[Frontiers in Bioscience 12, 771-782, January 1, 2007]

Fine urban atmospheric particulate matter modulates inflammatory gene and protein expression in human bronchial epithelial cells

Augustin Baulig [1], Sophie Blanchet [1], Melina Rumelhard [1], Ghislaine Lacroix [2], Francelyne Marano [1] and Armelle Baeza-Squiban [1]

[1] Laboratoire de Cytophysiologie et Toxicologie Cellulaire, Universite Paris 7, 2 Place Jussieu, Tour 53-54, 3e Etage, Case Courrier 7073, 75251, Paris Cedex 05, France, [2] Institut National de l'Environnement et des Risques Industriels (INERIS), Parc Technologique ALATA, Verneuil-en-Halatte, France

TABLE OF CONTENTS

1. ABSTRACT

Ambient particulate matter (PM) is known to induce inflammation in the respiratory tract of exposed subjects. The aim of the present study was to detect, in bronchial epithelial cells, candidate inflammatory genes exhibiting transcriptional modifications following urban $PM_{2.5}$ exposure. Paris urban $PM_{2.5}$ sampled either at a curbside or a background station in winter and in summer was tested in comparison with diesel exhaust particles (DEP) at 10 microg/cm² on human bronchial epithelial (16-HBE) cells (18 h of exposure). The gene profiling study performed using a 375 cDNA cytokine expression array highlighted the differential expression of certain genes, three of which were selected as genes of interest: the IL-1 alpha cytokine, the GRO-alpha chemokine, and amphiregulin, a ligand of the EGF receptor. Their increased expression was confirmed by RT-PCR and/or by Northern blotting in bronchial epithelial cells. In the culture medium of particle-treated cultures, increased release of GRO-alpha and amphiregulin was shown. The particle component responsible for protein release varied for the two genes. The organic extract seemed to be mainly involved in amphiregulin expression and secretion, whereas both the aqueous and organic extracts induced GRO-alpha release. In conclusion, in bronchial epithelial cells, Paris $PM_{2.5}$ increased mRNA and protein expression of GRO-alpha and AR involved in the chemoattraction process and bronchial remodeling, respectively.

2. INTRODUCTION

Atmospheric particulate pollution has become a major public health problem, particularly in urban areas with high traffic density. Many epidemiological studies have highlighted an increase in cardiopulmonary mortality and lung cancer associated with a high rate of fine particulate matter (PM) (1). In particular, PM collected

through a 2.5 microm size selective inlet with an efficiency of 50 % ($PM_{2.5}$) has been implicated in increased adverse respiratory effects (2).

The main acute effect induced by PM in the respiratory tract, the PM target organ, is the development of inflammation. *In vivo* studies have shown that PM induces an inflammatory cell influx (3, 4) and the release of proinflammatory cytokines (5, 6). In addition, *in vitro* studies have shown that primary cell targets of PM in the respiratory tract, such as macrophages and epithelial cells, respond to PM exposure by the release of inflammatory cytokines such as IL-1β, TNF-α and IL-6 (7, 8), erythropoietic cytokines such as G-CSF and GM-CSF (9, 10) and chemokines such as IL-8 (5, 11) .

Much research has been initiated in order to understand how particles induce these biological effects and to determine the particle components involved. Evidence has been obtained with particles rich in metals (residual oil fly ash, ROFA) or organic compounds (diesel exhaust particles, DEP), showing their ability to activate cellular signalling pathways and cytokine release by mechanisms involving reactive oxygen species (ROS). However, these particles differ in many respects from the PM found in the urban atmosphere and for which epidemiological data have been obtained. The mechanisms underlying the proinflammatory response induced by urban PM remain poorly understood. In a previous study, an attempt was made to compare the capacity to induce the release of GM-CSF and TNF-alpha by bronchial epithelial cells of four $PM_{2.5}$ samples collected at two different locations in Paris during winter and summer (12). However, those biomarkers did not succeed in distinguishing the different $PM_{2.5}$ samples, although they did exhibit chemical differences, particularly concerning their polycyclic aromatic hydrocarbons (PAH) and metal content (12). Different reasons might explain this result, such as the bioavailability of the different PM components but also the lack of sensitivity of the chosen biomarkers. Nevertheless, GM-CSF release was previously proven to be a sensitive biomarker for discriminating biological effects induced by DEP according to their organic compound content (13). The complex mixture present on PM probably triggers cross-talk between different cellular signalling pathways that does not converge towards differential GM-CSF or TNF-alpha secretion according to PM composition.

In this context, the aim of the present study was to elucidate, in bronchial epithelial cells, candidate inflammatory genes exhibiting transcriptional modifications upon urban $PM_{2.5}$ exposure. Our objective was to better characterize the inflammatory response and to find new, specific and more sensitive biomarkers of PM exposure. For this purpose, we used a macroarray containing 375 human cDNAs printed in duplicate and specific to inflammation, in order to analyze the response of the 16-HBE (human bronchial epithelial) cell line to non-cytotoxic concentrations of four $PM_{2.5}$ samples collected in Paris either at a curbside or a background station in winter and summer. Differential gene expression was confirmed by RT-PCR or northern blot. In addition, the resulting protein was analyzed by ELISA and the contribution of aqueous and organic fractions of $PM_{2.5}$ to protein secretion was assessed. A recent study highlighted the interest of toxicogenomic techniques for studying biological effects of particles (14). Until now, most of the studies using arrays to screen the effects of particles have been performed on rats (15, 16, 17), mice (18) and *in vitro* human macrophages with DEP or ROFA (19). But to our knowledge, up until now, no study has investigated the effects of urban $PM_{2.5}$ and their compounds on the expression of inflammatory genes in human bronchial epithelial cells.

3. MATERIALS AND METHODS

3.1. Particle collection

Urban atmospheric particulate matter of 2.5 microm aerodynamic diameter sampled with 50 % efficiency ($PM_{2.5}$), used for biological experiments, was collected at ground level with a high volume sampler machine (DA-80 Digitel, Cugy, Suisse), equipped with a $PM_{2.5}$ selective-inlet head, at two locations in Paris: (1) an urban background station at Vitry-sur-Seine, a suburb of Paris; and (2) a curbside station at Porte d'Auteuil bordering a large highway which is a ring road of Paris. The machine operated at the flow rate of 30 m^3/h and particles were recovered on 150 mm diameter nitrocellulose filters (HAWP, Millipore, Saint-Quentin-en-Yvelines, France). Particles were detached from filters as already described (12). Their polyaromatic hydrocarbon and metal content have been previously published (12).

$PM_{2.5}$ were compared with DEP standard reference materiel 1650a purchased from the National Institute of Standards and Technology (Gaithersburg, MD,USA). All particles were used at 10 microg/cm² (50 microg/mL) from stock solution of 2 mg/mL in 0.04 % dipalmitoyl phosphatidyl lecithin (DPL, Sigma, Saint-Quentin-Fallavier, France) water solution. DPL is a surfactant component which enables maintaining particles in suspension. Negative controls were done using DPL.

Native $PM_{2.5}$ were also compared to their respective organic and aqueous extracts. Organic extracts were obtained after extraction of $PM_{2.5}$ by dichloromethane with an accelerated solvent extractor (Dionex ASE 200, Sunnyvale, CA). The obtained extract was dried, then redissolved in dimethyl sulfoxide (DMSO, Sigma). Organic extracts were used at the concentration at which they are found on particles. This value was calculated according to the soluble organic fraction (SOF) determined for each particle sample (12).

The aqueous extract containing soluble components was obtained after washing of $PM_{2.5}$ with sterile ultrapure water. After two centrifugations at 12,000 g, the supernatants were filtered on a 0.2 microm filter to eliminate all particles. The aqueous fraction was used at the same volume used to treat cells at 10 microg/ cm² from the 2 mg/mL stock solution. In some experiments, soluble metals present in PM aqueous extracts were depleted by filtering on chelating ion exchange resin columns according

to the manufacturer's procedure (Chelex 100, Bio-Rad, Marnes-La-Coquette, France).

3.2. Chemicals and reagents

All chemicals were purchased from Sigma except when otherwise specified.

3.3. Cell culture and treatment

Dr. D. C. Gruenert (20) (Colchester, VT, USA) kindly provided the human bronchial epithelial cell subclone 16-HBE14o-. This transformed cell line was cultured in DMEM/F12 culture medium supplemented with penicillin (100 U/mL), streptomycin (100 microg/mL), glutamine (1%), fungizone (0.125 microg/mL, Invitrogen, Cergy-Pontoise, France) and UltroserG (UG) (2%, Bio Sepra, Cergy-Saint-Christophe, France). Cells were cultured at 20,000 cells/cm² on collagen (type I, 4 microg/cm²)-coated 75 cm² flasks (Costar, Cambridge, MA, USA) for RNA extraction and coated 6-well plates (Costar, Cambridge, MA, USA) for cytokine release experiments. At the time of treatment, UG was not added to DMEM/F12. Cultures were incubated in humidified 95% air with 5% CO_2 at 37°C.

3.4. RNA isolation

Total RNA were isolated from subconfluent 16-HBE cells cultured in 75 cm² flasks, using Tri Reagent™ (Sigma-Aldrich, Saint-Quentin-Fallavier, France) according to the manufacturer's instructions. RNA quantitation was realized by measuring the absorbance at 260 nm and 280 nm of an appropriate dilution in an Ultrospec 2100 Pro spectrophotometer, (Amersham Biosciences, Orsay, France); RNA purity was determined by calculating the 260/280 ratio, and integrity was checked by agarose gel electrophoresis. mRNA (poly $(A)^+$ RNA) was purified using the Dynabead mRNA purification kit (Dynal Biotech, Oslo, Norway) according to the manufacturer's instructions.

3.5. Reverse transcription and labeling for array hybridization and post-hybridization processes

We used a human cytokine array from R&D Systems (Abingdon, UK) containing 375 different genes specific to inflammation, printed in duplicate on a nylon membrane and using the manufacturer's instructions. Human cytokine-specific labeling primers were annealed to 0.5 microg mRNA by heating to 90°C for 2 min and then ramping to 42°C. 20 microCi [alpha-^{33}P]-dCTP (Perkin Elmer NEN® Zaventem, Belgium), 333 microM cold dATP, dGTP, dTTP and 1.67microM cold dCTP, 20 U RNase inhibitor (Promega, Charbonnières, France) and 50 U AMV reverse transcriptase were added to initiate cDNA synthesis at 42°C for 2 h in a total volume of 30 microl. After incubation, unincorporated radiolabeled nucleotides were removed by centrifugation at 1,100 g for 4 min on a Sephadex™ G-25 spin column (Amersham Biosciences, Orsay, France). Then, the incorporation percentage of labeled nucleotides into cDNA was estimated by counting an aliquot of the sample before and after column purification in a scintillation counter. All labelled cDNA were heat-denatured for 10 min at 95°C, chilled on ice and added to the hybridization solution. The hybridizations were performed in roller bottles at 6 rpm in a hybridization oven (SI 20H, Stuart Scientific, ATGC, Noisy-Le-Grand, France). The arrays were rinsed in 50 mL 2X SSPE at room temperature for 5 min, pre-hybridized (2 h at 65°C) and hybridized (overnight at 65°C) in 5 mL hybridization solution (5X SSPE, 2% SDS, 5X Denhardt's reagent and 100 microg/mL heat-denatured salmon testes DNA).

After hybridization, arrays were washed three times in 50 mL of 0.5X SSPE, 1% SDS at room temperature for 3 min, twice in 100 mL 0.5X SSPE, 1% SDS at 65°C for 20 min and once in 100 mL 0.1X SSPE, 1% SDS at 65°C for 20 min. The arrays were air-dried for 5 min, wrapped in clear plastic and subjected to a phosphor screen overnight.

3.6. Signal detection

After exposure, the phosphor screen was scanned by the Storm 860 imaging system (Amersham Biosciences, Orsay, France) and image analysis was performed with ArrayVision™ software (Imaging Research Inc., St. Catherine, Ontario, Canada).

3.7. Validation of hybridization signals and data analysis

The average intensity (i) of the duplicate spots of each cDNA was calculated and the hybridization signal was validated if the standard deviation was less than 10%. Values were normalized to the average value of all printed cDNA. The general background of the experiment was the mean of the positions without cDNA. The effect of treatment on gene expression was considered relevant if the ratio i(treated)/i(control) was higher than 1.70 (overexpression) or lower than 0.59 (downregulation) and if at least one of the 2 considered signal intensities (treated or control) was stronger than the general background of the experiment. The latter criterium was included in order to avoid considering differentially expressed genes whose signal was below the background. The expression of genes positively or negatively regulated by treatment described in this study was verified by RT-PCR and/or northern blot.

3.8. RT-PCR

2 microg of total RNA were reverse-transcribed into cDNA in the presence of 200 U of M-MuLV reverse transcriptase RNase H^-(Finnzyme Oy, Espoo, Finland), 500 ng of oligo-dT primer (Roche Applied Sciences, Meylan, France) and 40 U of RNase inhibitor (Promega, Charbonniere, France) for 1 h at 42°C in a 20 microl reaction volume. The RT reaction volume was completed to 50 microl with water. For semiquantitative experiments, $1/20^{th}$ of the RT reaction was PCR-amplified in a 25 microl reaction containing 1 x PCR buffer, 1.5 mM MgCl2, 200 microM each dATP, dTTP, dGTP, dCTP (Fermentas Life Science, Burlington, Ontario), 0.5 microM forward and reverse primers (Table 1, Operon Biotechnologies GmbH, Cologne, Germany) and 0.5 U DyNAzyme™II DNA polymerase (Finnzyme Oy, Espoo, Finland) in a PTC-100 thermocycler (MJ Research, Watertown, MA). The PCR reaction conditions were as follows: 94°C, 2 min 1 cycle; 94°C, 30 s ; 58°C, 30 s; 72°C, 1 min n cycles (Table 1); and 72°C, 5 min 1 cycle. Amplified products were then

Table 1. primer sequences for RT-PCR

Gene	N: number of cycles	Forward primer sequence	Reverse primer sequence
AR	25	5'-TGG TGC TGT CGC TCT TGA TA-3'	5'-ACA GCA ACA GCT GTG AGG AT-3'
GAPDH	21	5'-ACC ACA GTC CAT GCC ATC AC-3'	5'-TCC ACC ACC CTG TTG CTG TA-3'
GRO-alpha	27	5'-ATG GCC CGC GCT GCT CTC-3'	5'-GTT GGA TTT GTC ACT GTT-3'
IL-1 alpha	26	5'-AGA TGA AGA CCA ACC AGT GC-3'	5'-CAT AGT CAG TAC CTC TGG TC-3'

Table 2. Results of macro-array experiment realized with Diesel Exhaust Particles – exposed bronchial epithelial cells versus control cells

I_{DPL}	I_{DEP}	$I_{DEP/DPL}$	Genes	Gene family	Genbank access number
15.5	35.7	2.3	Amphiregulin	EGF family	M30704
0.5	1.5	2.5	IL-1alpha	Interleukin	M28983
6.84	3.97	0.58	IGF-BP5	Binding protein	M65062

Table 3. Results of macro-array experiment realized with Winter Urban Background $PM_{2.5}$ – exposed bronchial epithelial cells versus control cells

I_{DPL}	I_{WUB}	$I_{WUB/DPL}$	Genes	Gene family	Genbank access number
16.4	70.1	4.3	Amphiregulin	EGF family	M30704
0.8	3.4	4	IL-1 alpha	Interleukin	M28983
0.4	0.9	2.5	G-CSF	Cytokine	X03438
0.3	0.7	2.3	IL-1beta	Interleukin	M15330
0.9	2	2.2	Betacellulin	EGF family	NM_001729
0.4	0.8	1.9	VCAM-1	Adhesion molecule	X53051
1.2	2.3	1.9	TNF-R1	Cytokine receptor	M63121
0.2	0.3	1.8	IL-8	Chemokine	Y00787
1.70	1.00	0.59	TIMP-2	Protease	M32304

separated by 1 microg/mL ethidium bromide 2% agarose gel electrophoresis and visualized by 312 nm UV exposure.

3.9. Northern blotting

20 microg of total RNA were denatured at 65°C for 10 min in a solution containing 50% formamide, 6% formaldehyde and 1X MOPS at pH 7 and chilled on ice. RNA was separated by size by denaturing gel electrophoresis (1.2% agarose, 1X MOPS, 7% formaldehyde, 0.25 microg/mL ethidium bromide) at 15 mA for 12 h and transferred by capillarity onto a Hybond™-N+ membrane (GE healthcare, Orsay, France) for 18 h in 20X SSC solution (3 M NaCl and 0.3 M sodium citrate at pH 7). Then, RNA was fixed on membranes by heating at 80°C for 2 h.

Radiolabeled probes were generated by Rediprime™ II random priming (GE healthcare) according to the manufacturer's protocol. Membrane pre-hybridization, hybridization and washes were performed in roller bottles in a hybridization oven using Rapid-hyb™ buffer (GE healthcare) recommendations. The membranes were air-dried for 5 min, wrapped in clear plastic wrap, subjected to a phosphor screen for 2 days and development was carried out with a Storm 860 imaging system (Amersham Biosciences, Orsay, France). Signals were quantified with Bio-1D software (Vilber Lourmat, Marne-La-Vallee, France).

3.10. Cytokine assay

Subconfluent cultures were exposed for 24 h to particles. Culture supernatants were recovered and frozen at –80°C until use. The concentrations of amphiregulin (AR) and growth-related oncogen-alpha (GRO-alpha) released into the culture supernatants were measured with human AR and GRO-alpha Duo-set ELISA respectively (R&D Systems). Color development was measured at 450 nm with an MRX 5000 microplate photometer (Dynex Technologies, Issy-les-Moulineaux, France).

3.11. Statistical analysis

All data were expressed as the means ± SD of three cultures for *in vitro* studies. Means were compared by analysis of variance. The equal variance test was significant with alpha = 0.05 and all pairwise multiple comparisons were made with the Student Newman-Keuls method (SPSS software).

4. RESULTS

4.1. Macroarray results

The exposure of 16-HBE cells for 18 h to 10 microg/cm² of DEP, winter urban background, summer urban background, winter curbside and summer curbside particles versus DPL caused 3, 10, 15, 13 and 3 significant changes in gene expression, respectively, according to the relevant ratios considered (Tables 2-6).

The most striking changes induced by DEP treatment (Table 2) concerned the upregulation of 2 genes: amphiregulin (AR*)*, an EGF receptor ligand that exhibits highly constitutive expression further increased by DEP treatment (2.3-fold induction) and interleukin 1 alpha (IL-1 alpha), a cytokine well known to participate in the proinflammatory process, showing a 2.5-fold induction.

With winter urban background particles (WUB) (Table 3), AR and IL-1 alpha were again the most strongly upregulated genes (4.3- and 4.0-fold induction respectively) in addition to other differentially expressed genes with an induction factor exceeding 2, such as granulocyte colony-stimulating factor (G-CSF), IL-1 beta and betacellulin (BTC), another member of the EGF family.

Table 4. Results of macro-array experiment realized with Summer Urban Background $PM_{2.5}$ – exposed bronchial epithelial cells versus control cells

I_{DPL}	I_{SUB}	$I_{SUB/DPL}$	Genes	Gene family	Genbank access number
1.3	5.3	4	TIMP-2	Protease	M32304
0.8	2.9	3.4	CCR-3	Chemokine receptor	U28694
0.8	2.3	2.7	SMDF	Cytokine	L41827
1.0	2	2	Neuropilin-1	Neurotrophic factor	AF018956
1	1.8	1.9	L-selectin	Adhesion molecule	M25280
1.6	0.7	0.4	C-met	Cytokine receptor	X54559
2.6	1.2	0.5	Integrin-beta 6	Integrin	M35198
2.6	1.3	0.5	CD14	Cell surface protein	X06882
1.3	0.6	0.5	IL-11 R alpha	Interleukin receptor	Z38102
2	1	0.5	Integrin- alpha 5	Integrin	X06256
5.3	2.8	0.5	Integrin- beta 1	Integrin	X07979
1.5	0.8	0.5	IGF-BP5	Binding protein	M65062
1.6	0.9	0.5	ALCAM	Adhesion molecules	L38608
0.9	0.5	0.5	BMP-7	Chemokine	NM_001719
3.3	1.8	0.5	IK	Cytokine	S74221

Table 5. Results of macro-array experiment realized with Winter Curbside $PM_{2.5}$ – exposed bronchial epithelial cells versus control cells.

I_{DPL}	I_{WC}	$I_{WC/DPL}$	Genes	Gene family	Genbank access number
1.1	4.1	3.7	IL-1 alpha	Cytokine	M28983
0.6	1.3	2.3	GRO- alpha	Chemokine	J03561
1.1	2.1	2	IGF-BP5	Binding protein	M65062
0.6	1.2	2	GRO-gamma	Chemokine	M36821
24.3	46.3	1.9	Amphiregulin	EGF family	M30704
0.7	1.3	1.8	IL-10	Interleukin	M57627
0.4	0.8	1.8	SARP-1	Cytokine	AF017986
1.1	2	1.8	Betacellulin	EGF family	NM_001729
0.8	0.2	0.2	BLC/BCA-1	Chemokine	AF044197
0.6	0.3	0.4	ST-2	Cell surface protein	D12763
0.8	0.4	0.5	IGF-I R	Cytokine receptor	X04434
1.5	0.8	0.6	C-met	Cytokine receptor	X54559

Table 6. Results of macro-array experiment realized with Summer Curbside $PM_{2.5}$ – exposed bronchial epithelial cells versus control cells.

I_{DPL}	I_{SC}	$I_{SC/DPL}$	Genes	Gene family	Genbank access number
16.1	44.1	2.7	Amphiregulin	EGF family	M30704
1.4	2.9	2	IL-1alpha	Interleukin	M28983
1	2.5	1.8	Betacellulin	EGF family	NM_001729

Summer urban background particles (SUB) (Table 4) gave a very different expression profile in which genes were mainly downregulated (10 genes were involved), and upregulated genes differed from those previously identified with DEP and WUB (TIMP-2, CCR-3, SMDF, neuropilin-1 and L-selectin).

With winter curbside particles (WC) (Table 5), the most strongly upregulated genes were IL-1 alpha and GRO-alpha (3.7- and 2.3-fold induction respectively) in addition to other differentially expressed genes, but with an induction factor lower than 2, among them AR and BTC. One gene, BLC/BCA-1, was clearly downregulated.

Finally, with summer curbside particles (SC) (Table 6), only 3 genes appeared to be upregulated: AR, IL-1 alpha and BTC, with AR and IL-1 alpha exhibiting the highest induction factor (2.7 and 2.0 respectively).

4.2. Functional classification

A functional classification has been constituted for genes modulated for at least one kind of $PM_{2.5}$. Six families were created: interleukins and interleukin receptors (Figure 1A), cytokines and cytokine receptors (Figure 1B), chemokines and chemokine receptors (Figure 1C), the EGF family (Figure 1D), adhesion molecules/binding proteins (Figure 1E) and other genes (Figure 1D). Altogether, these data reveal that modulation of inflammatory genes in bronchial epithelial cells in response to particle stress mainly involves overexpression of a subset of genes. AR and IL-1 alpha are among the most frequently and most substantially upregulated genes by the different particle samples.

4.3. Confirmation by RT-PCR and/or northern blot of PM-induced expression of genes of interest

The upregulation of IL-1 alpha and its differential expression according to $PM_{2.5}$ samples observed with the macroarray was confirmed by northern blot with RNA extracted from cells treated for 18 h (Figure 2A). The highest induction effects were again observed with the $PM_{2.5}$ winter samples, but they were also observed with summer urban background particles (SUB), whereas this was not the case with the macroarray. In addition, a kinetic study carried out by RT-PCR and northern blot with DEP and the winter urban background samples showed that increased IL-1 alpha expression occurred from 6 h and was maintained up to 30 h (Figure 2B and 2C). The differential expression revealed by the macroarray was also confirmed by northern blot for AR (Figure 3A). For AR, early (within 6 h) and sustained (to 30 h) overexpression in DEP and winter urban background $PM_{2.5}$-treated epithelial cells was

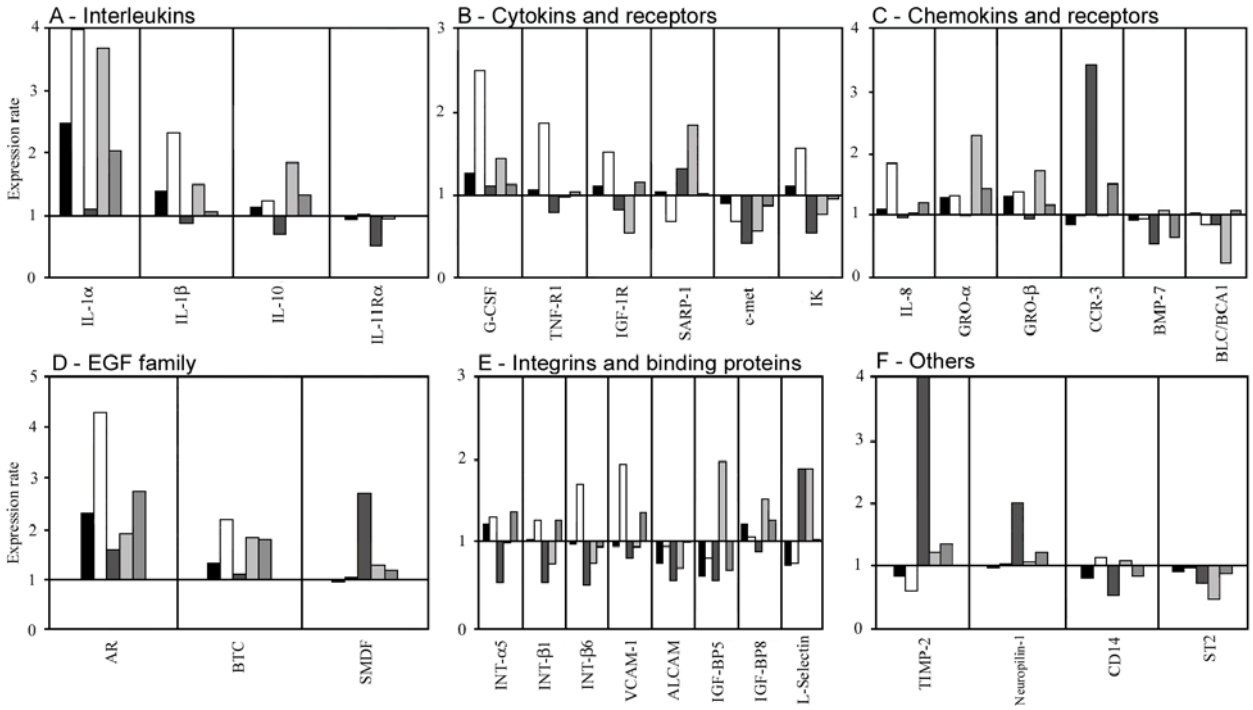

Figure 1. Functional classification of particle-modulated genes. Five families were created: A. Interleukin. B. Cytokines and receptors. C. Chemokines and receptors. D. EGF family. E. Adhesion molecules. F. Other genes. 1st black bar: diesel exhaust particles, 2nd white bar: winter urban background $PM_{2.5}$, 3rd high grey bar: summer urban background $PM_{2.5}$, 4th low grey bar: winter curbside $PM_{2.5}$, 5th medium grey bar: summer curbside $PM_{2.5}$.

already shown in our previous study (21). Moreover AR overexpression is observed with native summer curbside particles (SC) and their respective organic extract but not with its respective aqueous extract (Figure 3B). GRO-alpha gene upregulation observed for winter curbside $PM_{2.5}$ was confirmed by northern blot, in addition to its occurrence with other $PM_{2.5}$ samples not identified by the macroarray approach (Figure 4). Surprisingly, the differential expression of some genes (IGF-BP5, TIMP-2, BTC) in response to several PM samples has not been confirmed by RT-PCR and northern blot experiments (data not shown).

4.4. *In vitro* inflammatory mediator secretion

All native $PM_{2.5}$ induced similar AR secretion by bronchial epithelial cells, but this release was significantly greater (WB: 93%; SB: 78%; WC: 103%; and SC: 89%) than the release induced by DEP (46%) (Figure 5A). Aqueous extracts of $PM_{2.5}$ induced slight AR secretion for winter $PM_{2.5}$, but were without effect for summer $PM_{2.5}$ (Figure 5A). Moreover, this aqueous extract-induced AR secretion was decreased after metal removal from the aqueous extract by filtration over a Chelex column (Figure 5B). In contrast, organic extracts induced AR release which was similar to that induced by native particles (Figure 5A). The coexposure of epithelial cells to both the aqueous and organic extracts induced a slight but significant potentiation of the organic extract effect.

Native particles induced a similar increase in GRO-alpha chemokine release after 24 h of treatment (Figure 6A) This increase was low but statistically significant. The aqueous extract generally induced more substantial GRO-alpha release than native particles. However, higher GRO-alpha release was induced by the organic extracts (Figure 6A). In addition, metal removal in aqueous extract by Chelex filtration reduced GRO-alpha release (Figure 6B). Finally, DEP had no effect on GRO-alpha release (Figure 6A). The combination of the aqueous extract with the organic extract of WUB induced a reduction in GRO-alpha release compared to each extract, but which was similar to the respective native particles (Figure 6B).

5. DISCUSSION

For all tested $PM_{2.5}$, this gene profiling study carried out with $PM_{2.5}$ pointed to upregulation, in bronchial epithelial cells, of a gene, amphiregulin, a ligand of the EGF receptor that we had already described in the context of particle exposure (21), as well as upregulation of interleukin genes such as IL-1 alpha and chemokine genes such as GRO-alpha. Such increased gene expression also led to an increase in the corresponding AR and GRO-alpha proteins. Interestingly, the particle fraction responsible for protein release varied for both genes. The organic fraction seemed to be mainly involved in amphiregulin release, whereas both the aqueous and organic fractions were implicated in GRO-alpha release.

In order to evaluate the Paris $PM_{2.5}$ ability to elicit inflammation, a preliminary experiment was performed by intratracheal instillation in rats. It was observed an increase in the cell number in the bronchoalveolar lavage fluid, especially that of neutrophil

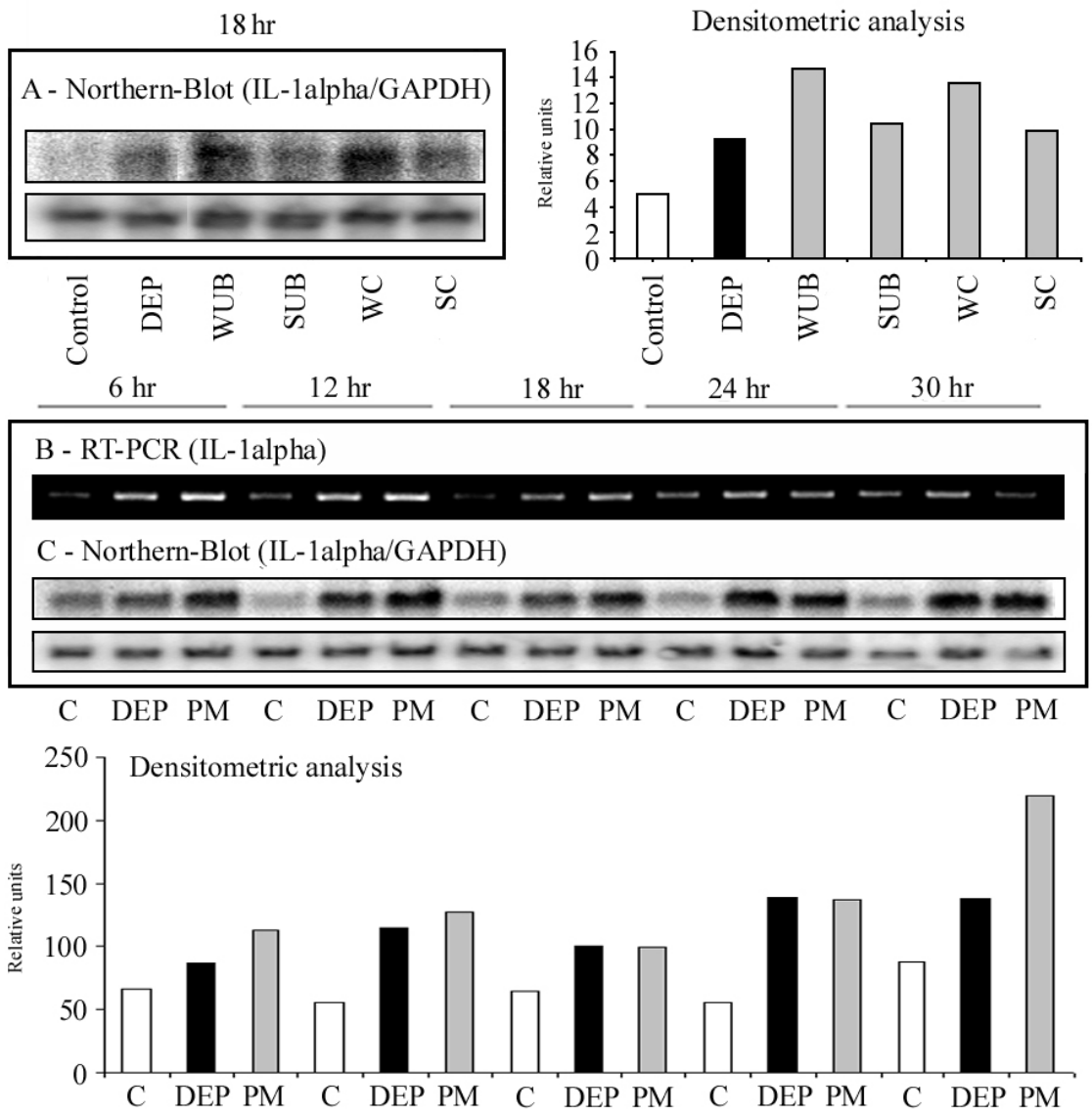

Figure 2. Expression of interleukin-1-alpha in 16-HBE cells. A- Treated with the four $PM_{2.5}$ (Winter Urban Background, Summer Urban Background, Winter Curbside, Summer Curbside or Diesel Exhaust Particles at 10 microg/cm². Interleukin-1-alpha expression was analyzed by northern blot and correlated with the GAPDH housekeeping gene expression by densitometric analysis. B- and C-. Treated with Winter Urban Background $PM_{2.5}$ at 10 microg/cm² from 6 h to 30 h: the time course study of interleukin-1-alpha expression was analyzed by RT-PCR (B) and northern blot (C). All treatments were conducted in triplicate and the results were verified twice.

cells and macrophages that release GRO-alpha chemokine (data not shown). In this context, and in addition to the $PM_{2.5}$-induced GM-CSF secretion previously shown (12), it was scientifically relevant to use a macroarray specific to inflammatory genes with these $PM_{2.5}$. $PM_{2.5}$ was compared with DEP, for which the mechanisms of action have been thoroughly investigated, and particularly the role of organic compounds in the induction of pro-inflammatory cytokine release (22, 23, 24, 25). $PM_{2.5}$ and DEP effects were tested on the transformed 16HBE cell line that we had previously shown to respond to DEP exposure similarly to normal cells (13). Furthermore airway epithelial cells are with macrophages, the first cells encountering particles and their proinflammatory response characterized by the release of cytokines, chemokines and growth factors is likely capable to exacerbate inflammatory associated-lung diseases (e.g. asthma, COPD, airway remodeling...).

Initially, the macroarray technique was used to screen the inflammatory genes most frequently and most strongly modulated by the different $PM_{2.5}$ samples. They were further considered as candidate genes depending on confirmation of their modulated expression by RT-PCR or northern blot experiments, and their biological relevance.

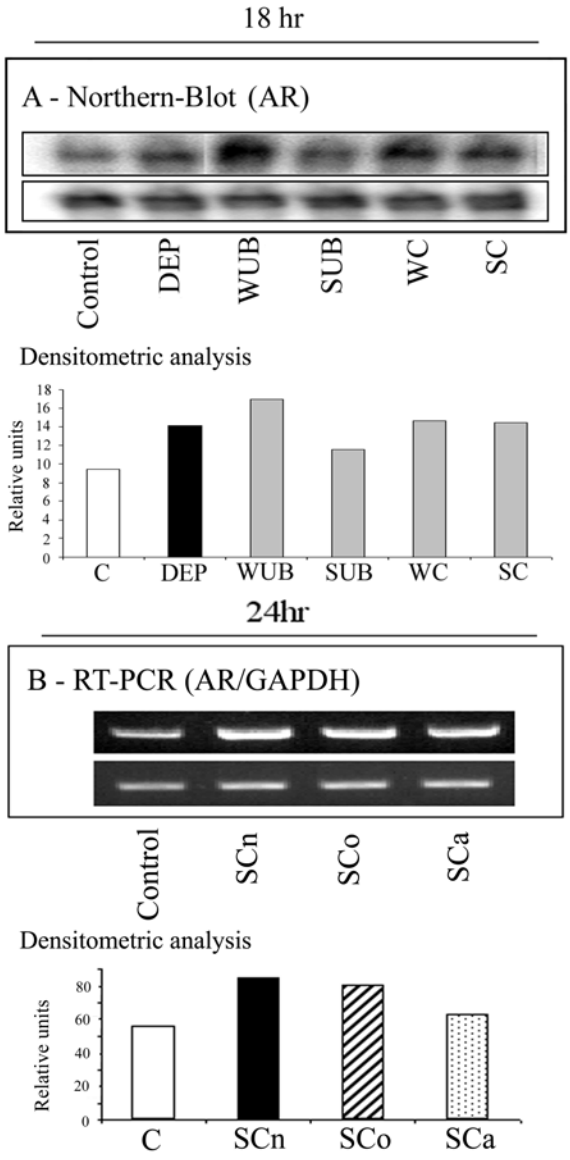

Figure 3. Confirmation of AR expression in 16-HBE cells. A- treated with the four $PM_{2.5}$ (Winter Urban Background, Summer Urban Background, Winter Curbside, Summer Curbside or Diesel Exhaust Particles at 10 microg/cm² for 18 h by northern blot. B- treated with native summer curbside particles (SCn) at 10 microg/cm^2 and their respective aqueous (SCa) and organic extract (SCo) by RT-PCR. AR mRNA expression was correlated with the GAPDH housekeeping gene expression. All treatments were conducted in triplicate and the results were verified twice.

From these considerations, we focused on 3 upregulated genes (AR, IL-1alpha and GRO-alpha). With the exception of the PM-SUB sample, all PM samples and DEP systematically and drastically induced the expression of both the AR and IL-1 alpha genes that were chosen for further investigations. In addition, GRO-alpha involved in neutrophil chemoattraction was selected due to its relevance based on *in vivo* data (data not shown, 26).

AR belongs to the EGF receptor ligand family. We have described for the first time the transient upregulation of this gene in relation to particle exposure (21), and we now confirm that this upregulation is a common event in $PM_{2.5}$ mechanisms. In addition, it is a specific event, as it does not occur with carbon black or titanium dioxide particles (21). AR is probably involved in regulation of GM-CSF release as previously described (21), but could also be implicated in bronchial remodeling by an autocrine effect on epithelial cells and paracrine effects on surrounding cells, i.e. fibroblasts and airway smooth muscle cells. Indeed, AR has a known mitogenic effect on bronchial epithelial cells (27) as well as on vascular smooth muscle cells (28, 29). Airway remodeling associated with the presence of particles has been described in women living in a highly polluted city (30, 31). AR expression is differential according to $PM_{2.5}$ samples, but these differences are no longer evident when considering the protein secretion level. These results could be explained by post-translation mechanisms, since AR is first expressed as a membrane precursor further matured after cleavage by tumor-necrosis-factor-alpha-converting enzyme (TACE) (32). In addition to an effect on AR gene expression, $PM_{2.5}$ could also act on TACE activity, as already suggested in the mechanism of action of cigarette smoke (33).

IL-1 alpha is a proinflammatory cytokine controlling the expression of chemokines and adhesion molecules. Moreover, it is involved in the stabilization and lengthening of the half-life of GM-CSF mRNA (34), a cytokine we had previously shown to be induced by DEP and $PM_{2.5}$ (12, 24). Northern blot experiments confirmed the macroarray results for 4 out of 5 tested $PM_{2.5}$ samples and revealed that IL-1 alpha upregulation is also induced by SUB, in contrast to macroarray data. IL-1 alpha overexpression is maintained from 6 h to 30 h.

GRO-alpha is a C-X-C chemokine with neutrophil-attractant properties. GRO-alpha levels are increased in sputum from patients with COPD (35). In addition, this chemokine has already been shown to be upregulated *in vivo* by DEP (26), but not by particulate matter. As for IL-1 alpha, the expression profiling obtained by macroarray was confirmed by northern blot, for which higher induction factors were observed. In contrast, the protein secretion profile according to $PM_{2.5}$ samples does not follow the mRNA expression profile, as we had already observed with AR expression and secretion. A slight but significant increase in GRO-alpha release is observed for all $PM_{2.5}$ samples, but not with DEP. GRO-alpha is known to be regulated by the NF-kappaB transcription factor, and our previous investigations have shown that DEP activates NF-kappaB in 16-HBE cells through its organic component (24) . Altogether, these data suggest that different cellular pathways leading to GRO-alpha secretion are triggered by $PM_{2.5}$ and DEP.

Experiments with DEP and ROFA have evidenced the involvement of organic compounds and

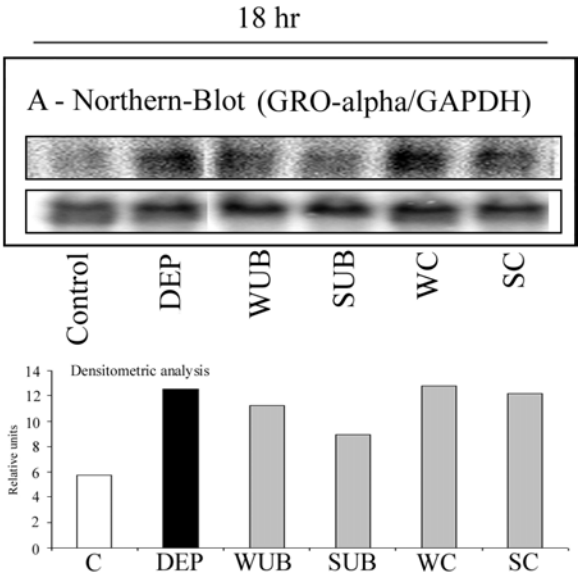

Figure 4. Confirmation by northern blot of GRO-alpha expression in 16-HBE cells treated with the four $PM_{2.5}$ (Winter Urban Background, Summer Urban Background, Winter Curbside, Summer Curbside or Diesel Exhaust Particles at 10 microg/cm² for 18 h. GRO-alpha mRNA expression was correlated with the GAPDH housekeeping gene expression. All treatments were conducted in triplicate and the results were verified twice.

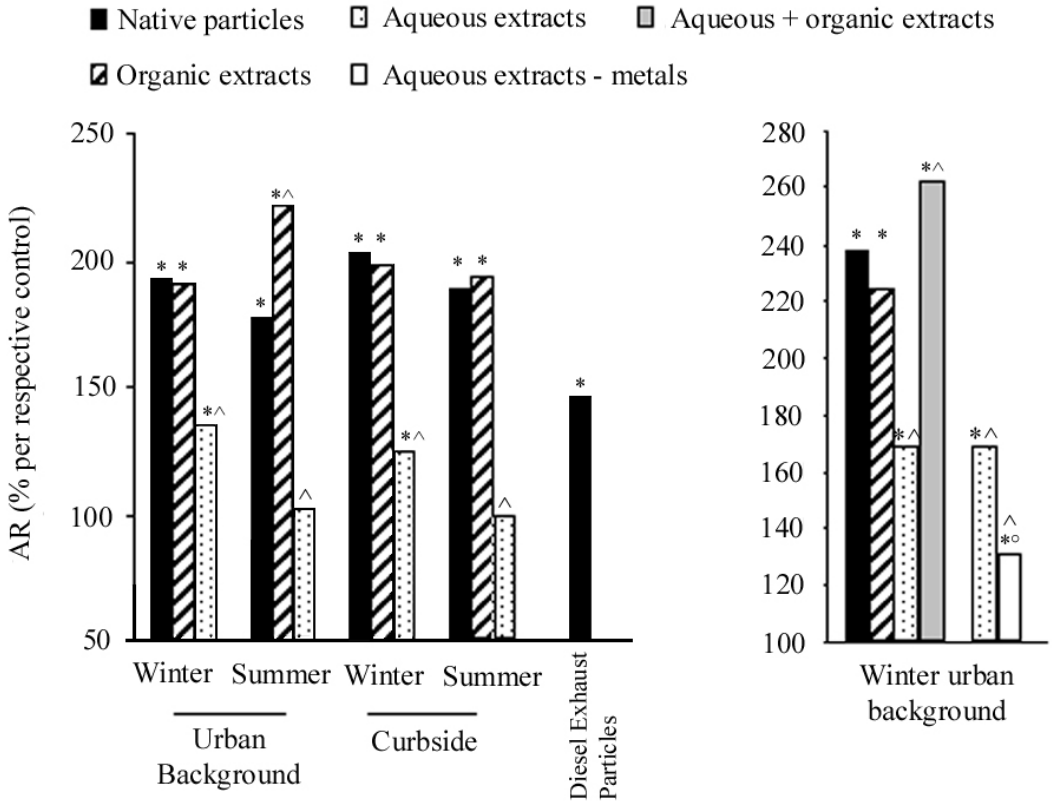

Figure 5. Release of AR by 16-HBE cells exposed for 24 h in triplicate to: A- 10 microg/cm² of the four $PM_{2.5}$ and their respective aqueous and organic extracts or of DEP. B- a combination of the aqueous and organic extracts or the aqueous extract after metal removal. Values are means ± SD (n=3). * : different from control, ^ : different from respective native particles, ° : different from winter urban background aqueous extract. All these experiments were repeated 3 times.

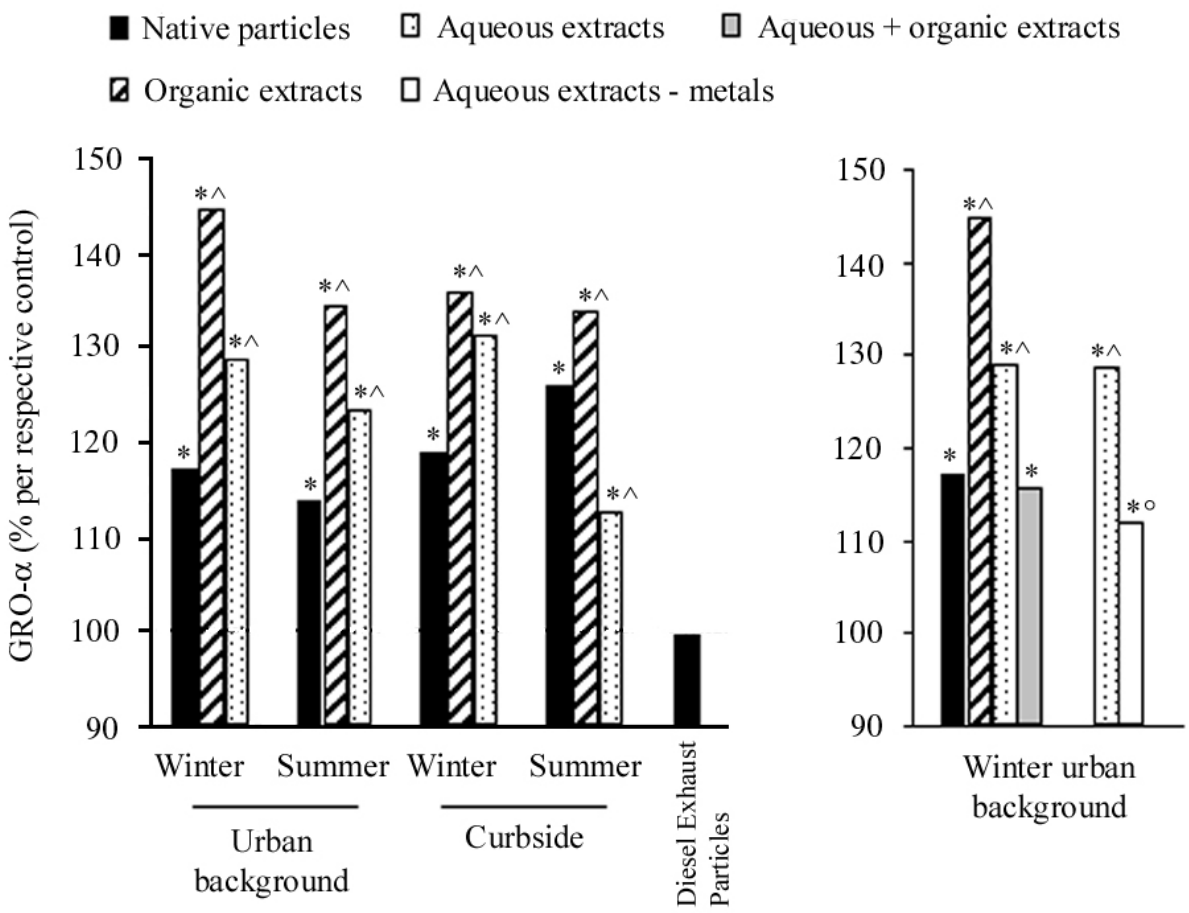

Figure 6. Release of GRO-alpha by 16-HBE cells exposed for 24 h in triplicate to: A- 10 microg/cm² of the four $PM_{2.5}$ and their respective aqueous and organic extracts or of DEP**.** B- a combination of the aqueous and organic extracts or the aqueous extract after metal removal**.** Values are means ± SD (n=3). *** :** different from control, ^ : different from respective native particles, ° : different from winter urban background aqueous extract. All these experiments were repeated 3 times.

metals, respectively, in the proinflammatory response of airway epithelial cells, likely due to their ability to elicit oxidative stress (22, 24, 5). Concerning AR, analysis of results obtained with particle fractions (aqueous and organic fractions) reveals a major effect of organic extract in AR expression and release that was similar to or slightly greater than with native PM. In contrast, both the aqueous extract and the organic extract induced GRO-alpha release. In addition GRO-alpha release induced by PM aqueous extract is abrogated when metals are removed. Interestingly, this is the first time we observe the involvement of metals in the induction of a proinflammatory gene by $PM_{2.5}$ in 16-HBE cells.

As GRO-alpha release induced by aqueous and organic extracts alone is stronger than GRO-alpha release induced by native particles, it could be due to interactions between metals and organic compounds or metals and organic compounds metabolizing enzymes. Indeed, we had previously shown that DEP induces cytochrome P450 1A1 (CYP 1A1) gene expression and enzymatic activity, suggesting that PAH desorbs from particles (22). It is known that metals such as copper inhibit CYP 1A1 activity (36). This hypothesis was investigated by studying the biological effects of a combination of both aqueous and organic extracts. A reduction in the biological effect induced by a single extract was observed at a level similar to that obtained with native particles, suggesting interactions between metallic and organic compounds. The higher effect induced by aqueous and organic extracts in comparison with native particles could be explained by bioavailability differences. Experiments using electron spin resonance have shown that metals are solubilized in the aqueous extracts, since they induce production of hydroxyl radicals in the presence of H_2O_2 (personal communication). Concerning organic compounds, especially PAH, they exhibit differences in their bioavailability according to PM samples (personal communication).

Finally, macroarray experiments provide useful tools for simultaneously studying a large variety of genes involved in the inflammatory process and in detecting those specifically overexpressed by $PM_{2.5}$ exposure. It provides potential explanations for *in vivo* observations, particularly

in the chemoattraction process and bronchial remodeling induced by particulate matter, which warrant further investigations.

6. ACKNOWLEDGMENTS

Augustin Baulig, and Sophie Blanchet contributed equally to this work. We acknowledge Dr. D.C. Gruenert for the human bronchial cell line. This work was supported by Renault, Ademe and Primequal grants. This work was also supported by the Caisse d'Assurance Maladie des Professions Liberales de Provinces, Paris, France.

7. REFERENCES

1. Pope, C. A., 3rd, R. T. Burnett, M. J. Thun, E. E. Calle, D. Krewski, K. Ito & G. D. Thurston: Lung cancer, cardiopulmonary mortality, and long-term exposure to fine particulate air pollution. *Jama*, 287, 1132-41 (2002)
2. Schwartz, J. & L. M. Neas: Fine particles are more strongly associated than coarse particles with acute respiratory health effects in schoolchildren. *Epidemiology*, 11, 6-10 (2000)
3. Holgate, S. T., R. B. Devlin, S. J. Wilson & A. J. Frew: Health effects of acute exposure to air pollution. Part II: Healthy subjects exposed to concentrated ambient particles. *Res Rep Health Eff Inst*31-50; discussion 51-67 (2003)
4. Li, X. Y., P. S. Gilmour, K. Donaldson & W. MacNee: *In vivo* and *in vitro* proinflammatory effects of particulate air pollution (PM10). *Environ Health Perspect*, 105 Suppl 5, 1279-83 (1997)
5. Carter, J. D., A. J. Ghio, J. M. Samet & R. B. Devlin: Cytokine production by human airway epithelial cells after exposure to an air pollution particle is metal-dependent. *Toxicol Appl Pharmacol*, 146, 180-8 (1997)
6. van Eeden, S. F. & J. C. Hogg: Systemic inflammatory response induced by particulate matter air pollution: the importance of bone-marrow stimulation. *J Toxicol Environ Health A*, 65, 1597-613 (2002)
7. Fujii, T., S. Hayashi, J. C. Hogg, R. Vincent & S. F. Van Eeden: Particulate matter induces cytokine expression in human bronchial epithelial cells. *Am J Respir Cell Mol Biol*, 25, 265-71 (2001)
8. Jimenez, L. A., E. M. Drost, P. S. Gilmour, I. Rahman, F. Antonicelli, H. Ritchie, W. MacNee & K. Donaldson: PM (10)-exposed macrophages stimulate a proinflammatory response in lung epithelial cells via TNF-alpha. *Am J Physiol Lung Cell Mol Physiol*, 282, L237-48 (2002)
9. Baulig, A., M. Sourdeval, M. Meyer, F. Marano & A. Baeza-Squiban: Biological effects of atmospheric particles on human bronchial epithelial cells. Comparison with diesel exhaust particles. *Toxicol In vitro*, 17, 567-73 (2003)
10. Reibman, J., Y. Hsu, L. C. Chen, A. Kumar, W. C. Su, W. Choy, A. Talbot & T. Gordon: Size fractions of ambient particulate matter induce granulocyte macrophage colony-stimulating factor in human bronchial epithelial cells by mitogen-activated protein kinase pathways. *Am J Respir Cell Mol Biol*, 27, 455-62 (2002)
11. Gilmour, P. S., I. Rahman, K. Donaldson & W. MacNee: Histone acetylation regulates epithelial IL-8 release mediated by oxidative stress from environmental particles. *Am J Physiol Lung Cell Mol Physiol*, 284, L533-40 (2003)
12. Baulig, A., J. J. Poirault, P. Ausset, R. Schins, T. Shi, D. Baralle, P. Dorlhene, M. Meyer, R. Lefevre, A. Baeza-Squiban & F. Marano: Physicochemical characteristics and biological activities of seasonal atmospheric particulate matter sampling in two locations of Paris. *Environ Sci Technol*, 38, 5985-92 (2004)
13. Boland, S., A. Baeza-Squiban, T. Fournier, O. Houcine, M. C. Gendron, M. Chevrier, G. Jouvenot, A. Coste, M. Aubier & F. Marano: Diesel exhaust particles are taken up by human airway epithelial cells *in vitro* and alter cytokine production. *Am J Physiol*, 276, L604-13 (1999)
14. Reynolds, L. J. & R. J. Richards: Can toxicogenomics provide information on the bioreactivity of diesel exhaust particles? *Toxicology*, 165, 145-52 (2001)
15. Koike, E., S. Hirano, N. Shimojo & T. Kobayashi: cDNA microarray analysis of gene expression in rat alveolar macrophages in response to organic extract of diesel exhaust particles. *Toxicol Sci*, 67, 241-6 (2002)
16. Nadadur, S. S. & U. P. Kodavanti: Altered gene expression profiles of rat lung in response to an emission particulate and its metal constituents. *J Toxicol Environ Health A*, 65, 1333-50 (2002)
17. Sato, H., M. Sagai, K. T. Suzuki & Y. Aoki: Identification, by cDNA microarray, of A-raf and proliferating cell nuclear antigen as genes induced in rat lung by exposure to diesel exhaust. *Res Commun Mol Pathol Pharmacol*, 105, 77-86 (1999)
18. Leikauf, G. D., S. A. McDowell, S. C. Wesselkamper, C. R. Miller, W. D. Hardie, K. Gammon, P. P. Biswas, T. R. Korfhagen, C. J. Bachurski, J. S. Wiest, K. Willeke, E. Bingham, J. E. Leikauf, B. J. Aronow & D. R. Prows: Pathogenomic mechanisms for particulate matter induction of acute lung injury and inflammation in mice. *Res Rep Health Eff Inst*5-58; discussion 59-71 (2001)
19. Verheyen, G. R., J. M. Nuijten, P. Van Hummelen & G. R. Schoeters: Microarray analysis of the effect of diesel exhaust particles on *in vitro* cultured macrophages. *Toxicol In vitro*, 18, 377-91 (2004)
20. Cozens, A. L., M. J. Yezzi, K. Kunzelmann, T. Ohrui, L. Chin, K. Eng, W. E. Finkbeiner, J. H. Widdicombe & D. C. Gruenert: CFTR expression and chloride secretion in polarized immortal human bronchial epithelial cells. *Am J Respir Cell Mol Biol*, 10, 38-47 (1994)
21. Blanchet, S., K. Ramgolam, A. Baulig, F. Marano & A. Baeza-Squiban: Fine particulate matter induces amphiregulin secretion by bronchial epithelial cells. *Am J Respir Cell Mol Biol*, 30, 421-7 (2004)
22. Baulig, A., M. Garlatti, V. Bonvallot, A. Marchand, R. Barouki, F. Marano & A. Baeza-Squiban: Involvement of reactive oxygen species in the metabolic pathways triggered by diesel exhaust particles in human airway epithelial cells. *Am J Physiol Lung Cell Mol Physiol*, 285, L671-9 (2003)
23. Boland, S., V. Bonvallot, T. Fournier, A. Baeza-Squiban, M. Aubier & F. Marano: Mechanisms of GM-CSF increase by diesel exhaust particles in human airway epithelial cells. *Am J Physiol Lung Cell Mol Physiol*, 278, L25-32 (2000)
24. Bonvallot, V., A. Baeza-Squiban, A. Baulig, S. Brulant, S. Boland, F. Muzeau, R. Barouki & F. Marano: Organic compounds from diesel exhaust particles elicit a

proinflammatory response in human airway epithelial cells and induce cytochrome p450 1A1 expression. *Am J Respir Cell Mol Biol*, 25, 515-21 (2001)
25. Nel, A. E., D. Diaz-Sanchez & N. Li: The role of particulate pollutants in pulmonary inflammation and asthma: evidence for the involvement of organic chemicals and oxidative stress. *Curr Opin Pulm Med*, 7, 20-6 (2001)
26. Salvi, S. S., C. Nordenhall, A. Blomberg, B. Rudell, J. Pourazar, F. J. Kelly, S. Wilson, T. Sandstrom, S. T. Holgate & A. J. Frew: Acute exposure to diesel exhaust increases IL-8 and GRO-alpha production in healthy human airways. *Am J Respir Crit Care Med*, 161, 550-7 (2000)
27. Lemjabbar, H., D. Li, M. Gallup, S. Sidhu, E. Drori & C. Basbaum: Tobacco smoke-induced lung cell proliferation mediated by tumor necrosis factor alpha-converting enzyme and amphiregulin. *J Biol Chem*, 278, 26202-7 (2003)
28. Kato, M., T. Inazu, Y. Kawai, K. Masamura, M. Yoshida, N. Tanaka, K. Miyamoto & I. Miyamori: Amphiregulin is a potent mitogen for the vascular smooth muscle cell line, A7r5. *Biochem Biophys Res Commun*, 301, 1109-15 (2003)
29. Shin, H. S., H. J. Lee, M. Nishida, M. S. Lee, R. Tamura, S. Yamashita, Y. Matsuzawa, I. K. Lee & G. Y. Koh: Betacellulin and amphiregulin induce upregulation of cyclin D1 and DNA synthesis activity through differential signaling pathways in vascular smooth muscle cells. *Circ Res*, 93, 302-10 (2003)
30. Brauer, M., C. Avila-Casado, T. I. Fortoul, S. Vedal, B. Stevens & A. Churg: Air pollution and retained particles in the lung. *Environ Health Perspect*, 109, 1039-43 (2001)
31. Churg, A., M. Brauer, M. del Carmen Avila-Casado, T. I. Fortoul & J. L. Wright: Chronic exposure to high levels of particulate air pollution and small airway remodeling. *Environ Health Perspect*, 111, 714-8 (2003)
32. Sunnarborg, S. W., C. L. Hinkle, M. Stevenson, W. E. Russell, C. S. Raska, J. J. Peschon, B. J. Castner, M. J. Gerhart, R. J. Paxton, R. A. Black & D. C. Lee: Tumor necrosis factor-alpha converting enzyme (TACE) regulates epidermal growth factor receptor ligand availability. *J Biol Chem*, 277, 12838-45 (2002)
33. Shao, M. X., T. Nakanaga & J. A. Nadel: Cigarette smoke induces MUC5AC mucin overproduction via tumor necrosis factor-alpha-converting enzyme in human airway epithelial (NCI-H292) cells. *Am J Physiol Lung Cell Mol Physiol*, 287, L420-7 (2004)
34. Ernst, T. J., A. R. Ritchie, G. D. Demetri & J. D. Griffin: Regulation of granulocyte- and monocyte-colony stimulating factor mRNA levels in human blood monocytes is mediated primarily at a post-transcriptional level. *J Biol Chem*, 264, 5700-3 (1989)
35. Traves, S. L., S. V. Culpitt, R. E. Russell, P. J. Barnes & L. E. Donnelly: Increased levels of the chemokines GROalpha and MCP-1 in sputum samples from patients with COPD. *Thorax*, 57, 590-5 (2002)
36. Ghosh, M. C., R. Ghosh & A. K. Ray: Impact of copper on biomonitoring enzyme ethoxyresorufin-o-deethylase in cultured catfish hepatocytes. *Environ Res*, 86, 167-73 (2001)

Abbreviations: AR: amphiregulin; DEP: diesel exhaust particles; DPL: dipalmitoyl phosphatidyl lecithin, GRO-alpha: growth-related oncogene-alpha; PBS: phosphate buffered saline; $PM_{2.5}$: particulate matter with an aerodynamic diameter < 2.5 μm; SC: summer curbside particles; SUB: summer urban background particles; WC: winter curbside particles; WUB: winter urban background particles; 16HBE: 16-HBE 14o- cell line.

Key Words: Particulate matter, inflammation genes, macroarray, amphiregulin, human bronchial epithelial cells.

Send correspondence to: Dr Armelle Baeza-Squiban, Laboratoire de Cytophysiologie et Toxicologie Cellulaire, Universite Paris 7, Denis Diderot, 2 Place Jussieu, Tour 53-54, 3^{e} etage, Case Courrier 7073, 75251 Paris Cedex 05, France, Tel: 33-1-44-27-37-08, Fax: 33-1-44-27-69-99, E-mail: baeza@paris7.jussieu.fr

http://www.bioscience.org/current/vol11.htm