



Expression and role of EGFR ligands induced in airway cells by PM_{2.5} and its components

M. Rumelhard, K. Ramgolam, R. Hamel, F. Marano and A. Baeza-Squiban

ABSTRACT: The aim of the current study was to establish the epidermal growth factor receptor (EGFR) ligand expression profile in human airway epithelial cells exposed to either particulate matter (PM) with an aerodynamic diameter $<2.5\ \mu\text{m}$ (PM_{2.5}) or its components and the involvement of EGFR ligands in PM_{2.5}-provoked airway inflammation.

EGFR ligand mRNA and protein expression were studied in a human bronchial epithelial cell line and normal nasal cells exposed to noncytotoxic concentrations of PM_{2.5} or its components. The autocrine role of EGFR ligands in airway epithelial cell pro-inflammation was determined by adding conditioned media from PM_{2.5}-treated cells to fresh cells and measuring the secretion of granulocyte-macrophage colony-stimulating factor (GM-CSF), a pro-inflammatory biomarker.

PM_{2.5} increased amphiregulin, transforming growth factor- α and heparin-binding EGF-like growth factor mRNA expression and protein secretion, with a slight contribution of aqueous metallic compounds and a strong participation of organic components putatively attributed to PM polyaromatic hydrocarbon content. PM_{2.5}-induced EGFR ligands were involved in cellular GM-CSF release.

The current study revealed upregulation of several epidermal growth factor receptor ligands by airway epithelial cells exposed to particulate matter with an aerodynamic diameter $<2.5\ \mu\text{m}$ and their contribution to bronchial epithelial cell granulocyte-macrophage colony-stimulating factor secretion by an autocrine action, suggesting that these ligands could elicit and sustain the particulate matter-induced airway pro-inflammatory response and contribute to bronchial remodelling.

KEYWORDS: Ambient particles, epidermal growth factor receptor ligands, inflammation, organic particulate matter with a diameter $<2.5\ \mu\text{m}$, respiratory epithelial cells

Considerable attention has been devoted to the health effects of atmospheric pollution, particularly in urban areas. Numerous epidemiological studies have correlated exposure to fine particulate matter (PM) with cardiovascular and respiratory morbidity and mortality, including the development of chronic bronchitis, asthma and lung cancer [1]. Urban PM with a diameter $<2.5\ \mu\text{m}$ (PM_{2.5}) is a complex mixture, including soot, produced by combustion with which organic, metallic and biological components are associated.

Human and animal studies have shown lung inflammation following brief exposure to diesel exhaust particles or to concentrated ambient particles [2, 3]. In addition, the *in vitro* release of

pro-inflammatory cytokines, *i.e.* granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-8, *etc.* by bronchial epithelial cells exposed to PM_{2.5}, has shown the role of epithelium in this airway pro-inflammatory response [4]. Long-term exposure to pollution has been associated with histopathological lung damage, such as bronchioli wall thickening, concomitant with the presence of numerous particles in the lungs of exposed individuals [5]. It is thus suspected that PM_{2.5} may provoke or exacerbate airway remodelling, a phenomenon characterised, especially in chronic obstructive pulmonary disease, by epithelial mucous metaplasia associated with airway wall fibrosis, inflammation, enlargement of bronchial smooth muscle mass and modifications of lung growth factor expression and/or functions

AFFILIATIONS

Laboratory of Cytophysiology and Cellular Toxicology, University of Paris Diderot, Paris, France.

CORRESPONDENCE

M. Rumelhard
Laboratory of Cytophysiology and Cellular Toxicology
Tour 53-54
Case Courrier 7073
University of Paris Diderot-Paris 7
2 place Jussieu
75 251 Paris Cedex 05
France
Fax: 33 144276999
E-mail: melina.rumelhard@univ-diderot-paris.fr

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STATEMENT OF INTEREST

A statement of interest for this study can be found at www.erj.ersjournals.com/misc/statements.shtml

(such as epidermal growth factor (EGF) and basal fibroblast growth factor, *etc.*) [6]. Furthermore, as recently shown in the current authors' laboratory, exposure of human bronchial epithelial cells to PM_{2.5} or diesel exhaust particles leads to the release of amphiregulin, a ligand of the EGF receptor (EGFR) [7].

EGFR is a 173 kD receptor tyrosine kinase predominantly located at the basolateral surface of polarised epithelial cells. Several ligands are able to bind to the mammalian EGFR (c-erbB1, human epidermal receptor 1) with various affinities: EGF, transforming growth factor (TGF)- α , heparin-binding EGF-like growth factor (HB-EGF), amphiregulin, betacellulin, epiregulin and epigen. EGFR ligands have autocrine properties, which may explain the preferential co-localisation of the ligands and their receptor at the basolateral surface. Indeed, TGF- α and amphiregulin are functionally involved in an EGFR autocrine growth loop in human normal bronchial epithelial cells [8].

EGFR and its ligands play a pivotal role in repair and maintenance of epithelial tissues [9]. Indeed, it has been shown that EGF and EGFR are involved in repair of scrape-wounded human bronchial epithelial (16HBE)14o- monolayers *in vitro* [10]. Moreover, EGFR is overexpressed in the airway epithelium of asthmatic subjects [10]. In addition to this implication in tissue repair, amphiregulin and HB-EGF have been associated with pro-inflammatory cytokine release (GM-CSF, IL-8) after exposure to PM or cigarette smoke, respectively [7, 11].

The aims of the current study were to establish the expression pattern of EGFR ligands induced in human respiratory epithelial cells by PM_{2.5} and its different components, and then to determine the resulting consequences, for airway epithelial cells, of increased EGFR ligand production, due to their potential involvement in pro-inflammatory responses.

MATERIALS AND METHODS

Particles

PM_{2.5} was collected in Vitry-sur-Seine, a suburban city near Paris (France), according to a previously published method [12].

The polyaromatic hydrocarbon (PAH) and metal content of the PM was characterised, respectively, by high-pressure liquid chromatography after extraction with dichloromethane, and by inductively coupled plasma-atomic emission spectrometry or flame photometry (table 1) according to previously described methods [12].

Stock suspensions of native particles (2 mg·mL⁻¹) were prepared in a 0.04% dipalmitoyl phosphatidyl lecithine (DPL; Sigma-Aldrich, Saint-Quentin-Fallavier, France) solution. DPL is a surfactant component used to maintain particles in suspension, and is consequently used as a negative control for particle exposure. For treatment, PM was sonicated six times for 10 s (Vibracell; Bioblock Scientific, Illkirch, France) and suspended in culture medium.

The organic extract was obtained by extraction of PM_{2.5} with dichloromethane in an accelerated solvent extractor (ASE 200; Dionex, Sunnyvale, CA, USA). The extract was dried and dissolved in dimethyl sulfoxide.

The aqueous extract containing hydrosoluble components was obtained after two 10-min centrifugations of the 2 mg·mL⁻¹

TABLE 1 Polyaromatic hydrocarbon (PAH) and metal content in particulate matter with a diameter <2.5 μ m

PAH μ g·g ⁻¹	
Naphtalene	8.12
Acenaphthalene	n.d.
Fluorene	0.41
Phenanthrene	2.51
Anthracene	0.58
Fluoranthrene	4.84
Pyrene	5.48
Benzo(a)anthracene	2.88
Chrysene	5.67
Benzo(b)fluoranthrene	14.64
Benzo(k)fluoranthrene	5.44
Benzo(a)pyrene	5.29
Dibenzo(a,h)anthracene	11.88
Benzo(ghi)perylene	13.30
Indeno pyrene	7.31
2-3 cycles	11.62
>3 cycles	76.72
Total	88.34
Soluble organic fraction %	9
Metals mg·g ⁻¹	
Na	20.7
Mg	4.3
Al	10.4
K	8
Ca	59.6
Fe	13.5
Zn	1.3
Ti	0.8
Mn	0.42
Cu	0.37
Sb	0.093
Pb	0.52
V	0.11
Cr	0.077
Ni	0.059
As	0.025
Cd	0.012
Total	120.29
Fraction %	12

n.d.: not done.

particle suspension, at 10,000 \times g, followed by filtration of the supernatant through a 0.22 μ m filter (Millex® GV, Carrigtohill, Cork, Ireland). Soluble metals present in the particle aqueous extract were depleted by filtration on chelating ion-exchange resins with Chelex® resin according to the manufacturer's instructions (Chelex 100, Bio-Rad, Marnes-la-Coquette, France).

Particles collected after the two centrifugations and suspended in a solution of DPL at 2 mg·mL⁻¹ constituted the washed PM_{2.5} devoid of hydrosoluble components.

Black carbon particles at 2 mg·mL⁻¹ (95 nm diameter; Degussa, Frankfurt, Germany) were used to mimic the carbonaceous

TABLE 2 Forward and reverse primers of epidermal growth factor ligands and reduced glyceraldehyde phosphate dehydrogenase (GAPDH)

	Forward primer	Reverse primer
Amphiregulin	5'TGG TGC TGT CGC TCT TGA TA3'	5'CCC TGA AGA CAT CTC ACT TC3'
Betacellulin	5'GAT GAA GGC TAC ATT GGA GC3'	5'TTA CGA CGT TTC CGA AGA GG3'
GAPDH	5'TCA CCA GGG CTG CTT TTA AC3'	5'GAC AAG CTT CCC GTT CTC AG3'
HB-EGF	5'GGA GAG GAG GTT ATG ATG TG3'	5'TTT GGC ACT TGA AGG CTC TG3'
TGF-α	5'CCC TGG CTG TCC TTA TCA TC3'	5'GTT TCT GAG TGG CAG CAA GC3'

HB-EGF: heparin-binding epidermal growth factor-like growth factor; TGF: transforming growth factor.

core of PM2.5, as production of PM2.5-devoid of its organic compounds was technically impossible.

Treatment

Cells were exposed to noncytotoxic concentrations of native PM2.5 (1, 5 or 10 $\mu\text{g}\cdot\text{cm}^{-2}$) or to a volume of washed PM2.5, aqueous extract and black carbon particles equivalent to the volume of native PM2.5. Organic extract was used at a concentration equivalent to that found on PM2.5, this value was calculated according to the proportion of soluble organic fraction in the total dry weight of PM2.5 (9%).

For the conditioned media, the following protocol was followed: cells were exposed for 24 h to PM2.5 and the culture medium was removed, centrifuged for 10 min at 10,000 g to eliminate particles, and the GM-CSF content measured. This constituted the conditioned media, which was applied for 24 h to fresh cells to further investigate the secreted GM-CSF, amphiregulin and TGF- α .

Anti-EGFR monoclonal antibody (clone LA1) was purchased from R&D Systems (Abingdon, UK) and used at 0.5 $\mu\text{g}\cdot\text{mL}^{-1}$. Anti-amphiregulin, anti-HB-EGF and anti-TGF- α polyclonal antibodies were also purchased from R&D Systems and used at respective concentrations of 20, 10 and 1 $\mu\text{g}\cdot\text{mL}^{-1}$.

Monoclonal mouse immunoglobulin (Ig)G antibody used as a control antibody was purchased from DakoCytomation (Dako France, Trappes, France). α -naphthoflavone (Sigma-Aldrich) was used as antagonist of the aryl hydrocarbon receptor (AhR). Benzo(a)pyrene (Sigma-Aldrich) at 1 μM was used as a positive control for AhR activation.

Cell cultures

16HBE14o- cells were kindly provided by D.C. Gruenert (Research Facility, California Pacific Medical Center, San Francisco, CA, USA). Primary cultures of normal human nasal epithelial (NHNE) cells were obtained from turbinates of patients undergoing turbinectomy (kindly supplied by J. Soudant, Service ORL, CHU La Pitié-Salpêtrière, Paris, France).

Cell culture details are provided in the online supplementary material. During treatment and for 4 h before cell exposure, 16HBE and NHNE cells were grown in DMEM/F12 without growth factors.

Gene expression analysis

Total RNA were extracted from 16HBE14o- cells using an SV Total RNA Isolation kit (Promega, Charbonnières, France)

according to the manufacturer's instructions. Reverse transcription (RT) of 1 μg of total RNA was then performed as described by RUMELHARD *et al.* [13], one-tenth of the RT reaction was then amplified using quantitative PCR in the presence of 1X Brilliant® SYBR® Green Master mix (Stratagene, La Jolla, CA, USA) and forward and reverse primers (table 2). Amphiregulin, betacellulin, HB-EGF and reduced glyceraldehyde phosphate dehydrogenase primers were purchased from Operon Biotechnologies (Cologne, Germany) and TGF- α primers from Invitrogen (Paisley, UK). Samples were amplified in a MX3000P® QPCR System (Stratagene) under the following conditions: 10 min at 94°C; 40 cycles of 30 s at 94°C, 1 min at 58°C and 45 s at 72°C; 1 min at 95°C; and a ramp from 55°C to 95°C.

The reduced glyceraldehyde phosphate dehydrogenase gene was used as an internal standard. The comparative cycle threshold method ($2^{-\Delta\Delta\text{Ct}}$ method) was used to analyse data and determine the relative abundance of the target genes. Ct is the threshold cycle at which SYBR® Green emission is determined to be statistically different above background. To obtain the $\Delta\Delta\text{Ct}$ values, the difference between Ct of a target gene (CtA) and house keeping gene (CtB) was calculated in treated cells ($\Delta\text{CtT}=\text{CtA,T}-\text{CtB,T}$) and in untreated cells ($\Delta\text{CtUT}=\text{CtA,UT}-\text{CtB,UT}$). Finally, the relative abundance was calculated as $2^{-\Delta\Delta\text{Ct}}$.

Analysis of EGFR ligand and GM-CSF release

Cell culture media was removed from cells and centrifuged for 10 min at 10,000 $\times g$ (4°C). Enzyme-linked immunosorbent assay kits for amphiregulin, TGF- α , betacellulin and GM-CSF (R&D Systems) were then used following the manufacturer's instructions.

Statistical analysis

Results are expressed as mean \pm SD. Data comparisons were carried out using one way ANOVA followed by Dunnett's post-test to compare treatments with their controls, or by Student–Newman–Keuls to compare the different treatment groups. Differences were considered significant for $p<0.05$.

RESULTS

Effects of PM2.5 on EGFR ligand mRNA and protein expression in respiratory epithelial cells

A time-course study (2, 6, 18, 24 and 48 h) with 16HBE cells treated with PM2.5 at 10 $\mu\text{g}\cdot\text{cm}^{-2}$, a noncytotoxic concentration (data not shown), revealed that among the four EGFR ligands

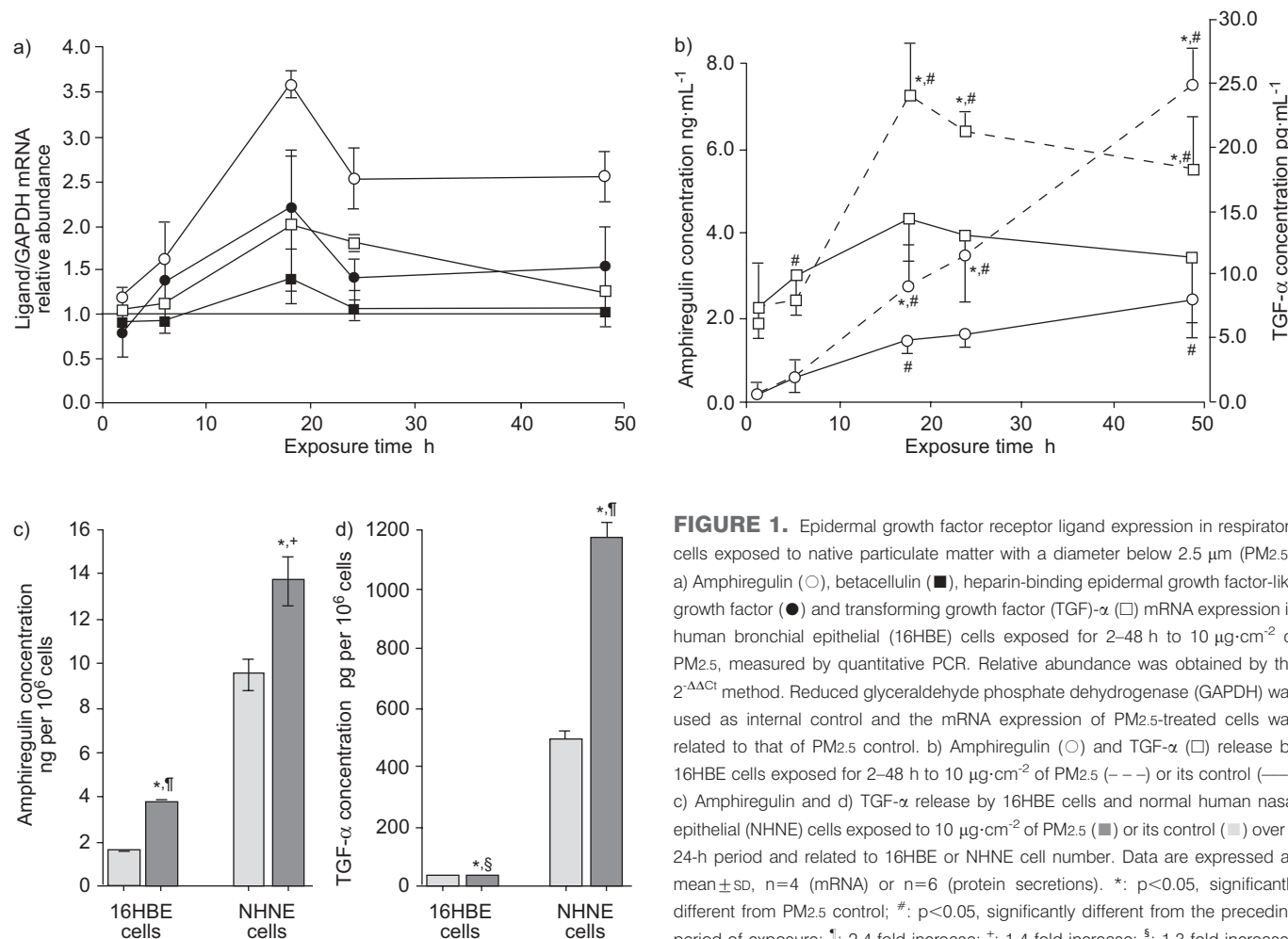


FIGURE 1. Epidermal growth factor receptor ligand expression in respiratory cells exposed to native particulate matter with a diameter below 2.5 μm (PM_{2.5}). a) Amphiregulin (○), betacellulin (■), heparin-binding epidermal growth factor-like growth factor (●) and transforming growth factor (TGF)- α (□) mRNA expression in human bronchial epithelial (16HBE) cells exposed for 2–48 h to 10 $\mu\text{g}\cdot\text{cm}^{-2}$ of PM_{2.5}, measured by quantitative PCR. Relative abundance was obtained by the $2^{-\Delta\Delta\text{CT}}$ method. Reduced glyceraldehyde phosphate dehydrogenase (GAPDH) was used as internal control and the mRNA expression of PM_{2.5}-treated cells was related to that of PM_{2.5} control. b) Amphiregulin (○) and TGF- α (□) release by 16HBE cells exposed for 2–48 h to 10 $\mu\text{g}\cdot\text{cm}^{-2}$ of PM_{2.5} (---) or its control (—). c) Amphiregulin and d) TGF- α release by 16HBE cells and normal human nasal epithelial (NHNE) cells exposed to 10 $\mu\text{g}\cdot\text{cm}^{-2}$ of PM_{2.5} (■) or its control (■) over a 24-h period and related to 16HBE or NHNE cell number. Data are expressed as mean \pm SD, $n=4$ (mRNA) or $n=6$ (protein secretions). *: $p<0.05$, significantly different from PM_{2.5} control; #: $p<0.05$, significantly different from the preceding period of exposure; †: 2.4-fold increase; ‡: 1.4-fold increase; §: 1.3-fold increase.

studied, the earliest upregulated genes were amphiregulin and HB-EGF from 6 h (fig. 1a). Amphiregulin and HB-EGF were further upregulated by 3.6- and 2.1-fold, respectively, up to 18 h, this then diminished in the following 30 h but remained sustained for amphiregulin (2.5-fold at 48 h). TGF- α mRNA expression was significantly increased to approximately the same extent (two-fold) as that of HB-EGF mRNA after 18–24 h of exposure, but was more transient as it descended to basal levels at 48 h. Betacellulin mRNA expression in 16HBE cells was virtually unmodulated by PM_{2.5} exposure.

The time-course study (2, 6, 18, 24 and 48 h) shown in figure 1b for 10 $\mu\text{g}\cdot\text{cm}^{-2}$ PM_{2.5} exposure revealed a significant induction of both amphiregulin and TGF- α secretion in 16HBE cell culture supernatants starting at 18 h of exposure. Amphiregulin secretion increased throughout the time, reaching a concentration of 7.4 $\text{ng}\cdot\text{mL}^{-1}$ after 48 h of exposure, whereas TGF- α secretion did not evolve and even decreased (24 $\text{pg}\cdot\text{mL}^{-1}$ at 18 h and 21 $\text{pg}\cdot\text{mL}^{-1}$ at 24 h). For constitutive and also inducible release, the amount of amphiregulin (in the $\text{ng}\cdot\text{mL}^{-1}$ range) was higher than that of TGF- α (in the $\text{pg}\cdot\text{mL}^{-1}$ range). The dose-dependent study (supplementary fig. 1) revealed a significant secretion of both ligands from 1 $\mu\text{g}\cdot\text{cm}^{-2}$ of PM_{2.5} that seemed to be maximal from 5 $\mu\text{g}\cdot\text{cm}^{-2}$ of PM_{2.5}. No release of betacellulin was detectable in the supernatants of

cells either at the basal level or after particle exposure (data not shown).

The comparison of EGFR ligand secretion induced by 10 $\mu\text{g}\cdot\text{cm}^{-2}$ of PM_{2.5} in 16HBE cells and in primary cultures of epithelial cells (fig. 1c) revealed that amphiregulin and TGF- α secretion was also induced by PM_{2.5}-treated NHNE cells. In addition, when relating secretion data to cell number, figure 1c revealed that the amphiregulin concentration was six-fold higher in NHNE than in 16HBE supernatants. TGF- α was also more strongly secreted by NHNE cells than 16HBE cells, but to a higher extent (16-fold). Again, betacellulin secretion was not detectable (data not shown).

Involvement of PM components in PM_{2.5}-induced EGFR ligand upregulation

Amphiregulin and TGF- α mRNA expression were measured by quantitative PCR after the treatment of 16HBE cells by noncytotoxic concentrations (data not shown) of PM_{2.5} organic and aqueous extracts. PM_{2.5} organic extract induced both amphiregulin and TGF- α mRNA expression (fig. 2a and b), to approximately the same extent as native PM over 24 h of exposure, but with a sustained upregulation up to 48 h of treatment. In contrast, PM_{2.5} aqueous extract has only a very

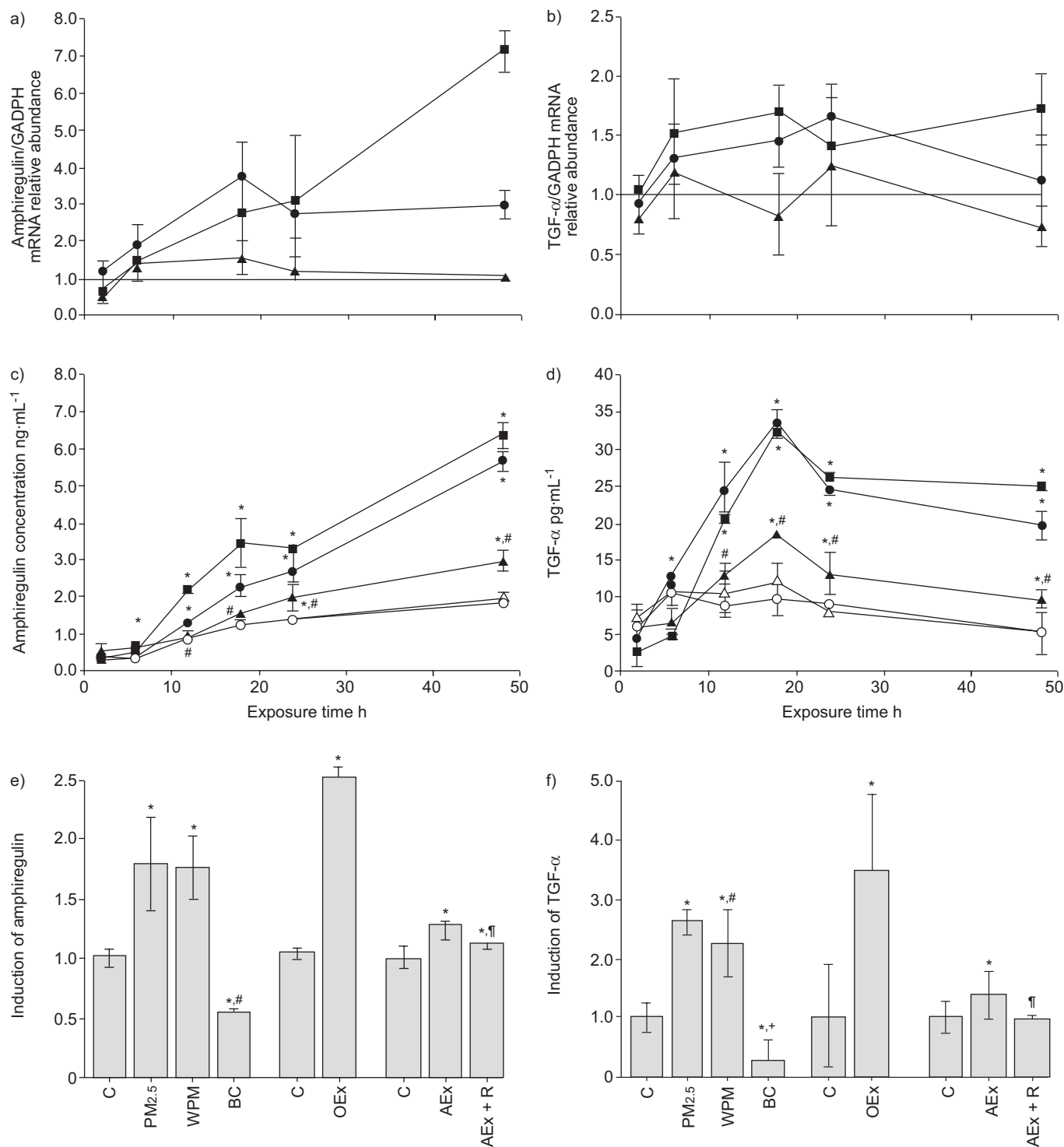


FIGURE 2. Contribution of particulate matter (PM) with a diameter below 2.5 µm (PM_{2.5}) components to epidermal growth factor receptor (EGFR) ligand upregulation. a) Amphiregulin and b) transforming growth factor (TGF)-α relative abundances compared with mRNA expression of reduced glyceraldehyde phosphate dehydrogenase (GAPDH) in human bronchial epithelial (16HBE) cells exposed to 10 µg·cm⁻² of PM_{2.5} (●), or to an equivalent concentration of PM organic extract (OEx; 4.27 µg·mL⁻¹, ■) or to an equivalent volume of PM aqueous extract (AEx; 19 µL, ▲) from 2–48 h of exposure. c) Amphiregulin and d) TGF-α release by 16HBE cells exposed from 2–48 h. △: particle control; ●: PM_{2.5}; ■: OEx; ○: OEx control; ▲: AEx. e) Amphiregulin and f) TGF-α release induction by 16HBE cells exposed for 18 h to 10 µg·cm⁻² of PM with a diameter <2.5 µm (PM_{2.5}), or to the equivalent volume of PM AEx (19 µL) alone or with pre-treatment by a Chelex resin (R), washed PM_{2.5} (WPM; 10 µg·cm⁻²), black carbon (BC; 10 µg·cm⁻²) and their relative control (C) or to the equivalent concentration of PM OEx (4.27 µg·mL⁻¹) and its control. Data are expressed as mean ± SD, n=3. *: p<0.05, significantly different from treatment control; #: p<0.05, significantly different from PM_{2.5} treatment; †: p<0.05, significantly different from AEx treatment.

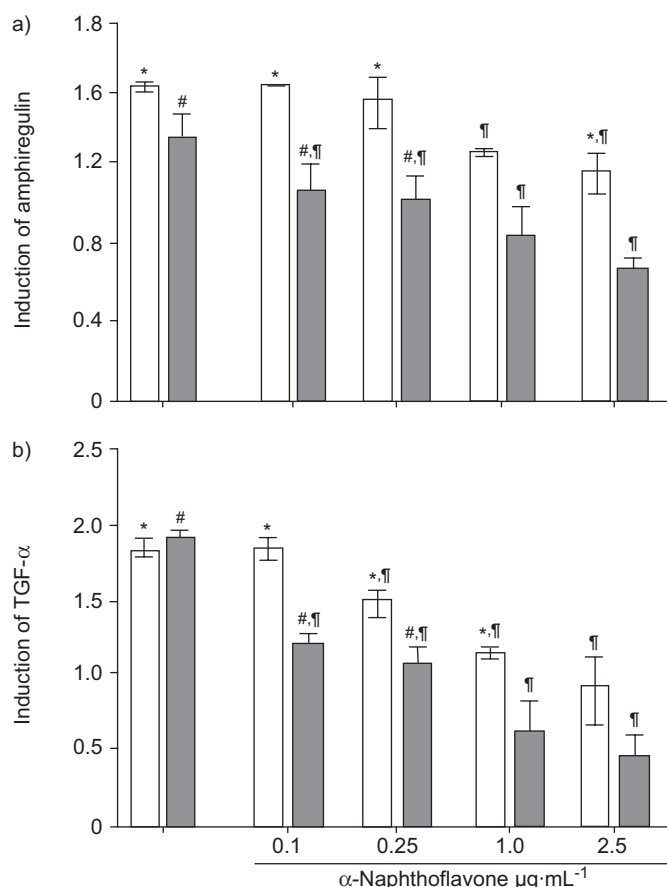


FIGURE 3. Involvement of the aryl hydrocarbon receptor (AhR) pathway in particulate matter (PM)-induced epidermal growth factor receptor ligand secretion. a) Amphiregulin and b) transforming growth factor (TGF)-α release induction by human bronchial epithelial cells exposed for 24 h to 10 μg·cm⁻² of PM with a diameter below 2.5 μm (PM_{2.5}: □) or 1 μM of benzo(a)pyrene (■) alone or with 30 min pre-treatment by 0.1–2.5 μg·mL⁻¹ of α-naphthoflavone, an AhR antagonist. Data are expressed as mean ± SD, n=3. *: p<0.05, significantly different from PM_{2.5} control; #: p<0.05, significantly different from benzo(a)pyrene control; †: p<0.05, significantly different from release without pre-treatment by α-naphthoflavone.

transient and weak effect on amphiregulin mRNA expression (fig. 2a) and no significant effect on TGF-α mRNA expression (fig. 2b).

A kinetic study (fig. 2c and d) revealed that amphiregulin and TGF-α release was significantly induced by the organic PM extract from 6 h of exposure, with a level of induction almost similar to the one of native PM. The aqueous extract also induced amphiregulin and TGF-α release with a significant induction from 18–24 h of exposure (fig. 2c and d). The level of induction was far weaker than that of native PM (fig. 2c–f) and was almost completely abrogated when metals were removed from the aqueous extract using a Chelex resin (fig. 2e and f).

Washed particles, devoid of their hydrosoluble compounds, induced amphiregulin and TGF-α to approximately the same level as native PM_{2.5} (1.8- and 2.5-fold for amphiregulin and TGF-α, respectively; fig. 2e and f), while there was virtually no effect of black carbon on amphiregulin and TGF-α release (fig. 2e and f). NHNE cells responded in a similar fashion to

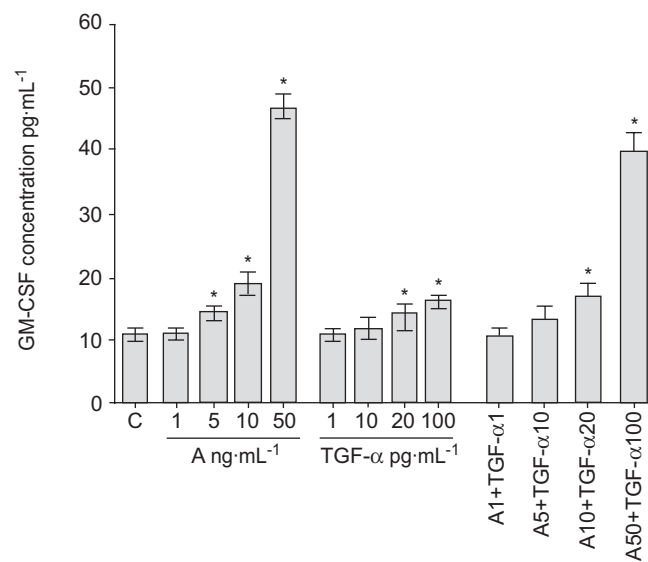


FIGURE 4. Pro-inflammatory response triggered in bronchial epithelial cells exposed to epidermal growth factor receptor (EGFR) ligands. Granulocyte-macrophage colony-stimulating factor (GM-CSF) release by human bronchial epithelial cells exposed for 24 h to 1–50 ng·mL⁻¹ of amphiregulin (A), 1–100 pg·mL⁻¹ of transforming growth factor (TGF)-α or to the combination of these two EGFR ligands. Data are expressed as mean ± SD, n=3. C: control. *: p<0.05, significantly different from control.

16HBE cells (fig. 2, supplementary online material) except for a slight increase or absence of response in TGF-α release by NHNE cells treated by PM organic and aqueous extracts (1.8- and 1.7-fold, respectively).

Since PM organic extract had the greatest effect on EGFR ligand expression and PAH represent constituents of this extract, the effect of PM_{2.5}-induced EGFR ligand secretion as mediated by the AhR pathway, was investigated. Figure 3 demonstrates that both amphiregulin and TGF-α release provoked by 10 μg·cm⁻² of PM_{2.5} diminished with the addition of α-naphthoflavone, an AhR antagonist, in a concentration-dependent manner. Furthermore, α-naphthoflavone decreased the amphiregulin and TGF-α secretion induced by 1 μM of benzo(a)pyrene (used as positive control) more than the secretion induced by PM_{2.5}.

EGFR ligands mediate a PM_{2.5}-induced epithelial cell autocrine pro-inflammatory response

When 16HBE cells were exposed for 24 h to human recombinant amphiregulin or TGF-α (fig. 4), the GM-CSF pro-inflammatory cytokine secretion was induced by an exposure to 5 ng·mL⁻¹ of amphiregulin or 20 pg·mL⁻¹ of TGF-α. Co-treatment with both amphiregulin and TGF-α had no additive effect on GM-CSF release (fig. 4).

In order to determine whether GM-CSF release, known to be induced by PM_{2.5} [12], was regulated in an autocrine manner by EGFR ligands, conditioned media of cells previously exposed to PM_{2.5} for 24 h was used (fig. 5). Figure 5a demonstrates that a 24 h exposure to 10 μg·cm⁻² of PM_{2.5} induced GM-CSF secretion (increase of 35 pg·mL⁻¹ in treated cells *versus* control cells, fig. 5a). The transfer of the conditioned

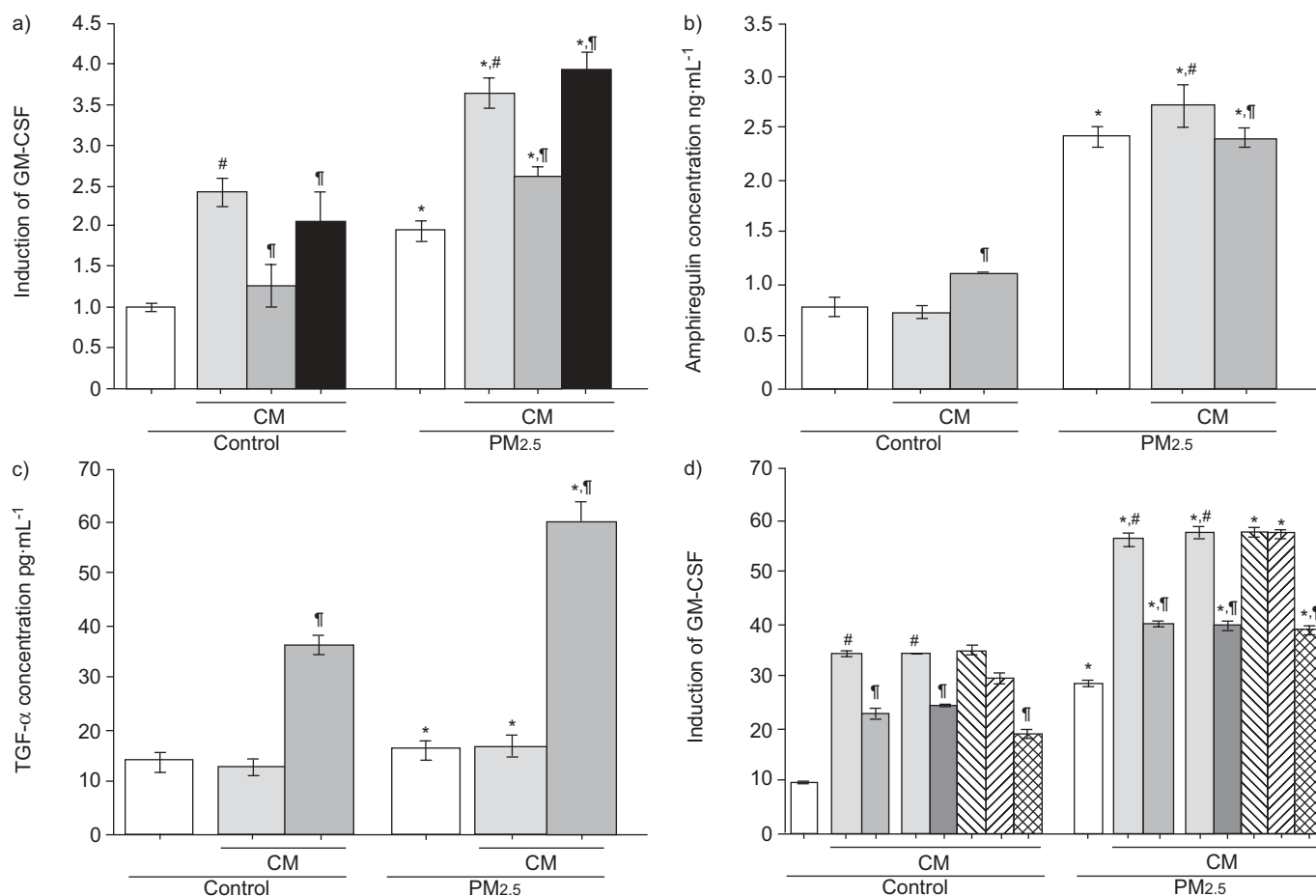


FIGURE 5. Pro-inflammatory response of bronchial epithelial cells exposed to particulate matter (PM) or conditioned media (CM). a and d) granulocyte-macrophage colony-stimulating factor (GM-CSF) release induction, b) amphiregulin or c) transforming growth factor (TGF)- α release by human bronchial epithelial (16HBE) cells exposed directly to 10 $\mu\text{g}\cdot\text{cm}^{-2}$ of PM with a diameter below 2.5 μm (PM_{2.5}) or PM_{2.5} control for 24 h (□). Fresh cells were then exposed to either control- or PM_{2.5}-CM alone (■) or CM following: 0.5 $\mu\text{g}\cdot\text{mL}^{-1}$ anti-epidermal growth factor (EGF) receptor antibody treatment (■); treatment with 0.5 $\mu\text{g}\cdot\text{mL}^{-1}$ of immunoglobulin G antibody (■); pre-treatment with 20 $\mu\text{g}\cdot\text{mL}^{-1}$ of anti-amphiregulin (AR) antibody; 1 $\mu\text{g}\cdot\text{mL}^{-1}$ of anti-TGF- α antibody (▨); 10 $\mu\text{g}\cdot\text{mL}^{-1}$ of anti-heparin-binding EGF-like growth factor antibody (▧); or the combination of anti-heparin-binding EGF-like growth factor, anti-AR and anti-TGF- α antibodies (▩). GM-CSF induction is normalised by the cells directly exposed to control. Data are expressed as mean \pm SD, n=6 or n=3. *: p<0.05, significantly different from treatment control; #: p<0.05, significantly different from release by cells directly exposed to PM_{2.5}; †: p<0.05, significantly different from release by cells exposed to CM.

media onto fresh cells further increased GM-CSF secretion by cells exposed to either control conditioned media or to PM_{2.5} conditioned media, but the increase in GM-CSF secretion was greater in cells exposed to PM_{2.5} conditioned media (increase of 46 $\text{pg}\cdot\text{mL}^{-1}$ in PM_{2.5}-conditioned media *versus* control conditioned media; fig. 5a). Interestingly, when conditioned media was applied to fresh cells in addition to 0.5 $\mu\text{g}\cdot\text{mL}^{-1}$ of an anti-EGFR antibody (fig. 5a), the conditioned media-induced GM-CSF release was strongly reduced both for control and PM_{2.5}-conditioned media, in contrast to the addition of 0.5 $\mu\text{g}\cdot\text{mL}^{-1}$ of control (IgG) antibody, which had almost no effect on GM-CSF release (fig. 5a). Furthermore, the GM-CSF secretion induced by conditioned media was concentration dependent, occurring from conditioned media of cells exposed to 1 $\mu\text{g}\cdot\text{cm}^{-2}$ of PM_{2.5} (fig. 3 in the online supplementary material). The measurements of amphiregulin and TGF- α release in parallel to GM-CSF release (fig. 5b and c) revealed that: 1) secretion levels of these two ligands were increased only slightly or not at all

by fresh cells exposed for 24 h to conditioned media; and 2) the addition of an anti-EGFR antibody only slightly modulated secretion levels of amphiregulin, whereas a strong increase in TGF- α release was observed, especially in PM-treated cultures.

In order to determine the respective roles of the different EGFR ligands in GM-CSF release, antibodies against amphiregulin, TGF- α or HB-EGF were used either alone or in combination in conditioned media (fig. 5d). Only the anti-amphiregulin antibody alone or in combination with the other antibodies significantly reduced the GM-CSF secretion, to the same extent as the anti-EGFR antibody (fig. 5d).

DISCUSSION

PM_{2.5} upregulates several EGFR ligands in respiratory epithelial cells

The current study demonstrates for the first time that urban PM_{2.5} differentially regulates by respiratory epithelial cell

mRNA expression of EGFR ligands, with upregulation of TGF- α , amphiregulin and HB-EGF but unchanged betacellulin expression. The three induced growth factors have similar gene expression kinetics, but different levels of induction. These differences also occur with protein release, since amphiregulin exhibits higher expression both at the mRNA and protein level (3.6- and 2–3.5-fold, respectively) than TGF- α (2.1- and 1.4–1.7-fold for mRNA and protein, respectively). These data suggest that increased EGFR ligand secretion results from increased gene expression. To the current authors' knowledge, this effect of PM on EGFR ligand secretion by respiratory epithelial cells has not been published previously. However, TGF- α , amphiregulin and HB-EGF gene upregulation has already been shown in the lung epithelial cell line (NCI-H292) exposed to cigarette smoke, where amphiregulin secretion was stronger than that of TGF- α [11]. Similarly, the present protein release kinetic study revealed that TGF- α induction is weaker than that of amphiregulin and does not accumulate in the cell medium. This weaker induction may be misleading, as underlined by the experiment performed in the presence of an anti-EGFR antibody, which blocks binding of TGF- α to EGFR, leading to a dramatic increase in TGF- α in the culture medium (fig. 5c). This suggests that TGF- α is quickly consumed by cells due to its strong affinity for EGFR. Moreover, this would explain the picogram amounts of TGF- α that were measured *in vitro* as well as those obtained in airway epithelial cells exposed to cigarette smoke [14].

EGFR ligand secretion may be a common response of respiratory epithelial cells to stress, as underlined by miscellaneous data obtained with human bronchial epithelial cells exposed to cigarette smoke [11] or after inhalation of asbestos fibres by rats [15]. These data, combined with the present study, performed with a bronchial cell line and nasal primary cells, underline the fact that induced release of EGFR ligands is not limited to cell lines and occurs throughout the respiratory tract: nasal and bronchial cells responding in a similar fashion. Furthermore, amphiregulin [16] and TGF- α [17] have been described as being preferentially released towards the basolateral compartment, while EGFR is localised in the basolateral membrane [13], which suggests that PM_{2.5}-induced EGFR ligands could mediate responses of bronchial epithelial cells *via* an autocrine effect.

PM_{2.5}-induced EGFR ligands mediate a pro-inflammatory response of bronchial epithelial cells but not their proliferation

Airway inflammation is the most frequently described and most well-known effect of short-term exposure of healthy volunteers to PM and diesel exhaust particulate (DEP) [18, 19], and can be explained by the capacity of these particles to induce the release of pro-inflammatory cytokines such as IL-8, TNF- α and GM-CSF [4, 16]. Mechanistic studies performed to elucidate signalling pathways triggered by PM in order to induce cytokine release have underlined the involvement of EGFR activation [7, 20]. Such EGFR activation could be a direct effect of particles, as shown for asbestos [21], but it is tempting to hypothesise that the PM_{2.5}-induced epithelial cell pro-inflammatory response could be mediated and/or amplified *via* EGFR activation by PM_{2.5}-induced EGFR ligands. This assumption was assessed using conditioned media in the

presence or absence of an antibody to block the binding of EGFR ligands to their receptor. These data highlight the maintenance of GM-CSF release by cells, particularly when exposed to PM, and the strong participation of EGFR ligands in constitutive and PM-enhanced GM-CSF sustained release. Amphiregulin and TGF- α may be specifically involved in this response, as both ligands appeared to induce GM-CSF secretion by cells when used at concentrations found in PM_{2.5}-treated cells (fig. 4), and to be entirely consumed by 16HBE cells exposed to conditioned media, as shown in figure 5b and c. Nevertheless, the use of antibodies directed towards the different EGFR ligands suggest that GM-CSF secretion is mainly driven by amphiregulin. These results suggest that PM_{2.5} could exacerbate and sustain bronchial epithelial cell inflammation. The autocrine effects of EGFR ligands may thus be a parameter explaining exacerbation of asthma by PM [22], since asthmatic subjects exhibit over-expression of EGFR in bronchial epithelium [10].

Beside the regulation of a pro-inflammatory response in epithelial cells, the current authors speculated whether EGFR ligands secreted after PM_{2.5} treatment could also mediate a mitogen effect on bronchial epithelial cells, since EGFR ligands are known to exhibit growth factor properties. Nevertheless, the culture of bronchial epithelial cells with conditioned media of PM-treated cells did not reveal any modification of cell proliferation (data not shown).

PM_{2.5} organic components mainly contribute to amphiregulin and TGF- α upregulation

Fractionation of PM_{2.5} reveals that PM aqueous extract induces weak but significant amphiregulin and TGF- α release by 16HBE cells that is abolished when metals are trapped by a Chelex resin, suggesting a main contribution of metals to growth factor induction by PM aqueous components. These results, already reported for amphiregulin [23], corroborate findings linking pro-inflammatory cytokine production by epithelial cells to the metal content of PM [24]. The present study sheds new light on the contribution of organic particle compounds to PM_{2.5}-induced amphiregulin and TGF- α gene expression and protein secretion. Indeed, extracted organic PM components, as well as water-washed PM_{2.5} still bearing its organic content, both appear to induce amphiregulin and TGF- α secretion, whereas inorganic carbonaceous compounds have no effect on such growth factor release (fig. 2e and f and supplementary fig. 2). This strong involvement of organic components in PM_{2.5}-induced EGFR ligand expression has already been shown for amphiregulin [13, 23].

The organic component of PM_{2.5} contains PAH, known to have biological activities, especially through specific binding to the AhR. α -naphthoflavone, an AhR antagonist, diminished PM-induced secretion of amphiregulin and TGF- α (fig. 3), suggesting AhR-dependent transcriptional regulation of amphiregulin and TGF- α , which has already been described for another EGFR ligand, epiregulin, in 2,3,7,8-tetrachlorodibenzo-p-dioxin-treated mouse cells [25]. This hypothesis is reinforced by the identification of consensus xenobiotic responsive element sequences upstream of the transcriptional start site in human amphiregulin and TGF- α promoters, to which a transcriptional complex including PAH and AhR binds to transactivate genes. But the presence of cyclic adenosine

monophosphate (cAMP) responsive element (CRE) sequences in amphiregulin and TGF- α promoters could also explain this AhR-dependent signalling pathway; indeed, DU *et al.* [26] recently described induced amphiregulin expression triggered by cigarette smoke in bronchial cells that involves an AhR \rightarrow cAMP \rightarrow PKA \rightarrow CRE pathway. An additional regulation pathway could be linked to the ability of PM components to induce oxidative stress. This pathway could activate the oxidative stress-sensitive transcription factor, nuclear factor- κ B, previously shown to be activated in DEP- and PM_{2.5}-exposed epithelial cells [27, 28], and present consensus binding sites in amphiregulin and TGF- α promoters.

In conclusion, the current study reports upregulation of several epidermal growth factor receptor ligands by human airway epithelial cells exposed to urban particulate matter with a diameter <2.5 μ m, and in particular, the strong participation of organic components of particulate matter with a diameter <2.5 μ m in amphiregulin and transforming growth factor- α upregulation and the weaker contribution of aqueous components through mechanisms that require further investigation. The current study demonstrates for the first time that particulate matter with a diameter <2.5 μ m induced amphiregulin and transforming growth factor- α release contribute to the bronchial pro-inflammatory response *via* an autocrine action. Amplification of bronchial inflammation is highly suspected to be responsible for bronchial remodelling, notably found in asthma or chronic obstructive pulmonary disease. The present findings may thus have clinical implications, especially since amphiregulin and transforming growth factor- α appear significantly released after exposure to only 1 μ g \cdot cm⁻² of native particulate matter with a diameter <2.5 μ m, a concentration easily reached in the tracheobronchial region of individuals with respiratory diseases living in polluted urban areas [22]. In addition to the role of particulate matter-induced epidermal growth factor receptor ligands in sustained bronchial inflammation, they may also have paracrine effects on other cellular actors involved in airway remodelling, such as fibroblasts or vascular smooth muscle cells previously described to be sensitive to such ligands [29, 30].

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