

Calcium signaling and β 2-adrenergic receptors regulate 1-nitropyrene induced CXCL8 responses in BEAS-2B cells.

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1	Title:			
2	Calcium Signaling and β2-Adrenergic Receptors Regulate 1-Nitropyrene Induced			
3	CXCL8 Responses in BEAS-2B Cells			
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1 Abstract

2 Nitro-polycyclic aromatic hydrocarbons (nitro-PAHs) are widespread environmental 3 pollutants, generated from reactions between PAHs and nitrogen oxides during combustion processes. In the present study we have investigated the mechanisms of CXCL8 (IL-8) 4 5 responses induced by 1-nitropyrene (1-NP) in human bronchial epithelial BEAS-2B cells. with focus on the possible importance of Ca^{2+} -signaling and activation of β 2-adrenergic 6 receptors (β 2AR). Ca²⁺-chelator treatment obliterated 1-NP-induced CXCL8 (IL-8) responses. 7 1-NP at 10 µM (but not 1 µM) induced a rapid and sustained increase in intracellular Ca²⁺-8 levels ($[Ca^{2+}]_i$). The early but not the later, sustained phase of 1-NP-induced $[Ca^{2+}]_i$ was 9 suppressed by beta-blocker treatment (carazolol). Moreover, inhibition of β2AR by blocking-10 11 antibody, beta-blocker treatment (ICI 118551) or siRNA transfection attenuated CXCL8 responses induced by 1-NP. The results confirm that PAHs may induce Ca^{2+} -signaling also in 12 BEAS-2B cells, at least partly through activation of β 2AR, and suggest that both β 2AR- and 13 Ca²⁺-signaling may be involved in 1-NP-induced CXCL8 responses in bronchial epithelial 14 15 cells.

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17 Key words: Lung; Inflammation; Chemokines; Polycyclic Aromatic Hydrocarbons; Calcium;
18 Adrenergic Receptors.

1

2 Introduction

Nitro-polycyclic aromatic hydrocarbons (nitro-PAHs) are ubiquitous air pollutants associated
with combustion particles, in particular diesel exhaust particles. Nitro-PAHs have long been
considered among the main contributors to the mutagenic effects of DEP (Hayakawa et al.,
1997; Scheepers et al., 1995). Nitro-PAHs may also exhibit considerable pro-inflammatory
potential, by inducing cytokine and chemokine responses in epithelial lung cells (Øvrevik et
al., 2010; Øvrevik et al., 2009; Park and Park, 2009; Pei et al., 2002).

9

10 Previous studies have shown that 1-nitropyrene (1-NP) and 3-nitrofluoranthene (3-NF) are 11 particularly potent inducers of the neutrophil attracting chemokine CXCL8 (IL-8), compared to their amine counterparts (1-AP and 3-AF), un-substituted pyrene or benzo[a]pyrene 12 13 (B[a]P) (Øvrevik et al., 2010; Øvrevik et al., 2009; Øvrevik et al., 2013). This marked effect 14 of 1-NP and 3-NF on CXCL8 induction seems to be independent of aryl-hydrocarbon 15 receptor (AhR) activation, although it appears to involve formation of reactive oxygen species 16 (ROS) and/ or reactive electrophilic metabolites at least partly formed via CYP-mediated 17 metabolism (Øvrevik et al., 2010; Øvrevik et al., 2013).

18

19 Of notice, it has recently been shown that certain PAHs may induce calcium signaling in 20 endothelial cells through an aryl-hydrocarbon receptor (AhR)-independent mechanism 21 (Mayati et al., 2011). The effects were reported to be due to direct PAH-mediated activation 22 of β 2-adrenergic receptors (β 2AR), leading to a G protein/adenylyl cyclase/cyclic-AMP-23 mediated calcium release from the endoplasmic reticulum (Mayati et al., 2012). Pyrene 24 seemed to be a particular potent inducer of this pathway and caused considerably stronger 25 calcium response in endothelial cells compared to other PAHs including B[*a*]P, chrysene and

1 benzo [e] pyrene (Mayati et al., 2011). Besides, we previously showed that the cell-permeable 2 calcium chelator BAPTA-AM attenuated CXCL8 responses in BEAS-2B cells by a panel of 3 environmental pollutants, including 1-NP (Øvrevik et al., 2011). Thus, in the present study we 4 have investigated the 1-NP-induced effects on intracellular calcium levels in relation to 5 CXCL8 responses in BEAS-2B cells, in order to test the hypothesis that β 2AR-mediated 6 calcium-signaling may be a central mechanism for the pro-inflammatory effects of nitro-7 PAHs. Our results suggest that 1-NP is able to induce β 2AR-mediated calcium responses in BEAS-2B cells, and that both β 2AR- and Ca²⁺-signaling are involved in 1-NP-induced 8 9 CXCL8 responses. However, 1-NP also appeared to affect intracellular calcium levels through additional mechanisms, and β 2AR-mediated Ca²⁺-signaling alone may not be 10 MAT 11 sufficient for CXCL8-induction.

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- 13

14 Materials and methods

15 Reagents

B[a]P, 1-NP, dimethyl sulphoxide (DMSO), ICI 118551 and carazolol were purchased from 16 17 Sigma-Aldrich (St. Louis, MO, USA). LHC-9 cell culture medium and fura-2 acetoxymethyl 18 ester (Fura-2-AM) were from Invitrogen (Carlsbad, CA, USA). Cytokine ELISA assay for 19 CXCL8 (Human IL-8 Cytoset) was purchased from Biosource International (Camarillo, CA, 20 USA). Antibodies against β2AR (sc-569) were from Santa Cruz Biotechnology (Santa Cruz, 21 CA, USA), whereas antibodies against β -actin were from Sigma-Aldrich. Short interfering 22 RNA (siRNA) against β 2AR (sc-35147) with corresponding non-targeting control siRNA (sc-23 37007) were from Santa Cruz Biotechnology (CA, USA). All other chemicals used were 24 purchased from commercial sources at the highest purity available.

1 Cell cultures and exposures

2 The BEAS-2B cell line, a SV40 hybrid (Ad12SV40) transformed human bronchial epithelial 3 cell line, was from European Collection of Cell Cultures (ECACC, Salisbury, UK). Cells 4 were maintained in LHC-9 medium in collagen-coated (PureColTM, Inamed Biomaterials, Fremont, CA, USA) flasks in a humidified atmosphere at 37°C with 5% CO₂, and passaged 5 6 twice per week. Prior to exposure, cells were plated in 12-well culture dishes, grown to near 7 confluence in serum-free LHC-9 medium and exposed to PAHs and inhibitors as described 8 elsewhere. Controls for 1-NP -exposed cells were treated with vehicle (DMSO) only. DMSO 9 concentrations in all samples were below 0.5%.

10

11 Chemokine release

12 CXCL8 protein levels in cell-supernatants were determined by ELISA (Biosource
13 International, Camarillo, CA, USA), as described elsewhere (Øvrevik et al., 2010).
14 Absorbance was measured using a plate reader (TECAN Sunrise, Phoenix Research Products,
15 Hayward, CA, USA) complete with software (Magellan V 1.10).

16

17 Gene silencing by siRNA

18 Cells were reverse-transfected with siRNAs against β 2AR or non-targeting siRNAs, using 19 HiPerFect transfection reagent as recommended by the manufacturer (Qiagen, Germany: Fast-20 Forward protocol for adherent cells). SiRNAs and HiPerFect were mixed by vortexing in 21 LHC-9 medium, incubated at room temperature (5-10 min) to form transfection complexes, 22 and added drop-wise to the cell cultures (100 µl/well) immediately after seeding (at a final 23 siRNA concentration of 10 nM and 2.75 µl of HiPerFect in a total of 1 ml growth medium). 24 Gene silencing was monitored by measuring β 2AR protein levels by Western blotting.

1 Calcium measurements

2	Variations in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) were analyzed by
3	microspectrofluorimetry using the acetoxymethyl ester form of the Ca^{2+} -sensitive probe Fura-
4	2, , as previously reported (Le et al., 2002; N'Diaye et al., 2006). Cells were then submitted
5	alternatively to 340 and 380 nm excitation wavelengths and the fluorescence from the trapped
6	dye was measured at 510 nm. The F340/F380 ratio, i.e., ratio of fluorescence intensities after
7	excitation at 340 nm and 380 nm, respectively, was used to estimate $[Ca^{2+}]_i$. To avoid
8	potential problems with autofluorescence, PAH-concentrations were restricted to 10 μM in
9	the $[Ca^{2+}]_i$ -assay.
10	
11	Statistical analysis
12	Statistical significance was evaluated by GraphPad Prism software (GraphPad Software Inc.,
13	San Diego, CA, USA), using analysis of variance (ANOVA) with Bonferroni post-test.
14	
15	
16	Results
17	Role of calcium signaling in 1-NP-induced CXCL8 responses in BEAS-2B cells
18	We have previously shown that 1-NP induces a concentration-dependent increase in CXCL8
19	release from BEAS-2B cells from 10 μ M (Øvrevik et al., 2010; Øvrevik et al., 2013). In line
20	with this, we observed that 1-NP induced a strong increase in CXCL8 gene expression and
21	protein release in BEAS-2B cells (Fig 1 A and B). To assess the role of Ca ²⁺ -signaling in 1-
22	NP-induced CXCL8 responses, BEAS-2B cell were pre-treated with the cell permeable Ca ²⁺ -

- 23 chelator BAPTA-AM for 30 min prior to 1-NP exposure for 18 h. BAPTA-AM completely
- 24 blocked the 1-NP-induced CXCL8-response in BEAS-2B cells without affecting basal

CXCL8 levels (Fig. 1B). Thus, 1-NP-induced CXCL8 responses seemed to depend on
 intracellular Ca²⁺-levels in BEAS-2B cells.

3

We next assessed whether 1-NP could affect intracellular Ca^{2+} -concentrations ($[Ca^{2+}]_i$) in 4 BEAS-2B cells. Cells were loaded with the Ca²⁺-sensitive fluorescent probe Fura-2 prior to 1-5 NP exposure, and changes in $[Ca^{2+}]_i$ were assessed by microspectrofluorimetry. As seen from 6 Fig 2A, 10 μ M 1-NP induced a time-dependent increase in [Ca²⁺], in BEAS-2B cells. The 7 kinetics of 1-NP-induced $[Ca^{2+}]_i$ in BEAS-2B cells resembled that reported for B[a]P in 8 9 endothelial HMEC cells (Mayati et al., 2011; Mayati et al., 2012) with a small, transient peak 10 around 5 min after exposure, followed by a gradual increase that reached maximum after about half an hour exposure. Moreover, it has recently been reported that PAHs may induce 11 increases in $[Ca^{2+}]_i$ in HMEC cells through direct activation of $\beta 2AR$ (Mayati et al., 2012). 12 Thus, to assess whether similar mechanisms could be involved in the present PAH-induced 13 Ca^{2+} response, BEAS-2B cells were pre-incubated with the β -blocker carazolol for 30 min 14 prior to exposure with 10 µM 1-NP. Carazolol almost completely blocked the early phases of 15 1-NP induced increases in $[Ca^{2+}]_i$ (Fig. 2B). However, 1-NP induced a sustained increase in 16 $[Ca^{2+}]_i$ lasting at least up to 6 h (Fig. S1A, online supplementary materials). The later phases 17 of 1-NP-induced (from 45 min to 6 h) seemed unaffected by Carazolol treatment (Fig. 2B). 18

19

Of notice, previous studies have shown that PAHs such as B[*a*]P and un-substituted pyrene may stimulate β 2AR-mediated [Ca²⁺]_{*i*} already at 1 µM concentration (Mayati et al., 2011; Mayati et al., 2012). In line with this we observed that 1 µM B[*a*]P was able to induce increased [Ca²⁺]_{*i*} in BEAS-2B cells. However, 1-NP failed to affect Ca²⁺ signaling at this low concentration. Moreover, at 10 µM B[*a*]P appeared to induce considerably higher effects on [Ca²⁺]_{*i*} compared to 1-NP (Fig S1B and C, online supplementary materials). Thus, although 1-

1 NP was able to stimulate β 2AR-induced [Ca²⁺]_{*i*}, it appeared to be a less potent activator 2 compared to other PAHs.

3

4 Role of β 2AR in 1-NP-induced CXCL8 responses in BEAS-2B cells

5 Next we wanted to assess whether $\beta 2AR$ could be involved in nitro-PAH-induced CXCL8 6 responses in BEAS-2B cells. Therefore we pre-incubated the cells with a β 2AR-blocking 7 antibody (Mayati et al., 2012) for 30 min prior to exposure with 1-NP. The β 2AR-blocking 8 antibody suppressed 1-NP-induced CXCL8 responses by approximately 50% at both tested 9 concentrations (Fig 3A). To further examine the role of $\beta 2AR$ in 1-NP-induced CXCL8 10 responses, we assess the effects of the selective β 2AR-antagonist ICI 118551 and of silencing 11 β 2AR by siRNA (si β 2AR). Both ICI treatment and transfection with si β 2AR resulted in a 12 partial, but statistically significant suppression of 1-NP-induced CXCL8 (Fig 3B and C), 13 comparable to the effect obtained with the β 2AR-blocking antibody. Thus β 2AR-signaling 14 seemed to be involved in 1-NP-induced chemokine responses.

15

16

17 Discussion

18 In the present study we have assessed the mechanisms of the 1-NP induced CXCL8 response with emphasis on the importance of Ca^{2+} -signaling and the $\beta 2AR$ -receptor. Previously, Ca^{2+} -19 chelation was found to suppress CXCL8 in BEAS-2B cells by a variety of different air 20 21 pollution constituents including 1-NP (Øvrevik et al., 2011). In coherence with this, the present results confirmed that the cell-permeable Ca2+-chelator BAPTA-AM almost 22 23 completely block 1-NP-induced CXCL8. Of interest, PAHs may increase $[Ca^{2+}]_i$ in 24 endothelial cells through AhR-independent mechanisms (Mayati et al., 2011). Recent findings 25 suggest that this effect may be regulated through PAH-mediated binding and activation of the

β2AR (Mayati et al., 2012). In support of this, we observed that 1-NP (10 μ M) exposure induced an increase in $[Ca^{2+}]_i$ in BEAS-2B cells that could be suppressed by beta-blocker treatment. Thus, PAH-induced Ca²⁺-signaling through β2AR-activation may extend to several cell types. However, only the early phase of the Ca²⁺-response seemed to depend on signaling through β-adrenergic receptors, as beta-blocker treatment had no effect on the 1-NP induced increase in $[Ca^{2+}]_i$ after 40 min exposure. Thus it is conceivable that 1-NP may induce Ca²⁺signaling through multiple mechanisms.

8

9 Interference with B2AR-signalling by use of blocking antibodies, a pharmacological betablocker, or by silencing β 2AR-expression by siRNA-transfection, led to a suppression of 1-10 NP-induced CXCL8 responses. Thus 1-NP-induced CXCL8 release in BEAS-2B cells 11 12 seemed at least partly dependent on β 2AR-activation. In line with this, the β 2AR agonists salbutamol and salmeterol have been found to enhance CXCL8 and interleukin-6 (IL-6) 13 responses by IL-1ß or virus infections in BEAS-2B cells and primary human bronchial 14 epithelial cells (Edwards et al., 2007; Holden et al., 2010). However, salbutamol and 15 16 salmeterol had no effect on CXCL8 or IL-6 responses alone, suggesting that β2AR-signalling 17 alone may be insufficient for activation of cytokine/chemokine responses. Indeed, B[a]Pappeared to be a more potent inducer of β 2AR-dependent [Ca²⁺],-responses than 1-NP, but is 18 19 nevertheless unable to induce CXCL8 or other chemokines in BEAS-2B cells (Øvrevik et al., 20 2010; Øvrevik et al., 2013). Thus, additional 1-NP-induced mechanisms are likely required 21 for the response. This notion is supported by previous findings suggesting that 1-NP-induced 22 CXCL8 responses also involve metabolic activation by CYP-enzymes and possibly ROS 23 (Øvrevik et al., 2013).

While β2AR-interference only partly attenuated 1-NP-induced CXCL8 release, Ca²⁺-chelation 1 2 by BAPTA-AM completely abrogated the CXCL8 response. It should therefore be considered 3 that the role of β 2AR in 1-NP-induced CXCL8-release could be linked to other signaling mechanisms such as cAMP or β-arrestin. Indeed, salbutamol-induced exacerbation of virus-4 5 induced IL-6 responses in BEAS-2B cells seemed to be mediated through a cAMP-dependent 6 mechanism (Edwards et al., 2007). If so, the effects of BAPTA-AM on 1-NP-induced CXCL8 7 may be also related to interference with the later, sustained, $\beta 2AR$ -independent $[Ca^{2+}]_{i}$ 8 response.

9

The strong CXCL8-induction by 1-NP seems predominately to occur at high concentrations 10 11 $(\geq 10 \ \mu\text{M})$ in BEAS-2B cells (Øvrevik et al., 2013). However, we recently observed that low 12 concentrations of 1-NP (1 µM; giving no cytokine release alone) potentiated CXCL8-13 responses induced by priming the cells with a Toll-like receptor 3-agonist. Similar effects 14 were observed with low concentrations of 1-AP and un-substituted pyrene (Øvrevik et al., 15 2013). Since 1-NP may exacerbate CXCL8 responses at concentrations that are insufficient to induce β 2AR-dependent $[Ca^{2+}]_i$, other cellular targets seem to exist that are more sensitive 16 17 towards 1-NP than the β 2AR. Such low-concentration targets may be more important 18 scenarios of real-life exposure to pyrene and pyrene-derivatives. Other PAHs may have 19 considerably higher affinity for β 2AR. As reported in the present study and elsewhere (Mayati et al., 2012), B[a]P may induce β 2AR-dependent [Ca²⁺]_i-responses already at 1 μ M. In fact, 20 21 the affinity of B[a]P (Kd = 10 nM) appears to be among the highest reported for $\beta 2AR$ 22 ligands (Mayati et al., 2012). However, it should also be noted that 1-NP concentrations in 23 diesel exhaust particles may be up to 10-fold higher than the concentration of B[a]P 24 (Totlandsdal et al., 2012; Totlandsdal et al., 2014).

1	In extension of previous observations from endothelial cells (Mayati et al., 2011; Mayati et				
2	al., 2012), the present results suggest that PAHs may induce increased $[Ca^{2+}]_i$ through $\beta 2AR$ -				
3	activation also in epithelial lung cells. Moreover, $\beta 2AR$ -signalling and $[Ca^{2+}]_i$ appeared to be				
4	involved in the regulation of 1-NP-induced CXCL8 in the BEAS-2B cells. Although other yet				
5	unidentified cellular targets may be more sensitive to 1-NP exposure, β 2AR appears to be a				
6	highly sensitive for other PAHs (Mayati et al., 2012). PAHs have also been reported to impair				
7	β 2AR-function and interfere with asthma treatment (Factor et al., 2011). Thus, further studies				
8	are warranted to clarify the role of $\beta 2AR$ in PAH-induced responses and its possible				
9	implications in lung toxicity.				
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17 18	Reference List				
19 20 21	Edwards, M.R., Haas, J., Panettieri, R.A., Jr., Johnson, M., Johnston, S.L., 2007. Corticosteroids and beta2 agonists differentially regulate rhinovirus-induced interleukin-6 via distinct Cis-acting elements. J Biol Chem 282, 15366-15375.				
22 23 24 25	Factor, P., Akhmedov, A.T., McDonald, J.D., Qu, A., Wu, J., Jiang, H., Dasgupta, T., Panettieri, R.A., Jr., Perera, F., Miller, R.L., 2011. Polycyclic aromatic hydrocarbons impair function of beta2-adrenergic receptors in airway epithelial and smooth muscle cells. Am J Respir.Cell Mol Biol 45, 1045-1049.				
26 27 28	Hayakawa, K., Nakamura, A., Terai, N., Kizu, R., Ando, K., 1997. Nitroarene concentrations and direct-acting mutagenicity of diesel exhaust particulates fractionated by silica-gel column chromatography. Chem.Pharm Bull.(Tokyo) 45, 1820-1822.				
29 30 31 32	Holden, N.S., Rider, C.F., Bell, M.J., Velayudhan, J., King, E.M., Kaur, M., Salmon, M., Giembycz, M.A., Newton, R., 2010. Enhancement of inflammatory mediator release by beta(2)-adrenoceptor agonists in airway epithelial cells is reversed by glucocorticoid action. Br J Pharmacol 160, 410-420.				

1 2 3 4	Le, F.E., Lagadic-Gossmann, D., Rauch, C., Bardiau, C., Maheo, K., Massiere, F., Le, V.M., Guillouzo, A., Morel, F., 2002. Transcriptional induction of CYP1A1 by oltipraz in human Caco-2 cells is aryl hydrocarbon receptor- and calcium-dependent. J Biol Chem 277, 24780-24787.
5 6 7 8	Mayati, A., Le Ferrec, E., Lagadic-Gossmann, D., Fardel, O., 2011. Aryl hydrocarbon receptor-independent up-regulation of intracellular calcium concentration by environmental polycyclic aromatic hydrocarbons in human endothelial HMEC-1 cells. Environ.Toxicol.
9 10 11 12 13	Mayati, A., Levoin, N., Paris, H., N'Diaye, M., Courtois, A., Uriac, P., Lagadic-Gossmann, D., Fardel, O., Le, F.E., 2012. Induction of intracellular calcium concentration by environmental benzo(a)pyrene involves a beta2-adrenergic receptor/adenylyl cyclase/Epac-1/inositol 1,4,5-trisphosphate pathway in endothelial cells. J Biol Chem 287, 4041-4052.
14 15 16 17	N'Diaye, M., Le, F.E., Lagadic-Gossmann, D., Corre, S., Gilot, D., Lecureur, V., Monteiro, P., Rauch, C., Galibert, M.D., Fardel, O., 2006. Aryl hydrocarbon receptor- and calcium-dependent induction of the chemokine CCL1 by the environmental contaminant benzo[a]pyrene. J Biol Chem 281, 19906-19915.
18 19 20 21	Øvrevik, J., Arlt, V.M., Oya, E., Nagy, E., Mollerup, S., Phillips, D.H., Lag, M., Holme, J.A., 2010. Differential effects of nitro-PAHs and amino-PAHs on cytokine and chemokine responses in human bronchial epithelial BEAS-2B cells. Toxicol.Appl.Pharmacol. 242, 270-280.
22 23 24	Øvrevik, J., Lag, M., Holme, J.A., Schwarze, P.E., Refsnes, M., 2009. Cytokine and chemokine expression patterns in lung epithelial cells exposed to components characteristic of particulate air pollution. Toxicology 259, 46-53.
25 26 27	Øvrevik, J., Refsnes, M., Holme, J.A., Schwarze, P.E., Lag, M., 2013. Mechanisms of chemokine responses by polycyclic aromatic hydrocarbons in bronchial epithelial cells: sensitization through toll-like receptor-3 priming. Toxicol Lett. 219, 125-132.
28 29 30	Øvrevik, J., Refsnes, M., Totlandsdal, A.I., Holme, J.A., Schwarze, P.E., Lag, M., 2011. TACE/TGF-alpha/EGFR regulates CXCL8 in bronchial epithelial cells exposed to particulate matter components. Eur Respir.J 38, 1189-1199.
31 32	Park, E.J., Park, K., 2009. Induction of pro-inflammatory signals by 1-nitropyrene in cultured BEAS-2B cells. Toxicol.Lett. 184, 126-133.
33 34 35	Pei, X.H., Nakanishi, Y., Inoue, H., Takayama, K., Bai, F., Hara, N., 2002. Polycyclic aromatic hydrocarbons induce IL-8 expression through nuclear factor kappaB activation in A549 cell line. Cytokine 19, 236-241.
36 37 38 39	Scheepers, P.T., Martens, M.H., Velders, D.D., Fijneman, P., van Kerkhoven, M., Noordhoek, J., Bos, R.P., 1995. 1-Nitropyrene as a marker for the mutagenicity of diesel exhaust- derived particulate matter in workplace atmospheres. Environ.Mol.Mutagen. 25, 134- 147.

Totlandsdal, A.I., Herseth, J.I., Bolling, A.K., Kubatova, A., Braun, A., Cochran, R.E., 1 2 Refsnes, M., Øvrevik, J., Lag, M., 2012. Differential effects of the particle core and

3 organic extract of diesel exhaust particles. Toxicol Lett. 208, 262-268.

4 Totlandsdal, A.I., Øvrevik, J., Cochran, R.E., Herseth, J.I., Bolling, A.K., Lag, M., Schwarze, 5 P., Lilleaas, E., Holme, J.A., Kubatova, A., 2014. The occurrence of polycyclic 6 aromatic hydrocarbons and their derivatives and the proinflammatory potential of 7 fractionated extracts of diesel exhaust and wood smoke particles. J Environ Sci Health 8 A Tox.Hazard.Subst.Environ Eng 49, 383-396.

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2 Figure legends

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FIGURE 1. CXCL8 gene expression and protein release in 1-NP-exposed BEAS-2B cells, and role of Ca²⁺-chelation by BAPTA-AM. Cells were exposed to 20 μ M 1-NP or vehicle (DMSO) alone. CXCL8 gene expression was measured after 6 h by real-time PCR (A). Cells were pre-incubated with 10 μ M of the cell-permeable Ca²⁺-chelator BAPTA-AM for 30 min prior to exposure to 20 μ M 1-NP or vehicle (DMSO) alone for 18 h (B). The figure depicts mean ± SEM of three independent experiments. *Significantly different from unexposed controls (P < 0.05); †Significant down-regulation by chelator-treatment (P < 0.05)..

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FIGURE 2. Intracellular Ca²⁺-levels and role of **B2AR**-signaling in 1-NP exposed BEAS-12 13 **2B cells.** Cells were pre-incubated with 10 μ M of the β -blocker carazolol (Cara) prior to incubation with 10 µM 1-NP or vehicle (DMSO) alone (A and B). Intracellular Ca2+-14 concentrations ($[Ca^{2+}]_i$) were measured by incubation with the Ca²⁺-sensitive probe Fura-2 15 and the ratio of fluorescence intensities after excitation at 340 nm and 380 nm, respectively, 16 was used to estimate $[Ca^{2+}]_i$. Figure A depicts the mean of normalized $[Ca^{2+}]_i$ from two 17 independent continuous recordings, while figure B depicts the mean \pm SEM of three 18 independent experiments after 6 h (B) exposure. 19

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FIGURE 3. Role of β 2AR-signaling in CXCL8-responses in 1-NP-exposed BEAS-2B cells. Cells were pre-incubated for 30 min with a β 2AR-blocking antibody (A) or the β blocker ICI 118551 (B), or transfected with siRNA against β 2AR (si β 2AR) or non-targeting control siRNA (siNT) (C), prior to exposure with 20 μ M 1-NP or vehicle (DMSO) alone for 18 h. CXCL8 release were measured by ELISA. Efficiency of β 2AR knock-down by siRNA

1 was assessed by Western blotting (C). The figures depict mean ± SEM of three or more

- 2 independent experiments. *Significantly different from unexposed controls (P < 0.05);
- Acctebric 3 +Significant down-regulation by inhibitor/antibody/siRNA treatment (P < 0.05).







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- 2 Highlights
 - We examined mechanisms of 1-NP induced CXCL8 responses in BEAS-2B cells •
- Acceleration Treatment with a Ca²⁺-chelator abrogated the 1-NP-induced CXCL8 response •
 - 1-NP induced a rapid increase in intracellular Ca²⁺-levels •