ORIGINAL ARTICLE

Expression of vascular remodelling markers in relation to bradykinin receptors in asthma and COPD

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ABSTRACT

Background Vascular remodelling plays a central role in asthma and chronic obstructive pulmonary disease (COPD). Bradykinin (BK) is a vasoactive proinflammatory peptide mediating acute responses in asthma. We investigated the role of angiogenic factors in relation to BK receptors in asthma and COPD.

Methods Bronchial biopsies from 33 patients with COPD, 24 old (≥50 years) patients with (≥50 years) asthma, 18 old control smokers, 11 old control non-smokers, 15 young (≤40yrs) patients with (≤40 years) asthma and 10 young control non-smokers were immunostained for CD31, vascular endothelial growth factor-A (VEGF-A), angiogenin and BK receptors (B2R and B1R). Fibroblast and endothelial co-localisation of relevant molecules were performed by immunofluorescence. BK-induced VEGF-A and angiogenin release was studied (ELISA) in bronchial fibroblasts from subjects with asthma and COPD.

Results In bronchial lamina propria of old patients with asthma, CD31 and VEGF-A$^+$ cell numbers were higher than old control non-smokers ($p<0.05$). Angiogenin$^+$, B2R$^+$ and B1R$^+$ cell numbers in old patients with asthma were higher than in old control non-smokers, control smokers and patients with COPD ($p<0.01$). Angiogenin$^+$ cell numbers were higher in patients with COPD than both old control groups ($p<0.05$). In all patients with asthma the number of B2R$^+$ cells was positively related to the numbers of B1R$^+$ ($r_s=0.43$), angiogenin$^+$ ($r_s=0.42$) and CD31 cells ($r_s=0.46$) ($p<0.01$). Angiogenin$^+$ cell numbers were negatively related to forced expiratory volume in 1 s ($r_s=-0.415$, $p=0.008$). Double immunofluorescence revealed that CD31 cells of capillary vessels coexpressed B2R and that fibroblasts coexpressed B2R, VEGF-A and angiogenin. BK (10^{-6}M) induced significant angiogenin release in fibroblasts from asthma and to a lesser extent in COPD.

Conclusions Unlike COPD, this study suggests the involvement of BK receptors in bronchial vascular remodelling in asthma.

Key messages

What is the key question?
- Are bradykinin receptors in relation to vascular remodelling in asthma and chronic obstructive pulmonary disease?

What is the bottom line?
- Bradykinin receptors are overexpressed in the bronchial wall of old patients with asthma in conjunction with an increased expression and fibroblast-derived release of vascular growth factors.

Why read on?
- The reader will figure out the bradykinin-associated mechanisms of increased vascularity and of overexpressed vascular growth factors in old asthma.

INTRODUCTION

Asthma and chronic obstructive pulmonary disease (COPD) are chronic inflammatory and obstructive respiratory diseases associated with structural alterations in the airways.[1] Angiogenesis has been recognised as a fundamental event in the development of airway remodelling that leads to irreversible airway obstruction.[2 3]

Several proteins are potentially involved in angiogenesis, like vascular endothelial growth factor-A (VEGF-A), angiogenin and bradykinin (BK). VEGF-A is a potent multifunctional cytokine that has several effects on angiogenesis and is associated with asthma and COPD.[2 4] VEGF-A stimulates endothelial cell migration and proliferation and it is widely expressed in highly vascularised organs, including the lung.[5] VEGF-A is modulated by many factors including nitric oxide and fibroblast growth factor.[2 6] Some studies have shown elevated levels of VEGF-A in bronchial biopsies, induced sputum and bronchoalveolar lavage fluid (BALF) from patients with asthma, and these correlated with increased total airway vascular area [7–9] and smaller airway calibre.[7 9] Furthermore, VEGF-A expression is increased in small airways of COPD.[2] In addition, all VEGF isoforms showed decreased expression with age in the anterior cruciate ligament of rabbits.[10]

Angiogenin is a member of the ribonuclease superfamily, normally present in the blood circulation.[11] It has been implicated as mitogen for vascular endothelial cells, immune modulator, activator of certain protease cascades, as well as adhesion molecule.[11] Like VEGF-A, it induces vascular endothelial cell proliferation, migration and tubule formation,[12] and its levels are increased in asthma.[7 13]

BK and the related peptide kallidin are formed from high and low molecular weight kininogen precursors following the activation of plasma and tissue kallikreins by pathophysiological stimuli leading to tissue inflammation and damage.[14]

The biological actions of kinins are mediated via interaction with B2 receptors (B2R) and B1 receptors (B1R). The B2R is constitutively expressed on most cell types and its activation leads to various intracellular events, including nitric oxide release.[15] By contrast, B1Rs can be induced during inflammatory insults.[14] BK induces acute inflammatory airway responses, including plasma protein extravasation/vasodilation and smooth muscle contraction,[16] leading to bronchoconstriction.[14] Increased levels of BK have been reported in BALF from patients with asthma,[14] and it upregulates the production of VEGF-A in vitro either in human airway smooth muscle cells[17] or in bronchial epithelial cells.[18] Despite BK, upon stimulation of its receptors, showed a vasoactive role[19] and vascular growth effect,[18] no reports have been published describing possible associations between BK and angiogenesis in asthma and COPD. No data are available for BK B1 and B2 receptors expression in the bronchial mucosa of patients with asthma and COPD. We investigated the expression of BK B1 and B2 receptors in bronchial biopsies of patients with asthma and COPD and their association with angiogenic factors and we examined the capability of BK to release VEGF-A or angiogenin from asthmatic and COPD bronchial fibroblasts.

METHODS

Study design

This cross-sectional study has been primarily designed in order to compare COPD (≥50 years old), asthma and control groups comparable for age. Since an age-dependent expression of VEGF isoforms and receptors in rabbits has been demonstrated,[10] we decided to also include in the present study young patients with asthma (YA) and young controls (≤40 years old) in order to evaluate potential differences related to age.

Subjects

We examined bronchial biopsies from 111 subjects by immunohistochemistry (IHC) and confocal analysis: 24 non-smoking (2 ex-smokers) old patients with asthma (OA; age≥50 years); 15 non-smoking young patients with asthma (YA; age≤40 years); 33 smoking (9 ex-smokers) patients with COPD (age≥50 years), 18 old control smokers (2 ex-smokers) (OCS; age≥50 years), 11 old control non-smokers (OCNS; age≥50 years) and 10 young control non-smokers (YCNS; age≤40 years) (table 1). Patients with asthma and the severity of asthma were identified and treated according to the Global Initiative for Asthma and American Thoracic Society criteria.[20 21] Patients with COPD were staged and treated using Global Initiative for Chronic Obstructive Lung Diseases criteria.[22] All control subjects had no history of respiratory disease and no airflow limitation. The clinical and demographic characteristics of all the subjects are shown in table 1. All subjects were in stable condition (see also online supplementary material). The study conformed to the Declaration of Helsinki, was approved by the local ethics committees (A.O.U. San Luigi Hospital: n. of protocol 1759, 22 January 2008; S. Maugeri Foundation: n. of protocol p81, 20 May 2009), bronchial biopsies were performed according to the local Ethics Committee Guidelines and written informed consent was obtained from each subject.

Measurements of lung function, fibre-optic bronchoscopy, IHC, immunofluorescence and confocal microscopy, scoring system for IHC, primary cultures of bronchial fibroblasts, and ELISA tests for in vitro angiogenic factor release are described in online supplementary material.

Data analysis

Group data were expressed as mean±SD for lung functional data or median (range) and IQR for morphological data. We assumed a normal distribution for functional data (ie, forced expiratory volume in 1 s (FEV_1), forced vital capacity, age, etc) and a non-normal distribution for morphological parameters.

Table 1 Subjects' characteristics

Patients						
Characteristics	**Young control**	**Old control**	**Control smokers**	**COPD**	**Old asthma**	**Young asthma**
Subjects, N°						
Total	10	11	18	33	24	15
Male	7	4	15	27	10	5
Female	3	7	3	6	14	10
Age, years	30±7	69±7	61±7	66±8	62±8	26±5
Smoking history						
No	10	11	0	0	22	15
Ex	0	0	2	9	2	0
Current	0	0	16	24	0	0
Atopy						
Yes	0	0	0	0	16	14
No	10	11	18	33	8	1
FEV_1 pre,% predicted	104±16	118±12	104±13	49±19*	74±21**	96±17***
FEV_1 post,% predicted	ND	ND	ND	55±20	83±22	105±12
FEV_1/FVC ratio,%	82±8	85±10	80±6	51±12*	74±14****	83±8****

Values given as mean±SD.
*p<0.0001 (analysis of variance) significantly different from young/old controls, control smokers and old/young patients with asthma.
**p<0.0001 (analysis of variance) significantly different from young/old controls, control smokers, patients with COPD and young patients with asthma.
***p<0.0001 (analysis of variance) significantly different from young/old controls, control smokers, patients with COPD and old patients with asthma.
****p<0.0001 (analysis of variance) significantly different from patients with COPD.
COPD, chronic obstructive pulmonary disease; FEV_1, forced expiratory volume in 1 s; FVC, forced vital capacity; ND, not determined

For this reason we applied the analysis of variance in comparing subgroups of patients and control subjects for functional data. The non-parametric Kruskal-Wallis test was applied for multiple comparisons when morphological data were analysed followed by the Mann-Whitney U test for comparison between groups. The correlation coefficient between functional-morphological data was calculated using the Spearman rank method. Data from in vitro experiments are expressed as mean±SEM of three independent experiments (each experiment was performed in duplicate) for each fibroblast culture. Differences between two groups were compared by using unpaired t tests. Probability values of $p<0.05$ were considered statistically significant. Data analysis was performed by using the Graph Pad Prism program (GraphPad V.5.00 Software Inc, San Diego, California, USA).

RESULTS

Clinical findings

Subjects' characteristics are shown in table 1. See also online supplementary material.

Inflammatory cells

Quantification of inflammatory cells in bronchial biopsies is summarised in table 2. Data are reported in online supplementary material. IHC of all the inflammatory cells from OA is shown in online supplementary figure E1.

Immunoreactivity of VEGF-A, CD31 and angiogenin

VEGF-A was mainly present in fibroblasts of lamina propria and in epithelial cells (figure 1A). Cells expressing VEGF-A immunoreactivity were significantly higher in lamina propria of OA than OCNS ($p=0.0267$) but not higher than other groups (figure 1B). VEGF-A expression was significantly higher in YA than in YCNS ($p=0.0171$) (figure 1B). In OA ($p=0.0103$), and also in patients with asthma as a whole ($p=0.0269$), non-atopic subjects showed increased expression of VEGF-A in lamina propria compared with atopic subjects. Furthermore, COPD as well as OCS had higher numbers of VEGF-A$^+$ cells compared with OCNS without reaching statistical significance (figure 1B). CD31 was present in endothelial cells of lamina propria (figure 1C). The number of CD31 cells was significantly increased in OA compared with OCNS ($p=0.0378$) and OCS ($p=0.0414$) but it did not significantly differ with COPD (figure 1D). A significant increase of CD31 cells was observed in YCNS compared with OCNS ($p=0.0279$).

Immunostaining for angiogenin was localised in endothelial cells and fibroblasts of lamina propria and, to a lesser extent, in epithelial cells (figure 2A). The number of angiogenin$^+$ cells was significantly higher in OA compared with OCNS ($p=0.0002$), OCS ($p<0.0001$) and COPD ($p<0.0001$) (figure 2B). Similarly, the number of angiogenin$^+$ cells were also significantly elevated in patients with COPD compared with OCNS ($p=0.0090$) and OCS ($p=0.0451$) (figure 2B). Furthermore, angiogenin expression in lamina propria of OA was significantly higher than in YA ($p=0.0003$) (figure 2B). Finally, angiogenin$^+$ cells were significantly lower in lamina propria of OCNS compared with YCNS ($p=0.0496$) (table 2). Data are reported in online supplementary material.

Immunoreactivity of BK B2R and B1R

B2R was mainly present in fibroblasts within the lamina propria, in epithelial cells and in endothelial cells of capillary vessels (figure 3A). Cells expressing B2R immunoreactivity were significantly higher in lamina propria of OA compared with OCNS ($p=0.0004$), OCS ($p<0.0001$) and COPD ($p<0.0001$) (figure 3B). Similarly, immunostaining for B1R was localised in fibroblasts within the lamina propria and in endothelial cells and epithelial cells (figure 3C). Quantitative analysis showed significantly greater B1R expression in OA compared with OCNS ($p=0.0014$), OCS ($p<0.0001$) and COPD ($p<0.0001$) (figure 3D), as well as in comparison with YA ($p=0.0160$) (figure 3D). No significant differences were observed in the numbers of cells expressing B2R or B1R between any other groups studied (figure 3B,D). Data are reported in online supplementary material.

Inflammatory cells, angiogenic factors, B2R and B1R expression in severe versus mild asthma

We also investigated the differences in inflammatory cells, angiogenic markers and BK receptors expression in the groups of severe (n=16) and mild (n=23) asthma (irrespective of age). Data are reported in online supplementary material (see also online supplementary figure E2A).

Angiogenin$^+$ cells (see online supplementary figure E2B) were significantly increased in the patients with severe asthma compared with the patients with mild asthma ($p=0.019$), whereas

Table 2 Quantification of inflammatory cells in the lamina propria of patients with COPD, asthma, control smokers and control non-smoking subjects

Patients							
	Young control	Old control	Control smokers	COPD	Old asthma	Young asthma	p Value (Kruskal-Wallis)
CD4	104 (34–194)	133 (63–175)	105 (68–175)	119 (86–139)	180 (120–296)*	189 (86–298)	0.0371
CD8	91 (64–193)	111 (82–168)	129 (96–214)	196 (146–267)**	116 (83–155)	52 (20–111)	0.0010
CD68	258 (177–271)	235 (128–370)	299 (215–520)	480 (273–673)***	226 (151–303)	264 (214–375)	0.0016
Mast cells	50 (47–64)	52 (32–63)	38 (21–73)	75 (42–127)	38 (28–97)	94 (69–132)	0.1276
Neutrophils	124 (90–140)	88 (62–133)	113 (84–146)	151 (104–290)****	241 (147–309)****	195 (86–247)	0.0006
Eosinophils	8 (0–21)	15 (0–26)	4 (0–21)	6 (0–35)	39 (35–60)*	32 (10–115)*	0.0002

Data are presented as median (lower quartile, LQ—upper quartile, UQ).
The Kruskal-Wallis test was applied for multiple comparisons. For comparison between groups the Mann-Whitney U test was applied.
The exact p values for comparison between groups are given in the Results section.
*p<0.05 significantly different from young/old controls, control smokers and patients with COPD.
**p<0.05 significantly different from old controls and young/old patients with asthma.
***p<0.05 significantly different from young/old controls, control smokers and young/old patients with asthma.
****p<0.05 significantly different from young/old controls and control smokers.
COPD, chronic obstructive pulmonary disease.

no difference was found in the number of VEGF-A$^+$ cells, CD31 cells, B2R$^+$ cells and B1R$^+$ cells (data not shown).

Correlations between angiogenic markers, BK receptors, inflammatory cells and clinical parameters

In the whole asthma group, the number of angiogenin$^+$ cells was negatively related to FEV_1 (r_s=−0.415, p=0.008, figure 4A) and positively related to the number of B2R$^+$ cells in lamina propria (r_s=0.417, p=0.008, figure 4B). Moreover, the number of B2R$^+$ cells in lamina propria was significantly related to the number of B1R immunoreactive cells (r_s=0.432, p=0.0060, figure 4C) as well as the number of CD31 cells (r_s=0.465, p=0.004, figure 4D). In all patients with asthma, the number of neutrophils was positively related to CD31 cells (r_s=0.47, p=0.02), to angiogenin$^+$ cells (r_s=0.34, p=0.04) and to B2R$^+$ cells in the lamina propria (r_s=0.44, p=0.007).

No other statistically significant correlations were found between angiogenic markers, BK receptors, inflammatory cells or any clinical parameters.

Immunofluorescence with confocal microscopy double staining for localisation of BK B2 receptor in CD31 cells

Double immunofluorescence staining of B2R with CD31 was performed using laser-scanning confocal microscopy. Endothelial cells of capillary vessels in lamina propria of OA were immunoreactive for CD31 (figure 5A). Epithelial cells and endothelial cells of capillary vessels in lamina propria were immunoreactive for B2R (figure 5B). Merging of the red (B2R) and green (CD31) images revealed that CD31 cells of capillary vessels coexpressed B2R (figure 5C).

Immunofluorescence with confocal microscopy double staining for identification of 5B5 cells coexpressing BK B2 receptor and angiogenic growth factors

Double immunofluorescence staining of B2R, VEGF-A or angiogenin with 5B5 (marker for fibroblast) was performed using laser-scanning confocal microscopy. The large majority of cells in lamina propria of OA was immunoreactive for 5B5 (figure 6A,E,I). Merging of the red (B2R, VEGF-A or angiogenin) and green (5B5) images revealed that 5B5$^+$ cells in lamina propria coexpressed B2R (C), VEGF-A (G) or angiogenin (M). Co-localisation quantitative analysis (figure 6D,H,N) confirmed 5B5 and B2R (72±12%) coexpression as well as 5B5 and VEGF-A (87±2%) or angiogenin (80.5±3.5) co-localisation in lamina propria of OA.

BK-induced VEGF-A and angiogenin release by HBF in vitro

Unstimulated human bronchial fibroblasts (HBF) from OA (n=3) and COPD (n=3) release VEGF-A and angiogenin (figure 7). Incubation with BK (10^{-6}M) induced a significant

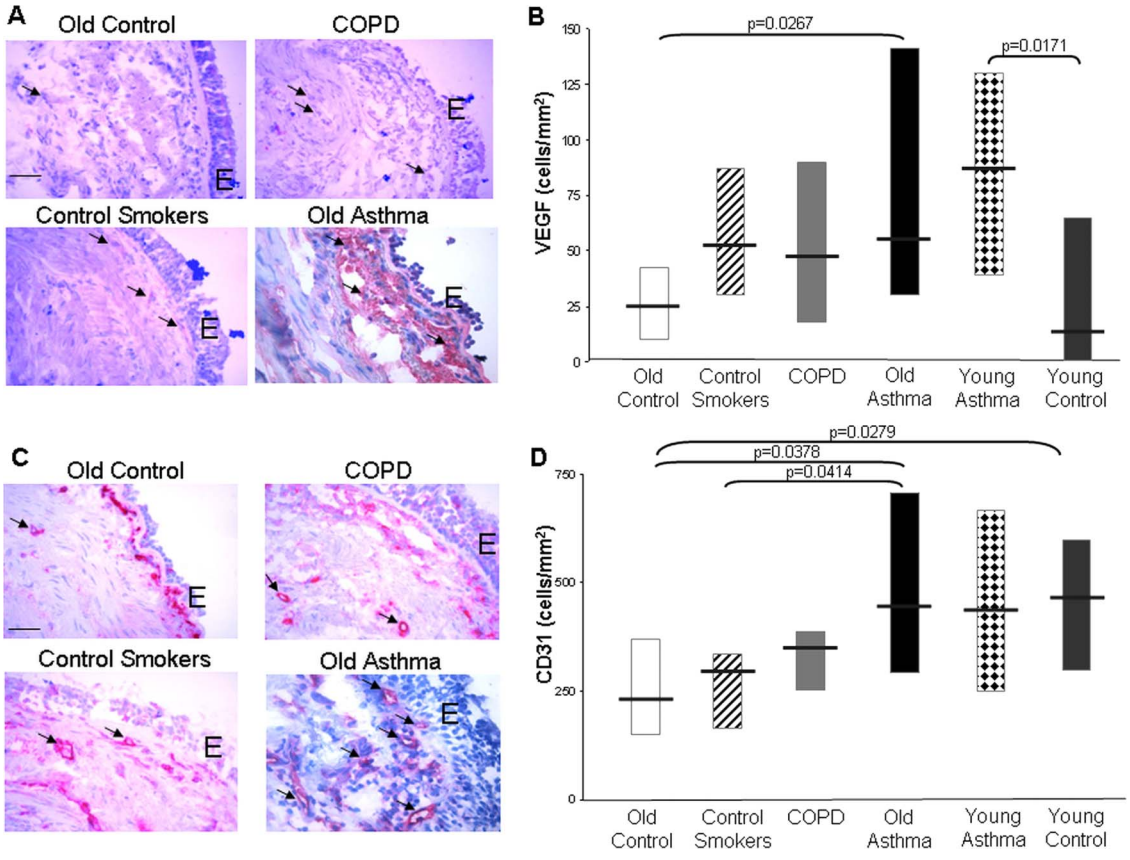

Figure 1 Vascular endothelial growth factor-A (VEGF-A) (A and B) and CD31 (C and D) expression in bronchial biopsy specimens obtained from old control non-smokers, healthy smokers, patients with chronic obstructive pulmonary disease (COPD), old and young patients with asthma, young control non-smokers. (A and C) Photomicrographs showing the bronchial immunostaining for VEGF-A and CD31, respectively, are representative of those from 11 old control non-smokers, 18 healthy smokers, 33 patients with COPD and 24 old patients with asthma. Original magnification 400×. Internal scale: 25 μm. E, epithelium. (B and D) Number of VEGF-A$^+$ and CD31 cells/mm^2 of bronchial lamina propria from old control non-smokers, healthy smokers, patients with COPD, old and young patients with asthma, young control non-smokers. Each bar indicates the median value in the respective groups and boxes represent lower and upper quartiles. The comparisons are made on the basis of age (old groups or young groups) and between asthma or control groups. Access the article online to view this figure in colour.

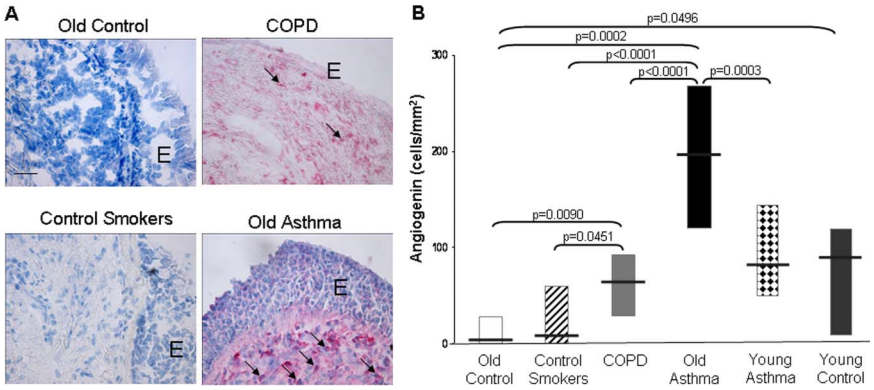

Figure 2 Angiogenin expression in bronchial biopsy specimens obtained from old control non-smokers, healthy smokers, patients with chronic obstructive pulmonary disease (COPD), old and young patients with asthma, young control non-smokers. (A) Photomicrographs showing the bronchial immunostaining for angiogenin are representative of those from 11 old control non-smokers, 18 healthy smokers, 33 patients with COPD and 24 old patients with asthma. Original magnification 400×. Internal scale: 25 μm. E, epithelium. (B) Number of angiogenin$^+$ cells/mm^2 of bronchial lamina propria from old control non-smokers, healthy smokers, patients with COPD, old and young patients with asthma, young control non-smokers. Each bar indicates the median value in the respective groups and boxes represent lower and upper quartiles. The comparisons are made on the basis of age (old groups or young groups) and between asthma or control groups. Access the article online to view this figure in colour.

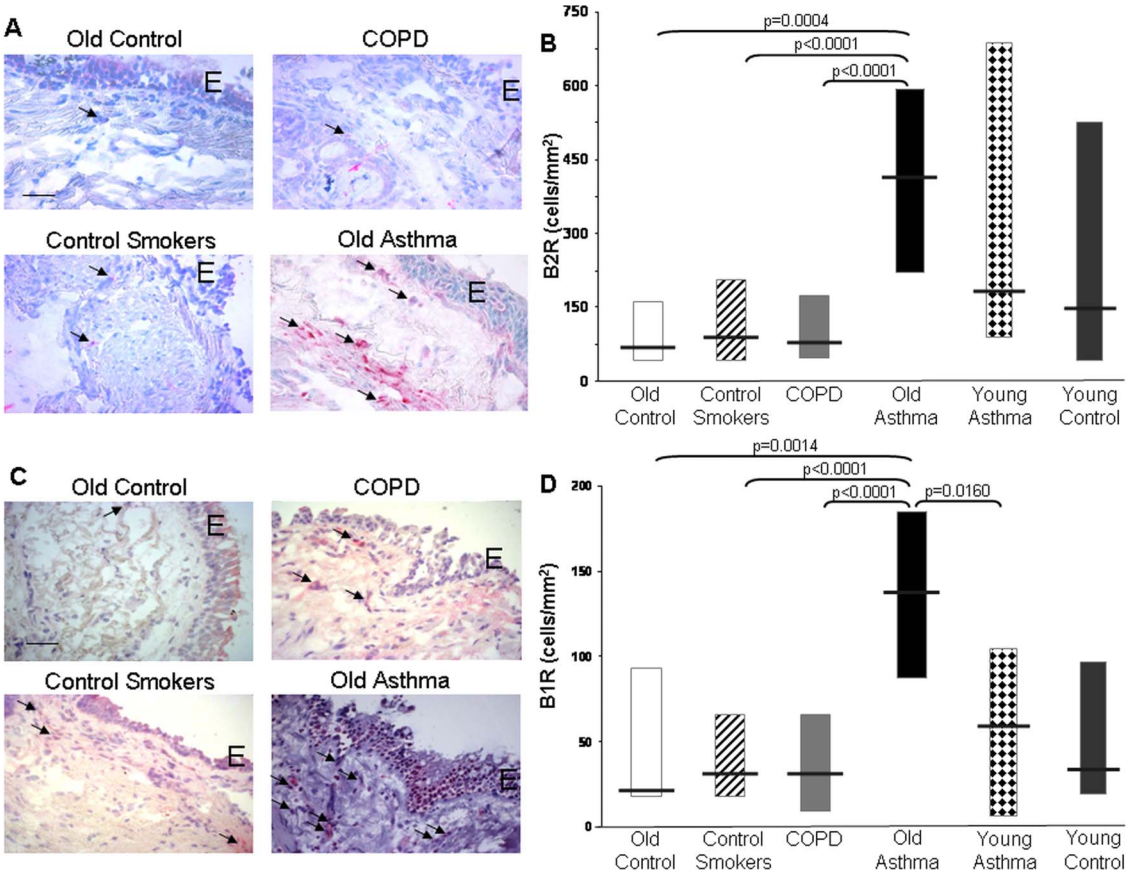

Figure 3 Bradykinin B2 receptor (B2R) (A and B) and bradykinin B1 receptor (B1R) (C and D) expression in bronchial biopsy specimens obtained from old control non-smokers, healthy smokers, patients with chronic obstructive pulmonary disease (COPD), old and young patients with asthma, young control non-smokers. (A and C) Photomicrographs showing the bronchial immunostaining for B2R and B1R, respectively, are representative of those from 11 old non-smokers, 18 healthy smokers, 33 patients with COPD and 24 old patients with asthma. Original magnification 400×. Internal scale: 25 μm. E=epithelium. (B and D) Number of B2R$^+$ and B1R$^+$ cells/mm^2 of bronchial lamina propria from old control non-smokers, healthy smokers, patients with COPD, old and young patients with asthma, young control non-smokers. Each bar indicates the median value in the respective groups and boxes represent lower and upper quartiles. The comparisons are made on the basis of age (old groups or young groups) and between asthma or control groups. Access the article online to view this figure in colour.

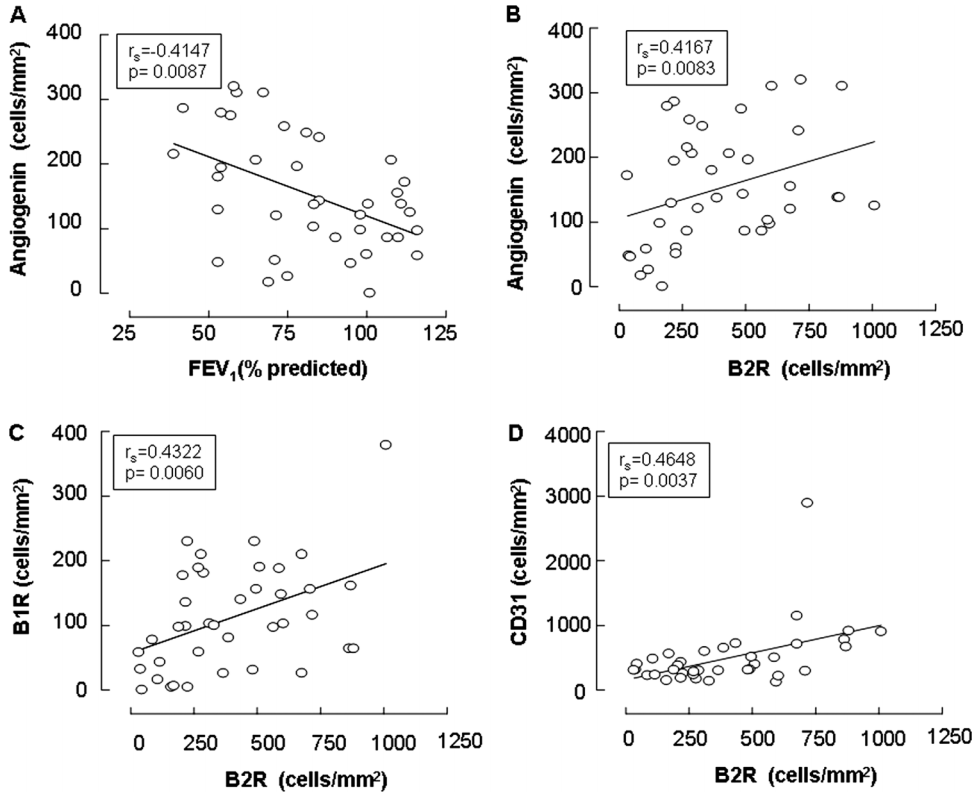

Figure 4 Relationship between angiogenin positive cells and forced expiratory volume in 1 s (FEV_1) (% predicted) levels (A) or the number of $B2R^+$ cells (B) in the bronchial lamina propria of all patients with asthma. In (C–D) the correlation between $B2R^+$ cells number and B1R immunoreactive cells (C) or the number of CD31 cells (D) in the bronchial lamina propria of all patients with asthma are shown. The correlation coefficient was obtained using the Spearman rank method (r_s).

increase in VEGF-A (figure 7A: p=0.0248; figure 7C: p=0.016) and angiogenin release (figure 7B: p<0.0001; figure 7D: p<0.0001) in HBF from patients with asthma and COPD, respectively. BK (1 h exposure) similarly increased VEGF-A levels in HBF from patients with asthma (160±25%) and COPD (208±34%; p>0.05), while BK elevated angiogenin levels differently in asthma fibroblasts (669±111%) compared with COPD fibroblasts (280±10%; p=0.013). Details of these experiments are reported in online supplementary material.

DISCUSSION

This study demonstrates that BK receptors (B2R and B1R) are upregulated in the lamina propria of OA compared with age-comparable healthy control subjects and COPD in conjunction with increased number of vessels (CD31 cells) and expression of angiogenin and, to a lesser extent, VEGF-A. Furthermore, angiogenin expression was significantly increased in patients with severe compared with mild asthma. Angiogenin was also negatively related to FEV_1 in all patients with asthma and positively related to B2R expression. Finally, we showed localisation of B2R, VEGF-A and angiogenin in bronchial fibroblasts from OA and the ability of BK to release VEGF-A and angiogenin in bronchial fibroblasts from patients with asthma and, to a lesser extent, from patients with COPD.

Asthma and COPD are chronic inflammatory diseases of the lung associated with structural remodelling that contributes to airway obstruction (see also online supplementary material). We found significant and positive correlations between numbers of neutrophils and $B2R^+$, CD31 or $angiogenin^+$ cells suggesting a role for this inflammatory cell in vascular remodelling of OA.

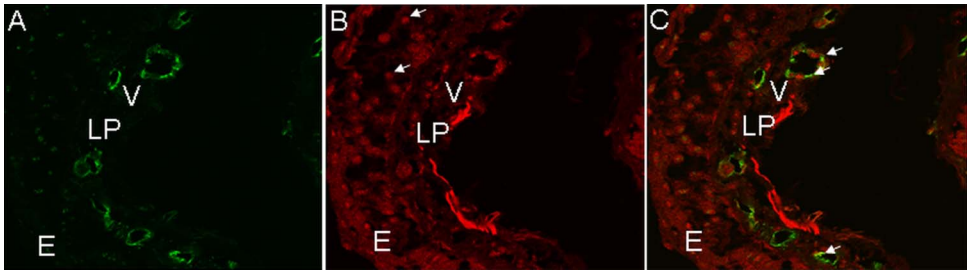

Figure 5 Representative photomicrographs obtained by confocal microscopy after immunofluorescence staining of CD31 and bradykinin B2 receptor (B2R) in bronchial lamina propria from an old patient with asthma. In (A) Alexa Fluor 488-green staining represents CD31, in (B) Alexa Fluor 647-red staining represents B2R whereas co-localised pixels are displayed in yellow (C). Arrows indicate B2R and B2R/CD31 positive cells (B and C, respectively). Original magnification: 630×. E, epithelium; LP, lamina propria; V, vessel. Access the article online to view this figure in colour.

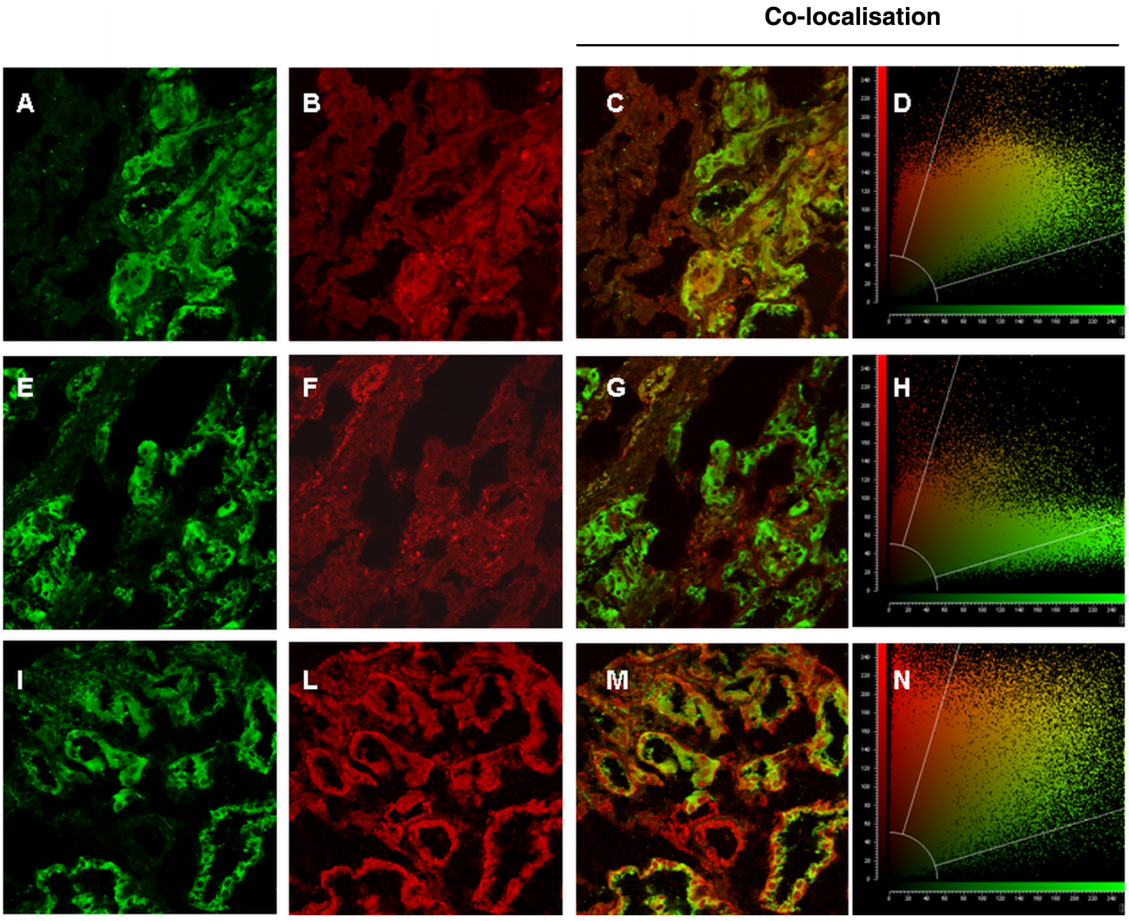

Figure 6 Representative photomicrographs obtained by confocal microscopy after immunofluorescence staining of prolyl 4-hydroxylase (5B5), bradykinin B2 receptor (B2R), vascular endothelial growth factor-A (VEGF-A) and angiogenin expression in bronchial lamina propria from an old patient with asthma. (A, E and I) show, the Alexa Fluor 488-tagged anti-5B5 (green) whereas (B, F and L) represent Alexa Fluor 647-tagged anti-B2R, anti-VEGF-A or antiangiogenin (red) respectively. In (C, G and M) the co-localised pixels representing coexpression of 5B5 and B2R (C), VEGF-A (G) or angiogenin (M) are displayed in yellow. (D, H and N) show the correlation cytofluorogram of the images in (A and B, or E and F or I and L), respectively. Original magnification 630×. Access the article online to view this figure in colour.

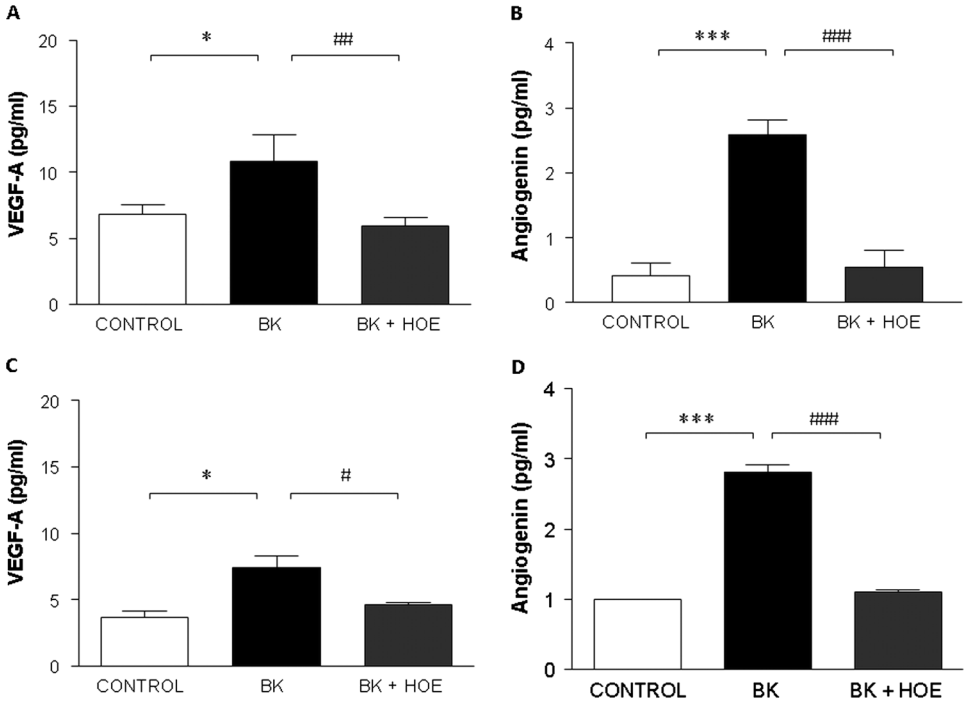

Figure 7 Effect of bradykinin (BK) B2 receptor antagonist HOE140 on BK-induced vascular endothelial growth factor-A (VEGF-A) release (A and C) and angiogenin release (B and D) by human bronchial fibroblasts from patients with asthma (n=3; A and B) and from chronic obstructive pulmonary disease (n=3; C and D). Cells were pretreated with or without HOE140 (1 μM) for 30 min before incubation with BK (10^{-6}M) for 1 h. At the end of the incubation period, the culture supernatants were collected and the VEGF-A or angiogenin contents were evaluated by ELISA. Data are presented as mean ±SEM of three independent experiments for each fibroblast culture. *p<0.05, ***p<0.001 versus unstimulated cells; and $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 versus cells stimulated with BK.

In a previous study BK-induced interleukin 8 production due to B2R stimulation in lung fibroblasts is in part dependent on the activation of ERK1/2 and p38 MAPK-pathway.[23] These data, together with our report on positive correlations between neutrophils and angiogenic molecules suggest a role for neutrophil-released mediators (including proteases) in modulating angiogenesis in asthma through activation of protease activated receptors.[24]

Vascular remodelling and reactivity of the airways including angiogenesis, vasodilation and microvascular leakage are the most prominent and uniform findings in asthma.[2] Furthermore, bronchial vascular changes may also occur in COPD, particularly in the small airways.[2 25] Microvascular changes in asthma and COPD may contribute to an increase in airway wall thickness which may be associated with severity of airway obstruction and disease progression.[25] Angiogenesis is a complex multiphase process involving a great number of growth factors, cytokines, chemokines and other factors. Some bronchial biopsy studies[7 8] as well as induced sputum studies[9] observed higher VEGF-A levels, related to vascularity, in asthmatic airways than those of healthy controls.[7 8] Angiogenin induces vascular endothelial cell proliferation, migration and tubule formation. In biopsies from patients with asthma, a higher expression of basic fibroblast growth factor and angiogenin was reported, with significant correlations between the vascular area and the number of angiogenic factor-positive cells within the airways.[7] Furthermore, VEGF-A and angiogenin are able to promote in vitro angiogenesis expressed as induction of primitive vascular tubule structures in culture with BALF collected from patients with mild asthma.[13] A recent study also showed increased levels of VEGF-A and angiogenin in induced sputum from stable COPD compared with healthy smokers and non-smokers.[26] We extend these observations by comparing patients with asthma, COPD and controls in relation to age. This is the first study in humans showing that the number of CD31 and angiogenin$^+$ cells was significantly reduced in old compared with young controls demonstrating that vascularity is probably influenced by age per se. VEGF-A appears to be more expressed in lamina propria of OA while angiogenin is significantly higher in OA (also in comparison with YA) and in COPD. Furthermore, angiogenin$^+$ cell expression in lamina propria negatively correlated with the level of FEV_1 in the group of patients with asthma, suggesting that angiogenin expression in lamina propria is associated with the severity of asthma, confirming previous findings.[25] The modest increase of angiogenin in the bronchi of our COPD may be related to apoptosis of endothelial cells induced by VEGF-A reduction due to oxidative stress as described elsewhere.[27] Finally, we showed for the first time a marked increase of VEGF-A expression in the lamina propria of non-atopic patients with asthma compared with atopic patients with asthma suggesting that in 'intrinsic' asthma higher release of VEGF-A could specifically contribute to the formation of vascular remodelling scenario.

BK exerts several effects in airways through direct and indirect pathways which are mediated by activation of two main BK receptor subtypes, B1 and B2, that differ in expression and ligand specificity. In tissues B1 receptors are inducible, whereas B2 receptors are constitutively expressed.[14] The distribution of B2 receptors has been mapped out in human and guinea pig lung by autoradiography with [^{3}H] BK.[28] See also online supplementary material. In this study, for the first time we demonstrated the expression of BK B1R and B2R in fibroblasts, epithelial cells and endothelial cells of the human bronchial lamina propria (by IHC and immunofluorescence analysis), and that their total expression is significantly increased in OA compared with OCNS, OCS and COPD (and for B1R also compared with YA), suggesting a potential role for these receptors in the vascular remodelling of old asthma. BK promotes angiogenesis by upregulation of basic fibroblast growth factor through the B1 receptor or by stimulation of VEGF-A formation following B2 receptor stimulation.[17 29] In our study, B2R showed a positive correlation with B1R and with CD31 endothelial cells and angiogenin suggesting that B2R may promote angiogenesis leading to blood vessel neoformation. We also found by immunofluorescence that the large majority of bronchial fibroblasts in OA coexpressed B2R, angiogenin and VEGF-A indicating a major role for fibroblasts in producing vascular growth factors potentially released upon stimulation of BK B2R.

BK is involved in the repair processes inducing fibroblast proliferation and differentiation.[30] BK also regulates angiogenic growth factors expression (angiogenin and VEGF-A) in human dental pulp fibroblasts.[31] Other authors showed that human airway smooth muscle cells secrete VEGF-A and this secretion is induced by BK via B2R.[17] In our in vitro study, we demonstrated for the first time that BK induced VEGF-A and angiogenin release in primary cultures of HBF from patients with asthma and that this effect is mediated by B2R, as confirmed by the use of the B2R antagonist HOE140 which completely blocked BK-induced VEGF-A and angiogenin release. We also showed similar effects, but to a lesser extent, in bronchial fibroblasts from COPD suggesting that bronchial fibroblasts from patients with asthma are more prone to release angiogenic factors.

In conclusion, this study demonstrates an increased expression of BK receptors in relation to vascular remodelling in asthma and suggests a role for bronchial asthmatic fibroblasts in releasing vascular growth factors upon activation of BK B2 receptors. Our study showed that markers of vascular remodelling are major features of OA and are potentially involved in fixed airway obstruction by increasing airway wall thickness and stiffness. The increased vascular reactivity of asthmatic airways, due to stimuli like BK,[32] in disease-related vascular remodelling might participate in airway variability which is a typical pattern of airway patency in asthma. Previous observations showed kinin involvement in airway hyperresponsiveness in asthma,[33] and the capability of selective B2R antagonists to treat acute attacks of hereditary angioedema,[34] and to improve pulmonary function in patients with asthma.[35] These data, combined, lead us to encourage the planning of long term studies using these selective antagonists in asthma treatment in order to inhibit BK B2 receptor-mediated angiogenic effects and its related remodelling processes.

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Contributors All authors included in this paper fulfil the criteria of authorship. In addition we assure that there is no one else who fulfils the criteria that has not been included as an author.

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Competing interests None.

Patient consent Obtained.

Ethics approval The study conformed to the Declaration of Helsinki. Ethics consent was obtained, bronchial biopsies were performed according to the local ethics committee guidelines.

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Supplementary material

EXPRESSION OF VASCULAR REMODELING MARKERS IN RELATION TO BRADYKININ RECEPTORS IN ASTHMA AND COPD

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METHODS

Subjects

We examined bronchial biopsies from 111 subjects by immunohistochemistry and confocal analysis: 24 non-smoking (2 ex-smokers) old asthmatics (age≥50 years); 15 young non-smoking asthmatics (age≤40 years); 33 smoking (9 ex-smokers) COPD patients (age≥50 years), 18 old control smokers (2 ex-smokers) (age≥50 years), 11 old control non-smokers (age≥50 years) and 10 young control non-smokers (age≤40 years) (Table 1). Asthmatics and the severity of asthma were identified and treated according to the GINA and ATS criteria.[E1, E2] Patients with COPD were staged and treated using GOLD criteria.[E3] All control subjects had no history of respiratory disease and no airflow limitation. The clinical and demographic characteristics of all the subjects are shown in Table 1. All subjects were in stable condition and none of them was treated with theophylline, antibiotics, antioxidants, mucolytics, and/or systemic glucocorticoids in the 6 weeks prior to bronchial biopsy. The study, conformed to the Declaration of Helsinki, was approved by the local Ethics Committees (A.O.U. San Luigi Hospital: n. of protocol 1759,

January 22nd, 2008; S. Maugeri Foundation: n. of protocol p81, May 20th, 2009), bronchial biopsies were performed according to the local Ethics Committee Guidelines, and written informed consent was obtained from each subject.

Lung function tests and volumes

FEV_1 and FEV_1/FVC were performed as previously described [E4] according to published guidelines. In order to assess the reversibility of airflow obstruction and post-bronchodilator functional values, the FEV_1 and FEV_1/FVC% measurements in the groups of subjects with FEV_1/FVC%≤70% pre-bronchodilator was repeated 20 min after the inhalation of 0.4 mg of salbutamol.

Fiberoptic bronchoscopy, collection and processing of bronchial biopsies

Fiberoptic bronchoscopy was performed according to a previously described protocol.[E5] Premedication consisted of atropine (0.5 mg) and diazepam (10 mg), both given by intramuscular injection. Using local anaesthesia with lidocaine (4%) to the upper airways and larynx, a fiberoptic bronchoscope (Pentax FB-18P; Asahi Optical Co. LTD, Tokyo, Japan) was passed through the nasal passages into the trachea. Further lidocaine (2%) was sprayed into the lower airways, and four bronchial biopsy specimens were taken from segmental and subsegmental airways of the right lower and upper lobes using size 19 cupped forceps. Bronchial biopsies were gently extracted from the forceps and processed for light microscopy as previously described.[E4] Two samples were embedded in Tissue Tek II OCT (Miles Scientific, Naperville, IL), frozen within 15 min in isopentane pre-cooled in liquid nitrogen, and stored at –80°C. Six old asthmatics, six young asthmatics and four young control non-smokers were collected in

Groningen following a slightly modified protocol.[E6] No premedication was given. After local anesthesia with lidocaine 2 or 4%, six bronchial biopsies were obtained using a flexible bronchoscope (type Olympus BF P20 or BF XT20; Olympus, Center Valley, PA) from subsegmental divisions of the right and left lower lob, using fenestrated forceps (FB-21C, Olympus). Two biopsies were mounted in Tissue Tek® (Sakura, Tokyo, Japan) and snap-frozen by immersion in isopentane at –80°C.

For all samples six μm thick cryostat sections were cut for immunohistochemistry and confocal microscopy analysis. Bronchial biopsies, to establish human bronchial fibroblast primary cultures, were immediately placed in high-glucose Dulbecco's modified Eagle's medium (DMEM, Sigma, Milan, Italy) supplemented with 10% foetal calf serum (FCS, Euroclone Ltd., Paignton, Devon, UK) and penicillin/streptomycin (Sigma) and processed as described below.

Immunohistochemistry

Sections, obtained by selection of the best oriented biopsy for each patient after preliminary analysis, were stained with immunohistochemical methods.[E5] One immunostained section for each antigen was used for quantitative purposes. Cracks of tissue or artificial mechanical areas were excluded from the quantification process and quantification was performed in a blinded fashion.[E7] Primary antibodies directed against the following markers were used: lymphocytes (CD4 1:100, mouse; CD8 1:200, mouse; Dakocytomation, Milan, Italy), macrophages (CD68 1:200, mouse; NeoMarkers, Freemont, CA), mast cells (tryptase 1:50, mouse; Millipore, Temecula, CA), neutrophils (anti-elastase 1:100, mouse; Dako), eosinophils (Eosin/Haematoxylin staining), bradykinin B2 receptor (B2R 1:100, mouse; BD Biosciences Inc.,Milan, Italy), bradykinin B1 receptor (B1R 1:50, rabbit; Santa Cruz Biotechnology,

Wembley, UK), endothelial cells (CD31 1:50, mouse; Dakocytomation, Milan, Italy), vascular endothelial growth factor-A (VEGF-A 1:100, goat; R&D Systems, Abingdon, UK) and angiogenin (1:100, goat; R&D Systems).

After blocking non-specific binding sites using serum derived from the same animal species as the secondary antibody, primary antibodies were applied at optimal dilutions in TRIS-buffered saline (0.15 M saline containing 0.05 M TRIS-hydrochloric acid at pH 7.6) and incubated (1 h) at room temperature in a humidified chamber. Antibody binding was demonstrated with the use of secondary antibodies anti mouse (Vector, BA 2000), anti rabbit (Vector, BA 1000) or anti goat (Vector, BA 5000) followed by Strept AB Complex/AP (Dako, K0391) and fast-red substrate. Lamina propria capillary vessels were identified using both a morphologic criterion (at least half of the vessel circumference had to be identified) and an immunohistochemical criterion: vessels had to be positively stained with anti-CD31 primary antibody.[E8] Thus, on the basis of these criteria we may consider the count of CD31 positive cells as an indirect index of the density of blood vessels. Control slides were included in each staining run using nasal polyps as positive controls for all immunostaining performed (Figure E3). For negative control slides, normal goat, rabbit or mouse non-specific immunoglobulins (Santa Cruz Biotechnology) were used at the same concentrations of the primary antibodies analysed (Figure E3).

Immunofluorescence staining with confocal microscopy

For confocal microscopy, sections were fixed with 4% paraformaldehyde, washed with phosphate-buffered saline (PBS) and incubated for 1 h with PBS containing 5% bovine serum albumin (BSA) and 5% goat serum.[E5] After blocking, the preparations were incubated for 1 h with the primary antibodies diluted in PBS containing 5% BSA. The following antibodies were used: mouse anti-human Prolyl 4- hydroxylase (5B5, 1:50; DakoCytomation, Milan, Italy), rabbit

anti-human B2R (1:100; Affinity Bioreagents, Golden, CO, USA), goat anti-human VEGF-A (1:50; R&D Systems), goat anti-human angiogenin (1:100; R&D System), mouse anti-human CD31 (1:50; Dakocytomation). [E9] After washing with PBS, the preparations were incubated for a further 30 min with the appropriate secondary Alexa Fluor 488 or Alexa Fluor 647 (Molecular Probes, Invitrogen S.R.L., Milan, Italy) conjugated antibodies diluted 1:200 in PBS. Nasal polyps were used as positive and negative controls (Figure E4). Negative controls included irrelevant mouse, rabbit and goat immunoglobulins revealed as for primary antibodies (Figure E4, panels A, B, C). Alexa Fluor 647-tagged anti-B2R, VEGF-A or angiogenin were visualized in red and Alexa Fluor 488-tagged anti-5B5 or CD31 were showed in green. The slides were mounted using a specific mounting medium (Fluka 10979, Sigma).

Scoring system for immunohistochemistry and confocal microscopy

Morphometric measurements were performed with a light microscope (Leitz Biomed, Leica Cambridge, UK) connected to a video recorder linked to a computerised image system (Quantimet 500 Image Processing and Analysis System, Software Qwin V0200B, Leica). Light-microscopic analysis was performed at a magnification of 630X. Immunostained cells were quantified in the area 100 μm beneath the epithelial basement membrane (lamina propria) in several non-overlapping high power fields until all the available area was covered. The final result, expressed as the number of positive cells/mm^2, was calculated as the average of all the cellular counts performed in each biopsy. We quantified the immunostained cells with at least a portion of the nucleus seen close to immunopositivity.[E7] A mean ± SD of 0.700 ± 0.260 millimeters of epithelium was analyzed in all subjects.

The slides for confocal microscopy were analyzed using a three-channel Leica TCS SP2 laser scanning confocal microscope. The Leica LCS software package was used for acquisition, storage, and visualization. Co-localized pixels representing co-expression of 5B5 and B2R, VEGF-A or angiogenin were displayed in yellow and the relative correlation were reported in cytofluorograms.[E10]

Primary culture of human asthmatic/COPD bronchial fibroblasts

Primary lines of human asthmatic/COPD bronchial fibroblasts were established by enzymatic digestion [E11] of bronchial biopsy specimens obtained from asthmatics (n=3) and COPD (n=3) patients. The fibroblast cultures were characterized by flow cytometry using the specific mouse IgG1 monoclonal antibody ASO2 (Dianova, Hamburg, Germany) that, according to the manufacturer, reacts specifically with a membrane-bound protein of human fibroblasts. In addition, using immunofluorescence staining, the presence of cytokeratin (Immunotech, Marseille, France), vimentin (Biogenex, CA, USA), fibronectin (Sigma) and α-smooth muscle actin (α-SMA, Dako) was assessed.

Both types of fibroblasts were negative for cytokeratin, positive for vimentin (>50%), ASO2 (100%), fibronectin (>50%) and α-SMA (<5%). There was no contamination with smooth muscle cells.

The cells were cultured in 75-cm^2 tissue culture flasks with high-glucose medium DMEM supplemented with 10% FCS and penicillin/streptomycin. Fibroblasts used in the present study were between cell passages 2 and 10.

VEGF-A and angiogenin release by human asthmatic and COPD bronchial fibroblasts *in vitro*

The effect of BK on VEGF-A and angiogenin release by human asthmatic and COPD bronchial fibroblasts was evaluated by enzyme-linked immunosorbent assay (ELISA). After being seeded into 24-well plates (7.0×10^3 cells/well), cells were cultured in serum-free DMEM for 48 h. At the end of incubation, medium was removed and fibroblasts were incubated for 1 h in serum-free DMEM in presence of BK 1 μM. Unstimulated fibroblasts were used as negative control. The involvement of B2R was investigated using the specific B2R antagonist HOE140 (1 μM, Sigma). Cells were preincubated for 30 minutes with HOE140 before being treated with BK for 1 h. At the end of incubation, culture supernatants were collected and kept frozen until being tested. The evaluation of VEGF-A and angiogenin in fibroblast supernatants was carried out according to the manufacturer's instructions (RayBiotech, Inc., Norcross GA, USA).

RESULTS

Clinical findings

Subjects' characteristics are shown in Table 1. As expected from the selection criteria, the four groups of old subjects (COPD, asthma, control non-smokers and control smokers) were age-matched, but were different in age compared to the groups of young control and young asthma (Table 1). The values of forced expiratory volume in 1 s (FEV_1 % predicted) were significantly lower in the groups with COPD and asthma (young and old) compared with all control groups (healthy smokers and young or old healthy never-smokers). FEV_1 (% predicted) in patients with COPD was also significantly lower than young and old asthma subjects (for overall groups, $p<0.001$). Compared with old asthmatics, young asthmatics had significantly higher FEV_1 values ($p<0.001$). FEV_1/FVC was significantly lower in subjects with COPD when compared with both

asthmatic subjects and all control groups (for overall groups, $p<0.001$). There were no significant differences in FEV_1 and FEV_1/FVC among the control groups (healthy smokers and young or old healthy never-smokers).

Inflammatory cells

Quantification of inflammatory cells in bronchial biopsies is summarised in Table 2. The number of CD4 lymphocytes was significantly higher in the bronchial lamina propria of old asthmatics than old control non-smokers ($p=0.044$), control smokers ($p=0.016$) and COPD ($p=0.011$). The number of $CD4^+$ cells in old asthmatics did not differ significantly from young asthmatics, but it was significantly higher than young controls ($p=0.044$) (Table 2). Subjects with COPD had a greater number of CD8 lymphocytes than young ($p=0.0051$) and old control non-smokers ($p=0.007$), old asthmatics ($p=0.003$) and young asthmatics ($p= 0.002$). In addition, $CD68^+$ cells were significantly increased in the COPD group compared with all other groups ($p<0.05$). No significant differences were observed in lamina propria numbers of mast cells in the six groups studied (Table 2) even though a tendency to higher values in young compared to old asthma ($p=0.074$) was noticed, but the number of mast cells was significantly higher in young asthmatics than in young controls ($p=0.03$).

Neutrophils were increased in the COPD group as well as in old asthmatics compared with young non-smoking control subjects ($p=0.027$ and $p=0.002$, respectively), old non-smoking control subjects ($p=0.020$ and $p=0.001$, respectively) or smoking control subjects ($p=0.019$ and $p=0.001$, respectively). The number of eosinophils was significantly higher in old asthmatics than in young non-smoking control subjects ($p=0.001$), old non-smoking control subjects ($p=0.001$), smoking control subjects ($p=0.003$) and COPD ($p=0.0003$). Furthermore, young asthmatics had

significantly more eosinophils than young non-smoking control subjects (p=0.024), old non-smoking controls (p=0.029), smoking control subjects (p=0.046) and COPD (p=0.034).

Immunohistochemistry of all the inflammatory cells examined in bronchial sections from old asthmatics is shown in figure E1.

Immunoreactivity of VEGF-A, CD31 and angiogenin

VEGF-A was present in structural cells, mainly in fibroblasts of bronchial lamina propria and in epithelial cells (Figure 1A). Cells expressing VEGF-A immunoreactivity were significantly higher in the bronchial lamina propria of old non smoking asthmatics (54 [30-140] cells/mm^2) than old control non-smokers (24 [11-45] cells/mm^2, p=0.0267) but not than other groups (Figure 1B). VEGF-A expression in young asthmatics (86 [38-129] cells/mm^2) was significantly higher than in young non smoking controls (14 [0-66] cells/mm^2, p=0.0171) (Figure 1B). In the group of old asthmatics (p=0.0103), and also in asthmatics as a whole (p=0.0269), non-atopic subjects showed increased expression of VEGF-A (130.0 [65.4-187.5] cells/mm^2) in the bronchial lamina propria compared to atopic subjects (34.5 [14.3-76.0] cells/mm^2). Furthermore, COPD patients as well as control smokers had higher numbers of VEGF-A^+ cells compared to old control non-smokers without reaching statistical significance (Figure 1B). $CD31^+$ was present in endothelial cells of bronchial lamina propria (Figure 1C). The number of $CD31^+$ cells was significantly increased in old asthmatics (449 [295-702] cells/mm^2) compared with old control non-smokers (234 [154-387] cells/mm^2, p=0.0378) and control smokers (299 [169-343] cells/mm^2, p=0.0414) but it did not significantly differ with COPD patients (Figure 1D). A significant increase of $CD31^+$ cells was observed in young controls compared to old control non-smokers (p=0.0279).

Immunostaining for angiogenin was localized in endothelial cells and fibroblasts of bronchial lamina propria and, to a lesser extent, in epithelial cells (Figure 2A). The number of angiogenin$^+$

cells was significantly higher in old asthmatics (201 [122-271] cells/mm^2) compared with old control non-smokers (9 [0-28] cells/mm^2, p=0.0002), control smokers (12 [0-62.5] cells/mm^2, p<0.0001) and COPD patients (69 [29-92] cells/mm^2, p<0.0001) (Figure 2B). Similarly, the number of angiogenin$^+$ cells were significantly elevated also in COPD patients compared to old control non-smokers (p=0.0090) and control smokers (p=0.0451) (Figure 2B). Furthermore, angiogenin expression in the bronchial lamina propria of old asthmatics was significantly higher than in young asthmatics (p=0.0003) (Figure 2B). Finally, angiogenin$^+$ cells were significantly lower in the bronchial lamina propria of old control non-smokers compared with young control non-smokers (p=0.0496) (Table 2).

Immunoreactivity of bradykinin B2R and B1R

B2R was present in structural cells, mainly in fibroblasts within the bronchial lamina propria, in epithelial cells and in endothelial cells of capillary vessels (Figure 3A). Cells expressing B2R immunoreactivity were significantly higher in the bronchial lamina propria of old asthmatic subjects (411 [268-593] cells/mm^2) compared with old control non-smokers (71 [44-157] cells/mm^2, p=0.0004), control smokers (93 [44-179] cells/mm^2, p<0.0001) and COPD patients (83 [32-172] cells/mm^2, p<0.0001) (Figure 3B). Similarly, immunostaining for B1R was localized in fibroblasts within the bronchial lamina propria and in endothelial cells and epithelial cells (Figure 3C). Quantitative analysis showed significantly greater B1R expression in old asthmatic subjects (138 [85-186] cells/mm^2) compared to old control non-smokers (22 [18-95] cells/mm^2, p=0.0014), control smokers (32 [18-59] cells/mm^2, p<0.0001) and COPD patients (32 [6.4-64.5] cells/mm^2, p<0.0001) (Figure 3D), as well as in comparison with young asthmatics (p=0.0160) (Figure 3D). No significant differences were observed in the numbers of cells expressing B2R or B1R between any other of the groups studied (Figures 3B, 3D).

Inflammatory cells, angiogenic factors, B2R and B1R expression in severe versus mild asthma

We also investigated the differences on inflammatory cells, angiogenic markers and BK receptors expression in the groups of severe (n=16) and mild (n=23) asthma (irrespective of age).

The number of $CD4^+$ cells (p=0.044), neutrophils (p=0.038) and eosinophils (p=0.013) was significantly higher in severe than in mild asthmatics (Figure E2A); whereas no difference was found in the number of the other inflammatory cells.

$Angiogenin^+$ cells (Figure E2B) were significantly increased in the severe asthma group (201 [122-278] cells/mm^2) compared to the mild asthma group (121 [86-155] cells/mm^2, p=0.019), whereas no difference was found in the number of VEGF-A^+ cells, $CD31^+$ cells, $B2R^+$ cells and $B1R^+$ cells (data not shown).

BK–induced VEGF-A and angiogenin release by human bronchial fibroblast *in vitro*

Unstimulated human bronchial fibroblasts (HBF) from old asthmatics (n=3; mean±SD: age, 58±9; sex 2M/1F; pack/years, 0; FEV_1%predicted, 56±4; FEV_1%predicted post bronchodilator, 64±4; FEV_1/FVC%, 60±8; FEV_1/FVC% post bronchodilator, 64±9) and COPD patients (n=3; mean±SD: age, 66±11; sex 3M; pack/years, 53±13; FEV_1%predicted, 65±21; FEV_1%predicted post bronchodilator, 67±26; FEV_1/FVC%, 49±12; FEV_1/FVC% post bronchodilator, 51±13) are able to release both VEGF-A and angiogenin (Figure 7) (asthma: VEGF-A: 6.9±0.46 pg/ml; angiogenin: 0.4±0.12 pg/ml) (COPD: VEGF-A: 3.7±1.05 pg/ml; angiogenin:1.0±0.02 pg/ml).

Incubation with BK (10^{-6}M) induced a statistically significant increase in release of VEGF-A (Figure 7A: p=0.0248; Figure 7C: p=0.016) and angiogenin (Figure 7B: p<0.0001; Figure 7D: p<0.0001) in HBF from asthmatics and COPD patients respectively. BK (1 h exposure) similarly increased VEGF-A levels in HBF from asthmatics (160±25%) and COPD patients (208±34%;

p>0.05), whilst BK elevated angiogenin levels differently in asthma fibroblasts (669±111%) compared to COPD fibroblasts (280±10%; p=0.013). The B2R selective antagonist HOE140 completely blocked BK-induced VEGF-A (p=0.0016 in asthma; p=0.0017 in COPD) and angiogenin (p<0.0001) release by HBF from old asthmatics and COPD patients *in vitro* (Figure 7, panels A,B,C,D).

DISCUSSION

This study demonstrates that bradykinin receptors (B2R and B1R) are up-regulated in the bronchial lamina propria of old asthmatics compared to age-comparable healthy control subjects and COPD patients in conjunction with increased number of vessels ($CD31^+$ cells) and expression of angiogenic growth factors including angiogenin and, to a lesser extent, VEGF-A. Furthermore, angiogenin and B1R expressions were higher in old than young asthmatics and only angiogenin expression was significantly increased in severe compared to mild asthmatics. Angiogenin was also negatively related to FEV_1 in all asthmatics and positively related to B2R expression. Finally, we showed localization of B2R, VEGF-A and angiogenin in bronchial fibroblasts from old asthmatics and the ability of BK to release VEGF-A and angiogenin in bronchial fibroblasts from asthmatic patients and, to a lesser extent, from COPD.

Asthma and COPD are both chronic inflammatory diseases of the lung associated with structural remodeling that contributes to airway obstruction. However, as expected, and in agreement with the literature,[E1] immunohistology of bronchial biopsies demonstrated differences in the predominant inflammatory cells: old asthmatics having increased numbers of activated $CD4^+$ lymphocytes, eosinophils and neutrophils when compared to old control non-smokers, control smokers and COPD; young asthmatics having increased numbers of eosinophils and mast cells

compared to young controls. In contrast, COPD inflammation was predominantly characterized by $CD8^+$ lymphocytes, macrophages and neutrophils.[E12]

We found significant and positive correlations between numbers of neutrophils and $B2R^+$, $CD31^+$ or $angiogenin^+$ cells suggesting a role for this inflammatory cell in vascular remodeling of old asthmatics. In previous studies BK increased the release of IL-8 in airway epithelial cells through an autocrine generation of endogenous prostanoids [E13] and BK-induced IL-8 production, due to B2R stimulation, is in part dependent on the activation of ERK1/2 and p38 MAPK-pathway.[E14] These data, together with our report on positive correlations between neutrophils and angiogenic molecules may suggest a role for neutrophil-released mediators (including proteases) in modulating angiogenesis in asthma through activation of protease activated receptors.[E15]

Vascular remodeling and reactivity of the airways including angiogenesis, vasodilation and microvascular leakage are the most prominent and uniform findings in asthma.[2] Furthermore, bronchial vascular changes may also occur in COPD, particularly in the small airways.[2, E16] Microvascular changes in asthma and COPD may contribute to an increase in airway wall thickness which may be associated with severity of airway obstruction and disease progression.[E16, E17] Angiogenesis is a complex multiphase process, potentially involving a great number of growth factors, cytokines, chemokines and other factors. Some bronchial biopsy studies [E18, E19] as well as induced sputum studies [E20, E21] observed higher VEGF-A levels in asthmatic airways than those of healthy controls. In particular, immunohistochemical studies demonstrated close relationships between VEGF-A expression and vascularity.[E16, E17] Angiogenin, like VEGF-A, induces vascular endothelial cell proliferation, migration and tubule formation. In biopsies from asthmatic patients, a higher expression of basic fibroblast growth factor (bFGF) and angiogenin was reported, with significant correlations between the vascular

area and the number of angiogenic factor-positive cells within the airways.[E16] Furthermore, both VEGF-A and angiogenin are able to promote in vitro angiogenesis expressed as induction of primitive vascular tubule structures in culture with BALF collected from mild asthmatic subjects.[E22] It has been demonstrated additionally that COPD is associated with increased expression of VEGF-A in the bronchial, bronchiolar and alveolar epithelium, particularly in macrophages and in vascular and airway smooth muscle cells.[E23] In addition, a recent study showed also increased levels of VEGF-A and angiogenin in induced sputum from stable COPD compared to healthy smokers and non-smokers.[E24] We extend these observations by directly comparing patients with asthma, COPD and controls in relation to age. This is the first study in humans showing that the number of $CD31^+$ cells and $angiogenin^+$ cells was significantly reduced in old compared to young controls demonstrating that vascularity is probably influenced by age per se. VEGF-A appears to be expressed especially in the bronchial lamina propria of old asthmatics while angiogenin is significantly higher in old asthmatics and in COPD, though values were lower in COPD. Furthermore, $angiogenin^+$ cell expression in bronchial lamina propria negatively correlated with the level of FEV_1 in the group of asthmatics, suggesting that angiogenin expression in bronchial lamina propria is associated with the severity of asthma, confirming previous findings.[E16] The modest increase of angiogenin in the bronchi of our COPD patients, may be related to apoptosis of endothelial cells induced by VEGF-A reduction due to oxidative stress as described elsewhere.[E25] Finally, we showed for the first time a marked increase of VEGF-A expression in the bronchial lamina propria of non-atopic asthmatics compared to atopic asthmatics suggesting that in “intrinsic” asthma higher release of VEGF-A could specifically contribute to the formation of vascular remodeling scenario. Taken together, these results strengthen the hypothesis that enhanced vascularity of bronchial lamina propria is closely related to the expression of angiogenic factors, which may then contribute to the

pathogenesis or progression of asthma by the development of chronic alterations and airflow obstruction.[E18, E26]

Bradykinin exerts several effects in airways through both direct and indirect pathways which are mediated by activation of two main bradykinin receptor subtypes, B1 and B2, that differ in expression and ligand specificity. In tissues B1 receptors are inducible, whereas B2 receptors are constitutively expressed.[E27] The distribution of B2 receptors has been mapped out in human and guinea pig lung by autoradiography with [^{3}H] bradykinin.[E28] There is a high density of binding sites in bronchial and pulmonary vessels, particularly on endothelial cells. Epithelial cells, airway smooth muscle, submucosal glands, and nerves are also labelled, indicating that bradykinin may have diverse effects on airway function. A particularly high density of labelling is observed in the lamina propria immediately beneath the epithelium; the subepithelial binding sites link to nerves and superficial blood vessels.[E29] An immunohistochemical study in human nasal mucosa revealed the expression of both bradykinin B1 and B2 receptors in human nasal epithelial cells, submucosal glands, fibroblasts, vascular smooth muscle, vascular endothelial cells and macrophages; B2R expression, but not B1R, was found also in peripheral nerve fibers.[E30] Finally, the presence of B1R and B2R was confirmed both on neutrophils [E31] and on eosinophils [E32] of asthmatic and non-asthmatic subjects; a significantly greater protein expression of B1R and B2R was observed in eosinophils of asthmatics compared with those of non-asthmatic subjects. In the present study, for the first time we demonstrated the expression of bradykinin B1R and B2R in fibroblasts, epithelial cells and endothelial cells of the human bronchial lamina propria (by IHC and immunofluorescence analysis), and that their total expression is significantly increased in old asthmatic subjects compared with old control non-smokers, control smokers and COPD patients, suggesting a potential role for these receptors in the remodeling process in these patients.

BK promotes angiogenesis by up-regulation of bFGF through the B1 receptor or by stimulation of VEGF-A formation following B2 receptor stimulation.[E33, E34] In our study, B2R showed a positive correlation not only with B1R but also with CD31 endothelial cells and angiogenin suggesting that B2R may promote angiogenesis leading to blood vessel neo-formation. In particular, we also found by means of confocal analysis that the large majority of submucosal bronchial fibroblasts in old asthmatics co-expressed B2R, angiogenin and VEGF-A indicating a major role for fibroblasts in producing vascular growth factors potentially released upon stimulation of bradykinin B2R.

Bradykinin is involved in the repair processes inducing fibroblast proliferation and differentiation.[E35, E36] BK-induced fibroblast contraction, associated with differentiation into α-SMA myofibroblasts, is mediated through the activation of B2R.[E36] Bradykinin also regulates angiogenic growth factors expression (angiogenin and VEGF-A), as shown in human dental pulp fibroblasts in vitro.[E37] Other authors showed that human airway smooth muscle cells secrete VEGF-A and this secretion is induced by BK via B2R.[E33] In our in vitro study, we demonstrated for the first time that bradykinin induced VEGF-A and angiogenin release in primary cultures of human bronchial fibroblasts from asthma patients (1.5-fold and 6-fold increase, respectively, in comparison with unstimulated fibroblasts) and that this effect is mediated by B2R, as confirmed by the use of the B2R specific antagonist HOE140 which completely blocked BK-induced VEGF-A and angiogenin release. In addition, we showed similar effects, but to a lesser extent, in bronchial fibroblasts from COPD patients suggesting that bronchial fibroblasts from asthmatics are more prone to release angiogenic factors.

In conclusion, this study demonstrates an increased expression of bradykinin receptors in relation to vascular remodeling in asthma and suggests a role for bronchial asthmatic fibroblasts in releasing vascular endothelial growth factor and angiogenin upon activation of bradykinin B2

receptors. Our study showed that markers of vascular remodeling are major features of old asthmatics and are potentially involved in fixed airway obstruction by increasing airway wall thickness and stiffness. The increased vascular reactivity of asthmatic airways, due to different stimuli like bradykinin, [E38] in disease-related vascular remodeling might participate to excessive airway variability which is a typical pattern of airway patency in asthma. Previous observations showed kinin involvement in airway hyperresponsiveness in asthma,[E39] and the capability of selective B2R antagonists to treat acute attacks of hereditary angioedema,[E40] and to improve pulmonary function in asthmatics.[E41] These data, combined, lead us to encourage the planning of long term studies using these selective antagonists in asthma treatment in order to inhibit bradykinin B2 receptor-mediated angiogenic effects and its related remodeling processes.

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FIGURE LEGENDS

Figure E1. Photomicrographs showing staining of the bronchial lamina propria biopsy specimens from old asthmatics with antibodies to inflammatory cells: CD4 lymphocytes (A), CD8 lymphocytes (B), $CD68^+$ cells (C), neutrophils (D), eosinophils (E) and mast cells (F). Arrows indicate some of the cells that react with each inflammatory cell antibody**.** The results are representative of those from 24 old asthmatics. E=epithelium. Original magnification: 400X.

Figure E2. Individual counts for CD4 lymphocytes, neutrophils, eosinophils (A) and $angiogenin^+$ cells (B) in the bronchial lamina propria of severe asthmatics (solid circles) and mild asthmatics (open circles). The results are expressed as the number of positive cells/mm^2 of bronchial lamina propria.

Figure E3. Photomicrographs showing staining by IHC of nasal polyps sections (panels A-H) with normal goat (A), rabbit (B) or mouse (C) non-specific immunoglobulins (negative controls) and with antibodies to VEGF (D), angiogenin (G), CD31 (F), B2R (H) and B1R (E) (positive controls). Arrows indicate some of the cells that react with each antibody**.** Original magnification: 400X.

Figure E4. Photomicrographs showing staining by immunofluorescence of nasal polyps sections (panels A-H) with Alexa Fluor 488-anti mouse, Alexa Fluor 647-anti rabbit and Alexa Fluor 647-anti goat antibodies in the following conditions: A: Alexa Fluor 488-anti mouse staining in presence of normal mouse non-specific immunoglobulins (negative control); B: Alexa Fluor 647-anti rabbit staining in presence of normal rabbit non-specific immunoglobulins (negative

control); C: Alexa Fluor 647-anti goat staining in presence of normal goat non-specific immunoglobulins (negative control); D,G: Alexa Fluor 488-anti mouse staining in presence of mouse anti human 5B5 (D) or mouse anti human CD31 (G); E: Alexa Fluor 647-anti rabbit staining in presence of rabbit anti human B2R; F,H: Alexa Fluor 647-anti goat staining in presence of goat anti human VEGF (F) or goat anti human angiogenin (H). Original magnification: 200X.