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Chronic obstructive pulmonary disease and neutrophil infiltration: role of cigarette smoke and cyclooxygenase products

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Profita M, Sala A, Bonanno A, Riccobono L, Ferraro M, La Grutta S, Albano GD, Montalbano AM, Gjomarkaj M. Chronic obstructive pulmonary disease and neutrophil infiltration: role of cigarette smoke and cyclooxygenase products. *Am J Physiol Lung Cell Mol Physiol* 298: L261–L269, 2010. First published November 6, 2009; doi:10.1152/ajplung.90593.2008.—Cigarette smoke is the main cause of chronic obstructive pulmonary disease (COPD), where it can contribute to the observed airway inflammation. PGE_2 is produced within human airways, and both pro- and anti-inflammatory activities have been reported. We quantitated PGE_2 concentrations in induced sputum supernatants from different groups of subjects and correlated the obtained values to neutrophil infiltration as well as to the expression of cyclooxygenase-2 (COX-2). Cigarette smoke extract (CSE) was used to evaluate the effect of smoking on COX-2 and PGE_2 receptor expression as well as on PGE_2 release in neutrophils and alveolar macrophages (AM) obtained from normal donors. The effects of PGE_2 and of PGE receptor agonists and antagonists were evaluated on the adhesion of neutrophil to a human bronchial epithelial cell line (16HBE). PGE_2 levels, COX-2 expression, and neutrophil infiltration were significantly higher in normal smokers and COPD smokers ($P < 0.0001$) compared with controls and COPD former smokers. Induced sputum supernatant caused neutrophil adhesion to 16HBE that was significantly reduced, in COPD smokers only, by PGE_2 immunoprecipitation. In vitro experiments confirmed that CSE increased PGE_2 release and COX-2 and PGE_2 receptor expression in neutrophils and AM; PGE_2 enhanced the adhesion of neutrophils to 16HBE, and a specific E-prostanoid 4 (EP_4) receptor antagonist blunted its effect. These results suggest that CSE promote the induction of COX-2 and contributes to the proinflammatory effects of PGE_2 in the airways of COPD subjects.

alveolar macrophages; neutrophils; cigarette smoke extract; cyclooxygenase-2

CHRONIC OBSTRUCTIVE PULMONARY disease (COPD) is characterized by a progressive and irreversible airflow obstruction as a result of a chronic inflammatory status of the airways.

Neutrophil accumulation in the lung is a prominent feature of COPD, and an important role is played by neutrophil chemoattractants being produced within the airways and/or by the increase in neutrophil adhesion molecules expression (41). The activation of resident alveolar macrophages (AM) by different factors may be involved in the release of neutrophil chemoattractants and plays a role in the recruitment of neutrophils into the airways (37).

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Cigarette smoke is a major risk factor for a number of diseases, including cancer, cardiovascular diseases, and COPD. Cigarette smoke can cause the formation and release of different inflammatory factors such as IL-8 (16), and it can increase the expression of the adhesion molecules MAC-1 and LFA-1 on peripheral blood neutrophils (10), suggesting a potential causative link between smoking, neutrophil recruitment, and adhesion within the airways of COPD subjects.

Among the mediators involved in the development of airway diseases, an important role is played by arachidonic acid (AA) metabolites such as cyclooxygenase (COX) and lipoxygenase metabolites (40). However, although the 5-lipoxygenase-derived leukotrienes are known to be involved through the interaction with their $CysLT_1$ receptor in asthma and allergic rhinitis (3), little attention has been paid, so far, to the potential involvement of COX metabolites, namely PG, in airway diseases.

The synthesis of PGE_2, the main inflammatory PG, takes place in several different cellular types within the airways, including epithelial cells, follicular dendritic cells, fibroblasts, and monocytes (36), but AM certainly represent a major source of PGE_2, in particular following LPS and granulocyte/macrophage colony-stimulating factor (GM-CSF)-dependent expression of the inducible form of COX, namely COX-2 (9). COX-2 expression is increased at the sites of inflammation, and its enhanced expression has been reported in airway cells from patients with COPD (36). Interestingly, increased PGE_2 concentration in the exhaled breath condensate of patients with COPD has also been reported (18).

In this study, we evaluated the concentrations of PGE_2 in the induced sputum obtained from COPD (smokers and former smokers), healthy smoker, and control subjects. In light of the results obtained and, in particular, of the apparent correlation between PGE_2 concentrations and neutrophil infiltration, we tested the role of PGE_2 in the recruitment of neutrophil and the effect of cigarette smoke on COX-2 and E-prostanoid (EP) receptors expression.

MATERIALS AND METHODS

Patients. We recruited four groups of subjects: COPD smokers ($n = 12$) and former smokers ($n = 24$), asymptomatic smokers with normal lung function ($n = 12$), and healthy asymptomatic nonsmoking subjects with normal lung function ($n = 15$).

COPD subjects were defined and classified according to the criteria reported by Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines (23) and were classified as stage ≥1. Patients with COPD and healthy smokers had a smoking history of 10 pack-year or more. COPD former smokers patients had quit smoking for at least 2 yr. Reversibility test to bronchodilator was performed to exclude an

asthmatic component, and the increase in forced expiratory volume in 1 s (FEV_1) after salbutamol was lower than 12% and 200 ml, compared with basal values, in all COPD subjects. All COPD subjects were under treatment with long-acting β-adrenergic agonists (salmeterol 50-μg bid).

All patients were characterized with respect to sex, age, smoking history, COPD symptoms, comorbidity, and current history of treatment. Exclusion criteria included the following: other systemic diseases; other lung diseases apart from COPD and lung tumors; upper respiratory tract infections; and treatment with glucocorticoids or anticholinergics within the 3 mo before the study. None of the subjects had received a prior or concomitant treatment with COX-2 inhibitors.

The local ethics committee approved the study, and participating subjects gave their informed consent.

Sputum induction and processing. Sputum induction and processing were performed according to the methods of Hargreave et al. (8) with minor modifications. Briefly, patients in a fasting condition were exposed for 20 min to an aerosol of 3% hypertonic saline solution early in the morning, and sputum was collected into previously weighted 50-ml sterile ampoules. The volume of the induced sputum was previously determined, and an equal volume of DTT (0.1% in saline; Sigma, St. Louis, MO) was added. After homogenization, sputum samples were centrifuged at 800 *g* for 10 min to separate the supernatants from the cell pellet. The supernatants were then aspirated and frozen at −20°C in separate aliquots for the subsequent biochemical analysis. The cells obtained from induced sputum were then cytocentrifuged (Cytospin 2; Shandon, Runcorn, United Kingdom) and stained with May-Grunwald-Giemsa. The slides were read blindly by 2 independent investigators who counted at least 400 cells per slide. The number of squamous cells was subtracted from the total cell count to get the corrected cell number. The cytospins for immunocytochemistry were prepared on 3-aminopropyltriethoxysilane (APTEX)-coated slides by adding 100 μl of cell suspension ($\sim 5 \times 10^5$ cells/ml) into Shandon II cytocentrifuge cups and centrifuging at 180 *g* for 5 min. The air-dry slides were fixed in paraformaldehyde-lysine-periodate (PLP) for 30 min and in 15% sucrose in Dulbecco's PBS for 30 min. The slides were stored at −80°C until use for immunocytochemical staining.

Analysis of prostanoids in induced sputum supernatant. The aliquots from the supernatants recovered from induced sputum samples were thawed, and PGE_2 was extracted according to Powell (24). The recovery was evaluated by using standard PGE_2 (1 ng), which was added to separate aliquots of induced sputum samples prepared and extracted in parallel to the original samples. PGE_2 concentrations were evaluated using a commercially available radioimmunoassay (RIA; Amersham International, Little Chalfont, Buckinghamshire, United Kingdom). DTT was not retained during the solid phase extraction, as verified using its rapid colorimetric reaction with the Ellman reagent, and therefore its presence in the sputum samples did not affect the quantitation of PGE_2. Results are expressed as picograms per milliliter induced sputum supernatant.

Immunocytochemistry. After thawing, immunostaining of COX-2 on sputum cells was performed using a mouse monoclonal anti-COX-2 (IgG_1) antibody (Cayman Chemical, Ann Arbor, MI) as previously described (25). The cell identification was based on cell morphology under light microscopy (×400 final magnification), carefully referring to the cell type distribution in corresponding Diff-Quik-stained slides; red staining identified positive cells. Two independent observers counted a minimum of 600 cells, and the mean value of the 2 observations was used ($r = 0.93$). The results were expressed as percentage of positively staining cells over the total cell number.

Preparation of cigarette smoke extract. Cigarette smoke solution was prepared as described previously (33) with some modifications. Each commercial cigarette (Marlboro) was smoked for 5 min, and two cigarettes were used per 25 ml of PBS to generate a cigarette smoke extract (CSE)-PBS solution. The CSE solution was filtered through a 0.22-μm pore sieve to remove bacteria and large particles. The smoke solution was then adjusted to pH 7.4 and used within 30 min of preparation. This solution was considered to be 100% CSE and diluted to obtain the desired concentration in each experiment. The concentration of CSE was calculated spectrophotometrically measuring the optical density as previously described (12). The pattern of absorbance, among different batches, showed very little differences.

Stimulation of AM from bronchoalveolar lavage and neutrophils from peripheral blood. AM were collected from the airways of subjects with no pulmonary and systemic inflammatory diseases who underwent bronchoscopy and bronchoalveolar lavage (BAL) for suspected lung cancer and who finally resulted cancer free. Briefly, the BAL was carried out in one of the subsegmental bronchi of the middle lobe by injection of several aliquots of sterile saline (up to a total volume of 0.2 l) reaspirated by gentle syringe suction. Immediately after lavage, mucus was removed from the fluid by filtration through a gauze, then BAL fluid was centrifuged at 400 *g* for 10 min at 4°C, and cells were resuspended in RPMI. The BAL cytology was conducted on cytocentrifuged slides (Cytospin; Shandon) stained by May-Grunwald-Giemsa, and macrophages were separated by adhesion.

Peripheral blood polymorphonuclear leukocytes were prepared from healthy subjects with the use of dextran sedimentation and centrifugation over Ficoll cushions, as previously described (26). AM and neutrophils were treated with CSE (10%) for different time of incubation (from 0 to 24 h) and added with calcium ionophore A23187 (2.5 μM; Sigma), and PGE_2 production was evaluated as described above. Expression of COX-1 and COX-2 isoforms was performed by Western blot on cellular lysates as described below. Furthermore, the expression of EP_1, EP_2, EP_3, and EP_4 receptors was evaluated in neutrophils treated with the CSE (10%).

Western blot analysis. Total protein extracts from AM and neutrophils treated with CSE (10%) were resuspended in 2× Laemmli buffer and separated by SDS-PAGE on 4–12% gradient gels followed by electroblotting onto nitrocellulose membranes. The following antibodies were used: mouse monoclonal anti-human COX-1 and

Table 1. *Patients' characteristics*

					P					
	Control Subjects	Healthy (S)	COPD (fS)	COPD (S)	Control Healthy (S)	Control COPD (fS)	Control COPD (S)	Healthy (S) COPD (fS)	Healthy (S) COPD (S)	COPD (fS) COPD (S)
Subject, *n*	15	12	24	12						
Sex, male/female	9/6	7/5	14/10	6/6						
Mean age, yr	62 (49–72)	59.4 (45–70)	65 (59–71)	70 (63–72)	NS	NS	NS	NS	NS	NS
FEV_1, % predicted	100 (99–106)	97 (92–106)	64 (57–78)	60 (45–71)	NS	<0.0001	<0.0001	<0.0001	0.0001	NS
FEV_1/FVC, %	98 (96–102)	94 (91–100)	71 (66–82)	69 (63–76)	NS	<0.001	<0.001	<0.001	<0.001	NS
Pack years	0	54.3 (26.2–30)	0	65 (42–55)	0.0001	NS	0.0001	0.0001	NS	0.0001

Results are expressed as medians (25th to 75th percentiles). Statistical analysis was performed by Mann-Whitney. NS, not significant; S, smoker; fS, former smoker; COPD, chronic obstructive pulmonary disease; FEV_1, forced expiratory volume in 1 s; FVC, forced vital capacity.

Table 2. *Total and differential cell count from induced sputum samples*

					P					
	Control Subjects	Healthy (S)	COPD (fS)	COPD (S)	Control Healthy (S)	Control COPD (fS)	Control COPD (S)	Healthy (S) COPD (fS)	Healthy (S) COPD (S)	COPD (fS) COPD (S)
Macrophages (10^4)	120.6 (52–147.7)	233.3 (147–330)	59.9 (28.1–167)	66.5 (56–93.6)	<0.01	NS	NS	<0.004	<0.003	NS
Neutrophils (10^4)	26.5 (7.3–43)	106.4 (51–255)	159 (72–413)	491 (241–783)	<0.02	<0.001	0.0001	NS	<0.02	0.055
Lymphocytes (10^4)	0 (0–1.8)	2.6 (0–5.5)	0.06 (0–4.6)	0 (0–2.7)	NS	NS	NS	NS	NS	NS
Eosinophils (10^4)	0 (0–0)	1.6 (0–4.61)	1.2 (0–12.2)	5.0 (0.1–10.9)	NS	<0.02	<0.02	NS	NS	NS
Epithelial cells (10^4)	0 (0–3.0)	0 (0–2.4)	2.0 (0–7.0)	0 (0–4.0)	NS	NS	NS	NS	NS	NS
Total cells (10^6)	1.5 (0.6–2.2)	3.7 (2.4–5.6)	2.6 (1.1–6.9)	5.7 (3.1–8.6)	<0.01	<0.05	<0.05	NS	NS	NS

Results are expressed as medians (25th to 75th percentiles). Statistical analysis was performed by Mann-Whitney.

COX-2 antibodies (Cayman Chemical) and rabbit polyclonal anti-human EP_1, EP_2, EP_3, and EP_4 receptor antibodies (Cayman Chemical). Primary antisera were visualized with horseradish peroxidase-conjugated secondary antibody (Sigma) and developed with an enhanced chemiluminescence system (Amersham International). Approximate molecular masses were determined using calibrated prestained standards (Amersham International). Negative controls were performed in the absence of primary antibody or including an isotype control antibody. β-Actin (Sigma) was used to normalize the amount of protein included in the Western blot analysis.

Real-time quantitative RT-PCR of muscarinic EP_1, EP_2, EP_3, and EP_4 receptors. Total cellular RNA was extracted from cells according to the method of Chomczynski and Sacchi using the RNAzol kit (Biotec Italia, Rome, Italy). Total RNA (4 μg) was reverse-transcribed into cDNA using Moloney murine leukemia virus (MMLV)-RT and oligo(dT)12–18 primers (Invitrogen) in a 25-μl reaction mixture. Real-time quantitative PCR of EP_1, EP_2, EP_3, and EP_4 receptor subtypes of human PGE_2 receptors was carried out using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) using specific FAM-labeled probes and primers (*Taq*Man Assays on Demand; Applied Biosystems). GAPDH gene expression was used as endogenous control. Gene expression levels were expressed as threshold cycle crossover points (30).

Adhesion assay. Purified peripheral blood neutrophils were resuspended in PBS (10^6 cells/ml), labeled for 45 min at 37°C with 50 μg/ml fluorochromic dye SFDA (Molecular Probes), washed, and resuspended in PBS (0.4×10^6 cells/ml). Neutrophil adhesion was performed according to Zeidler et al. (44) with minor modifications. The simian virus 40 (SV40) large T antigen-transformed human airway epithelial cell line (16HBE) was used for adhesion assay of neutrophils. 16HBE cell line was cultured as adherent monolayers in MEM supplemented with 10% heat-inactivated (56°C, 30 min) fetal calf serum + 100 U/ml penicillin and 100 mg/ml streptomycin. 16HBE cells have previously been used to study the functional properties of bronchial epithelial cells in inflammation (15). Immediately before addition of neutrophils, medium was removed from 16HBE cultures (70,000 cells/well) grown to confluence in standard 24-well culture plates, and cells were washed with warm PBS. Labeled neutrophils (0.2×10^6 cells/well) were added in a final volume of 0.5 ml. The plates were incubated at 37°C for 25 min, and total fluorescence was evaluated using an excitation wavelength of 485 nm and monitoring emission at 530 nm in a Wallac 1420 Victor multilabel counter (PerkinElmer). Subsequently, nonadherent cells were removed by washing, and fluorescence was measured to evaluate bound cells. Adhesion was expressed as percentage of the fluorescence ratio of bound cells to total cells. All test points were performed in triplicate.

Effect of induced sputum supernatant and PGE_2 on neutrophil adhesion. The induced sputum supernatants (1 ml) were added to neutrophils for 18 h. At the end of the incubation time, the neutrophils were centrifuged at 1,000 rpm for 10 min, and adhesion was assessed as described above. To determine the contribution of PGE_2 present in induced sputum supernatants to the observed effect on neutrophil adhesion, selected samples (6 COPD smokers and 6 COPD former smokers) were incubated in the presence or absence of PGE_2 affinity

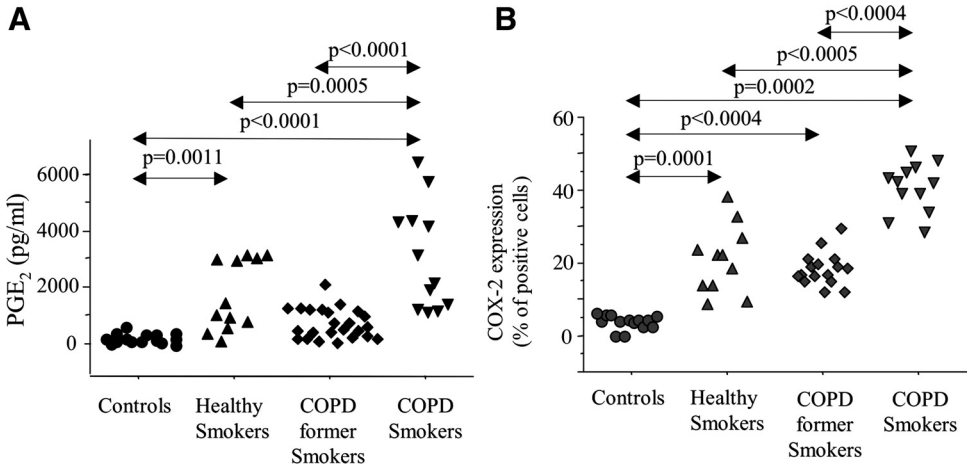

Fig. 1. PGE_2 concentrations and cyclooxygenase-2 (COX-2) expression in induced sputum samples. *A*: PGE_2 concentrations in supernatants of induced sputum samples obtained from controls, healthy smokers, chronic obstructive pulmonary disease (COPD) former smokers, and COPD smoker subjects. Samples were analyzed by specific radioimmunoassay (RIA) kits as described in MATERIALS AND METHODS. *B*: percentage of COX-2-expressing cells over the total number of cells obtained from induced sputum samples. Cells obtained from induced sputum samples of controls, healthy smokers, and COPD former smoker and COPD smoker subjects were immunostained using a COX-2-selective monoclonal antibody as described in MATERIALS AND METHODS. Data are expressed as medians and 25th to 75th percentiles (boxed) of PGE_2 concentrations or percentage of COX-2-positive cells. Minimum and maximum values are represented by error bars. Statistical analysis was performed by Kruskal-Wallis test and Dunn procedure.

Table 3. *COX-2 expression by different cell types in induced sputum samples*

	Control Subjects	Healthy (S)	COPD (fS)	COPD (S)	*P*					
					Control Healthy (S)	Control COPD (fS)	Control COPD (S)	Healthy (S) COPD (fS)	Healthy (S) COPD (S)	COPD (fS) COPD (S)
Macrophages, %	2.5 (2–3.2)	13.9 (11.1–15.8)	12.2 (9.1–15.3)	29.2 (28.3–35)	0.0001	0.0004	0.0002	NS	0.0002	0.0004
Neutrophils, %	1.0 (0–1.2)	6.0 (3–11.3)	6.0 (2.9–6.4)	12.3 (6.9–14.3)	0.0001	0.0004	0.0002	NS	0.05	0.005
Lymphocytes, %	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	NS	NS	NS	NS	NS	NS
Eosinophils, %	0 (0–0)	0 (0–0.2)	0 (0–0)	0 (0–0)	NS	NS	NS	NS	NS	NS
Epithelial cells, %	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	NS	NS	NS	NS	NS	NS
Total cells	3.8 (2.3–4.2)	21.6 (13.3–25.6)	17.4 (15.2–20)	46.0 (36.9–49.5)	0.0001	0.0004	0.0002	NS	0.0005	0.0004
PGE_2, ng/ml	0.3 (0.2–0.4)	1.3 (0.8–3.1)	0.6 (0.3–1.3)	3.2 (1.5–4.4)	0.004	0.002	<0.0001	<0.05	<0.05	<0.0001

Results are expressed as medians (25th to 75th percentiles). COX-2, cyclooxygenase-2.

sorbent (mouse anti-PGE_2 IgG covalently bound to Sepharose 4B; Cayman Chemical) as previously described (25).

The adhesion of neutrophils to 16HBE, with or without pretreatment with CSE (10%), was also tested on treatment of neutrophils with synthetic PGE_2 (Sigma) at different concentrations, the EP_2 receptor agonist Butaprost (Cayman Chemical), the specific EP_4 receptor antagonist AH-23848 (30 μM; Cayman Chemical), the potent and specific TxA_2-PGH_2 (TP) receptor antagonist GR-32191B (100 nM; Cayman Chemical), and PGE_2 (10 nM) in the presence of AH-23848 or GR-32191B. Controls were carried out incubating the neutrophils with the appropriate dilution of the solvent used for the tested compounds (DMSO).

Statistical analysis. The data relative to induced sputum are expressed as medians and 25th to 75th percentiles. Statistical analysis was performed using the nonparametric Mann-Whitney *U* test for comparison between groups. The Spearman rank test was used for correlation between data. A value of $P < 0.05$ was accepted as statistically significant. The results obtained from recovery experiments are expressed as means and SD or SE of *n* replication. The results of in vitro experiments were analyzed using ANOVA with Fisher test correction or *t*-test.

RESULTS

Patients and differential cell counts. As expected, pulmonary functions significantly decreased in COPD subjects (smokers or former smokers) compared with either control subjects or healthy smokers (Table 1).

In line with published data, the results of the differential cell counts performed on induced sputum samples showed a statistically significant increase in the number of cells both in healthy smokers and COPD subjects (either smokers or former smokers). In healthy smokers, this increase reflected an increase in the number of both macrophages and neutrophils, whereas in COPD subjects neutrophils showed a large increase (in particular in actual smokers) with the number of macrophages being significantly lower than healthy smokers and similar to normal controls (Table 2). In agreement with previous reports, eosinophils also resulted significantly higher in COPD subjects compared with controls (28), whereas lymphocytes did not show significant changes (11, 20, 28), possibly reflecting the relatively different sampling of the airways (bronchial vs. alveolar) obtained with the induced sputum compared with BAL.

PGE_2 and COX-2 expression in induced sputum samples. The recovery of PGE_2, as assessed in induced sputum samples analyzed with and without the addition of 1 ng of synthetic PGE_2, was 89 ± 10%. PGE_2 concentrations in supernatants of induced sputum samples obtained from COPD smokers were significantly higher than those observed in COPD former

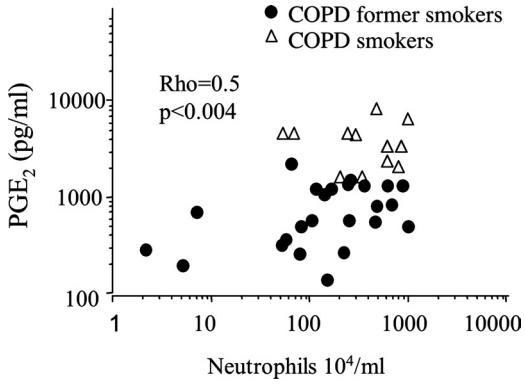

Fig. 2. Correlation between PGE_2 and neutrophil concentrations in the supernatants of induced sputum samples obtained from either current (●) or former (△) smoker COPD subjects. Statistical analysis was performed using the Spearman rank test.

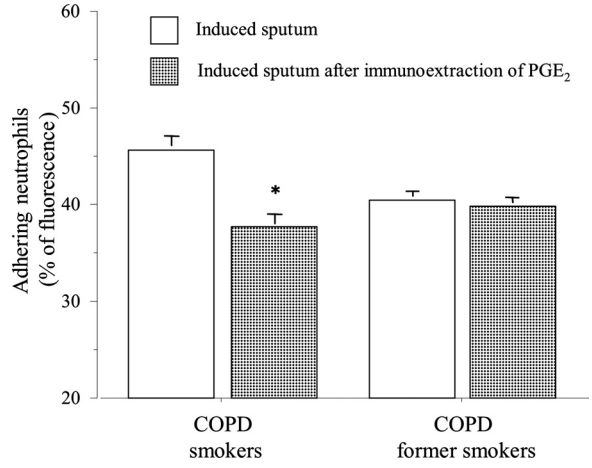

Fig. 3. Adhesion of neutrophils in the presence of supernatant from induced sputum samples. Neutrophils obtained from peripheral blood were incubated with the supernatant (1 ml) of induced sputum samples obtained from COPD smokers and COPD former smokers. Supernatant was used as obtained from induced sputum processing or after depletion of PGE_2 by immunoprecipitation with a specific affinity sorbent (mouse monoclonal anti-PGE_2 antibody coupled to Sepharose 4B). Adhesion was assessed by fluorescence measurements using a Wallac 1420 Victor multilabel counter (PerkinElmer) as described in MATERIALS AND METHODS. Results are expressed as means ± SD of percentage of fluorescence. Statistical analysis was performed using ANOVA with Fisher protected least significant differences correction for multiple comparisons. *$P < 0.05$ vs. induced sputum.

smokers, healthy smokers, and control subjects ($P < 0.0001$, $P = 0.0005$, and $P = 0.0011$, respectively); interestingly, healthy smokers also showed values higher than control subjects (Fig. 1*A*).

The percentage of total COX-2-positive cells recovered from induced sputum samples increased in COPD smokers compared with COPD former smokers, healthy smokers, and control subjects ($P < 0.0004$, $P < 0.0005$, and $P = 0.0002$, respectively; Fig. 1*B*); again, healthy smokers and COPD former smokers showed a higher number of COX-2-staining cells compared with controls. Macrophages and neutrophils represented the main cell types expressing COX-2 in all groups of subjects (Table 3).

In COPD nonsmokers, the correlation between PGE_2 and neutrophils has a ρ of 0.42 and is statistically significant ($P < 0.05$), whereas the same correlation in COPD smokers (with a much smaller *n*) is not. Aggregating the data relative to all COPD subjects, we still observed a significant correlation between PGE_2 concentrations and the number of neutrophils ($\rho = 0.5$; $P < 0.004$) in induced sputum samples (Fig. 2). We also carried out the analysis of the correlation between PGE_2 and neutrophils in the healthy smokers group or between PGE_2 and macrophages in the healthy smokers or COPD subjects and did not find any statistically significant correlation ($P > 0.1$).

Effect of induced sputum supernatant and PGE_2 on neutrophil adhesion. Based on the direct correlation between PGE_2 concentrations and the percentage of neutrophils in induced sputum, we evaluated the effect of supernatants of induced sputum samples obtained from COPD subjects on the adhesion of peripheral blood neutrophils to human airway epithelial cells (16HBE). Samples from six COPD smokers showing the highest concentrations of PGE_2 (>2 ng/ml, 0.6 nM) and six COPD former smokers with lowest concentrations of PGE_2 (<1 ng/ml) were used. The results obtained showed a significantly higher number of adhering neutrophils on incubation with induced sputum supernatants from COPD smoker subjects compared with samples obtained from COPD former smoker subjects ($P < 0.001$). Selective immunoprecipitation of PGE_2 from the same samples significantly reduced neutrophil adhesion induced by supernatants from COPD smokers but was basically ineffective on the activity of supernatants from COPD former smokers (Fig. 3), suggesting that PGE_2 contributes to the increased adhesivity of neutrophils incubated with supernatants from induced sputum from active smoker COPD subjects.

Stimulation of AM from BAL and neutrophils from peripheral blood. AM and neutrophils from normal subjects when pretreated with CSE (10%) significantly increased COX-2 expression and activity (as assessed by the production of PGE_2 after activation with the calcium ionophore A23187). In particular, AM increased COX activity (Fig. 4*A*) and COX-2 expression (Fig. 4, *C* and *E*), reaching the maximum after 24 h of incubation, and neutrophils increased their COX activity (Fig. 4*B*) and COX-2 expression (Fig. 4, *D* and *F*), reaching a maximum after 3 h of incubation with CSE, whereas at 24 h it

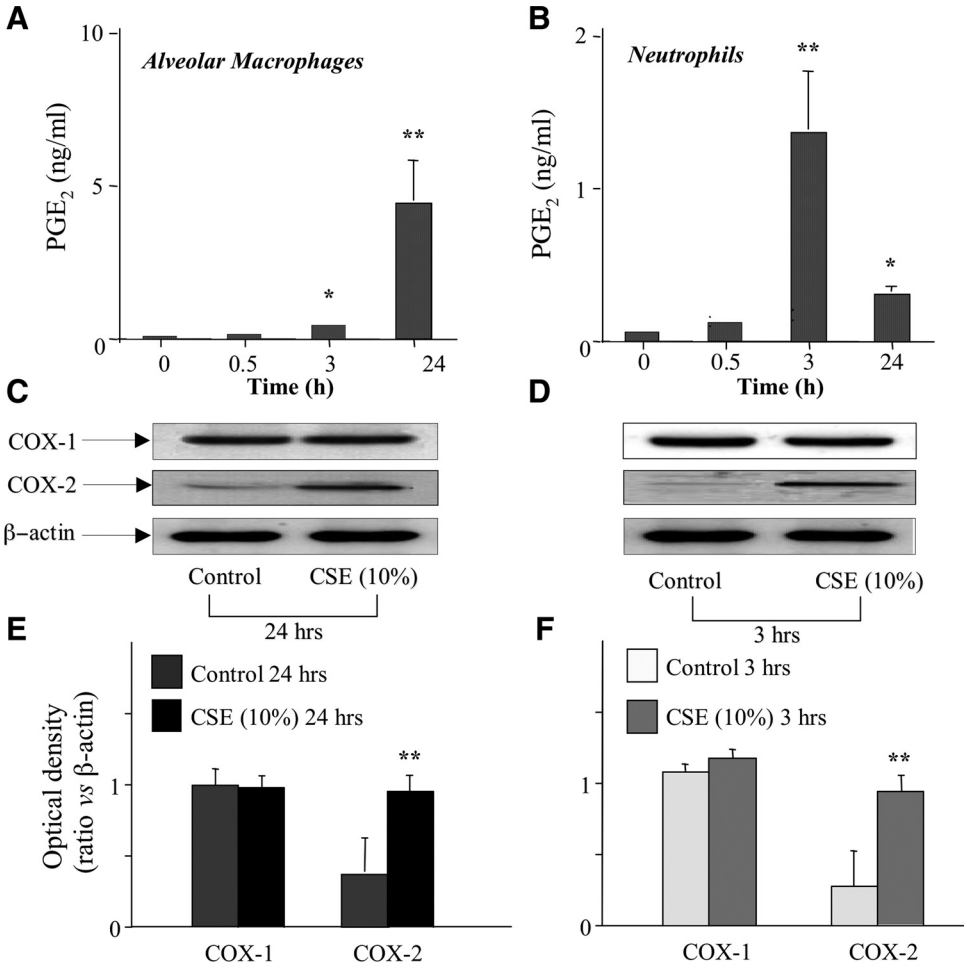

Fig. 4. COX activity and COX-1 and COX-2 expression in alveolar macrophages (AM) and neutrophils treated with cigarette smoke extract (CSE). AM (*A*) and neutrophils (*B*) from normal donors were treated with CSE (10%) for different times (0–24 h), challenged with 2.5 μM calcium ionophore A23187 for 5 min, and analyzed for PGE_2 production as described in MATERIALS AND METHODS. The cell lysates of AM stimulated for 24 h with 10% CSE (*C* and *E*) and of neutrophils stimulated for 3 h with 10% CSE (*D* and *F*) were analyzed for COX-1 and COX-2 expression using specific Western blots. Representative immunoblots are shown in *C* and *D*. Quantitative densitometric results are reported in *E* and *F* as means ± SE ($n = 3$) of the ratio vs. β-actin. PGE_2 concentrations are expressed as means ± SD. Statistical analysis was performed using ANOVA with Fisher protected least significant differences correction for multiple comparisons. *$P < 0.05$ and **$P < 0.01$ vs. Control.

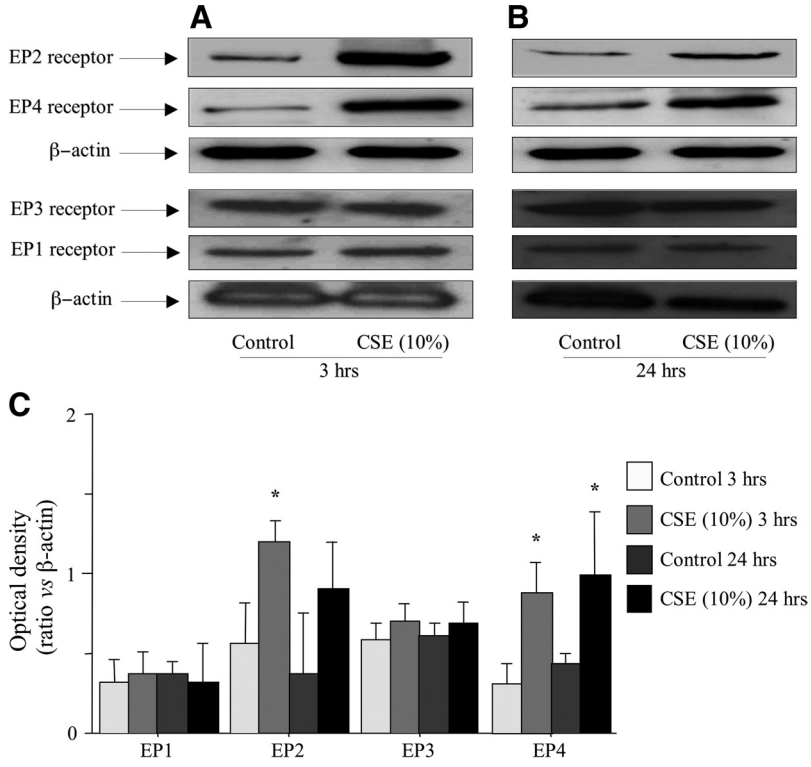

Fig. 5. E-prostanoid EP_1, EP_2, EP_3, and EP_4 receptor expression in peripheral blood neutrophils treated with CSE. Neutrophils from normal donors were stimulated with CSE (10%) for 3 or 24 h. The total cell lysates were analyzed for EP_1, EP_2, EP_3, and EP_4 expression using specific Western blots. Representative immunoblots are shown in *A* and *B*. Quantitative densitometric results are reported in *C* as means ± SE ($n = 3$) of the ratio vs. β-actin. $^*P < 0.05$ vs. the relevant Control.

was possible to observe a decrease in COX activity. Similarly, pretreatment with CSE markedly increased the expression of EP_2 and EP_4 receptors in neutrophils, an effect that was maximal at 3 h for EP_2 receptors (Fig. 5, *A* and *C*) but still remained quite significant at 24 h for both receptors (Fig. 5, *B* and *C*). EP_1 and EP_3 receptor expression was not affected by treatment with CSE (Fig. 5, *A–C*). These results were confirmed by quantitative RT-PCR analysis that showed increased amounts of EP_2 and EP_4 mRNA (as indicated by the lower number of amplification cycles required) after treatment with CSE, whereas EP_1 and EP_3 did not change in response to CSE (Fig. 6).

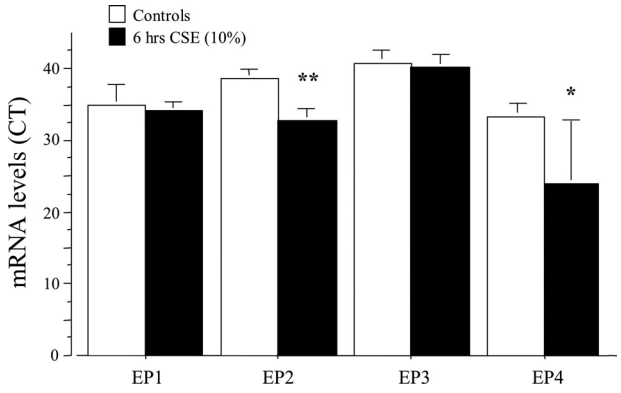

Fig. 6. Real-time quantitative RT-PCR of EP_1, EP_2, EP_3, and EP_4 receptors was performed in neutrophils treated with and without CSE (10%). Gene expression levels are expressed as CT (crossover cycle at the threshold of PCR amplification; the higher the number of cycles required to reach the CT, the lower the abundance of target mRNA in the reaction). The results represent the means ± SD of 3 different experiments performed with freshly purified normal human neutrophils. $^*P < 0.05$ and $^{**}P < 0.01$ vs. Control.

Effect of PGE_2 on neutrophil adhesion. To confirm the potential involvement of PGE_2 in the enhanced adhesivity of neutrophils observed in induced sputum samples, we tested the effect of PGE_2 on the adhesivity of CSE-treated, purified peripheral blood neutrophils, showing a significant enhancement at 1 and 10 nM but not at 100 nM (Fig. 7*A*). The EP_2 receptor agonist Butaprost also significantly enhanced the adhesivity of neutrophils to human airway epithelial cells, whereas the effect of PGE_2 was significantly blunted by the preincubation of neutrophils with the EP_4-selective antagonist AH-23848 (30 μM) (Fig. 7*B*). Pretreatment with a potent and selective TP receptor antagonist (GR-32191B, 100 nM) did not affect the enhanced adhesion induced by PGE_2 (Fig. 7*B*). Neither AH-23848 nor GR-32191B affected the adhesion of neutrophils observed in the absence of PGE_2 activation (Fig. 7*B*).

DISCUSSION

The present work provides evidence about a possible role of COX-2 and its metabolite PGE_2 on the neutrophil infiltration in COPD subjects and suggests that cigarette smoke may significantly affect the contribution of PGE_2 through the increased expression of both its biosynthetic enzyme and specific receptors in AM and/or neutrophils.

Induced sputum represents a well-accepted, minimally invasive approach to the sampling of the airways of normal as well as pathological subjects (21) and has been widely used in COPD patients (38). The concentrations of PGE_2, the main proinflammatory COX metabolite, in induced sputum samples from different group of subjects showed maximal values for COPD current smoker subjects, followed by the group of healthy smokers, whereas COPD former smoker had values that were not higher than normal subjects. A similar pattern, with increased expression in smokers (either healthy or COPD

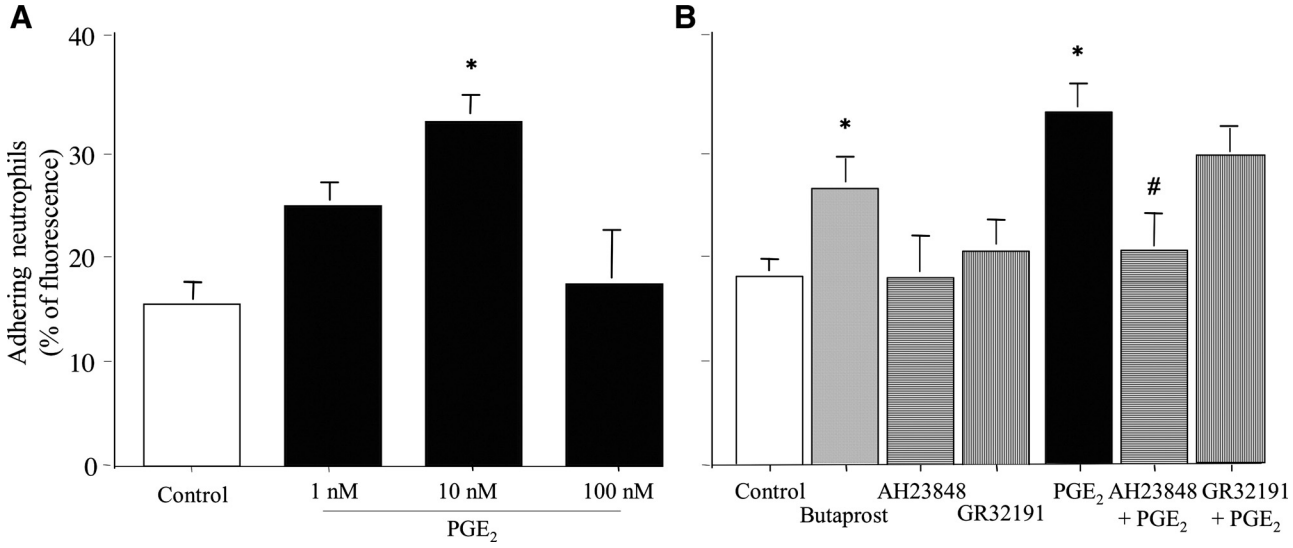

Fig. 7. Effect of PGE_2 on neutrophil adhesion on pretreatment with CSE. Purified peripheral blood neutrophils were treated for 24 h with CSE (10%) and then stimulated on human airway epithelial cells (16HBE) with different concentrations of PGE_2 (*A*), the EP_2-selective agonist Butaprost (100 nM), the selective EP_4 antagonist AH-23848 (30 μM), the potent and selective TxA_2-PGH_2 (TP) receptor antagonist GR-32191B (100 nM), and PGE_2 (10 nM) in the presence or absence of AH-23848 or GR-32191B (*B*). Adhesion was assessed by fluorescence measurements using a Wallac 1420 Victor multilabel counter (PerkinElmer) as described in MATERIALS AND METHODS. Results are expressed as means ± SD of percentage of fluorescence. $*P < 0.05$ vs. Control; $\#P < 0.05$ vs. 10 nM PGE_2.

subjects), emerged looking at the expression of COX-2 in cells obtained from induced sputum samples, although in this case COPD former smoker subjects also were clearly distinguishable from controls. Although it appears clear that smoke per se is able to induce COX-2 (and boost PGE_2 production), irrespectively from the development of COPD, the finding that COPD former smokers appear to have enhanced COX-2 expression is suggestive that long-lasting changes in the inflammatory status of airway cells are taking place specifically in COPD subjects, and COX-2 may represent an important contributor. Interestingly, PGE_2 concentrations correlated with the number of neutrophils present in induced sputum samples, suggesting a potential causal relationship between the inflammatory mediator and the inflammatory cell influx within the airways of COPD subjects. Indeed, preincubating normal peripheral blood neutrophils with the supernatant obtained from induced sputum of current smoker COPD subjects resulted in increased neutrophil adhesion to a human airway epithelial cell line, compared with the effect of supernatant from COPD former smoker subjects, characterized by lower concentrations of PGE_2. Neutrophil accumulation in the lung is a prominent feature of COPD, and the activation of these cells, producing proteases and oxygen-derived free radicals, is thought to be important in the pathogenesis of the disease (41). A critical passage is represented by the in situ formation of signal(s), which may cause the influx and trapping of neutrophils within the airways. Several factors have been considered for this role, including lipid mediators such as leukotriene B_4 and chemokines such as IL-8, and it is quite reasonable that the system may present significant redundancies, as cigarette smoke itself is able to increase neutrophil adhesion (32).

PGE_2 has been previously measured in exhaled breath condensate from exsmoker COPD subjects showing increased concentrations compared with controls (18) and resistance to treatment with COX-2-selective inhibitors (19), suggesting that PGE_2 in breath condensate may not be the result of COX-2 activity. Nevertheless, as the subjects participating in that study were all former smokers, this appears to be consistent with our data. The results of a very recent study carried out using induced sputum samples supported the role of COX-2-derived PGE_2 in airway inflammation, suggesting it may contribute to the severity of airflow limitation mediated by matrix metalloproteinase-2 (MMP-2) during progression of COPD (4). Interestingly, increased concentrations of the urinary metabolite of PGE_2 have been reported in smokers and former smokers compared with never-smoker subjects; in these subjects, treatment with celecoxib, a selective COX-2 inhibitor, caused a ≥50% decrease in the excretion of urinary PGE_2 metabolites, providing evidence for a critical involvement of COX-2 in the increased formation of PGE_2 in smokers (6).

Cigarette smoke represents the most important risk factor in the development of COPD given the compelling evidence that smoke represents a significant source of oxidant species (5), oxidative stress (17), and that the unbalance of oxidant and antioxidant within the lung has been long linked to COPD (27). Nevertheless, cigarette smoke is also known to induce the expression of COX-2, as well as downstream isomerases, in several cell types present within the airways (13, 39), and indeed in our experimental conditions CSE was able to induce COX activity, evaluated as maximal PGE_2 biosynthesis and COX-2 protein expression in both AM obtained from BAL of control subjects and peripheral blood neutrophils. This is well in agreement with the enhanced expression of COX-2 in induced sputum cells as well as with the concentrations of PGE_2 that we observed in induced sputum supernatants obtained from COPD and healthy smokers.

It is known that the activity of PGE_2 is mediated by four subtypes of EP receptors ($EP_{1\text{-}4}$; Ref. 22). Interestingly, CSE also significantly increased the expression of EP_2 and EP_4 receptors in purified human neutrophils, although leaving EP_1 and EP_3 receptors unaffected, as evaluated both by Western blot analysis and quantitative RT-PCR. Although the role of

each receptor has not been clearly established, an altered expression of EP_2 and EP_4 receptors was observed in cells from patients with asthma, supporting the hypothesis that these receptors may be involved in chronic airway inflammation (43). The activation of EP_2 and EP_4 receptors has mostly been described as leading to a decreased adhesion/chemotaxis of neutrophils (1, 2), but several differences can be noted between previous publications and the conditions used in the present study, namely: *1*) higher PGE_2 concentrations ($\geq$100 nM) and/or the concomitant use of phosphodiesterase inhibitors were commonly used; *2*) in most cases, the studied effect was the inhibition of FMLP-induced neutrophil adhesion on pretreatment with PGE_2; and *3*) no pretreatment with CSE was present. It is interesting to note that the common use of phosphodiesterase inhibitors seems to link the inhibitory effect on neutrophils to increased cAMP concentrations (7), whereas the effects observed in the present study may involve alternative signal transduction mechanisms. In our work, we could verify that low nanomolar (1–10 but not 100 nM) concentrations of PGE_2 were able to significantly enhance the adhesion of CSE-treated neutrophils to airway epithelial cells, an effect that was mimicked by the EP_2-selective agonist Butaprost and blunted by the EP_4-selective antagonist AH-23848, suggesting that both subtypes could play a role in the observed effect of PGE_2. The potential activity of PGE_2 onto the TP receptor was ruled out through the use of a specific antagonist. Previous works reported about the activation of neutrophil by PGE_2 (34) or neutrophil-like HL-60 possibly through a cAMP-independent mechanism (42), suggesting that the complex activities of PGE_2 within the context of the inflammatory reaction need additional investigation, in particular with respect to the airways. Indeed, evidence of possible anti-inflammatory activities of PGE_2 in the lung have been made available throughout the years, and the bronchoconstriction observed on treatment with COX inhibitors in aspirin-sensitive asthmatics (35) is clearly pointing to a protective role of PGE_2 in these subjects (29). Interestingly, selective COX-2 inhibitors such as rofecoxib appeared to be well-tolerated in these patients (14, 31), suggesting that COX-1 may be responsible for the biosynthesis of bronchoprotective PGE_2.

In conclusion, we found increased PGE_2 concentrations in induced sputum from healthy and COPD smokers that, in COPD subjects, correlated with the number of infiltrating neutrophils. Based on this correlation, we were able to observe a previously undescribed effect of COX-2-derived PGE_2 within the airways, namely its ability to increase the adhesion of neutrophils to human airway epithelial cells. CSE were shown to induce both COX-2 and specific EP receptor subtypes in AM and/or neutrophils, suggesting that smoking may play a causal role also with respect to the observed effects of COX-2-derived PGE_2 within the airways of COPD subjects. Additional work is required to clarify the transduction mechanisms involved in the observed effect of PGE_2 on neutrophils.

GRANTS

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DISCLOSURES

No conflicts of interest are declared by the author(s).

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