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**

FEV₁ and FEV₁/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 FEV₁ and FEV₁/FVC% measurements in the groups of subjects with FEV₁/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/mm², 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 \( \alpha \)-smooth muscle actin (\( \alpha \)-SMA, Dako) was assessed.

Both types of fibroblasts were negative for cytokeratin, positive for vimentin (>50%), ASO2 (100%), fibronectin (>50%) and \( \alpha \)-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/mm²) compared with old control non-smokers (9 [0-28] cells/mm², p=0.0002), control smokers (12 [0-62.5] cells/mm², p<0.0001) and COPD patients (69 [29-92] cells/mm², 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/mm²) compared with old control non-smokers (71 [44-157] cells/mm², p=0.0004), control smokers (93 [44-179] cells/mm², p<0.0001) and COPD patients (83 [32-172] cells/mm², 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/mm²) compared to old control non-smokers (22 [18-95] cells/mm², p=0.0014), control smokers (32 [18-59] cells/mm², p<0.0001) and COPD patients (32 [6.4-64.5] cells/mm², 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 FEV1 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 FEV₁ 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 \( \alpha \)-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.

REFERENCES


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/mm² 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.