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

3

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20

21 **Running Title**
22 Environmental PAH mixtures activate MAPK signaling

23

24 **Abbreviations**

25 AP-1, activator protein 1; BP, benzo[*a*]pyrene; ERK, extracellular regulated kinase; JNK, c-
26 Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEK4, MAPK kinase 4;
27 PAH, polycyclic aromatic hydrocarbon; PM, particulate matter; TEF, toxic equivalency
28 factor; TNF, tumor necrosis factor;
29

30 **Abstract**

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

47

48 **Key Words**

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

50

51 **1. Introduction**

52 Human exposure to environmental pollutants in air particulate matter (PM) has been
53 identified to cause a number of adverse health effects including cancer and various
54 cardiovascular and respiratory diseases [1, 2]. One important group of environmental

55 pollutants that are associated with PM and play an important role in the reported detrimental
56 health effects are the polycyclic aromatic hydrocarbons (PAHs) [3]. PAHs are ubiquitous
57 environmental pollutants that are naturally present as mixtures and are formed during
58 combustion of carbon-containing fuels. Both individual and mixtures of PAHs are classified
59 as carcinogens or probable carcinogens by the International Agency for Research on Cancer
60 [4]. Recent data from us and others have suggested synergistic effects due to interactions
61 between PAHs in complex mixtures on the genotoxic and carcinogenic properties of PAHs [5-
62 8], though conversely, strong antagonistic effects have also been observed in human cells,
63 probably resulting from competitive inhibition of metabolizing enzymes [9-11]. However, the
64 role of interactions between PAHs in complex mixtures in relation to the adverse health
65 effects of PAHs is poorly understood [12].
66 The activator protein-1 (AP-1) transcription factor is a dimeric complex comprising
67 members of the ATF, FOS, JUN and MAF protein families that has functions in many areas
68 of cellular homeostasis [13, 14]. In response to cellular stress stimuli AP-1 proteins are
69 activated by the mitogen-activated protein kinase (MAPK) family of proteins, including c-Jun
70 N-terminal kinase (JNK), p38 and extracellular signal-regulated kinase (ERK) [15, 16]. The
71 protein MAPK kinase 4 (MEK4/MKK4) specifically activates JNK and p38 [17] and has been
72 identified as an important metastasis suppressor in several organs [18] and a possible target
73 for small molecule inhibition in therapy for tumor necrosis factor alpha (TNF) mediated
74 diseases [19]. The present study focuses on activation of MAPK signaling in response to
75 environmental mixtures of PAHs compared to single benzo[*a*]pyrene (BP) exposure in
76 human-derived hepatoma (HepG2) cells. It has previously been shown that *in vitro* exposure
77 to BP leads to activation of MAPK signaling associated with apoptotic cell death via p53 [20,
78 21]. However, the role of MAPK signaling in the stress response after exposure to nanomolar
79 concentrations of PAHs in air PM remains unclear.

80 Using PAH-containing air PM extracts from Stockholm, Sweden and Limeira, Brazil
81 at nanomolar concentrations we studied the time-dependent activation of MAPK signaling
82 and downstream gene expression in HepG2 cells. Our results show that nanomolar
83 concentrations of PAH extracts more strongly activate MAPK signaling and proteins of the
84 AP-1 transcription factor than BP alone suggesting effects due to interactions and that this
85 activation is mediated via MEK4 and JNK. Furthermore, the transactivation of cellular stress
86 mediators including interleukin 8 in response to PAH extract was shown to be mediated
87 through a MAPK. To the authors knowledge this is the first study to demonstrate activation of
88 MEK4/JNK/AP-1 with downstream increased gene expression in the cellular stress response
89 after exposure to nanomolar levels of PAHs found in air PM.
90

91 **2. Materials and Methods**

92

93 **2.1. Reagents and antibodies**

94 Unless otherwise stated all chemicals, including BP, were of analytical grade and obtained
95 from Sigma Aldrich (Stockholm, Sweden). Detailed information on manufacturer and purity
96 of the standards and solvents used for PAH analysis have been published previously [22, 23].
97 Gibco (Invitrogen, Paisley, UK) supplied all cell culture reagents. Cell Signaling Technology
98 (Beverly, MA) provided the following antibodies: phospho-ATF2 Thr71, phospho-cJun
99 Ser63, JNK, phospho-JNK Thr183/Tyr185, MEK4, phospho-MEK4 Thr261. Santa Cruz
100 Biotechnology (Santa Cruz, CA, USA) provided the Cdk2, phospho-Erk Tyr204 and
101 phospho-p38 Thr180/Tyr182 antibodies, secondary anti-rabbit, anti-mouse and siRNA
102 against MEK4 and control siRNA-A. Calbiochem (Gibbstown, NJ, USA) provided the JNK
103 inhibitor VIII.
104

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 $\mu g/m^3$ (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 $\mu g/m^3$ [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.

130 Prior to exposure cells were seeded at 3×10^5 cells/ml in 6-well plates and cultured for 72 h
131 unless otherwise stated. Cells were exposed to solvent control (0.1 % DMSO), BP, or PAH
132 extracts for up to 24 h.
133

134 **2.4. RNA interference**

135 Transfection of cells was performed using Lipofectamine 2000 reagent (Invitrogen, Paisley,
136 UK). Briefly, cells were seeded into 35 mm culture dishes and after 24 h transfected with 50
137 nM siRNA. siRNA sequences are shown in Supplementary Table 1. After 48 h of incubation
138 cells were exposed to PAHs or solvent control, and then harvested for Western blot analysis.
139

140 **2.5. Western blotting**

141 Western blotting was performed as described previously [6]. Briefly, whole cell lysates were
142 subjected to standard SDS-PAGE and separated proteins transferred to a PVDF membrane
143 (Bio-Rad, Hercules, CA, USA) by wet electro-blotting. Non-specific antibody binding was
144 reduced by incubating membranes in 5 % non-fat dry milk. Signals were detected using
145 enhanced chemiluminescence (Amersham GE Healthcare Bio-Sciences AB, Uppsala,
146 Sweden). Experiments were performed at least in triplicate and analyzed separately.
147 Densitometric analysis was performed using ImageJ software version 1.45s (National Institute
148 of Health, USA).
149

150 **2.6. RNA purification and real-time RT-PCR**

151 Total RNA was prepared using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and 1 µg
152 used to generate cDNA with the High Capacity cDNA Reverse Transcription kit (Applied
153 Biosystems, Foster City, CA, USA) according to protocol. Subsequently, quantification of
154 gene expression was performed in duplicates using Maxima™ SYBR® Green qPCR Master

155 Mix (Fermentas, St. Leon-Rot, Germany) with detection on an Applied Biosystems 7500
156 Real-Time PCR System. The reaction cycles used were 95 °C for 2 min, and then 40 cycles at
157 95 °C for 15 s and 60 °C for 1 min followed by melt curve analysis using GAPDH as
158 housekeeping gene. Primer sequences are shown in Supplementary Table 1. Relative gene
159 expression quantification was analyzed with the mathematical model described in [26].

160

161 **2.7. Statistical analysis**

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

164

165 **3. Results and discussion**

166

167 **3.1. Analysis of PAH content in air PM extracts**

168 The PAH content in the two air PM extracts was determined using our recently developed
169 methodology that is capable of quantifying 42 unique PAHs of between 3 and 6 rings [6, 24]
170 (Supplementary Table 2). PAHs at the LIMEIRA sampling site mainly result from heavy
171 traffic (including cars and trucks), industrial emissions and biomass burning, whereas those at
172 the STHLM site can mainly be attributed to heavy traffic emissions. The results showed that
173 the air PM extract from Limeira (LIMEIRA) had approximately 3-fold higher total PAH
174 content than the extract from Stockholm (STHLM) (10169.2 and 3392.6 pg/m^3, respectively),
175 in agreement with recent data [24]. The level of BP was approximately 5-fold higher in the
176 LIMEIRA sample (807 pg/m^3) compared to the STHLM sample (160 pg/m^3). The level of the
177 highly potent dibenzo[*a,l*]pyrene was approximately 9-fold higher in the LIMEIRA sample
178 (9.07 pg/m^3) compared to the STHLM sample (1.05 pg/m^3).

179 In order to compare the cellular response to the two PAH extracts and with BP alone,
180 the extracts were prepared in DMSO to contain a concentration equivalent of 1 μM BP (BP_{eq})
181 (actual 0.91 and 1.10 μM for STHLM and LIMEIRA respectively, Supplementary Table 2).
182 Cells were exposed to the PAH extracts with a final concentration of 1 nM BP_{eq}, and hence,
183 cells were also exposed to 1 nM BP alone for comparative purposes. The final total PAH
184 concentration the cells were exposed to was 21.6 and 14.4 nM for the STHLM and LIMEIRA
185 extracts respectively. When assessing human exposure to PAHs in ambient air BP is often
186 used as a surrogate indicator [27, 28] and thus, normalizing the PAH extracts to a set
187 concentration of BP allows us to evaluate how well the BP content can predict the observed
188 effects. Neither the Stockholm [6] nor the Limeira extract (data not shown) demonstrated
189 cytotoxicity in the HepG2 cell line.
190

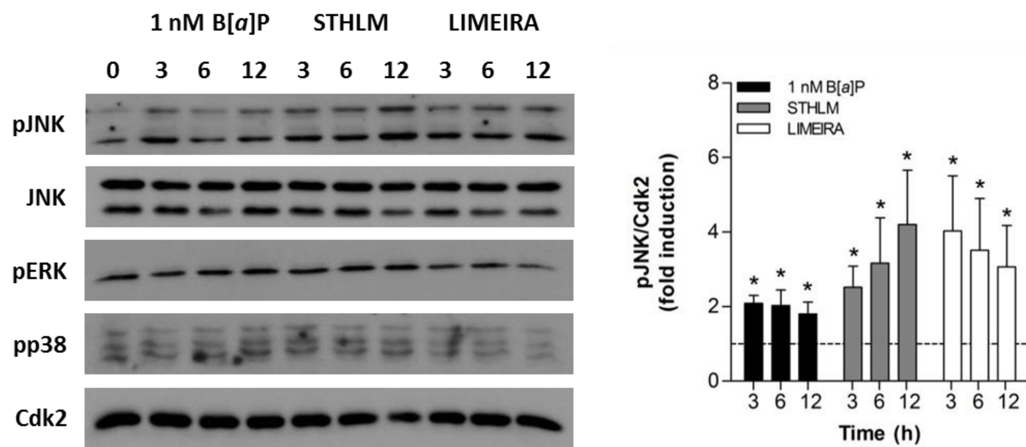
191 **3.2. Nanomolar levels of PAH extracts activate JNK signaling**

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

203 investigation is required to confirm this. Both extracts induced a stronger activation of JNK

204 than exposure to 1 nM BP alone (Figure 1).

205



206

207 **Figure 1.** Nanomolar concentrations of PAH extracts activate JNK signaling. Cells were

208 exposed to the PAH extracts (1 nM BP_{eq}) or 1 nM BP alone. Total and phosphorylated levels

209 of the indicated proteins were determined by Western blot at 3, 6 and 12 h. Cdk2 was used as

210 a loading control. Densitometric analysis is shown to the right. *p<0.05 compared with

211 DMSO control levels.

212

213 **3.3. AP-1 transcription factors are activated in response to nanomolar levels of PAH**

214 **extracts**

215 ATF2 and cJun are important proteins that form part of AP-1 and that are activated

216 downstream of JNK [33, 34]. In agreement with the increased levels of pJNK, we observed

217 increased phosphorylation of ATF2 and cJun (Figure 2A). Levels of phosphorylated ATF2

218 (pATF2) showed a significant increase in response to both BP_{eq} extracts, with the STHLM

219 extract inducing higher and more sustained levels of pATF2 than the LIMEIRA extract,

220 displaying >8-fold higher levels than control up to 12 h post-exposure. Levels of

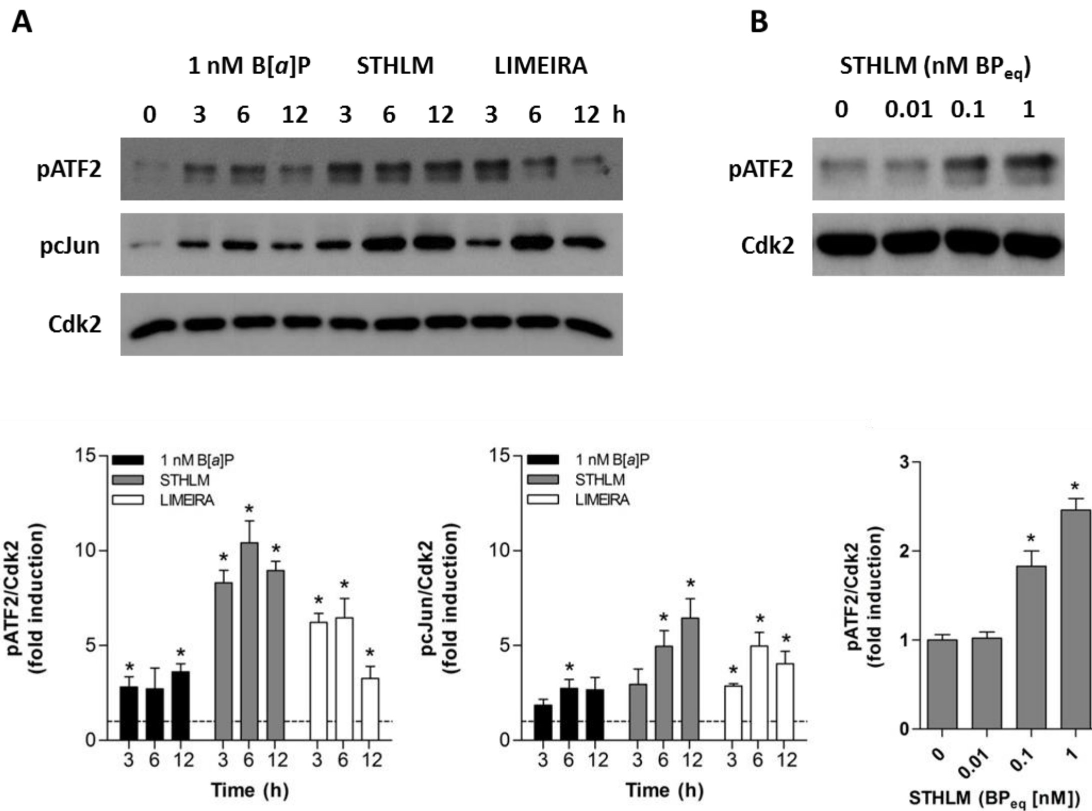
221 phosphorylated cJun (pcJun) displayed the same pattern as pATF2 with the highest levels in

222 response to the STHLM extract (Figure 2A). Similar to the effect on pJNK, both extracts

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

231 The results presented here show clear differences in the observed responses between
232 the PAH extracts. The signaling was also in general much stronger in response to the PAH
233 extracts compared to BP alone, similar to what we have reported earlier for DNA damage
234 signaling [6]. Since the total PAH content was quite similar for the 1 nM BP_{eq} STHLM and
235 LIMEIRA extracts this implies that the observed differences more likely are due to sample
236 composition. An explanation for this observation could be that there are different PAHs
237 present in the STHLM extract that are more potent MAPK inducers than BP. Alternatively,
238 substituted PAHs such as nitro-PAHs, which are known to induce stress signaling [36], might
239 also contribute to the effects of the extracts. In addition, non-PAH compounds could affect the
240 response of the extracts. The level of polychlorinated biphenyls were approximately 0.5 % of
241 the PAH level (pg/m^3) in the STHLM extract (unpublished data) and thus are predicted to
242 have minimal effects. Other polychlorinated compounds such as dibenzo-dioxins and furans
243 may also contribute to the overall effect of the extracts [37, 38]. Metals are also known to be
244 present in environmental air PM, and although the levels of metals in our extracts is unknown,
245 we speculate that due to the sampling and processing methods the levels are likely to be low
246 [11].

247



248

249 **Figure 2.** AP-1 transcription factors are activated in response to nanomolar levels of PAH

250 extracts. Cells were exposed to the PAH extracts (1 nM BP_{eq}) or 1 nM BP alone. Total and

251 phosphorylated levels of the indicated proteins were determined by Western blot at 3, 6 and

252 12 h (A). Cells were exposed to decreasing concentrations of the STHLM extract and level of

253 pATF2 was assessed at 12 h (B). Cdk2 was used as a loading control. Densitometric analysis

254 is shown below. *$p<0.05$ compared with DMSO control levels.

255

256 **3.4. Activation of JNK/AP-1 signaling is mediated through MEK4**

257 MEK4 has previously been shown to be involved in regulating pro-apoptotic signaling in

258 human 293T and HeLa cells and rat F258 cells in response to BP treatment, albeit at higher

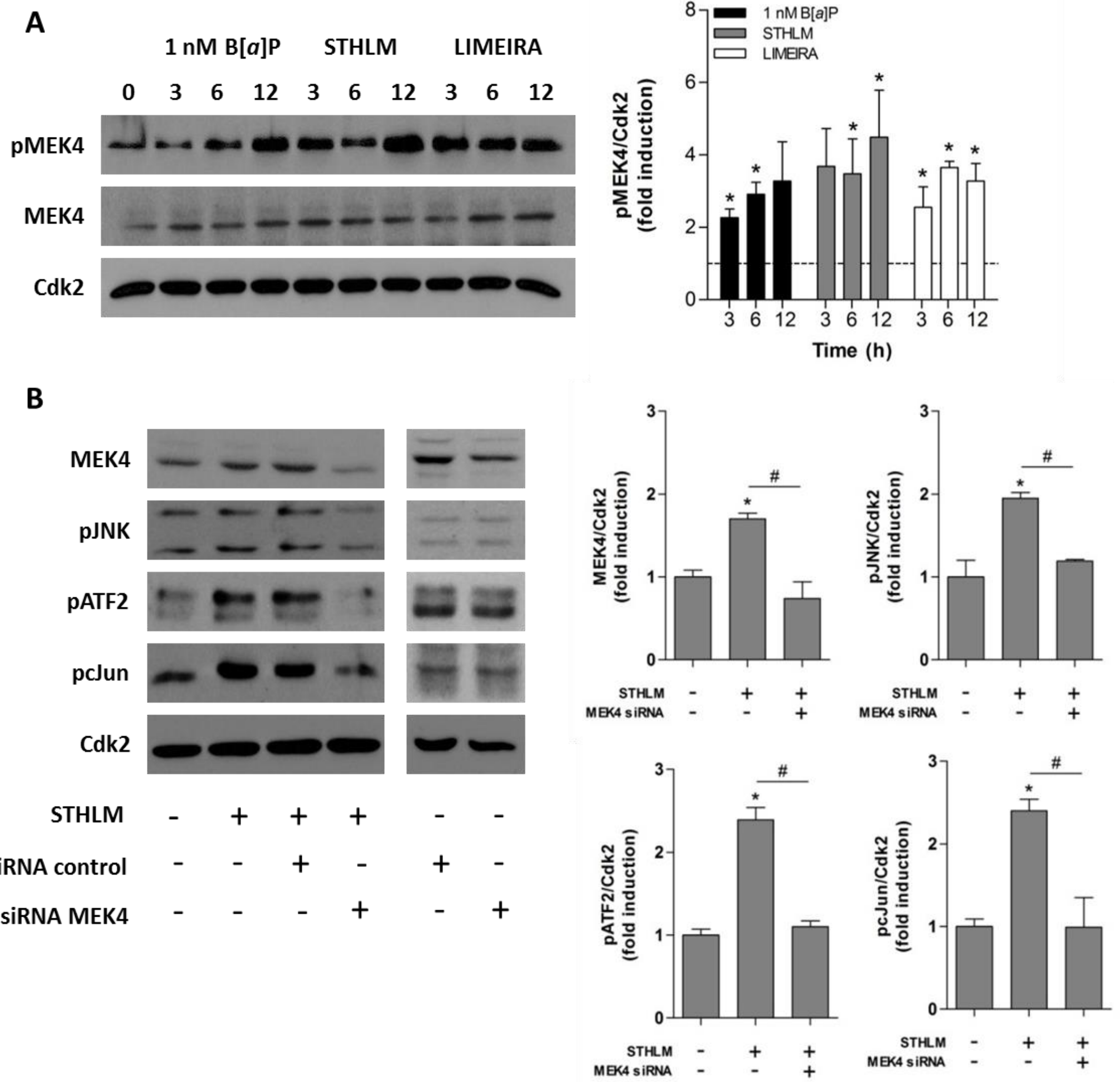
259 concentrations than used here, leading to downstream caspase activation [39, 40]. However, it

260 has not been established if MEK4 is activated in response to levels of PAHs found in urban

261 air. The results showed significantly increased levels of phosphorylated MEK4 (pMEK4)

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

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



278

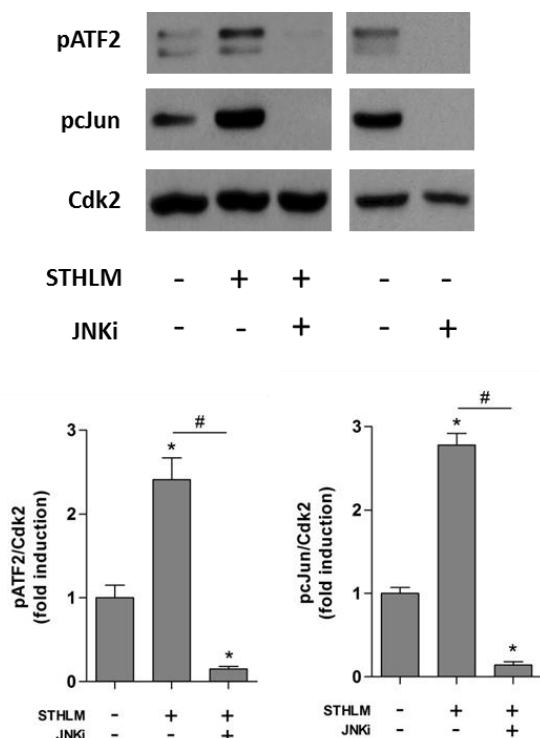
279 **Figure 3.** MEK4 mediates signaling through MAPK leading to activation of AP-1
280 transcription factors. Cells were exposed to the PAH extracts (1 nM BP_{eq}) or 1 nM BP alone
281 and total and phosphorylated levels of MEk4 were determined by Western blot at 3, 6 and 12
282 h (A). Cells transfected with either mock siRNA or siRNA against MEK4 were exposed to the
283 STHLM extract (1 nM BP_{eq}) for 12 h and effects on phosphorylation status of JNK, ATF2
284 and cJun were assessed by Western blot (B). Cdk2 was used as a loading control.
285 Densitometric analysis is shown to the right. *$p<0.05$ compared with DMSO control levels.
286 $^{\#}p<0.05$ compared with STHLM treatment.

287

288 **3.5. JNK mediates transactivation of cellular stress mediators through AP-1**

289 To confirm the role of activation of JNK signaling in activation of AP-1 proteins in response
290 to PAH extracts, HepG2 cells were pre-treated for 1 h with a JNK inhibitor (JNKi, 20 μM)
291 [41] followed by exposure to the STHLM extract for 12 h (Figure 4). Significantly reduced
292 levels of both pATF2 and pcJun were observed confirming the involvement of JNK in
293 activation of ATF2 and cJun in this study. JNK and cJun have previously been reported to be
294 mediators of cellular stress responses to PAHs [42, 43]. However, this is the first time that
295 activation of ATF2 can be linked to cellular stress signaling in response to PAHs.

296



297

298 **Figure 4.** Activation of AP-1 transcription factors is mediated through JNK. Cells pretreated
299 for 1 h with JNKi (20 μM in DMSO) were exposed to the STHLM extract (1 nM BP_{eq}) for 12
300 h and effects on phosphorylation status of ATF2 and cJun was assessed by Western blot.
301 Densitometric analysis is shown below. *$p<0.05$ compared with DMSO control levels.
302 $^{\#}p<0.05$ compared with STHLM treatment.

303

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

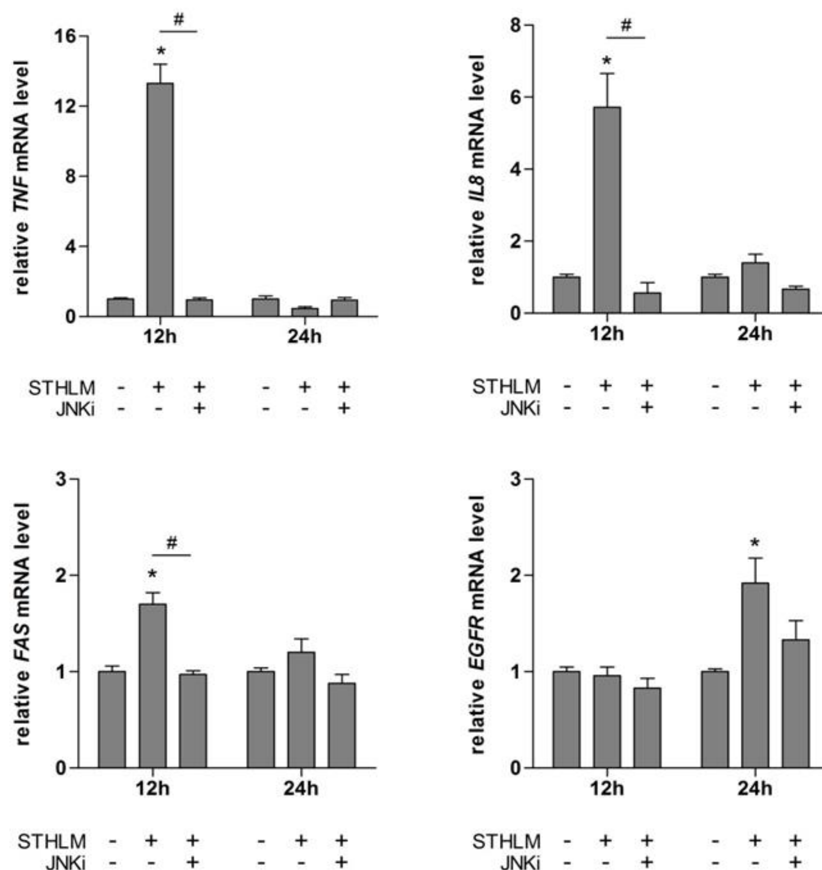


Figure 5. Induction of gene expression of stress response mediators is mediated through a MEK4/JNK/AP-1 signaling pathway. Cells were exposed to the STHLM extract (1 nM BP_{eq}) for 12 h with or without JNKi pretreatment and effects on gene expression were determined by qRT-PCR. *$p<0.05$ compared with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

333 seen, the fold differences for pATF2 are higher than predicted based on BP_{TEQ} concentrations
334 of the PAH extracts, emphasizing pATF2 as a sensitive biological marker for activation of
335 MAPK signaling following PAH exposure. An explanation for this observation is that there
336 could be different PAHs present in the extracts that are more potent inducers of the stress
337 response than BP. Fold differences were similar to what would be predicted for pcJun, pJNK
338 and pMEK4 based on BP_{TEQ} concentrations. However, it should be noted that there are a
339 number of important considerations and requirements that make the TEF-based approach
340 insufficient for mixture assessment as discussed recently [12].
341
342 **4. Conclusion**
343 Here we report that nanomolar concentrations of environmental PAH mixtures, extracted
344 from urban air PM collected in Stockholm, Sweden and Limeira, Brazil, induce a time-
345 dependent activation of MAPK signaling in HepG2 cells. We show that exposure to low
346 levels of PAH extracts more strongly activates signaling pathways compared to BP alone
347 suggesting possible effects from interactions. Exposure to the PAH extracts induces activation
348 of MEK4 signaling with down-stream increased expression of several important stress
349 response mediators. Abrogation of the MEK4-JNK pathway using siRNA and a specific
350 inhibitor against JNK significantly reduces the transactivation mediated through AP-1
351 transcription factors ATF2 and cJun. This is the first study showing activation of
352 MEK4/JNK/AP-1 pathway in in response to nanomolar levels of PAHs in environmental
353 mixtures.
354
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490 # Nanomolar levels of PAHs in extracts from urban air induce MAPK signaling in HepG2 cells

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509

510 **Running Title**
511 Environmental PAH mixtures activate MAPK signaling

512

513 **Abbreviations**

514 AP-1, activator protein 1; BP, benzo[*a*]pyrene; ERK, extracellular regulated kinase; JNK, c-

515 Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEK4, MAPK kinase 4;

516 PAH, polycyclic aromatic hydrocarbon; PM, particulate matter; TEF, toxic equivalency

517 factor; TNF, tumor necrosis factor;

518

519 **Abstract**

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

536

537 **Key Words**

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

539

540 **1. Introduction**

541 Human exposure to environmental pollutants in air particulate matter (PM) has been
542 identified to cause a number of adverse health effects including cancer and various
543 cardiovascular and respiratory diseases [1, 2]. One important group of environmental

544 pollutants that are associated with PM and play an important role in the reported detrimental
545 health effects are the polycyclic aromatic hydrocarbons (PAHs) [3]. PAHs are ubiquitous
546 environmental pollutants that are naturally present as mixtures and are formed during
547 combustion of carbon-containing fuels. Both individual and mixtures of PAHs are classified
548 as carcinogens or probable carcinogens by the International Agency for Research on Cancer
549 [4]. Recent data from us and others have suggested synergistic effects due to interactions
550 between PAHs in complex mixtures on the genotoxic and carcinogenic properties of PAHs [5-
551 8], though conversely, strong antagonistic effects have also been observed in human cells,
552 probably resulting from competitive inhibition of metabolizing enzymes [9-11]. However, the
553 role of interactions between PAHs in complex mixtures in relation to the adverse health
554 effects of PAHs is poorly understood [12].

555 The activator protein-1 (AP-1) transcription factor is a dimeric complex comprising
556 members of the ATF, FOS, JUN and MAF protein families that has functions in many areas
557 of cellular homeostasis [13, 14]. In response to cellular stress stimuli AP-1 proteins are
558 activated by the mitogen-activated protein kinase (MAPK) family of proteins, including c-Jun
559 N-terminal kinase (JNK), p38 and extracellular signal-regulated kinase (ERK) [15, 16]. The
560 protein MAPK kinase 4 (MEK4/MKK4) specifically activates JNK and p38 [17] and has been
561 identified as an important metastasis suppressor in several organs [18] and a possible target
562 for small molecule inhibition in therapy for tumor necrosis factor alpha (TNF) mediated
563 diseases [19]. The present study focuses on activation of MAPK signaling in response to
564 environmental mixtures of PAHs compared to single benzo[*a*]pyrene (BP) exposure in
565 human-derived hepatoma (HepG2) cells. It has previously been shown that *in vitro* exposure
566 to BP leads to activation of MAPK signaling associated with apoptotic cell death via p53 [20,
567 21]. However, the role of MAPK signaling in the stress response after exposure to nanomolar
568 concentrations of PAHs in air PM remains unclear.

569 Using PAH-containing air PM extracts from Stockholm, Sweden and Limeira, Brazil
570 at nanomolar concentrations we studied the time-dependent activation of MAPK signaling
571 and downstream gene expression in HepG2 cells. Our results show that nanomolar
572 concentrations of PAH extracts more strongly activate MAPK signaling and proteins of the
573 AP-1 transcription factor than BP alone suggesting effects due to interactions and that this
574 activation is mediated via MEK4 and JNK. Furthermore, the transactivation of cellular stress
575 mediators including interleukin 8 in response to PAH extract was shown to be mediated
576 through a MAPK. To the authors knowledge this is the first study to demonstrate activation of
577 MEK4/JNK/AP-1 with downstream increased gene expression in the cellular stress response
578 after exposure to nanomolar levels of PAHs found in air PM.
579

580 **2. Materials and Methods**

581

582 **2.1. Reagents and antibodies**

583 Unless otherwise stated all chemicals, including BP, were of analytical grade and obtained
584 from Sigma Aldrich (Stockholm, Sweden). Detailed information on manufacturer and purity
585 of the standards and solvents used for PAH analysis have been published previously [22, 23].
586 Gibco (Invitrogen, Paisley, UK) supplied all cell culture reagents. Cell Signaling Technology
587 (Beverly, MA) provided the following antibodies: phospho-ATF2 Thr71, phospho-cJun
588 Ser63, JNK, phospho-JNK Thr183/Tyr185, MEK4, phospho-MEK4 Thr261. Santa Cruz
589 Biotechnology (Santa Cruz, CA, USA) provided the Cdk2, phospho-Erk Tyr204 and
590 phospho-p38 Thr180/Tyr182 antibodies, secondary anti-rabbit, anti-mouse and siRNA
591 against MEK4 and control siRNA-A. Calbiochem (Gibbstown, NJ, USA) provided the JNK
592 inhibitor VIII.
593

594 **2.2. Air sampling, sample preparation and PAH analysis**

595 Air PM was collected at two sites: the campus of Stockholm University, Stockholm, Sweden
596 and the campus of the Faculty Technology at UNICAMP, Limeira, Brazil. At both collection
597 sites total PM was collected. The air PM sample from Stockholm was collected at roof-top
598 level on a Teflon-coated glass fiber filter (Ø149 mm, Pallflex Inc., Putnam, CT, USA) with an
599 average flow rate of 509 l min^{-1} for 170 h. The total PM concentration for this sample was not
600 determined, but PM concentration from other similar urban sites in Stockholm during the
601 same season ranged from 10.4 – 19.4 $\mu g/m^3$ (unpublished data). The air PM sample from
602 Limeira was collected at street level on a glass-fiber filter (254 × 233 mm, 0.33 mm pore size,
603 Energética Ind. Com. LTDA, Rio de Janeiro, RJ, Brazil) using a high-volume sampler
604 (Energética Ind. Com. LTDA, Rio de Janeiro, RJ, Brazil) operated at an average flow rate of
605 1130 l min^{-1} for 24 h. The total PM concentration in the Limeira sample was 95.8 $\mu g/m^3$ [24].
606 Extraction was performed using an ASE 200 accelerated solvent extraction system (Dionex
607 Corporation, Sunnyvale, CA, USA). Toluene was used as an extraction solvent at 200 °C and
608 3000 psi for five consecutive 30 min static extraction cycles as described previously [6]. PAH
609 content in the extracts was determined by HPLC-GC/MS as described previously [6, 22].

610

611 **2.3. Cell culture and exposure**

612 Human-derived hepatocellular carcinoma cells (HepG2) were obtained from the American
613 Type Culture Collection (Rockville, MD, USA). The motivation for the use of this cell line in
614 this study is the metabolic competence for PAHs [25] and a previously demonstrated response
615 to low levels of PAHs (in mixtures) extracted from environmental samples by ourselves and
616 others [5-7]. HepG2 cells were cultured in minimal essential medium supplemented with 10
617 % fetal bovine serum, sodium pyruvate (1 mM), non-essential amino acids (0.1 mM),
618 penicillin (100 units/ml) and streptomycin (0.1 mg/ml) and maintained at 37 °C in 5 % CO_2.

619 Prior to exposure cells were seeded at 3×10^5 cells/ml in 6-well plates and cultured for 72 h
620 unless otherwise stated. Cells were exposed to solvent control (0.1 % DMSO), BP, or PAH
621 extracts for up to 24 h.
622

623 **2.4. RNA interference**

624 Transfection of cells was performed using Lipofectamine 2000 reagent (Invitrogen, Paisley,
625 UK). Briefly, cells were seeded into 35 mm culture dishes and after 24 h transfected with 50
626 nM siRNA. siRNA sequences are shown in Supplementary Table 1. After 48 h of incubation
627 cells were exposed to PAHs or solvent control, and then harvested for Western blot analysis.
628

629 **2.5. Western blotting**

630 Western blotting was performed as described previously [6]. Briefly, whole cell lysates were
631 subjected to standard SDS-PAGE and separated proteins transferred to a PVDF membrane
632 (Bio-Rad, Hercules, CA, USA) by wet electro-blotting. Non-specific antibody binding was
633 reduced by incubating membranes in 5 % non-fat dry milk. Signals were detected using
634 enhanced chemiluminescence (Amersham GE Healthcare Bio-Sciences AB, Uppsala,
635 Sweden). Experiments were performed at least in triplicate and analyzed separately.
636 Densitometric analysis was performed using ImageJ software version 1.45s (National Institute
637 of Health, USA).
638

639 **2.6. RNA purification and real-time RT-PCR**

640 Total RNA was prepared using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and 1 µg
641 used to generate cDNA with the High Capacity cDNA Reverse Transcription kit (Applied
642 Biosystems, Foster City, CA, USA) according to protocol. Subsequently, quantification of
643 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**

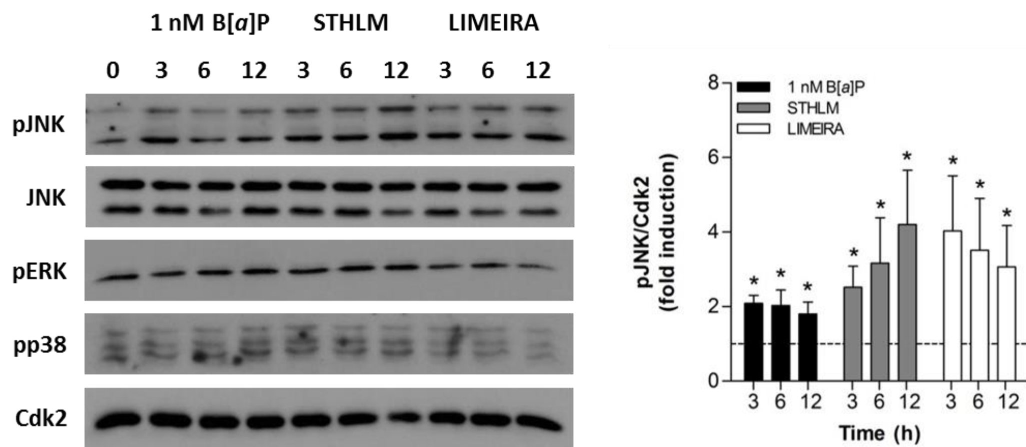
655

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).

668 In order to compare the cellular response to the two PAH extracts and with BP alone,
669 the extracts were prepared in DMSO to contain a concentration equivalent of 1 μM BP (BP_{eq})
670 (actual 0.91 and 1.10 μM for STHLM and LIMEIRA respectively, Supplementary Table 2).
671 Cells were exposed to the PAH extracts with a final concentration of 1 nM BP_{eq}, and hence,
672 cells were also exposed to 1 nM BP alone for comparative purposes. The final total PAH
673 concentration the cells were exposed to was 21.6 and 14.4 nM for the STHLM and LIMEIRA
674 extracts respectively. When assessing human exposure to PAHs in ambient air BP is often
675 used as a surrogate indicator [27, 28] and thus, normalizing the PAH extracts to a set
676 concentration of BP allows us to evaluate how well the BP content can predict the observed
677 effects. Neither the Stockholm [6] nor the Limeira extract (data not shown) demonstrated
678 cytotoxicity in the HepG2 cell line.
679
680 **3.2. Nanomolar levels of PAH extracts activate JNK signaling**
681 Activation of DNA damage signaling in cells after exposure to nanomolar concentrations of
682 the STHLM extract has been demonstrated previously [6]. To identify regulatory pathways
683 involved in different cellular responses including inflammation, apoptosis and cell cycle
684 control, we assessed activation of MAPK proteins that have previously been shown to be
685 activated in response to PAHs [29-32]. The results showed a significant increase in levels of
686 phosphorylated JNK (pJNK) whilst no significant effect was observed on total JNK protein or
687 on phosphorylation of Erk (pERK) or p38 (pp38) (Figure 1). Phosphorylation of JNK in
688 response to the two PAH extracts followed different kinetics with the STHLM extract
689 inducing a persistent increase in activation throughout the 12 hours whereas the LIMEIRA
690 extract induced the highest levels of pJNK after 3 h which decreased thereafter. It is probable
691 that this difference in trends is a result of different extract composition though further

692 investigation is required to confirm this. Both extracts induced a stronger activation of JNK
693 than exposure to 1 nM BP alone (Figure 1).
694



695

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

701

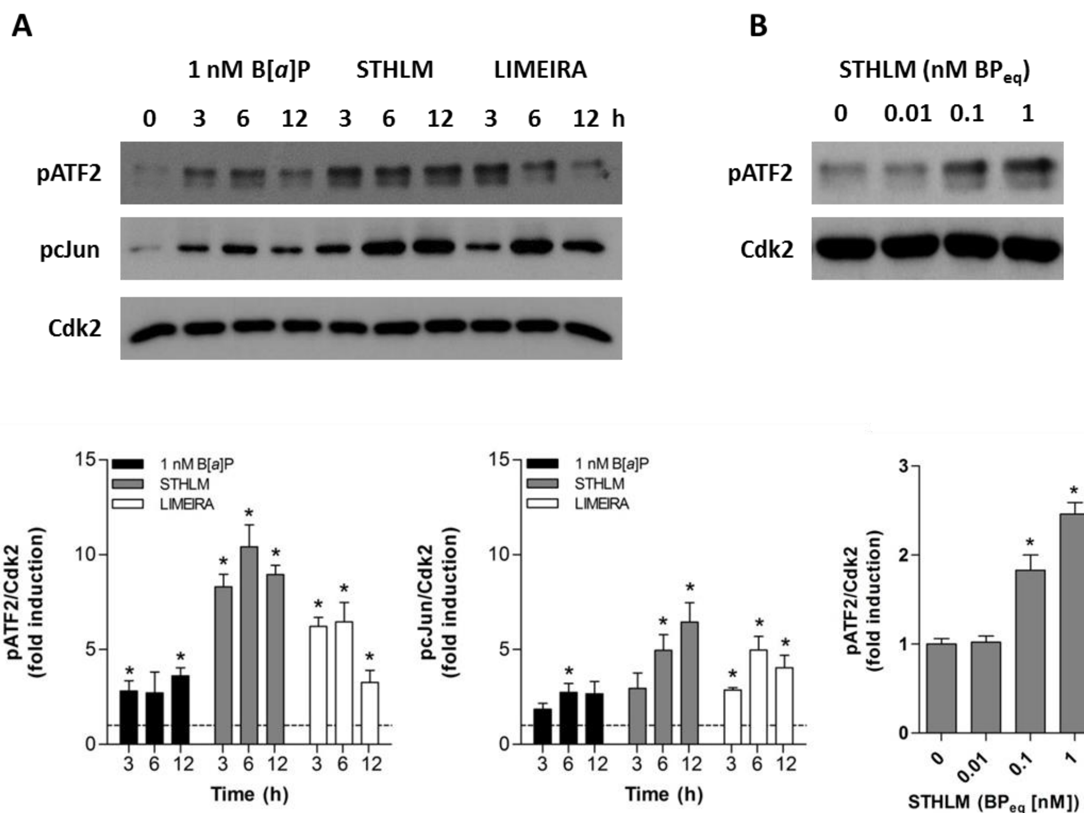
702 **3.3. AP-1 transcription factors are activated in response to nanomolar levels of PAH**
703 **extracts**

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

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

720 The results presented here show clear differences in the observed responses between
721 the PAH extracts. The signaling was also in general much stronger in response to the PAH
722 extracts compared to BP alone, similar to what we have reported earlier for DNA damage
723 signaling [6]. Since the total PAH content was quite similar for the 1 nM BP_{eq} STHLM and
724 LIMEIRA extracts this implies that the observed differences more likely are due to sample
725 composition. An explanation for this observation could be that there are different PAHs
726 present in the STHLM extract that are more potent MAPK inducers than BP. Alternatively,
727 substituted PAHs such as nitro-PAHs, which are known to induce stress signaling [36], might
728 also contribute to the effects of the extracts. In addition, non-PAH compounds could affect the
729 response of the extracts. The level of polychlorinated biphenyls were approximately 0.5 % of
730 the PAH level (pg/m^3) in the STHLM extract (unpublished data) and thus are predicted to
731 have minimal effects. Other polychlorinated compounds such as dibenzo-dioxins and furans
732 may also contribute to the overall effect of the extracts [37, 38]. Metals are also known to be
733 present in environmental air PM, and although the levels of metals in our extracts is unknown,
734 we speculate that due to the sampling and processing methods the levels are likely to be low
735 [11].

736



737

738 **Figure 2.** AP-1 transcription factors are activated in response to nanomolar levels of PAH
739 extracts. Cells were exposed to the PAH extracts (1 nM BP_{eq}) or 1 nM BP alone. Total and
740 phosphorylated levels of the indicated proteins were determined by Western blot at 3, 6 and
741 12 h (A). Cells were exposed to decreasing concentrations of the STHLM extract and level of
742 pATF2 was assessed at 12 h (B). Cdk2 was used as a loading control. Densitometric analysis
743 is shown below. *p<0.05 compared with DMSO control levels.

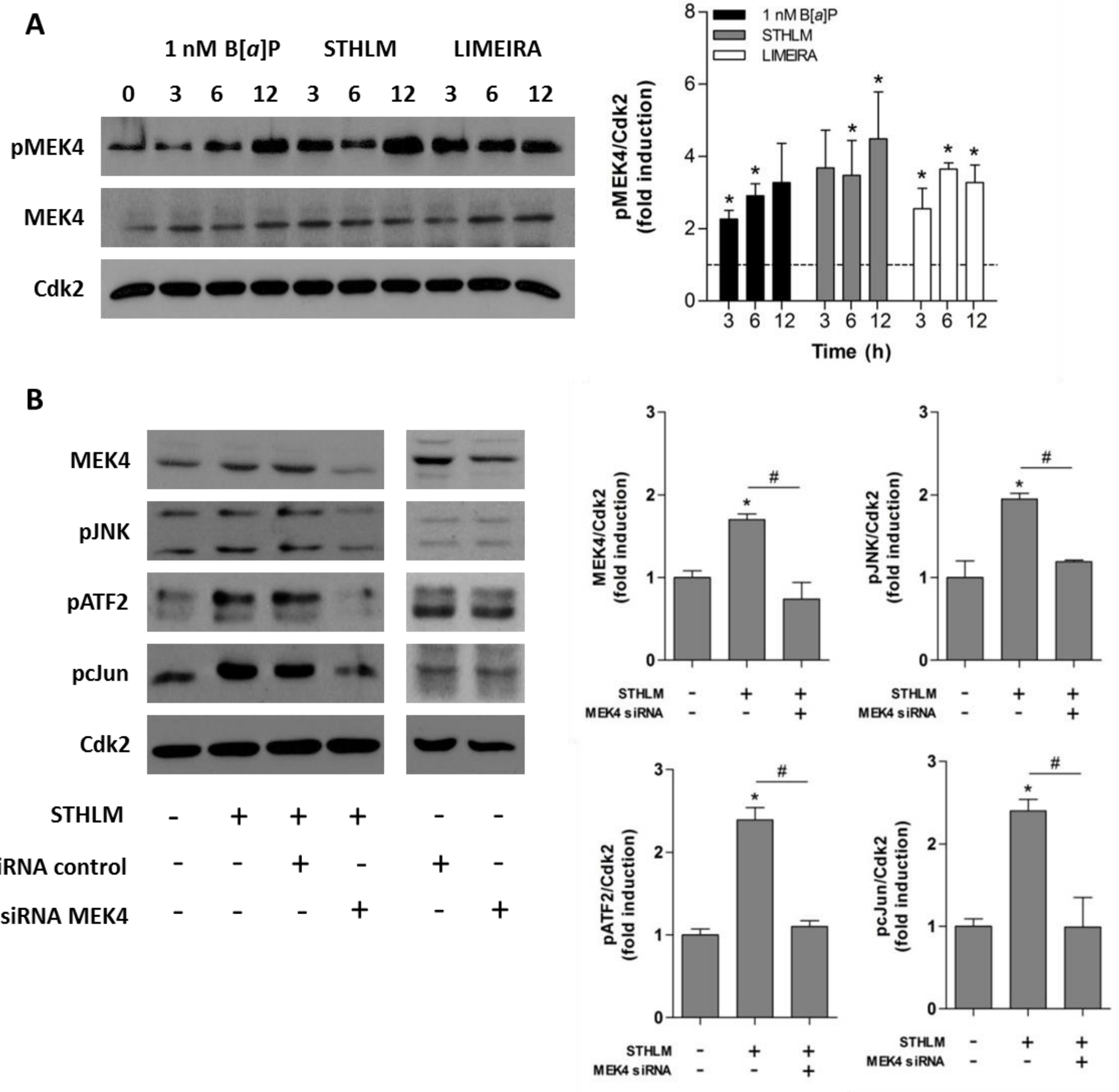
744

745 **3.4. Activation of JNK/AP-1 signaling is mediated through MEK4**

746 MEK4 has previously been shown to be involved in regulating pro-apoptotic signaling in
747 human 293T and HeLa cells and rat F258 cells in response to BP treatment, albeit at higher
748 concentrations than used here, leading to downstream caspase activation [39, 40]. However, it
749 has not been established if MEK4 is activated in response to levels of PAHs found in urban
750 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.



767

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

776

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.

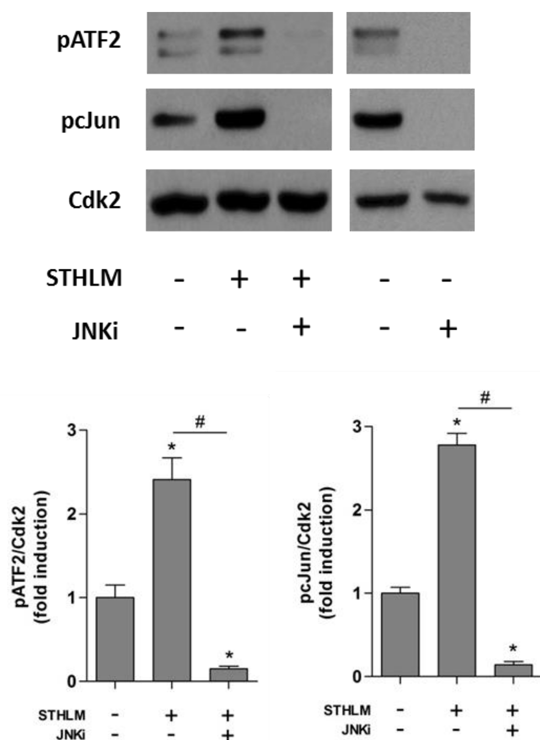
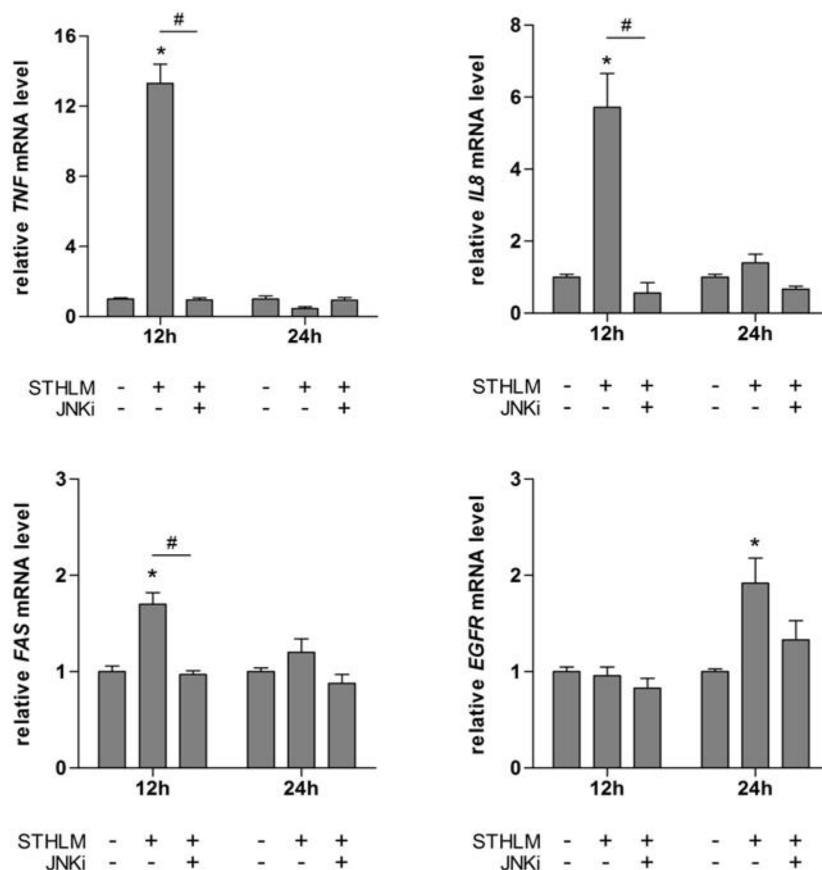


Figure 4. Activation of AP-1 transcription factors is mediated through JNK. Cells pretreated for 1 h with JNKi (20 μM in DMSO) were exposed to the STHLM extract (1 nM BP_{eq}) for 12 h and effects on phosphorylation status of ATF2 and cJun was assessed by Western blot. Densitometric analysis is shown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

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



808

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

814

815 **3.6. Comparison with toxic equivalency factors**

816 In order to compare the extracts with BP they were prepared to have a final exposure
817 concentration equivalent to 1 nM BP. An alternative approach is to derive BP equivalent
818 concentrations using toxic equivalency factor (TEF) scales. Using previously published TEF
819 values [44] we calculated BP_{TEQ} concentrations of 1.74 and 1.46 for the STHLM and
820 LIMEIRA samples. Data for fold difference for pATF2, pcJun, pJNK and pMEK4 are given
821 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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