Prostaglandin E_2 possesses different potencies in inducing Vascular Endothelial Growth Factor and Interleukin-8 production in COPD human lung fibroblasts

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Abbreviations:
COPD: Chronic Obstructive Pulmonary Disease; COX: Cyclooxygenase; EP: E-prostanoid receptors; VEGF:vascular endothelial growth factor; IL-8: Interleukin 8; PGE_2: prostaglandin E_2

Summary

We studied the role of PGE_2, its biosynthetic enzymes and its receptors, in regulating the functions of lung fibroblasts through the production of Vascular Endothelial Growth Factor (VEGF) and Interleukin-8 (IL-8) in COPD subjects.

Lung fibroblasts from Control (C) (n=6), Smoker (HS) (n=6) and COPD patients (n=8) were cultured, and basal PGE_2, VEGF, and IL-8 measured in supernatants by ELISA. COX-1/COX-2 and EP receptors expression were assessed by western blot and by RT-PCR. Release of VEGF and IL-8 by human fetal lung fibroblasts (HFL-1; lung, diploid, human) was evaluated under different conditions.

PGE_2, VEGF, and IL-8 levels, COX-2, EP2, and EP4 protein expression and mRNA were increased in COPD when compared to Controls. Low concentrations of synthetic PGE_2 increased the release of VEGF in HFL-1, but higher concentrations were needed to induce the release of IL-8. This effect was mimicked by an EP2 agonist and modulated by an EP4 antagonist.

In the airways of COPD subjects, fibroblast-derived PGE_2 may regulate angiogenesis and inflammation through the production of VEGF and IL-8 respectively, suggesting that the increase in expression of COX-2, EP2 and EP4 observed in COPD fibroblasts may contribute to steering the role of PGE_2 from homeostatic to pro-inflammatory.

Keywords

Prostaglandins; Immunology; Cyclooxygenase; Inflammation; Lung fibroblasts; Arachidonic acid; Prostaglandin E_2; Cyclooxygenase-2; EP receptors; Lung inflammation; Angiogenesis; COPD

1. Introduction

Prostaglandin E_2 (PGE_2), is a metabolite derived from arachidonic acid by the coordinated activity of cyclooxygenase and prostaglandin E synthase enzymes, and cigarette smoke is known to increase the expression of both cyclooxygenase-2 (COX-2) and prostaglandin E synthase in human fibroblasts [1]. PGE_2 has the potential to function as both an autocrine and a paracrine mediator in fibroblasts, affecting chemotactic recruitment [2][3], proliferation [4], matrix production [5,6], and matrix remodeling [7,8]. PGE_2 acts on four distinct G protein–coupled E-prostanoid (EP) receptors named EP1, EP2, EP3, and EP4, [9,10], and coupled to different transduction mechanisms such as the increase in intracellular cyclic adenosine monophosphate (cAMP, EP2 and EP4), the decrease in cAMP (EP3), and the increase in intracellular calcium (EP1) [11][12]. Through the interaction with these receptors, PGE_2 mediates a variety of physiologic responses [10,13,14].

Alterations in fibroblast functions could play a role in the pathogenesis of pulmonary emphysema during COPD, which is characterized by inadequate maintenance of tissue structure [15]. Fibroblasts are a major source of vascular endothelial growth factor (VEGF), which modulates pulmonary microvasculature promoting the angiogenesis by alveolar endothelial cells. Prostacyclin analogues stimulate the production of VEGF by human lung fibroblasts, an effect mediated by the I-prostanoid receptor acting through the cAMP/protein kinase–A (PKA) pathway [16]. Similarly, it has recently been observed that PGE_2 stimulates the production of VEGF through the activation of the Gs-coupled EP2 receptor in cultured human lung fibroblasts [4]. Furthermore, PGs are known to increase IL-8 release by fibroblasts [17], playing also a potentially important role in COPD airway chronic inflammation [18].

In this study, we evaluated the release of PGE_2, VEGF, and IL-8 as well as the expression of COX-2 and EP receptors in cultured fibroblasts obtained from COPD patients, smoker, and control subjects. Furthermore, since we observed a positive correlation between PGE_2 concentrations and VEGF or IL-8 in the supernatants of cultured fibroblasts obtained from COPD patients, we studied the effect of PGE_2 on VEGF and IL-8 production in human fibroblasts, in order to understand the potential contribution of PGE_2 to angiogenesis and inflammation within the airways of COPD subjects.

2. Materials and Methods

2.1. Study population

Three groups of subjects who underwent lung resection for a solitary peripheral lung cancer were recruited: Chronic obstructive pulmonary disease (COPD) (n=8), asymptomatic smokers with normal lung function (n=6) (HS), and asymptomatic non-smoking subjects with normal lung function (n=6) (C). COPD patients were defined accordingly to the GOLD guidelines [19] and were classified as stage I-II (Mild to Moderate COPD). Patients with COPD and HS subjects had a smoking history of 10 pack years or more. Four of the COPD patients are former smokers and had quit smoking for at least 2 yr before the lung resection. All patients were characterized with respect to sex, age, smoking history, COPD symptoms, co-morbidities and current 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 months prior to the study.

The study protocol was approved by the Ethic Committee (#217806-30/06/2008) and informed written consent was obtained from each patient.

2.2. Pulmonary function tests.

Pulmonary function tests were carried out according to the GOLD guidelines [19]. To assess the reversibility of the airway obstruction in subjects with a FEV1/FVC <70%, the FEV1 measurement was repeated 15 minutes after the inhalation of 200 μg of salbutamol.

2.3. Isolation and culture of lung fibroblasts from patients.

Tumor free material and nontraumatic tissue from surgical specimens were used. Human lung fibroblasts were isolated from surgical specimens of human bronchi as previously described [20]. To confirm the purity of the cultured fibroblasts, the recovered cells were identified by their morphology, adherent nature, expression of vimentin and types I and III collagen, and lack of expression of cytokeratin, α-smooth muscle actin, factor VIII

and CD45. Immunoreactivity for these markers was revealed using the LSAB method (Dako LSAB®, Glostrup, Denmark) following the manufacturer's instructions.

Purified lung fibroblasts from COPD, HS, and C subjects were grown in a humidified atmosphere containing 5% CO_2, and passaged by trypsinization at nearly confluence onto 100 mm culture plates as previously described [20]. Subsequently, after additional 24 hrs under FBS-free conditions (5% CO_2 at 37°C), supernatants of monolayer cultures were harvested and stored at -80°C until assayed for basal PGE_2, VEGF, and IL-8 levels. At the same time lung fibroblasts were detached and treated for protein and mRNA extraction. Only early passage cells (1-3) were used for each experiment to avoid problems that may occur in the higher number of cell passages and the biological analyses.

2.4. Measurement of PGE_2, VEGF, and IL-8.

PGE_2 levels were quantified by a commercial ELISA (GE Healthcare, Uppsala, Sweden) according to the manufacturer's protocol. The detection limit was 40 pg/ml. VEGF and IL-8 were quantified in the supernatants of monolayer cultures by commercial ELISA kits (R&D Systems Europe Ltd, Abington, UK). Sensitivity was 5.0 pg/ml and 3.5 pg/ml for VEGF and IL-8, respectively. Absorbance was measured using a Wallac 1420 Victor2 multilabel counter (Perkin-Elmer Life Sciences, Turku, Finland).

2.5. Western blot analyses of COX-1 and COX-2 proteins and EP receptors expression.

Total protein extracts from cultured lung fibroblasts of COPD, control smoker and control subjects were resuspended in 2x Laemmli buffer and separated by SDS-PAGE followed by electroblotting onto nitrocellulose membranes for COX-1, COX-2, and EP receptors protein expressions. The following antibodies were used: a mouse monoclonal antibody direct against human COX-1 or COX-2 (Cayman Chem, MI, Italy), a rabbit polyclonal antibody directed against the human EP1, EP2, EP3 or EP4 receptor (Cayman Chem, MI, Italy). Primary antisera were visualized with HRP-conjugated secondary antibody (Sigma St. Louis, MO) and developed with an enhanced chemiluminescence system (Amersham Life Sciences UK Limited). Approximate molecular masses were determined using calibrated pre-stained standards (Amersham Life Sciences UK Limited). Negative

controls were performed in the absence of primary antibody or including an isotype control antibody. β-actin (Sigma St. Louis, MO) was used as a housekeeping protein to normalize western blot analyses. Gel images were taken with an EPSON GT-6000 scanner and then imported to a National Institutes of Health Image analyses 1.61 program to determine band intensity. Data are expressed as arbitrary densitometric units corrected against the density of β-actin bands.

2.6. Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) of COX-2 and EP receptors.

Total RNA was extracted from patient fibroblasts with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions, and was reverse-transcribed into cDNA, using M-MLV-RT and oligo(dT)$_{12\text{-}18}$ primer (Invitrogen). Quantitative real-time PCR of the transcripts for COX-2 and for both EP2 and EP4 receptor subtypes of human PGE_2 receptors was carried out on Step One Plus Real-time PCR System (Applied Biosystems, Foster City, CA, USA) using specific FAM-labeled probe and primers (prevalidated TaqMan Gene expression assay for COX-2, Hs00153133m1, and for EP2, and EP4 receptors, Assays on Demand, Applied Biosystems). Gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control gene. Relative quantitation of gene expression level between C and HS or COPD subjects was evaluated with the comparative C_T method ($2^{-\Delta\Delta Ct}$). Relative expression levels were presented as the relative fold increase compared to the healthy subjects and calculated using the formula: $2^{-\Delta\Delta CT} = 2^{-(\Delta CT(HS \text{ or } COPD) - \Delta CT(C)}$, where each $\Delta CT = \Delta CT^{target} - \Delta CT^{GAPDH}$ [21].

2.7. HFL-1 culture.

Human fetal lung fibroblasts (HFL-1; lung, diploid, human) (from American Type Culture Collection, Manassas V.A.) were cultured in 100 mm tissue culture dishes (Falcon; Becton-Dickinson Labware, Lincoln Park, NJ) with RPMI1640 supplemented with 10% FCS, 2 mM glutamine, 25 mM HEPES buffer, 1% MEM non essential aminoacids, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2.5 μg/ml fungizone. Cells were refed

twice a week, subcultured weekly and maintained in 10% FCS RPMI1640. Fibroblasts between passages 15 and 20 were used for all experiments. The HFL-1 cell line was used in the present study because of difficulty in obtaining sufficient quantities of primary human lung airway fibroblasts for these experiments.

2.8. Stimulation of HFL-1 cells and PGE_2 depletion in supernatants from lung fibroblasts.

To evaluate the relationship between PGE_2 concentration and the release of VEGF and IL-8, HFL-1 cells (1.5×10^6/ml) were plated in 100 mm tissue culture dishes for 18 hrs in the presence or absence of exogenous synthetic PGE_2 at different concentrations (10^{-9} to 10^{-6} M, Sigma Aldrich, Milan, Italy). To test the contribution of different levels of paracrine PGE_2 present in cultured lung fibroblast supernatants on VEGF and IL-8 release, HFL-1 cells (1.5×10^6/ml) were stimulated for 18 hrs in the presence or absence of selected supernatants with low ($<10^{-9}$ M, n=3) or high ($>10^{-8}$ M, n=3) PGE_2 levels. Selected supernatants from lung fibroblasts were added diluted 1:2 with culture medium for HFL-1 stimulation.

To determine the specific effect of paracrine PGE_2 present in cultured lung fibroblast supernatant on VEGF and IL-8, selected samples showing high ($>10^{-8}$ M, n=3) PGE_2 concentrations were incubated in the presence or absence of PGE_2 affinity sorbent (mouse anti-PGE_2 IgG covalently bound to Sepharose 4B) (Cayman Chemical). The affinity sorbent was used to eliminate the PGE_2 from supernatants as previously described [22]. Finally, HFL-1 cells were stimulated for 18 hrs with selected supernatants from lung fibroblast depleted of PGE_2.

To evaluate the role of EP receptors, we stimulated HFL-1 cell line with Butaprost (Sigma, 10^{-6} M, EP2 agonist), Sulprostone (Sigma, 10^{-5} M, EP1/EP3 agonist)[23] or supernatants of lung fibroblasts from COPD in presence or absence of SC 19220 (Sigma, 10^{-5} M, EP1 antagonist) or AH23848 (Sigma, 10^{-5} M EP4 antagonist). Antagonists were added to the HFL-1 1 hr before the stimulation with supernatants of lung fibroblasts from COPD patients selected with a level of $PGE_2 > 10^{-8}$ M.

2.9. Statistical analysis.

Data are expressed as mean+SD. Statistical analysis for multiple comparison was carried out using Kruskal-Wallis and Mann-Whitney *U* test or non parametric ANOVA tests followed by Fisher's PLDS correction. A $p<0.05$ was considered as statistically significant.

3. **Results**

3.1. Demographic characteristic of the patients

The patients' characteristics are summarized in Table I. The differences in the median ages or smoking history of Controls, Smokers and subjects with COPD were not statistically significant. As expected, FEV1% after bronchodilators as well as FEV1%/FVC were significantly lower in COPD patients than HS ($p<0.001$) and C ($p < 0.001$).

3.2. PGE_2, VEGF, and IL-8 levels in human lung fibroblasts

The concentrations of PGE_2 in supernatants from lung fibroblasts of COPD patients were significantly higher than in supernatants obtained from C. HS showed higher PGE_2 levels compared to C, but no statistically significant difference was observed (Figure 1A).

VEGF concentration were higher in supernatants from lung fibroblasts of both HS and COPD when compared to C (Figure 1B), while only the concentrations of IL-8 in supernatants from lung fibroblasts of COPD subjects showed statistically significant difference from C (Figure 1C).

3.3. COX-1, COX-2, EP1, EP2, EP3 and EP4 protein expression and mRNA in human lung fibroblasts.

Western blot analyses of COX-1 and COX-2 proteins in cell lysates showed a significant increase in COX-2 expression by lung fibroblasts of Smokers and COPD subjects when compared to Controls (Figure 2A), while no differences were observed in COX-1 expression. The expression of EP2 and EP4 receptors was also significantly higher in the cell protein extracts from lung fibroblasts of COPD subjects compared to both HS and C (Figure 2B), while the expression of EP1 and EP3 receptor was not different among the three groups (Figure 2B). The changes in protein expression resulted associated with changes in transcription levels as confirmed by the analysis of COX-2, EP2 and EP4 mRNAs (Figure 3A-C).

3.4. VEGF and IL-8 release in stimulated HFL-1.

Synthetic PGE_2 (10^{-9} to 10^{-6} M, a range of concentrations likely to mimic the differences observed in the pulmonary microenvironment in the absence or presence of phlogosis and inflammatory cells [24]) dose-dependently increased the production of VEGF and IL-8 in stimulated HFL-1 (Figure 4A-B). Interestingly, the maximal release of IL-8 was observed at the highest concentration of PGE_2 tested (namely 10^{-6} M, Figure 4B), with the EC_{50} for this effect certainly higher than 10^{-7} M, while the maximal efficacy on VEGF release was already observed at the PGE_2 concentration of 10^{-7} M, with the concentration of 10^{-8} M representing the EC_{50} for this effect of PGE_2 (Figure 4A).

In order to assess if this differential effect could be observed also using biologically synthesized PGE_2 we used supernatants of lung fibroblasts presenting either low ($<10^{-9}$ M) or high ($>10^{-8}$ M) concentrations of PGE_2 to challenge HFL-1 cells. The results obtained showed that both COPD fibroblasts supernatants presenting low and high PGE_2 concentrations significantly increased the production of VEGF (Figure 5A-B top panel). The supernatants of COPD lung fibroblasts showing high levels of PGE_2 also increased the production of IL-8 in stimulated HFL-1 cells (Figure 5B bottom panel) while, according to the different potency observed using synthetic PGE_2, no increase was observed using supernatants with low PGE_2 concentrations (Figure 5A bottom panel). Finally, depleting the PGE_2 present in the supernatants of COPD lung fibroblasts significantly decreased their ability to induce the production and release of VEGF or IL-8 in HFL-1 stimulated with supernatants from lung fibroblasts showing high levels of PGE_2 (Figure 5C bottom panel).

3.5. EP receptors role in the production of VEGF and IL-8 by PGE_2

To confirm the role of EP receptors in the production of VEGF (1) and IL-8 generated by PGE_2 stimulation, we used specific agonists and antagonists for the different EP receptor subtypes. The specific EP2 receptor agonists Butaprost (10^{-6} M) significantly increased the production of VEGF as well as that of IL-8 in HFL-1, while the EP1/EP3 receptor agonist Sulprostone (10^{-5} M) did not affect the release of either VEGF or IL-8 in HFL-1 (Figure 6A). Interestingly, the preincubation of HFL-1 with the specific EP4 receptor antagonist AH-

23848 (10^{-5} M) caused a modest, but significant, reduction of the production of VEGF in HFL-1 stimulated with supernatants from COPD fibroblasts compared to baseline conditions, while the use of EP1 receptor antagonist SC-19220 (10^{-5} M) was ineffective (Figure 6B).

4. Discussion and Conclusions

The results of this study provide evidence that lung fibroblasts from COPD patients show increased expression of COX-2, EP2, and EP4 receptors, and are able to affect their own production of VEGF and IL-8 through the synthesis of different concentrations of PGE_2. Indeed, in an autocrine/paracrine fashion, nanomolar levels of PGE_2 are able to increase the production of VEGF, but over 20 folds higher concentrations are needed to also affect IL-8 release. Both effects appear to be associated to the activation of EP2, and possibly EP4, receptors on human fibroblasts. In light of these activities, we suggest that COX-2 expression/PGE_2 synthesis together with enhanced expression of EP2/EP4 receptors might represent a switch factor from modulation/maintenance of pulmonary microvasculature to inflammation in COPD airways. Important pharmacological implications of these findings are associated to cyclooxygenases inhibition by NSAIDs.

COPD is a disease state characterized by airflow limitation not fully reversible by bronchodilators. The airflow limitation is usually progressive and is associated to an abnormal inflammatory response of the lungs to noxious particles or gases, primarily cigarette smoking [25]. Pulmonary fibroblasts confer structural support to the lung connective tissue and play a role in stimulating and amplifying inflammatory signals through the expression of COX-2 and of microsomal prostaglandin E2 synthase in response to cigarette smoke [1]. Cigarette smoke, the major risk factor of COPD, also promotes the induction of COX-2 and PGE_2 receptor expression in neutrophils and alveolar macrophages (AM), therefore contributing to the proinflammatory effects of PGE_2 in the airways of COPD subjects [24,26]; indeed a significant overlap with minor, but statistically significant, differences was observed between HS and COPD subjects in terms of COX-2 and PGE_2, but clearcut increases, in agreement with previously published data [27], were observed in COPD subjects only for EP2 and EP4 expressions, both as mRNA and protein, suggesting that the increase in PGE_2-dependent IL-8 formation may be the result of concomitant higher PGE2 concentration and enhanced receptor expression when compared to C.,

Fibroblasts are the major mesenchymal cells present within the interstitium of the lung and have been shown to be a major source of VEGF [4]. VEGF in the lung is required to maintain endothelial cell survival of pulmonary capillaries and therefore a normal alveolar wall [28], but VEGF is also an extremely potent pro-angiogenic factor,

and relatively small changes in its concentrations may promote pathological blood vessel expansion. Nevertheless the concentrations of VEGF, while higher than C, were not different in supernatants from HS and COPD subjects leading to hypothesis that the observed increase may represent the homeostatic response to smoking-induced hypoxia, leaving the IL-8 as a marker of the crossing into the COPD chronic inflammatory state. We certainly cannot exclude that VEGF may also participate in the inflammatory and fibrotic response observed in COPD, but, as recently reported [29], a synergistic effect can be hypothesized to occur when both VEGF and IL-8 are on-board, while the role of VEGF as an homeostatic response factor may prevail in HS.

A relevant number of *ex vivo* and *in vitro* studies have shown PGE_2 to be important in airway inflammation and remodeling: PGE_2 can modulate extracellular matrix deposition and inflammatory cytokine release, such as IL-6 and IL-8, in primary human airway smooth muscle cells and fibroblasts [17]. Indeed, we confirmed that synthetic PGE_2 also stimulates human fibroblasts to release IL-8, but we found that higher concentrations were necessary when compared to that inducing the release of VEGF. These results were confirmed using supernatants from COPD lung fibroblasts containing lower ($<10^{-9}$ M) and higher ($>10^{-8}$ M) PGE_2 concentrations, to stimulate the production of VEGF and IL-8 in HFL-1. Indeed, while both groups of supernatants were able to generate consistent concentrations of VEGF in stimulated HFL-1 cells, only supernatants with higher concentrations of PGE_2 were capable of increasing the release of IL-8.

It must be noted that the final concentrations of VEGF and IL-8 observed using the COPD fibroblast supernatants to challenge the HFL-1 cells represents the sum of the amounts synthesized by HFL-1 cells and the amounts already present in the supernatant; nevertheless the concentrations observed using the fibroblast supernatant resulted higher (by approximately 25% for VEGF and 40% for IL-8) than those observed challenging HFL-1 cells with synthetic PGE_2. We certainly cannot exclude the presence in the supernatant of factor(s) that may potentiate the activity of PGE_2 or further contribute to stimulate the formation of VEGF and IL-8, and this may be particularly relevant for the latter, given that the depletion of PGE_2 from the supernatant only inhibited the increase in IL-8 formation by approximately 50%, even if the high variability observed in this measurement may limit the relevance of these considerations.

Although we cannot rule out the contribution of other eicosanoids (including lipoxygenase-derived autacoids [30]), taken together, the findings of our research work suggest and support a role of PGE_2 as mediator capable of switching the activity of fibroblast from homeostatic maintenance of alveolar endothelium through the production of VEGF to an inflammatory cytokine-releasing phenotype supporting the synthesis of IL-8.

HFL-1-cells are widely used normal human fetal lung fibroblasts known to express all four EP receptors [3]. Nevertheless, through the use of selective agonists and antagonist we confirmed the involvement of EP2 receptor, and possibly EP4, in the production of VEGF and IL-8 production from lung fibroblasts stimulated with PGE_2. These results provide additional support to the evidence that the increase of cAMP and, possibly, the activation of PKA, are involved in the activation of fibroblasts to release both VEGF and IL-8.

Previously published evidence showed the increased expression of EP2 and EP4 receptors in COPD [27], as well as the ability of cigarette smoke to induce VEGF release from fibroblasts [31] or the positive correlation between PGE_2 and VEGF [4], but with the present work we provide evidence that different levels of PGE_2 production, as a result of COX-2 expression, can differentiate between the homeostatic pro-angiogenic or the pro-inflammatory role of this prostanoid, underlining its critical role in normal physiology as well as in COPD pathophysiology.

In conclusion we found that PGE_2 biosynthesis and activity are enhanced in lung fibroblasts from COPD patients, as a result of an increase in COX-2, EP2 and, possibly, EP4 expression. PGE_2 appeared to differentially affect the production of VEGF and of IL-8, with higher concentrations needed to steer fibroblasts toward the production of inflammatory cytokines during airway inflammation in COPD patients. As a result, while a basal production of PGE_2 may have an homeostatic role driving the production of VEGF, the increased expression of COX-2 and of EP receptors may turn PGE_2 into a pro inflammatory factor in COPD subjects. Significant inhibition of PGE_2 production by NSAIDs may therefore result beneficial in reducing PGE_2 concentrations to levels observed in basal conditions, reducing IL-8 production without affecting VEGF levels.

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References

[1] C.A. Martey, S.J. Pollock, C.K. Turner, K.M.A. O'Reilly, C.J. Baglole, R.P. Phipps, et al., Cigarette smoke induces cyclooxygenase-2 and microsomal prostaglandin E2 synthase in human lung fibroblasts: implications for lung inflammation and cancer., Am. J. Physiol. Lung Cell. Mol. Physiol. 287 (2004) L981–91. doi:10.1152/ajplung.00239.2003.

[2] T. Kohyama, R.F. Ertl, V. Valenti, J. Spurzem, M. Kawamoto, Y. Nakamura, et al., Prostaglandin E(2) inhibits fibroblast chemotaxis., Am. J. Physiol. Lung Cell. Mol. Physiol. 281 (2001) L1257–L1263.

[3] Y.J. Li, X.Q. Wang, T. Sato, N. Kanaji, M. Nakanishi, M. Kim, et al., Prostaglandin E2 inhibits human lung fibroblast chemotaxis through disparate actions on different E-prostanoid receptors, Am. J. Respir. Cell Mol. Biol. 44 (2010) 99–107. doi:10.1165/rcmb.2009-0163OC.

[4] M. Nakanishi, T. Sato, Y. Li, A.J. Nelson, M. Farid, J. Michalski, et al., Prostaglandin E2 stimulates the production of vascular endothelial growth factor through the E-prostanoid-2 receptor in cultured human lung fibroblasts, Am J Respir Cell Mol Biol. 46 (2012) 217–223. doi:46/2/217 [pii]10.1165/rcmb.2010-0115OC.

[5] M. Kawamoto, D.J. Romberger, Y. Nakamura, Y. Adachi, L. Tate, R.F. Ertl, et al., Modulation of fibroblast type I collagen and fibronectin production by bovine bronchial epithelial cells, Am J Respir Cell Mol Biol. 12 (1995) 425–433. doi:10.1165/ajrcmb.12.4.7695922.

[6] L.E. Saltzman, J. Moss, R.A. Berg, B. Hom, R.G. Crystal, Modulation of collagen production by fibroblasts. Effects of chronic exposure to agonists that increase intracellular cyclic AMP., Biochem. J. 204 (1982) 25–30.

[7] T. Mio, X.D. Liu, Y. Adachi, I. Striz, C.M. Skold, D.J. Romberger, et al., Human bronchial epithelial cells modulate collagen gel contraction by fibroblasts, Am J Physiol. 274 (1998) L119–26. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids

=9458809.

[8] S. Huang, S.H. Wettlaufer, C. Hogaboam, D.M. Aronoff, M. Peters-Golden, Prostaglandin E(2) inhibits collagen expression and proliferation in patient-derived normal lung fibroblasts via E prostanoid 2 receptor and cAMP signaling., Am. J. Physiol. Lung Cell. Mol. Physiol. 292 (2007) L405–L413. doi:10.1152/ajplung.00232.2006.

[9] Y. Sugimoto, S. Narumiya, Prostaglandin E receptors, J. Biol. Chem. 282 (2007) 11613–11617. doi:10.1074/jbc.R600038200.

[10] S. Narumiya, Y. Sugimoto, F. Ushikubi, Prostanoid receptors: structures, properties, and functions., Physiol. Rev. 79 (1999) 1193–1226. doi:10.1016/0928-4680(94)90096-5.

[11] R.M. Breyer, C.K. Bagdassarian, S.A. Myers, M.D. Breyer, Prostanoid receptors: subtypes and signaling., Annu. Rev. Pharmacol. Toxicol. 41 (2001) 661–690. doi:10.1146/annurev.pharmtox.41.1.661.

[12] C.L. Bos, D.J. Richel, T. Ritsema, M.P. Peppelenbosch, H.H. Versteeg, Prostanoids and prostanoid receptors in signal transduction, Int. J. Biochem. Cell Biol. 36 (2004) 1187–1205. doi:10.1016/j.biocel.2003.08.006.

[13] T. Sanchez, J.J. Moreno, Role of EP(1) and EP(4) PGE(2) subtype receptors in serum-induced 3T6 fibroblast cycle progression and proliferation., Am. J. Physiol. Cell Physiol. 282 (2002) C280–C288. doi:10.1152/ajpcell.00128.2001.

[14] A.N. Hata, R.M. Breyer, Pharmacology and signaling of prostaglandin receptors: Multiple roles in inflammation and immune modulation, Pharmacol. Ther. 103 (2004) 147–166. doi:10.1016/j.pharmthera.2004.06.003.

[15] P.K. Jeffery, Structural and inflammatory changes in COPD: a comparison with asthma., Thorax. 53 (1998) 129–36.

http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1758710&tool=pmcentrez&rendertype=abstract (accessed November 12, 2014).

[16] K. Kamio, T. Sato, X. Liu, H. Sugiura, S. Togo, T. Kobayashi, et al., Prostacyclin analogs stimulate VEGF production from human lung fibroblasts in culture., Am. J. Physiol. Lung Cell. Mol. Physiol. 294 (2008) L1226–L1232. doi:10.1152/ajplung.00129.2007.

[17] D. Van Ly, J.K. Burgess, T.G. Brock, T.H. Lee, J.L. Black, B.G.G. Oliver, Prostaglandins but not leukotrienes alter extracellular matrix protein deposition and cytokine release in primary human airway smooth muscle cells and fibroblasts., Am. J. Physiol. Lung Cell. Mol. Physiol. 303 (2012) L239–50. doi:10.1152/ajplung.00097.2012.

[18] K.F. Chung, Cytokines as targets in chronic obstructive pulmonary disease, Curr Drug Targets. 7 (2006) 675–681. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16787167 (accessed November 11, 2014).

[19] R.A. Pauwels, A.S. Buist, P.M. Calverley, C.R. Jenkins, S.S. Hurd, Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease. NHLBI/WHO Global Initiative for Chronic Obstructive Lung Disease (GOLD) Workshop summary., Am. J. Respir. Crit. Care Med. 163 (2001) 1256–76. doi:10.1164/ajrccm.163.5.2101039.

[20] M. Profita, A. Bonanno, L. Siena, A. Bruno, M. Ferraro, A.M. Montalbano, et al., Smoke, choline acetyltransferase, muscarinic receptors, and fibroblast proliferation in chronic obstructive pulmonary disease., J. Pharmacol. Exp. Ther. 329 (2009) 753–63. doi:10.1124/jpet.108.145888.

[21] T.D. Schmittgen, K.J. Livak, Analyzing real-time PCR data by the comparative C(T) method., Nat. Protoc. 3 (2008) 1101–8. http://www.ncbi.nlm.nih.gov/pubmed/18546601 (accessed September 29, 2014).

[22] M. Profita, A. Sala, A. Bonanno, L. Riccobono, L. Siena, M.R. Melis, et al., Increased prostaglandin E2 concentrations and cyclooxygenase-2 expression in asthmatic subjects with sputum eosinophilia, J. Allergy Clin. Immunol. 112 (2003) 709–716. doi:10.1016/S0091-6749(03)01889-X.

[23] G. Tamma, The prostaglandin E2 analogue sulprostone antagonizes vasopressin-induced antidiuresis through activation of Rho, J. Cell Sci. 116 (2003) 3285–3294. doi:10.1242/jcs.00640.

[24] M. Profita, A. Sala, A. Bonanno, L. Riccobono, M. Ferraro, S. La Grutta, et al., Chronic obstructive pulmonary disease and neutrophil infiltration: role of cigarette smoke and cyclooxygenase products., Am. J. Physiol. Lung Cell. Mol. Physiol. 298 (2010) L261–L269. doi:10.1152/ajplung.90593.2008.

[25] J.D. Taylor, COPD and the response of the lung to tobacco smoke exposure, Pulm. Pharmacol. Ther. 23 (2010) 376–383. doi:10.1016/j.pupt.2010.04.003.

[26] C.-C. Lin, I.-T. Lee, Y.-L. Yang, C.-W. Lee, Y.R. Kou, C.-M. Yang, Induction of COX-2/PGE(2)/IL-6 is crucial for cigarette smoke extract-induced airway inflammation: Role of TLR4-dependent NADPH oxidase activation., Free Radic. Biol. Med. 48 (2010) 240–54. doi:10.1016/j.freeradbiomed.2009.10.047.

[27] S. Togo, O. Holz, X. Liu, H. Sugiura, K. Kamio, X. Wang, et al., Lung fibroblast repair functions in patients with chronic obstructive pulmonary disease are altered by multiple mechanisms., Am. J. Respir. Crit. Care Med. 178 (2008) 248–60. doi:10.1164/rccm.200706-929OC.

[28] Y. Kasahara, R.M. Tuder, L. Taraseviciene-Stewart, T.D. Le Cras, S. Abman, P.K. Hirth, et al., Inhibition of VEGF receptors causes lung cell apoptosis and emphysema, J. Clin. Invest. 106 (2000) 1311–1319. doi:10.1172/JCI10259.

[29] P. DelNero, M. Lane, S.S. Verbridge, B. Kwee, P. Kermani, B. Hempstead, et al., 3D culture broadly regulates tumor cell hypoxia response and angiogenesis via pro-inflammatory pathways, Biomaterials. 55 (2015) 110–118. doi:10.1016/j.biomaterials.2015.03.035.

[30] S.K. Huang, M. Peters-Golden, Eicosanoid lipid mediators in fibrotic lung diseases: Ready for prime time?, Chest. 133 (2008) 1442–1450. doi:10.1378/chest.08-0306.

[31] G. Volpi, F. Facchinetti, N. Moretto, M. Civelli, R. Patacchini, Cigarette smoke and α,β-unsaturated aldehydes elicit VEGF release through the p38 MAPK pathway in human airway smooth muscle cells and lung fibroblasts., Br. J. Pharmacol. 163 (2011) 649–61. doi:10.1111/j.1476-5381.2011.01253.x.

Tables

Table I: Demographic characteristics of patients

	Control n=6	Smoker n=6	COPD n=8	Overall p value
Sex, male/female	4/2	4/2	6/2	
Age, yr	64.5 ± 5.8	69.4 ± 7.5	66.3 ± 13.2	N.S.
FEV1, % predicted	100 ± 9.4	93.3 ± 8.2	72.8 ± 17.5	$p < 0.001$
FEV1/FVC, %	95.3 ± 4.9	92.4 ± 7.4	65.9 ± 3.5	$p < 0.001$
Smoking, pack/yr	0	54.3 ± 26.2	65.7 ± 21.8	$p < 0.001$

Data are shown as mean±S.D. Abbreviations: FEV1 = forced expiratory volume in 1 s; FVC = forced vital capacity. Statistical analysis for multiple comparisons was performed by Kruskal-Wallis test

Captions to Illustrations

Figure 1: PGE_2 (Panel A), VEGF (Panel B), and IL-8 (Panel C) concentrations in supernatants of cultured lung fibroblasts from control subjects (n=6), smoker (n=6) and COPD patients (n=8). Box plot represents median and IQ range. Statistical analysis was performed by ANOVA and Fisher's PLSD correction.

Figure 2: COX-1, COX-2, EP1, EP2, EP3, and EP4 protein expression in cultured lung fibroblasts from control subjects (n=6), smoker (n=6) and COPD patients (n=8). The results were expresses in arbitrary densitometric units (ADU) corrected against the density of β-actin bands. Panel A: COX-1 (top panel) and COX-2 (middle panel) and representative gel images of COX-1 and COX-2 (bottom panel). Panel B: EP1 and EP2 (top panels), EP3 and EP4 (middle panels) and representative gel images of EP1, EP2, EP3, and EP4 (bottom panel). Statistical analysis was performed by ANOVA and Fisher's PLSD correction.

Figure 3: COX-2, EP2, EP4 gene expression in cultured lung fibroblasts from control subjects (n=3), smoker (n=5) and COPD patients (n=6). Data are expressed as fold increase *versus* the control group that was chosen as the reference group (see Materials and Methods for details). Statistical analysis was performed by Kruskal-Wallis test and Mann-Whitney *U* test.

Figure 4: PGE_2 stimulates VEGF and IL-8 release in human fetal lung fibroblasts (HFL-1) in a dose-dependent manner. Cultured HFL-1 were stimulated with different concentrations of PGE_2 (10^{-9} M to 10^{-6} M) for 18 hrs. At the end of stimulation, cell supernatants were collected for VEGF (Panel A) and IL-8 (Panel B) analysis by ELISA (See Methods for details). The results were expressed as mean±SD from four independent experiments. Statistical analysis was performed by ANOVA and Fisher's PLSD correction. *$p<0.05$, **$p<0.01$, and ***$p<0.001$ vs basal;.

Figure 5: VEGF and IL-8 production from human fetal lung fibroblasts (HFL-1) stimulated with supernatants of lung fibroblasts with low PGE_2 level ($<10^{-9}$ M, n=3), and with high PGE_2 level ($>10^{-8}$ M, n=3), (see Methods for details). Values are expressed as mean±SD Panel A: VEGF (pg/ml) (top) and IL-8 (pg/ml) (bottom) production after stimulation with and without supernatants of lung fibroblasts with low PGE_2 level. Panel B: VEGF (pg/ml)

(top) and IL-8 (pg/ml) (bottom) production after stimulation with and without supernatants of lung fibroblasts with high PGE_2 level and with supernatants depleted from PGE_2. Statistical analysis was performed by ANOVA and Fisher's PLSD correction.

Figure 6: Effect of specific EP receptors agonists and antagonists on VEGF and IL-8 release from human fetal lung fibroblasts (HFL-1). HFL-1 cells were stimulated with the indicated agonists or with the supernatants of lung fibroblasts from COPD (see Methods for details). Values are expressed as mean±SD. Production of VEGF (Panel A) and IL-8 (Panel B) in the presence or absence of Butaprost (EP2 agonist) or Sulprostone (EP3 agonist). Panel C: Production of VEGF after stimulation with supernatants of lung fibroblasts from COPD in presence or absence of SC-19220 (EP1 antagonist) or AH-23848 (EP4 antagonist). Statistical analysis was performed by ANOVA and Fisher's PLSD correction.

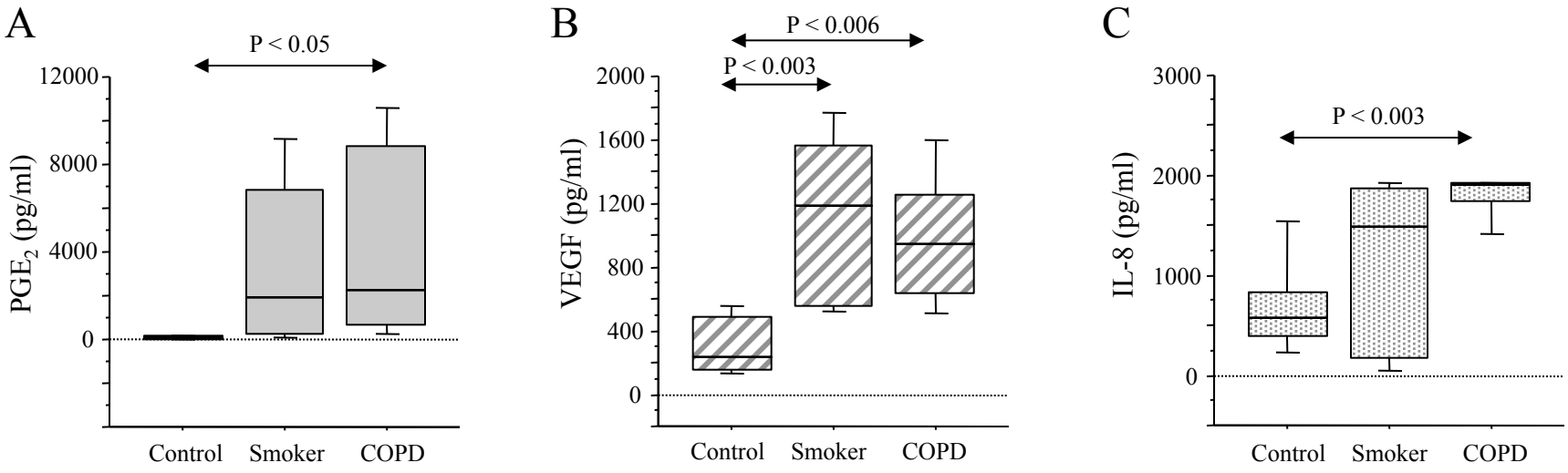
A
P < 0.05
12000
8000
4000
0
PGE2 (pg/ml)
Control
Smoker
COPD
B
P < 0.006
P < 0.003
2000
1600
1200
800
400
0
VEGF (pg/ml)
Control
Smoker
COPD
C
P < 0.003
3000
2000
1000
0
IL-8 (pg/ml)
Control
Smoker
COPD

Figure 1

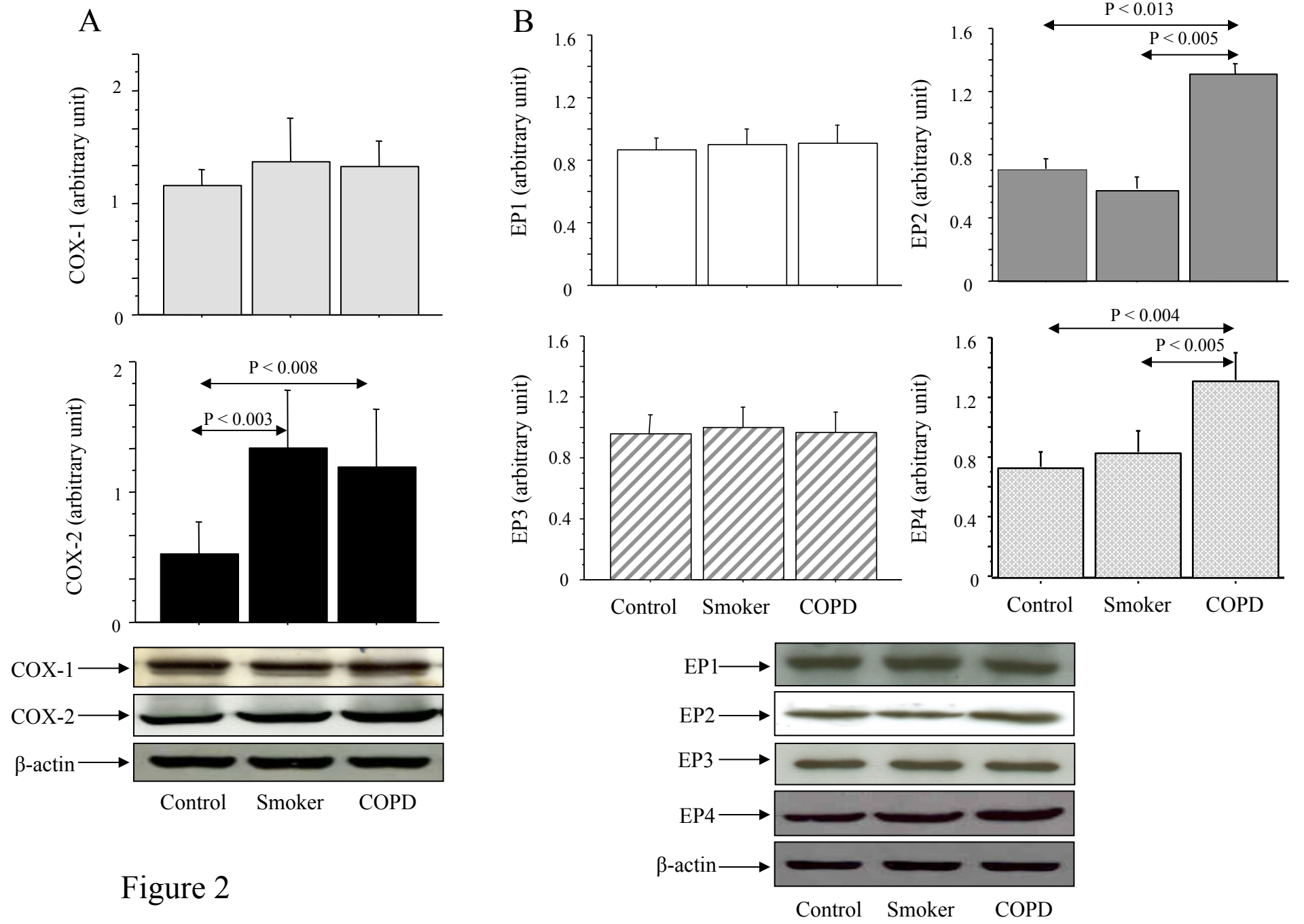
A
COX-1 (arbitrary unit)
2
1
0
COX-2 (arbitrary unit)
P < 0.008
P < 0.003
COX-1
COX-2
β-actin
Control
Smoker
COPD
B
EP1 (arbitrary unit)
1.6
1.2
0.8
0.4
0
EP2 (arbitrary unit)
P < 0.013
P < 0.005
EP3 (arbitrary unit)
EP4 (arbitrary unit)
P < 0.004
P < 0.005
Control
Smoker
COPD
EP1
EP2
EP3
EP4
β-actin
Control
Smoker
COPD

Figure 2

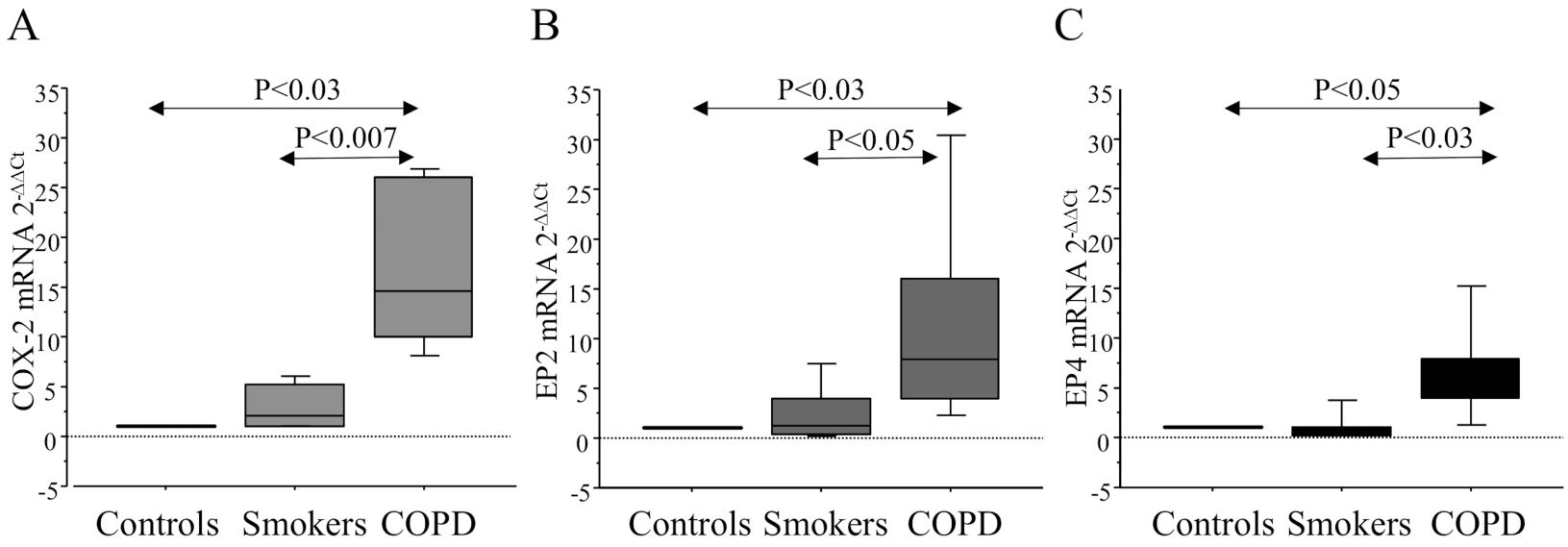
A
B
C
P<0.03
P<0.007
P<0.03
P<0.05
P<0.05
P<0.03
COX-2 mRNA 2-ΔΔCt
EP2 mRNA 2-ΔΔCt
EP4 mRNA 2-ΔΔCt
35
30
25
20
15
10
5
0
-5
Controls
Smokers
COPD

Figure 3

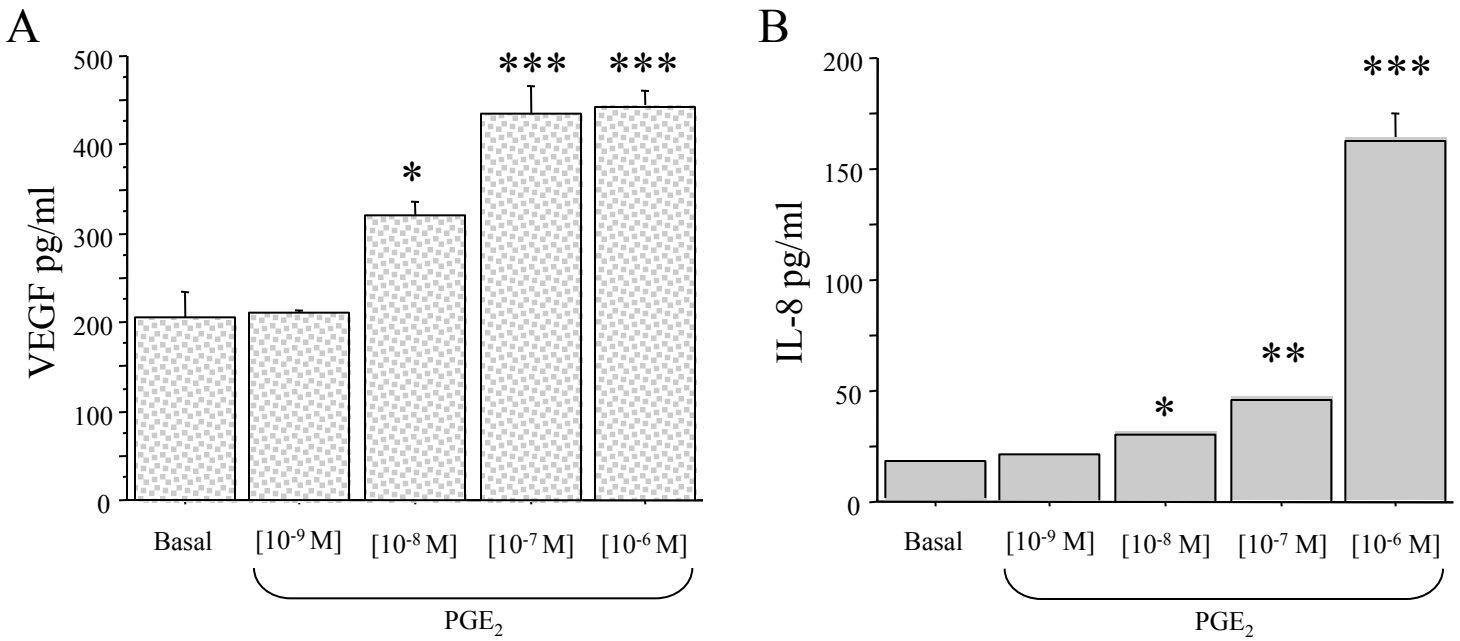
A
VEGF pg/ml
500
400
300
200
100
0
*
***
***
Basal
[10-9 M]
[10-8 M]
[10-7 M]
[10-6 M]
PGE2
B
IL-8 pg/ml
200
150
100
50
0
*
**
***
Basal
[10-9 M]
[10-8 M]
[10-7 M]
[10-6 M]
PGE2

Figure 4

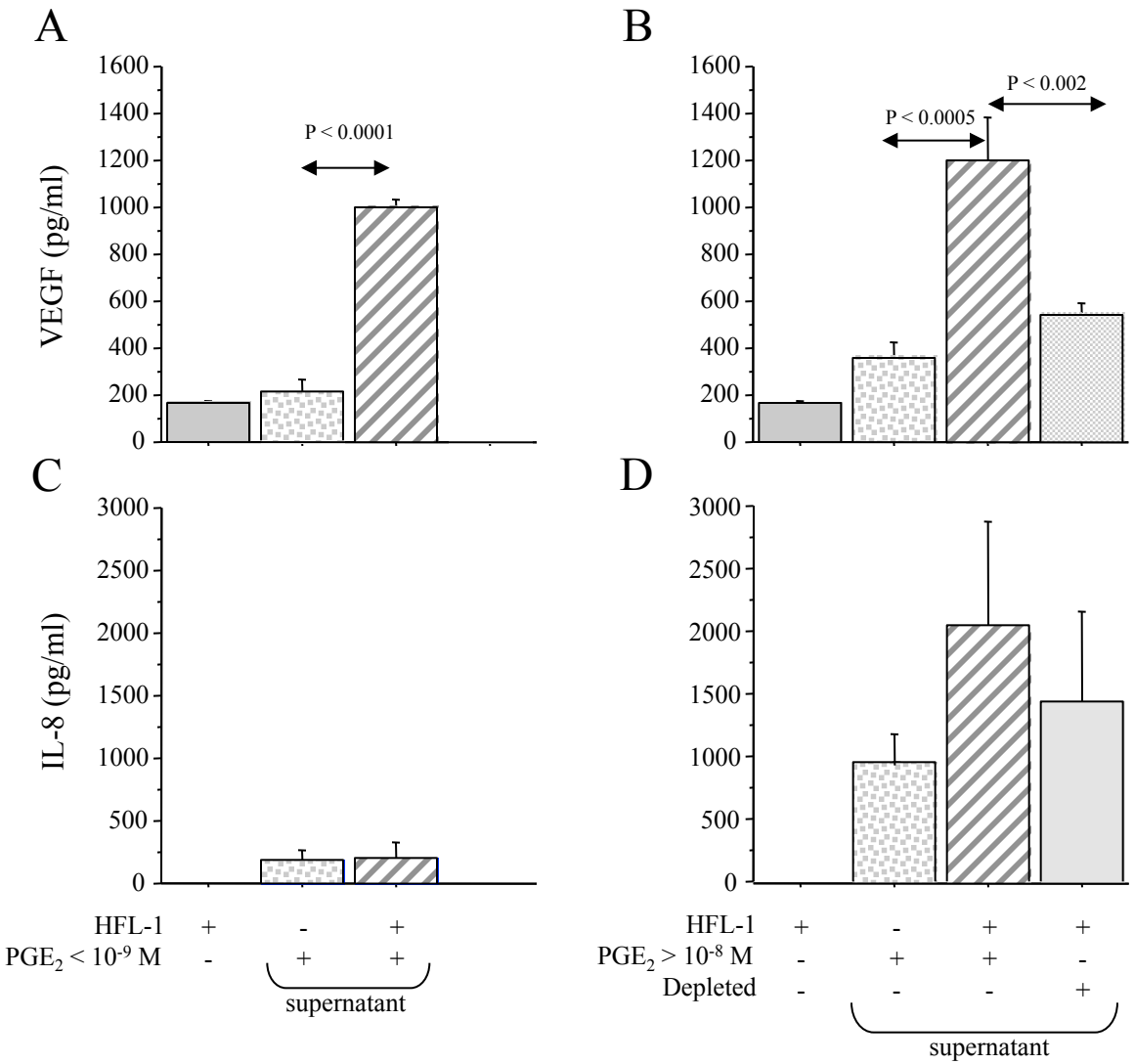
A
B
C
D
VEGF (pg/ml)
IL-8 (pg/ml)
P < 0.0001
P < 0.0005
P < 0.002
HFL-1
PGE2 < 10-9 M
PGE2 > 10-8 M
Depleted
supernatant

Figure 5

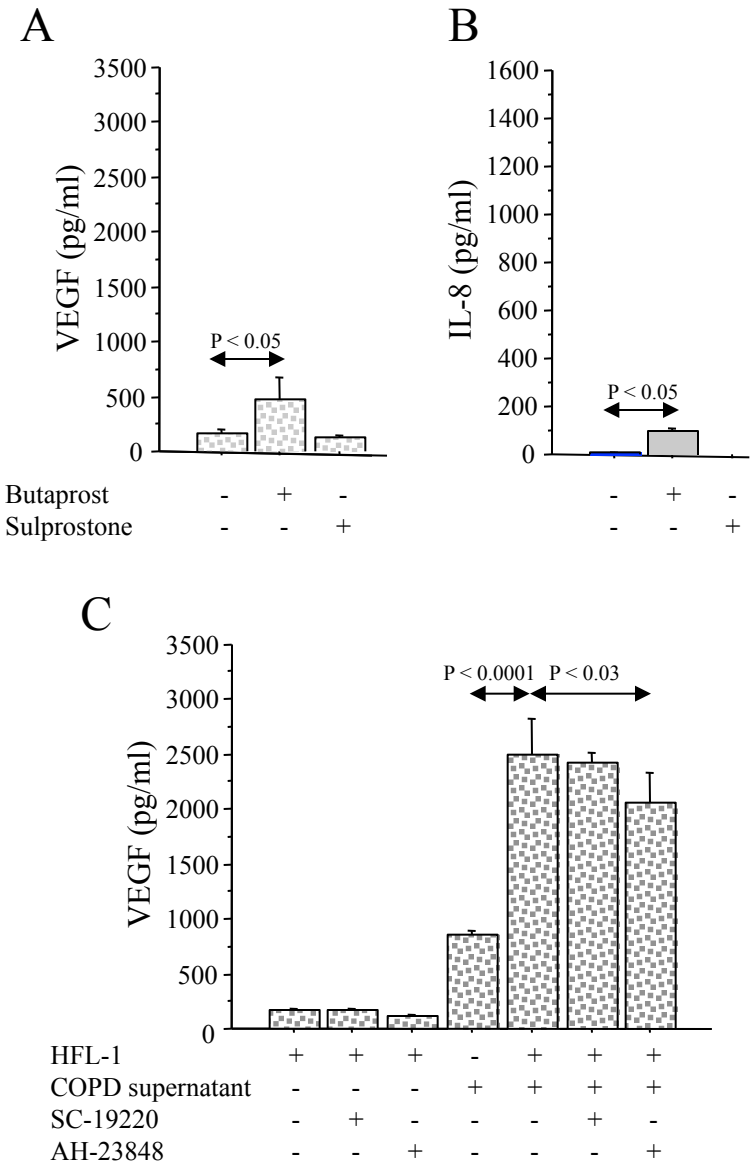
A
B
C
VEGF (pg/ml)
IL-8 (pg/ml)
P < 0.05
P < 0.0001
P < 0.03
Butaprost
Sulprostone
HFL-1
COPD supernatant
SC-19220
AH-23848

Figure 6