpha (TNF), interleukin 8 (IL-8), tumor necrosis factor receptor superfamily member 6 (TNFR6 or FAS) and, epidermal growth factor receptor (EGFR). Expression levels of TNF and IL-8 displayed the strongest response with 13.3- and 5.7-fold up-regulation at 12 h, respectively. These genes are transactivated via MAPK signaling and involved in stimulating inflammation, proliferation or apoptosis and have all been shown to be play important roles in regulation of human diseases such as cancer (reviewed in [13, 14]. Moreover, in the presence of JNKi, mRNA levels of TNF, IL-8 and FAS were significantly reduced 12 h post treatment to the level of the DMSO control. Although the mRNA levels of EGFR were reduced in response to blocked JNK signaling 24 h post treatment, no significant effects were observed. These results confirm the involvement of MAPK signaling in transactivation of cellular stress mediators in response to nanomolar levels of PAH mixtures.

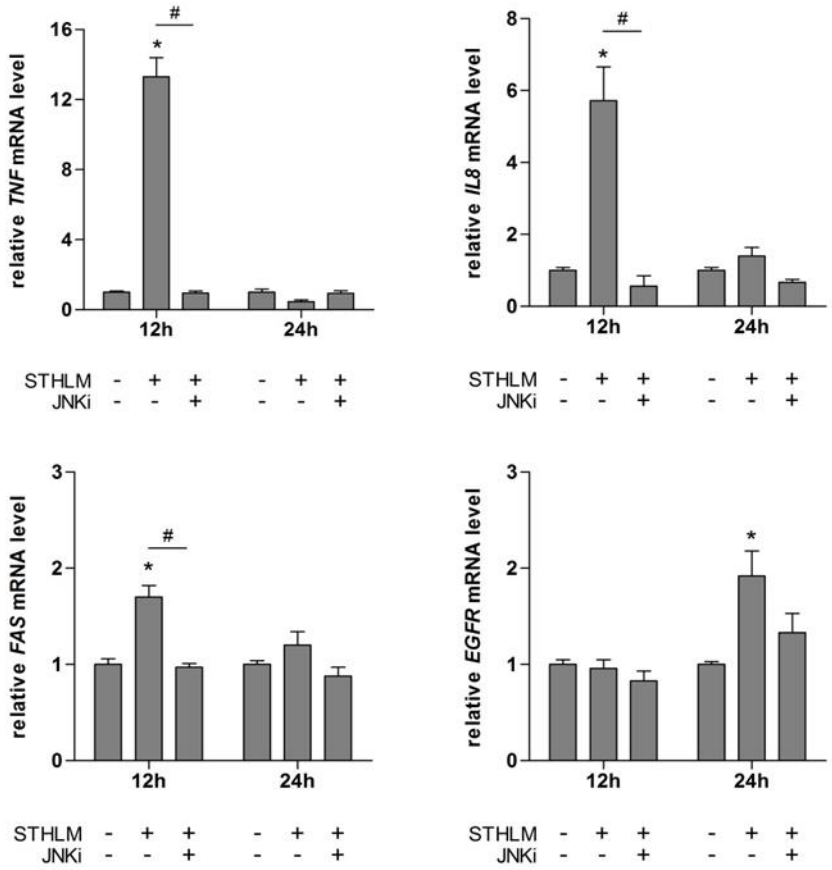

Figure 5. Induction of gene expression of stress response mediators is mediated through a MEK4/JNK/AP-1 signaling pathway. Cells were exposed to the STHLM extract (1 nM BP_{eq}) for 12 h with or without JNKi pretreatment and effects on gene expression were determined by qRT-PCR. *$p<0.05$ compared with DMSO control levels. $^{\#}p<0.05$ compared with STHLM treatment.

3.6. Comparison with toxic equivalency factors

In order to compare the extracts with BP they were prepared to have a final exposure concentration equivalent to 1 nM BP. An alternative approach is to derive BP equivalent concentrations using toxic equivalency factor (TEF) scales. Using previously published TEF values [44] we calculated BP_{TEQ} concentrations of 1.74 and 1.46 for the STHLM and LIMEIRA samples. Data for fold difference for pATF2, pcJun, pJNK and pMEK4 are given in Supplementary Table 3 and are presented as fold differences compared to BP. As can be

822 seen, the fold differences for pATF2 are higher than predicted based on BP_{TEQ} concentrations
823 of the PAH extracts, emphasizing pATF2 as a sensitive biological marker for activation of
824 MAPK signaling following PAH exposure. An explanation for this observation is that there
825 could be different PAHs present in the extracts that are more potent inducers of the stress
826 response than BP. Fold differences were similar to what would be predicted for pcJun, pJNK
827 and pMEK4 based on BP_{TEQ} concentrations. However, it should be noted that there are a
828 number of important considerations and requirements that make the TEF-based approach
829 insufficient for mixture assessment as discussed recently [12].
830
831 **4. Conclusion**
832 Here we report that nanomolar concentrations of environmental PAH mixtures, extracted
833 from urban air PM collected in Stockholm, Sweden and Limeira, Brazil, induce a time-
834 dependent activation of MAPK signaling in HepG2 cells. We show that exposure to low
835 levels of PAH extracts more strongly activates signaling pathways compared to BP alone
836 suggesting possible effects from interactions. Exposure to the PAH extracts induces activation
837 of MEK4 signaling with down-stream increased expression of several important stress
838 response mediators. Abrogation of the MEK4-JNK pathway using siRNA and a specific
839 inhibitor against JNK significantly reduces the transactivation mediated through AP-1
840 transcription factors ATF2 and cJun. This is the first study showing activation of
841 MEK4/JNK/AP-1 pathway in in response to nanomolar levels of PAHs in environmental
842 mixtures.
843
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