Toxicology Letters 228 (2014) 42–47

Contents lists available at ScienceDirect

Toxicology Letters

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

Toll like receptor-3 priming alters diesel exhaust particle-induced cytokine responses in human bronchial epithelial cells

Nicolai S. Bach[a,b], Marit Låg[a], Johan Øvrevik[a,*]

[a] Department of Air Pollution and Noise, Division of Environmental Medicine, Norwegian Institute of Public Health, P.O. Box 4404, Nydalen, N-0403 Oslo, Norway

[b] Department of Biology, Faculty of Mathematics and Natural Sciences, University of Oslo, P.O. Box 1066, Blindern, N-0316 Oslo, Norway

HIGHLIGHTS

- We investigated cytokine responses by diesel exhaust particles (DEP) in BEAS-2B cells.
- DEP induced IL-6 and CXCL8 but not CCL5 in unprimed BEAS-2B cells.
- DEP induced stronger IL-6/CXCL8 responses but suppressed CCL5 in TLR3-primed cells.
- Combinatory effects of DEP and TLR3-priming were also observed on MAPKs and NF-κB.
- TLR3-priming may affect susceptibility toward proinflammatory effects of DEP.

ARTICLE INFO

Article history:
Received 13 December 2013
Received in revised form 26 March 2014
Accepted 27 March 2014
Available online 4 April 2014

Keywords:
Air pollution
Diesel exhaust
Particulate matter
Lung cells
Inflammation
Cytokines

ABSTRACT

Inflammation is considered central in the pathology of health effects from airborne particulate matter (PM). Preexisting inflammatory disorders, such as asthma, but also pulmonary infections, appear to be a risk factor of adverse health effects from PM exposure. Thus, to assess whether and how preexisting inflammation may sensitize lung cells toward additional proinflammatory effects of PM, human bronchial epithelial cells (BEAS-2B) were primed with the highly proinflammatory Toll-like receptor 3 (TLR3) ligand, Poly I:C, prior to exposure with diesel exhaust particles (DEP). DEP-exposure alone induced increased gene-expression of interleukin-6 (IL-6) and CXCL8 (IL-8) but did not affect expression of CCL5 (RANTES), while TLR3-priming alone induced expression of IL-6, CXCL8 and CCL5. DEP-exposure exacerbated IL-6 and CXCL8 responses in TLR3-primed cells, while TLR3-induced CCL5 was suppressed by DEP. TLR3-priming and DEP-exposure resulted in possible additive effects on p38 phosphorylation and IκB-degradation, while DEP rather suppressed ERK and JNK-activation. However, TLR3-priming elicited a considerable increase in p65-phosphorylation at serine 536 which is known to enhance the transcriptional activity of NF-κB. DEP-exposure was unable to induce p65-phosphorylation. Thus TLR3-priming may affect susceptibility toward DEP by activating both shared and complementing pathways required for optimal expression of proinflammatory genes such as IL-6 and CXCL8. The study underscores that primed "sick" cells may be more susceptible toward effects of particle-exposure and respond both stronger and differently compared to unprimed "healthy" cells.

1. Introduction

Urban air particulate matter (PM) is associated with the development or exacerbation of a variety of adverse cardiopulmonary outcomes (Donaldson et al., 2001; Kelly and Fussell, 2011; Sacks et al., 2011). PM has been suggested to contribute to the pathogenesis of disease development by promoting chronic low-grade inflammation (Donaldson et al., 2001; Kelly and Fussell, 2011; Salvi and Holgate, 1999). Moreover, preexisting inflammation-related disorders such as chronic obstructive pulmonary disease (COPD) and asthma, but also virus infections, appear to represent susceptibility factors for adverse effects of PM-exposure (Sacks et al., 2011; Wong et al., 2010). Thus preexisting inflammation may possibly sensitize the airways toward additional proinflammatory effects of PM-exposure.

The pulmonary epithelium is a physical barrier to the outside environment, and is therefore a primary target of inhaled pollutants. In a recent study, we showed that priming bronchial

* Corresponding author at: Department of Air Pollution and Noise, Division of Environmental Medicine, Norwegian Institute of Public Health, P.O. Box 4404, Nydalen, N-0403 Oslo, Norway. Tel.: +47 21076408; fax: +47 21076686.
E-mail address: johan.ovrevik@fhi.no (J. Øvrevik).

http://dx.doi.org/10.1016/j.toxlet.2014.03.021
0378-4274/

epithelial cells (BEAS-2B) with the highly proinflammatory Toll-like receptor 3 (TLR3) ligand polyinosinic:polycytidylic acid (Poly I:C), sensitized the cells toward additional proinflammatory effects of polycyclic aromatic hydrocarbons (PAHs) from combustion-derived pollution (Ovrevik et al., 2013). Poly I:C, which is a synthetic double-stranded RNA analog often used to mimic virus infection, induced release of the neutrophil attracting chemokine CXCL8 (interleukin-8: IL-8) and the eosinophil attractant CCL5 (Regulated upon Activation Normal T-cell Expressed and Secreted: RANTES). Pyrene and pyrene derivatives, at concentrations unable to provoke chemokine responses in unprimed cells, exacerbated CXCL8 and CCL5 responses in TLR3-primed cells (Ovrevik et al., 2013). The mechanisms underlying this increased sensitivity of TLR3-primed cells has not been explored, but conceivably the TLR3-priming and PAH exposure acted in concert on common/shared signaling pathways involved in chemokine regulation, or perhaps more likely, TLR3-priming activated complementary pathways required for maximal chemokine induction by the PAHs.

Activation of the redox sensitive transcription factor nuclear factor (NF)-κB, appears to be indispensable for CXCL8 expression (Hoffmann et al., 2002). However, NF-κB activation alone may have little effect on CXCL8 unless additional pathways are activated. Thus, a combined activation of NF-κB along with the three main MAPK pathways ERK1/2, JNK1/2 and p38, may be required for optimal expression of CXCL8. ERK and JNK appears to elicit their effects by regulating additional transcription factors involved in CXCL8 regulation, such as activator protein (AP)-1, while p38 also promote mRNA stabilization (Hoffmann et al., 2002). In addition to their effects on CXCL8, NF-κB and MAPKs are known to be involved in regulation of a variety of other proinflammatory genes.

The present study aimed to assess whether a preexisting inflammation may represent a susceptibility factor for effects of combustion-derived PM in lung cells. We have previously shown that diesel exhaust particles (DEP), a major component of urban air PM, induce increased expression and release of CXCL8 and IL-6 from BEAS-2B cells (Totlandsdal et al., 2012). Thus, we hypothesized that (1) TLR3-priming would sensitize BEAS-2B cells toward additional proinflammatory effects of DEP-exposure, and (2) that any combinatory effects of TLR3-priming and DEP-exposure could be attributed to effects on MAPK-signaling and/or NF-kB activation.

2. Materials and methods

2.1. Reagents

LHC-9 cell culture medium was from Invitrogen (Carlsbad, CA, USA) and PureCol™ collagen from Inamed Biomaterials (Fremont, CA, USA). Polyinosinic:polycytidylic acid (Poly I:C), Ponceau S, phenylmethylsulfonyl fluoride (PMSF), aprotinin, ethylenediaminetetraacetic acid (EDTA), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and polyoxyethylene octyl phenyl ether (Triton X-100) were purchased from Sigma–Aldrich (St. Louis, MO, USA). All real-time RT-PCR reagents and TaqMan probes/primers were purchased from Applied Biosystems (Foster City, CA, USA). Bio-Rad DC protein assay was from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Pepstatin A was from Calbiochem (Cambridge, MA, CA, USA). Leupeptin was from Amersham Biosciences (Uppsala, Sweden). Antibodies against phospho- and total ERK1/2 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against IκB, phospho- and total p65, p38, JNK1/2, were from Cell Signaling Technology (Beverly, MA, USA). Antibodies against β-actin, as well as secondary antibodies horseradish peroxidase-conjugated goat-anti-rabbit IgG, were from Sigma–Aldrich Chemical Company (St. Louis, MO, USA). Horseradish peroxidase-conjugated rabbit anti-mouse IgG from Dako (Glostrup, Denmark) was applied. Mild antibody stripping solution® was from Chemicon International (Termecula, CA, USA). All other chemicals used were purchased from commercial sources at the highest purity available.

2.2. Culture of cells

BEAS-2B cells, an immortalized SV40-adenovirus-hybrid (Ad12SV40) transformed human bronchial epithelial cell line was from European Collection of Cell Cultures (ECACC, Salisbury, UK). Cells were grown at 37 °C in a humidified incubator with a 5% CO_2 atmosphere, where they were passaged twice per week. Cells were cultured in serum-free LHC-9 medium on collagen (PureCol™)-coated culture dishes and flasks. Prior to exposure, cells were plated in 6-well culture dishes, grown to near confluence in serum free LHC-9 medium and exposed as described below.

2.3. Particles

DEP (MAPCEL soot) were generated by an unloaded diesel engine (Deutz, 4 cylinder, 2.2 L, 500 rpm) using gas oil as described elsewhere (Totlandsdal et al., 2010). For each experiment, particles were suspended in fresh LHC-9 cell exposure medium (2 mg/ml) and stirred overnight in room temperature before exposure.

2.4. Exposure of cells

In all experiments, fresh medium was added the day after seeding and right before exposure and, depending on the experiments, the cells were exposed to 50 or 100 μg/ml DEP for 2, 4 and/or 6 h as described under the respective experiments. The controls were added medium that had been subjected to the same stirring procedure as the particle suspensions. In experiments with primed cells, Poly I:C (10 μg/ml) was added 30 min prior exposure to DEP, previously optimized in our laboratory.

2.5. Gene expression analysis by real-time RT-PCR

Total RNA was isolated using Absolutely RNA Miniprep Kit (Stratagene, La Jolla, CA, USA) and reverse transcribed to cDNA on a PCR System 2400 (PerkinElmer, Waltham, MA, USA) using a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed using pre-designed TaqMan Gene Expression Assays and TaqMan Universal PCR Master Mix and run on ABI 7500 fast (Applied Biosystems). Gene expression of IL-6 (Hs00174131_m1), CXCL8 (Hs00174103_m1) and CCL5 (Hs00174575_m1) were normalized against 18S rRNA (Hs99999901_s1), and expressed as fold change compared to untreated control as calculated by the $\Delta\Delta C_t$ method ($\Delta C_t = C_t$[Gene of Interest] – Ct[18S]; $\Delta\Delta C_t = \Delta C_t$[Treated] – ΔC_t[Control]; fold change = $2^{[-\Delta\Delta Ct]}$).

2.6. Examination of protein levels by Western blotting

DEP-induced phosphorylation of MAPK (ERK1/2, JNK1/2, p38) and p65, and degradation of IκB were measured by Western blot analysis. After exposure, cell culture medium was removed and the dishes were immediately rinsed with ice-cold PBS, and stored at −70 °C until further processing. Frozen cells were thawed, harvested and sonicated in lysis buffer (20 mM Tris–HCl, pH = 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.4 mM Na-pyrophosphate, 1.0 mM orthovanadate, 1 mM NaF, 21 μM leupeptin, 1.5 μM aprotinin, 15 μM pepstatin A, 1 mM PMFS and 1% Triton-X) prior to protein determination using the BioRad DC Protein Assay (BioRad Life Science, CA, USA). Subsequently glycerol, β-mercaptoethanol and SDS were added to all samples, and final sample protein concentrations were adjusted by adding more lysis buffer. Proteins (10–20 μg/well) from whole-cell lysates were separated by 10–15% SDS-PAGE and blotted onto nitrocellulose membranes. To ensure that the protein levels of each well were equal, Ponceau-staining was used for loading control. The membranes were then probed with antibodies for the respective phosphorylated kinases (p-ERK1/2, p-JNK1/2, p-p38), antibodies for IκB or antibodies for phosphorylated p65, prior to incubation with horseradish peroxidase-conjugated secondary antibodies. The blots were developed using the Super-Signal® West Dura chemiluminescence system (Pierce, Perbio Science, Sweden) according to the manufacturer's instructions. Finally, the membranes were stripped by incubation for 15 min at room temperature with mild antibody stripping solution, and re-probed with β-actin, or with the total amount of the respective kinases or p65. Optical quantification of the protein bands was performed by using Image Lab Analysis Software (BioRad).

2.7. Statistical analysis

Multiple comparisons were analyzed by the Holm–Sidak method. Results are expressed as means ± SEM. All calculations were performed using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA).

3. Results

3.1. DEP-induced cytokine and chemokine expression

As a measure of the proinflammatory effects of DEP-exposure we assessed the expression of IL-6, CXCL8 and CCL5 by real-time PCR. First we explored the time-course of DEP-induced cytokine/chemokine gene expression after 2, 4 and 6 h exposure. DEP (100 μg/ml) induced a statistically significant up-regulation of IL-6 mRNA at 2 and 4 h, while CXCL8 was statistically significantly increased at all tested time-points in DEP-exposed cells (Fig. 1).