Anti-angiogenic activity of carebastine: a plausible mechanism affecting airway remodelling

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ABSTRACT: Ebastine is a well-known selective second-generation histamine H_1 receptor antagonist, which is used for seasonal and perennial allergic rhinitis and chronic urticaria. Angiogenesis plays a crucial role in the development of airway inflammation and remodelling in allergic rhinitis and asthmatic patients, in whom, indeed, the mucosa displays increased vascularity and overexpression of vascular endothelial growth factor (VEGF). The aim of the present study was to evaluate the anti-angiogenic properties of carebastine, the active metabolite of ebastine.

The effects of carebastine on human umbilical vein endothelial cell (EC) (HUVEC) and human pulmonary artery EC (HPAEC) proliferation, migration and capillary-like tube formation were investigated *in vitro*, and in the chick embryo chorioallantoic membrane (CAM) assay *in vivo*. Moreover, the effect of carebastine on phosphorylation of the cell VEGF receptor fetal liver kinase-1, or VEGF receptor 2 (VEGFR-2), and Akt kinase (Akt) was evaluated by Western blotting.

Carebastine inhibited VEGF-induced HUVEC and HPAEC proliferation, migration and angiogenesis in a dose-dependent manner *in vitro*. Cell proliferation was inhibited by 42 and 64% in HUVECs and 62 and 75% in HPAECs upon exposure for 48 and 72 h, respectively, to 20 μM carebastine ($p \leqslant 0.03$), and even more with 30 μM carebastine. Cell migration was inhibited by 37 and 70% in HUVECs ($p \leqslant 0.03$) and 60 and 78% in HPAECs ($p \leqslant 0.01$) in the presence of 10 and 30 μM carebastine, respectively. Carebastine (20 μM) caused a significant reduction (70-86%; $p < 0.01$) in topological parameters of the capillary network produced *in vitro* by both EC lines on a basement membrane extract. Carebastine (30 and 50 μM) inhibited the VEGF-induced angiogenesis in the CAM assay *in vivo* two- and three-fold, respectively ($p < 0.001$). Finally, both EC lines, on exposure to 10 and 20 μM carebastine, showed a four- to six-fold reduction ($p \leqslant 0.01$) in both VEGF- and H_1 receptor-induced VEGFR-2 and Akt phosphorylation.

Overall, these data provide the first evidence regarding the anti-angiogenic activity of ebastine, and suggest its potential use as an anti-angiogenic molecule, besides its antihistaminic activity for the treatment of allergic diseases in which angiogenesis takes place.

KEYWORDS: Allergic diseases, anti-angiogenesis, asthma, carebastine, ebastine, endothelial cells

Histamine H_1 receptor antagonists are commonly used for symptomatic relief in the treatment of hypersensitive reactions, such as allergic rhinitis, rhinoconjunctivitis and urticaria. The ability of antihistamines to inhibit histamine release by mast cells is well known, whereas their additional properties, which contribute to their clinical efficacy, are incompletely understood. It has been reported that some antihistamines regulate the expression and/or release of cytokines, chemokines, adhesion molecules and other inflammatory mediators, and, consequently, that they inhibit the recruitment of inflammatory cells [1].

The anti-inflammatory effect of H_1 receptor antagonists may involve a receptor-dependent mechanism *via* the inhibition of secretion of nuclear factor-κB (NF-κB)-dependent cytokines, such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) 2 and hepatocyte growth factor, or the inhibition of

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adhesion molecules [2–4]. Others have described receptor-independent mechanisms involving the release of preformed mediators, such as histamine and eosinophil proteins, as well as production of eicosanoid and oxygen free radicals [5–7].

Ebastine (4-diphenylmethoxy-1[3-(4-terbutyl-benzoyl)-propyl] piperidine) is a second-generation selective and potent H_1 receptor antagonist with no anticholinergic or sedative effects, used for seasonal and perennial allergic rhinitis and chronic urticaria. Following oral administration, it is rapidly converted into its active metabolite, carebastine. It inhibits the secretion of interleukin (IL)-4, IL-5, IL-6 and tumour necrosis factor-α by inflammatory cells [8].

Recent findings indicate that angiogenesis (new vessel formation) is involved in both the development of airway inflammation and pathophysiology of airway remodelling during the course of allergic rhinitis and asthma [9, 10]. Indeed, major structural and functional changes in the airway microcirculation include angiogenesis, increased blood flow and microvascular permeability, and oedema formation in the airway wall [11]. Increased expression of angiogenic molecules, such as VEGF, FGF-2 and angiogenin and their receptors, has been correlated with disease severity and accelerated decline in lung function [12–20]. Others have shown that bronchoalveolar lavage fluid (BALF) from asthmatic patients exhibits enhanced *in vitro* angiogenic activity and increased FGF-2 [21] and VEGF levels [22]; the BALF angiogenic activity is blocked by an anti-VEGF antibody [22]. In addition, VEGF levels in the BALF of asthmatic patients were increased in proportion to the vessel number in the bronchial mucosa, which, indeed, showed intense VEGF, VEGF receptor (VEGFR)-1 and VEGFR-2 (or fetal liver kinase-1/kinase insert domain receptor) staining [20].

The direct contribution of VEGF to the allergic response in asthma has been proposed by LEE *et al.* [23], who generated lung-targeted $VEGF_{165}$ transgenic mice and evaluated the role of VEGF in antigen-induced type-2 T-helper-cell-mediated inflammation. Overexpression of VEGF induced angiogenesis, oedema, vascular remodelling, leukocyte infiltration into the lung, collagen deposition, overexpression of IL-13 (which promotes the collagen deposition into the basement membrane), increased mucus production and smooth muscle cell hyperplasia.

The aim of the present study was to investigate the anti-angiogenic activity of carebastine by using *in vitro* and *in vivo* assays. The former include human umbilical vein endothelial cell (EC) (HUVEC) and human pulmonary artery EC (HPAEC) proliferation, migration and capillary-like tube formation; the latter is the chick embryo chorioallantoic membrane (CAM) assay. Moreover, the effect of carebastine on cell VEGFR-2 and Akt kinase (Akt) phosphorylation was evaluated by Western blotting.

MATERIALS AND METHODS
Cell culture and in vitro functional assays
HUVECs were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA), and HPAECs were from Lonza (Walkersville, MD, USA). Both cell lines were cultured in EC basal medium EBM-2® supplemented with SingleQuots® (Lonza) and 10% fetal bovine serum (Gibco®,

Invitrogen Corporation, Carlsbad, CA, USA) (complete medium) at 37°C in a 5% CO_2 atmosphere.

HUVEC and HPAEC proliferation was evaluated as previously described [24]; 5×10^4 cells·well^{-1} in 96-well plates were cultured for 24, 48 and 72 h in starvation serum-free medium (SFM; negative control) or complete medium admixed with 10 ng·mL^{-1} $VEGF_{165}$ (recombinant human $VEGF_{165}$; Sigma-Aldrich, Milwaukee, WI, USA; positive control) or supplemented with 5–30 μM carebastine (kindly supplied by Almirall, Barcelona, Spain) in five independent experiments performed in triplicate. Cells were counted using the 3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyl tetrazolium bromide (MTT) cell proliferation assay (ATCC), and expressed as mean±SD percentage of control. The EC number was also estimated using a slightly modified [24] crystal violet colorimetric method [25]. No aspecific cytotoxic effects (nuclear pyknosis, cytoplasmic vescicles and granules, round cell shape or cell detachment from supports) were seen in either HUVECs or HPAECs with carebastine, even at 30 μM.

The chemotactic assay was performed in Boyden microchambers, as previously described [26], towards SFM alone (negative control) or admixed with 10 ng·mL^{-1} $VEGF_{165}$ alone (positive control) or together with the carebastine doses in five independent experiments performed in triplicate. After 4 h, migrated cells were counted on five 1,000× magnification oil-immersion fields·membrane^{-1}, and presented as mean±SD percentage.

In vitro angiogenesis assay
HUVECs and HPAECs (1×10^5 cells) were placed in 24-well plates pre-coated with Cultrex® basement membrane extract (Trevigen, Inc., Gaithersburg, MD, USA; 400 μL·well^{-1}) in 1 mL·well^{-1} of SFM alone (negative control) or complete medium supplemented with 10 ng·mL^{-1} $VEGF_{165}$ alone (positive control) or together with carebastine doses in five independent experiments performed in duplicate, and incubated at 37°C in a 5% CO_2 atmosphere. After an 18-h incubation, tube and mesh formation were examined using a reverted phase-contrast microscope (Leitz DM IRB; Leica Microsystems, Wetzlar, Germany). Then the skeletonisation of the mesh was followed by measurement of its topological parameters, *i.e.* 1) number of areas, 2) vessel length, and 3) number of branching points, using computer image analysis as previously described [27], and presented as mean±SD percentage inhibition *versus* the positive control. Representative fields were photographed using a Leica D-Lux3 digital camera (Leica Camera, Solms, Germany).

Chorioallantoic membrane assay
The CAM assay was performed in fertilised White Leghorn chicken eggs incubated at 37°C at constant humidity as previously described [28]. On incubation day 3, a square window was opened in the shell and 2–3 mL albumen was removed in order to detach the CAM. On day 8, the CAMs were implanted with 1-mm^3 sterilised gelatin sponges (Gelfoam Upjohn, Kalamazoo, MI, USA) loaded with 1 μL medium alone (negative control) or added with 250 ng $VEGF_{165}$ alone (positive control) or combined with 30 or 50 μM carebastine in five independent experiments for each dose and control performed in duplicate. No embryo death

was seen, even with 50 μM carebastine. The angiogenic response was evaluated on day 12 as the number of vessels converging toward the sponge at $50\times$ magnification, and photographed *in ovo* using an Olympus stereomicroscope (Olympus, Rozzano, Italy).

Western blotting

HUVECs and HPAECs were grown in six-well plate dishes in SFM containing 0.1% bovine serum albumin for 18–20 h. Then the cells were exposed to 10 ng·mL^{-1} VEGF$_{165}$ alone or combined with 30 μM histamine (Sigma-Aldrich) followed by 10 and 20 μM carebastine, and then lysed in a buffer containing 25 mM tris(hydroxymethyl)aminomethane (Tris)/HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium dodecylsulfate (SDS), 1 mM NaF, 1 mM Na$_3$VO$_4$ and a protease inhibitor cocktail (Sigma-Aldrich). Total protein concentrations were determined by the Bradford method using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA). Aliquots (30 μg protein) of the cell lysate were then separated by SDS-polyacrylamide gel electrophoresis (PAGE) using precast 4–12% NuPAGE® Novex® Bis-Tris gels (Invitrogen Corporation) under reducing conditions, electrotransferred to a polyvinylidene difluoride membrane (PerkinElmer Life Sciences, Inc., Boston, MA, USA) and incubated with the primary and secondary antibodies (antibodies directed against VEGFR-2, Akt and phospho-Akt (serine 473) were from Cell Signaling Technology (Danvers, MA, USA), against phospho-VEGFR-2 (tyrosine 1054) were from Abcam (Cambridge, UK), against β-actin, and goat immunoglobulin (Ig)G (horseradish peroxidase-conjugated) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and against mouse and rabbit IgG (horseradish peroxidase-conjugated) were from Bio-Rad). Then bands were visualised by enhanced chemiluminescence (SuperSignal® West Pico substrate; Thermo Scientific, Inc., Waltham, MA, USA) using the Gel Logic 1500 Imaging System (Eastman Kodak Co., Rochester, NY, USA) and their intensity quantified using the Kodak Molecular Imaging Software, and expressed as mean±SD arbitrary optical density units based on five independent experiments.

For inhibition experiments, ECs were treated with 5 μg·mL^{-1} anti-human VEGFR-2 neutralising monoclonal antibody (mAb) (R&D Systems, Inc., Minneapolis, MN, USA) for 24 h, and then exposed sequentially to 30 μM histamine for 10 min and 10 μM carebastine for 1 h. In separate experiments, ECs were treated with 20 μM mepyramine (H$_1$ receptor inhibitor; Sigma Chemical Co., St Louis, MO, USA) for 1 h, and then exposed sequentially to 10 ng·mL^{-1} VEGF$_{165}$ for 10 min and carebastine as above. The dose of mepyramine was chosen on the basis of a molar excess *versus* carebastine. Cells were then lysed and processed for Western blotting.

RESULTS

Effect of carebastine on in vitro *HUVEC and HPAEC proliferation, chemotaxis and angiogenesis*

The HUVEC and HPAEC proliferative response to VEGF$_{165}$ was significantly reduced by carebastine in a dose-dependent fashion, being maximal after a 72 h exposure to 30 μM carebastine in both HUVECs (65% reduction) and HPAECs (80% reduction) (fig. 1a and b). A significant lowering of cell proliferation was also achieved with 20 μM carebastine after

both 48 h (HUVECs 42% reduction and HPAECs 62% reduction; $p \leqslant 0.03$ (two-way ANOVA)) and 72 h (HUVECs 64% reduction and HPAECs 75% reduction; $p \leqslant 0.01$), with median inhibitory concentrations of 28.7 ± 6.9 and 16.5 ± 4.7 μM, respectively. These results were obtained using the MTT method and confirmed with the crystal violet colorimetric method (fig. 1 of online supplementary material). Carebastine exerted an inhibitory effect on the VEGF$_{165}$-induced HUVEC and HPAEC migration in the chemotaxis assay (fig. 1c and d). The cell migration was inhibited by 12 and 45%, 37, ($p<0.03$ (one-way ANOVA)) and 60% ($p<0.01$), 46 and 68% ($p<0.01$), and 70 and 78% in the presence of 5, 10, 20 and 30 μM carebastine, respectively.

Carebastine significantly inhibited HUVEC and HPAEC angiogenesis *in vitro* on the basement membrane extract. As shown in figure 2, after an 18-h incubation VEGF$_{165}$-exposed HUVECs produced a closely knit capillary network with thin, branching and anastomosing tubes linked through numerous junctions; mesh area counts were 31.2 ± 7, vessel length $6,814 \pm 618$ μm and branching point counts 37 ± 5. Exposure to carebastine at 1 μM, and even more at 20 μM, gave rise, in a dose-dependent manner, to a poorly organised plexus with few straight and disorganised tubes with scarce junctions; mesh area counts were 19.6 ± 4.2 (37% reduction) and 6.3 ± 2.8 (79% reduction), vessel length $4,225 \pm 313$ (38% reduction) and $1,135 \pm 126$ μm (83% reduction), and branching point counts 24 ± 4 (37% reduction) and 11 ± 3 (70% reduction) respectively ($p \leqslant 0.01$ (paired t-test)). Overlapping changes were observed in the HPAEC capillary network following exposure to 20 μM carebastine (82% reduction in mesh areas, 86% reduction in vessel length and 75% reduction in branching points; data not shown).

Effect of carebastine on in vivo *angiogenesis in the CAM assay*

CAMs treated with sponges loaded with VEGF$_{165}$ (positive control) were surrounded by allantoic vessels as newly formed capillaries converged radially towards the sponge in a spoke-wheel pattern (27 ± 3 vessels; fig. 3b). No vascular response was detectable around the sponges loaded with vehicle alone (6 ± 2 vessels; fig. 3a). When 30 and 50 μM carebastine (fig. 3c) were added to VEGF$_{165}$, a significant reduction in the angiogenic response was found (14 ± 2 and 8 ± 2 vessels respectively; $p \leqslant 0.01$ (paired t-test).

Effect of carebastine on VEGF-induced phosphorylation of VEGFR-2 and Akt

In vivo experiments provided evidence that carebastine exerts an inhibitory effect on the VEGF-induced angiogenic response. In order to determine whether carebastine was able to inhibit VEGFR-2 and Akt phosphorylation, Western blotting experiments were performed in VEGF$_{165}$-treated HUVECs. As shown in figure 4a, 10 μM carebastine markedly blocked the VEGF-dependent phosphorylation of VEGFR-2 (tyrosine 1054) 30 min after treatment, and the inhibitory effect was still detectable after a 24-h exposure to the drug. Moreover, the inhibition of receptor phosphorylation correlated with a marked decreased in the ability of VEGF to induce Akt phosphorylation under the same experimental conditions. As shown in figure 4b and c, 10 and 20 μM carebastine significantly
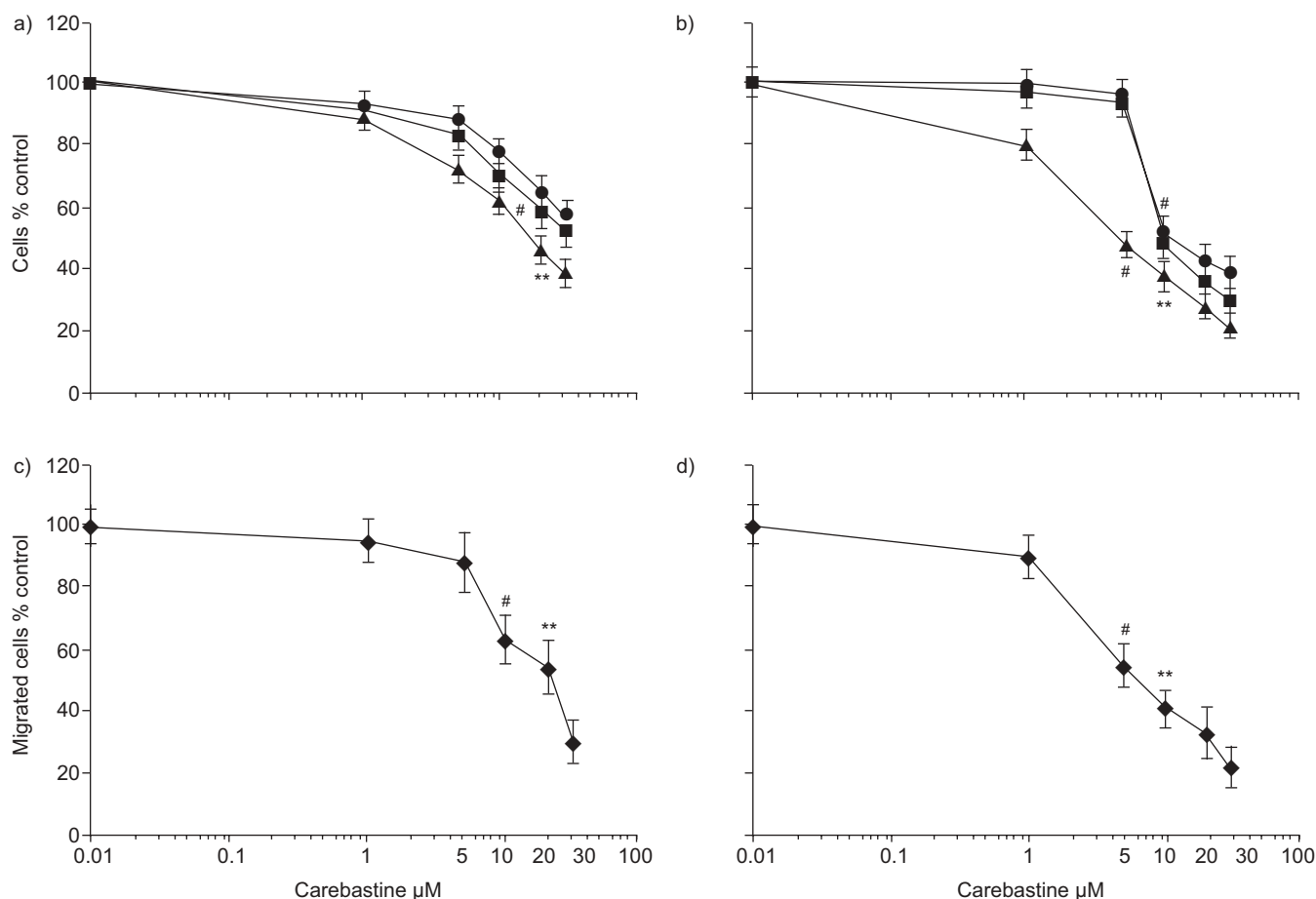
FIGURE 1. Effect of carebastine on the proliferation and migration of endothelial cells (ECs). Effects of different carebastine doses on vascular endothelial growth factor (VEGF)$_{165}$-induced cell proliferation of: a) human umbilical vein ECs (HUVECs); and b) human pulmonary artery ECs (HPAECs) at 24 (●), 48 (■) and 72 h (▲). Effects of the same doses on VEGF$_{165}$-induced chemotaxis of: c) HUVECs; and d) HPAECs. Both cell types were plated on a collagen-coated polycarbonate membrane in a Boyden microchamber assay, and exposed to VEGF$_{165}$ and carebastine at the indicated doses for 4 h. Data are presented as mean $\pm$ SD from five independent experiments performed in triplicate. **: $p < 0.01$; #: $p < 0.03$ (two-way ANOVA).

inhibited the VEGF-induced phosphorylation of both VEGFR-2 and Akt in a dose-dependent manner, and consequently blocked HUVEC survival.

Effect of carebastine on the histamine signalling pathway mediated by the H$_1$ receptor in HUVECs and HPAECs

In order to verify whether the H$_1$ receptor was involved in the activation of VEGFR-2 and Akt, the effect of histamine treatment on protein phosphorylation was investigated. Western blotting analysis showed that stimulation of both ECs with VEGF$_{165}$ followed by histamine produced a rapid (after a 10-min exposure) and sizeable increase in the phosphorylation of VEGFR-2 and Akt. When 10 and 20 μM carebastine was added, it significantly blocked the histamine-stimulated phosphorylation of both VEGFR-2 and Akt in HUVECs (fig. 5a, c and e) and HPAECs (fig. 5b, d and f), demonstrating that inhibition of the VEGFR-2 and Akt pathways by carebastine in both EC types is mediated by the blocking of H$_1$ receptor activity.

In order to investigate whether carebastine was able to inhibit VEGFR-2 phosphorylation *via* simultaneous interference with

the H$_1$ receptor and VEGFR-2, inhibition experiments were performed with a neutralising anti-VEGFR-2 mAb and, separately, with mepyramine, an inhibitor of the H$_1$ receptor. As shown in figure 6, blockade of VEGFR-2 produced marked inhibition of VEGFR-2 phosphorylation in histamine-stimulated HUVECs and HPAECs. In addition, slight inhibition was observed in VEGF$_{165}$-stimulated cells blocked in the H$_1$ receptor with mepyramine and exposed to carebastine. The data suggest that the direct inhibitory activity of carebastine is mediated mostly by the H$_1$ receptor and, to a lesser extent, by VEGFR-2.

DISCUSSION

Enhanced angiogenesis is involved in many aspects of tissue inflammation and remodelling, and contributes to abnormal airway function, including the increased mucosal reactivity to agents in allergic rhinitis and fixed airflow obstruction with progressive decline in lung function in asthma [9, 10]. The complex network between allergic inflammation and angiogenesis and the limited benefit of the classic therapeutic approaches to airway remodelling in inflammatory airway diseases, such as allergic rhinitis and asthma, have led to
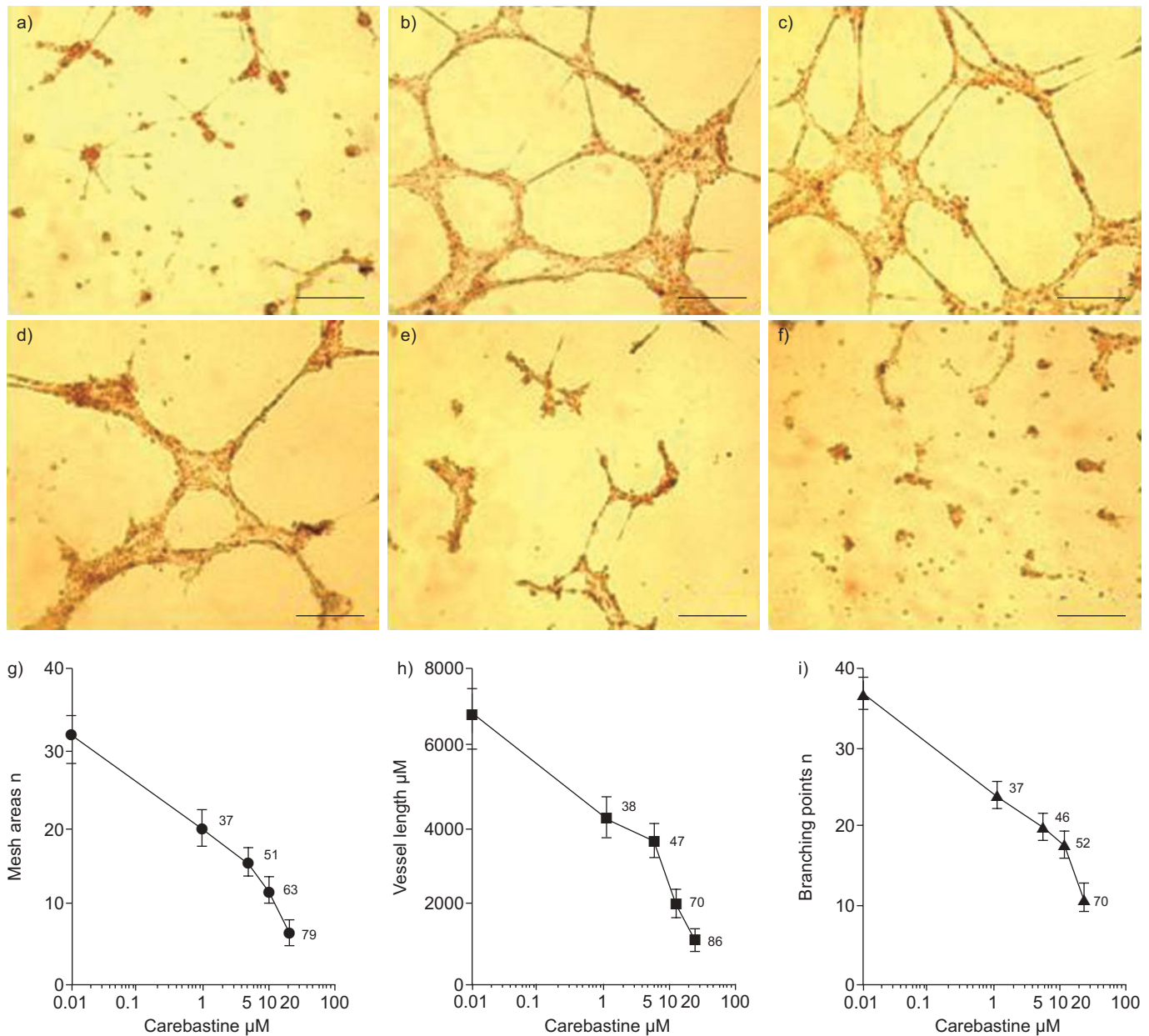
FIGURE 2. Effect of carebastine on *in vitro* angiogenesis. Human umbilical vein endothelial cells were seeded on Cultrex® basement membrane extract (Trevigen, Inc., Gaithersburg, MD, USA) in: a) serum-free medium (SFM) alone; and b) SFM supplemented with vascular endothelial growth factor$_{165}$ alone; and together with: c) 1 μM carebastine; d) 5 μM carebastine; e) 10 μM carebastine; and f) 20 μM carebastine. Tube formation was observed periodically using a phase-contrast microscope and photomicrographs were taken after an 18-h incubation. One representative experiment is shown. Skeletonisation of the mesh was followed by measurement of its topological parameters: g) mesh areas; h) vessel length; and i) branching points. Data are presented as mean ± SD from five independent experiments performed in duplicate. Numbers indicate percentage inhibition. Scale bars=15 μm.

consideration of the use of angiogenesis inhibitors to control inflammation, angiogenesis and remodelling in allergic disorders.

Herein, it has been demonstrated for the first time that ebastine, a well-known selective and potent H_1 receptor antagonist widely used for seasonal and perennial allergic rhinitis and chronic urticaria, exhibits potent anti-angiogenic activity, as demonstrated by *in vitro* and *in vivo* assays. Experiments performed using the *in vivo* CAM assay have

provided evidence that carebastine (the active metabolite) exerts an inhibitory effect on the VEGF-induced angiogenic response. These results were confirmed by Western blotting, indicating that the inhibition of VEGFR-2 phosphorylation by carebastine interferes with the signalling pathways triggered by VEGF. Moreover, the inhibition of receptor phosphorylation correlated with a marked decrease in the ability of VEGF to induce Akt activation under the same experimental conditions. Finally, carebastine was also able to significantly block histamine-stimulated phosphorylation of both VEGFR-2 and

FIGURE 3. Effect of carebastine on *in vivo* angiogenesis (chorioallantoic membrane (CAM) assay). CAMs were implanted with sponges loaded with: a) serum-free medium (SFM) alone; b) SFM supplemented with vascular endothelial growth factor (VEGF)$_{165}$ alone; and c) with 50 µM carebastine. VEGF induced a strong angiogenic response in the form of newly formed capillary vessels, whereas no vascular response was detectable around the sponges loaded with SFM alone. Carebastine inhibited the VEGF$_{165}$-induced angiogenic response. One representative experiment of five independent experiments performed in duplicate for the carebastine dose and controls are shown. Scale bars=1 mm.
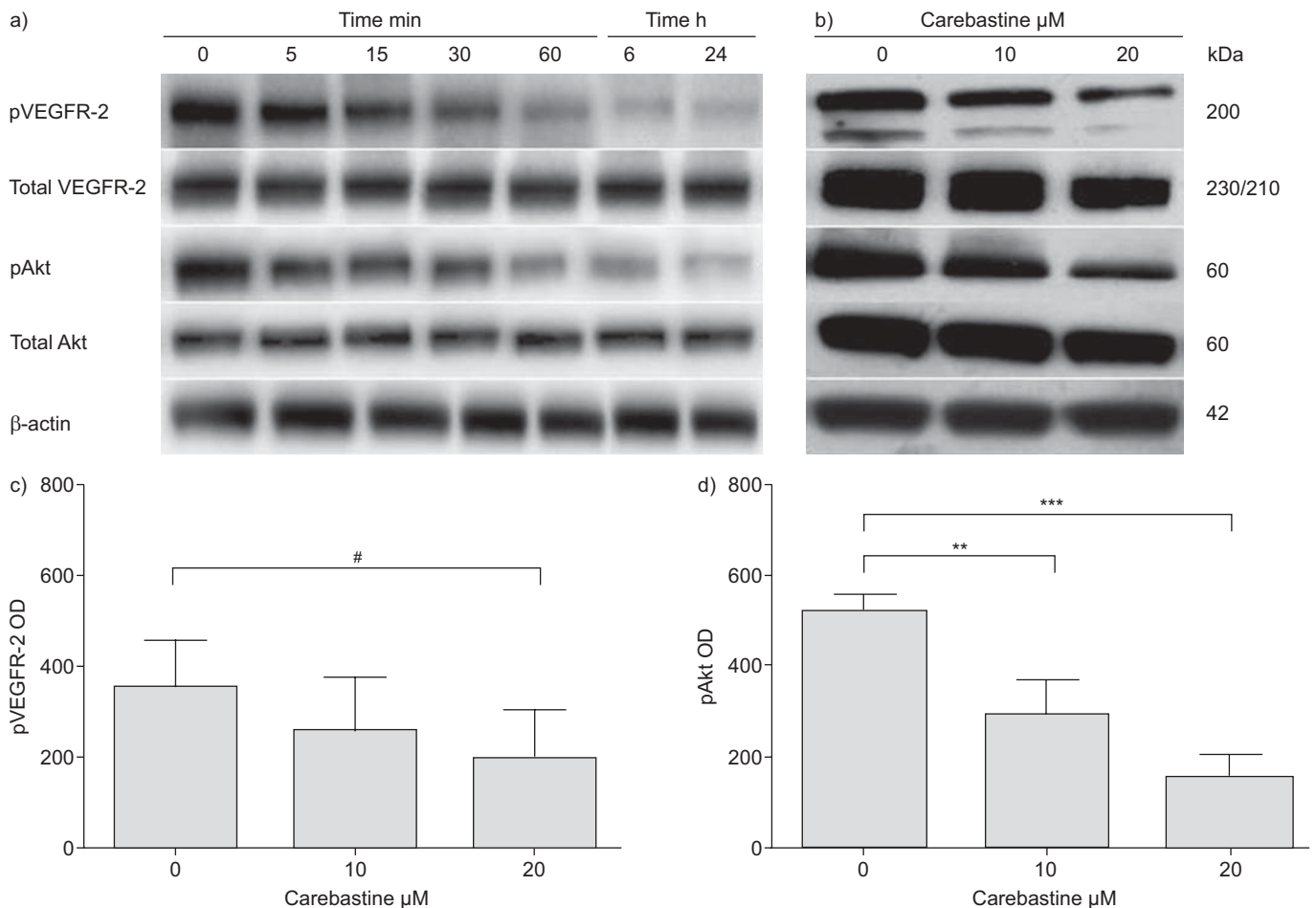

FIGURE 4. Western blotting demonstrating the effect of carebastine on phosphorylation of vascular endothelial growth factor (VEGF) receptor (VEGFR)-2 and Akt kinase (Akt). a) Time-course of phosphorylation of VEGFR-2 (pVEGFR-2) and Akt (pAkt) in response to carebastine. Human umbilical vein endothelial cells were synchronised in serum-free medium for 18–20 h and then treated with VEGF$_{165}$ and 10 µM carebastine for 24 h. Samples were subsequently analysed on a separate blot using antibodies against nonphosphorylated (total) VEGFR-2 and Akt in order to confirm equal loading in each lane. b) Dose-dependence of the inhibitory effect on VEGFR-2 and Akt phosphorylation of 10 and 20 µM carebastine. The lanes shown are representative of five independent experiments. Densitometric analysis of: c) pVEGFR-2; and d) pAkt. The analysis was performed with the Kodak Molecular Imaging Software (Eastman Kodak Co., Rochester, NY, USA) and is presented using mean±SD arbitrary optical density (OD) units. **: $p<0.01$; ***: $p<0.001$; #: $p<0.03$ (one-way ANOVA).
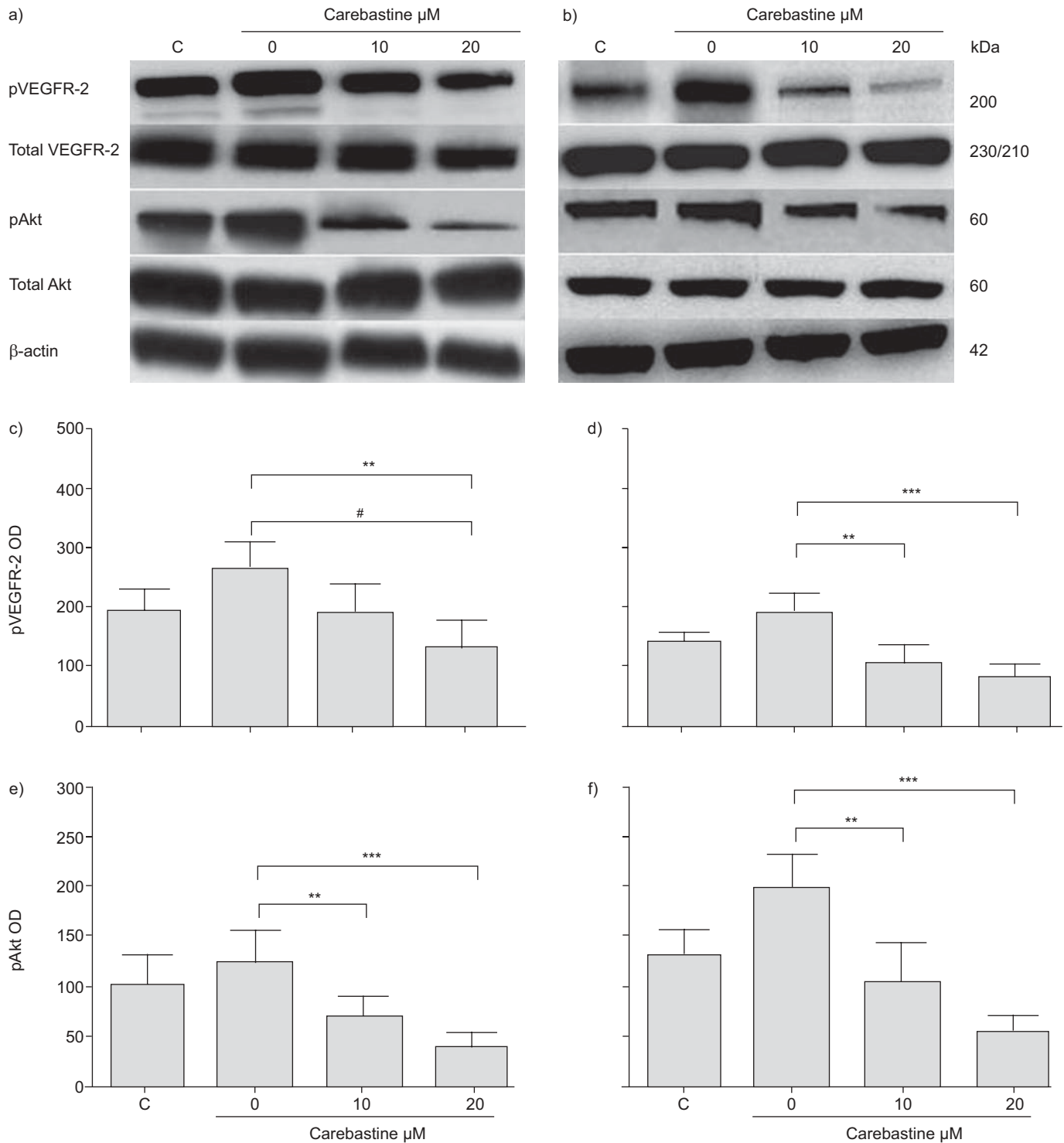
FIGURE 5. Western blotting demonstrating the effect of carebastine on vascular endothelial growth factor (VEGF) receptor (VEGFR)-2 and Akt kinase (Akt) phosphorylation mediated by histamine H_1 receptor. Increased VEGFR-2 and Akt phosphorylation induced by 30 μM histamine, and inhibition of H_1 receptor-induced phosporylation by 10 and 20 μM carebastine in: a) human umbilical vein endothelial cells (HUVECs); and b) human pulmonary artery endothelial cells (HPAECs). Cells were synchronised in serum-free medium for 18–20 h and then treated with $VEGF_{165}$ alone (control (C)) or in combination with 30 μM histamine alone or added with 10 or 20 μM carebastine. Samples were subsequently analysed on a separate blot using antibodies against nonphosphorylated (total) VEGFR-2 and Akt in order to confirm equal loading in each lane. The lanes shown are representative of five independent experiments. Densitometric analysis of: c, d) pVEGFR-2; and e, f) pAkt in: c, e) HUVECs; and d, f) HPAECs. The analysis was performed with the Kodak Molecular Imaging Software (Eastman Kodak Co., Rochester, NY, USA) and is presented using mean$\pm$SD arbitrary optical density (OD) units. **: $p<0.01$; ***: $p<0.001$; #: $p<0.03$ (one-way ANOVA).
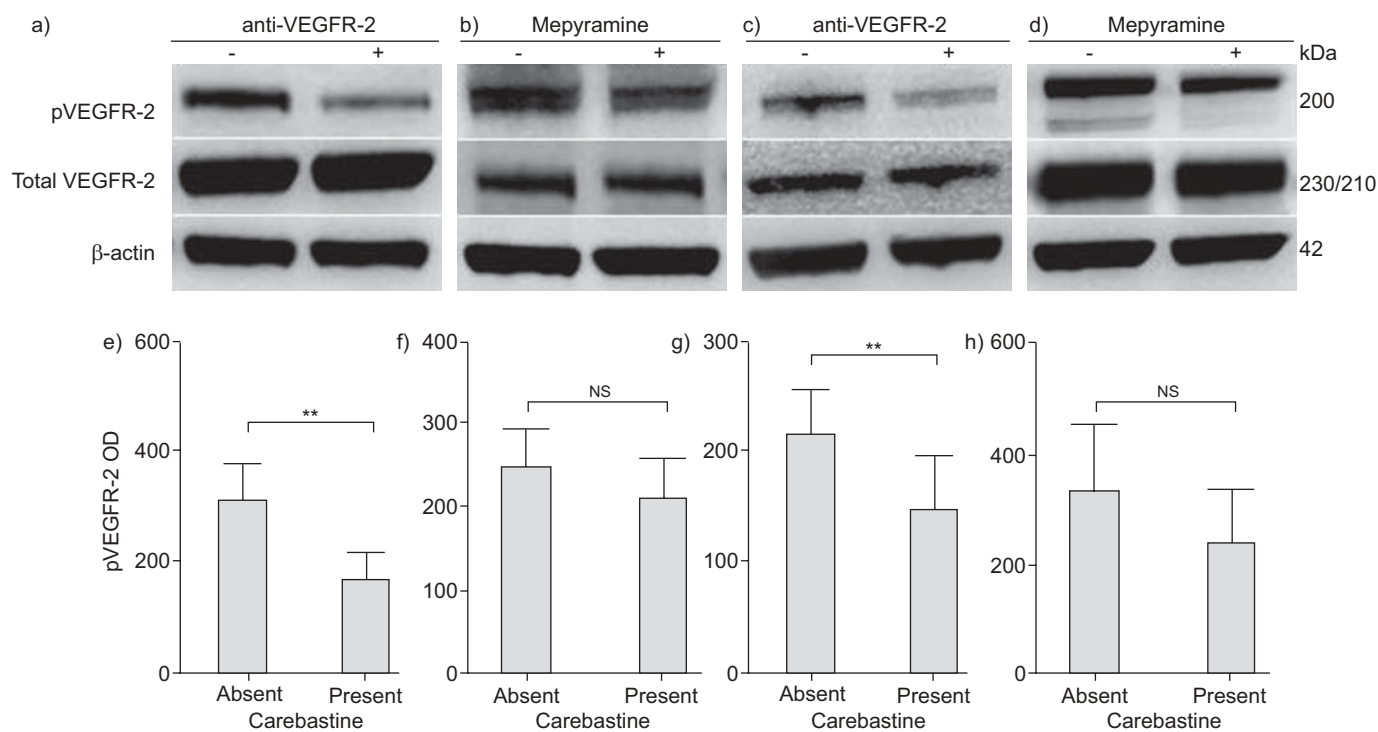
FIGURE 6. Separate blockade in: a, b) human umbilical vein endothelial cells (HUVECs); and c, d) human pulmonary artery endothelial cells (HPAECs) of vascular endothelial growth factor receptor (VEGFR)-2 and histamine H_1 receptor by: a, c) a neutralising anti-VEGFR-2 antibody; and b, d) mepyramine with histamine in the absence (-) and presence (+) of 10 µM carebastine. It should be noted that the greatest inhibition of VEGFR-2 phosphorylation (pVEGFR-2) in both HUVECs (a) and HPAECs (c) is caused by interference of carebastine with the H_1 receptor. In contrast, interference with VEGFR-2 produces minimal inhibitory effects (b and d). The lanes shown are representative of five independent experiments. Densitometric analysis of pVEGFR-2 in: e, f) HUVECs; and g, h) HPAECs. The analysis was performed with the Kodak Molecular Imaging Software (Eastman Kodak Co., Rochester, NY, USA) and is presented using mean ± SD arbitrary optical density (OD) units. NS: nonsignificant. **: $p < 0.01$ (one-way ANOVA).

the downstream Akt, suggesting that the inhibition of the VEGFR-2 and Akt pathways is mediated through blocking H_1 receptor activity. In separate inhibition experiments involving VEGFR-2 and H_1 receptor, it was found that carebastine inhibited VEGFR-2 phosphorylation mostly *via* interference with the H_1 receptor. Thus it may well be that signalling downstream of the H_1 receptor and VEGFR-2 are closely interrelated, perhaps *via* Akt. In addition, it was found that carebastine inhibited, albeit to a lesser extent, VEGFR-2 phosphorylation following H_1 receptor blockade. Thus an interference of carebastine with VEGFR-2, perhaps *via* aspecific mechanisms, can be hypothesised. Overall, the data suggest that the anti-angiogenic activity of carebastine is mediated through both a H_1 receptor-dependent and -independent mechanism, though the former is prevalent.

In addition, it is conceivable to hypothesise that the H_1 receptor signalling affected by carebastine activates protein kinase (PK) C instead of PKA, since a PKC activator (phorbol-12-myristate-13-acetate) enhances H_1 receptor activation, whereas an activator of PKA does not [29]. Furthermore, histamine-induced activation of the H_1 receptor is fully suppressed by the PKC inhibitor Ro-31-8220 [30]. Downregulation of NF-κB may be also involved in the mechanism of action of carebastine. Indeed, low concentrations of H_1 receptor antagonists (cetirizine and azelastine) have been demonstrated to downregulate

NF-κB expression in parallel to inhibition of pro-inflammatory cytokines [7].

VEGF seems to be the most important factor involved in the angiogenic processes taking place in allergic rhinitis [9] and asthma [10], and plays an important role in disease development and persistence. Indeed, both VEGF and expression of its receptor VEGFR-2 are increased in the nasal mucosa of patients with allergic rhinitis compared to nonallergic controls [12]. Moreover, in the bronchial mucosa of asthmatic patients, the number of VEGF-positive cells is significantly increased and correlates with the vessel counts and vascular area, and expression of VEGFR-1 and VEGFR-2 inversely correlates with airway function [16–20, 23]. VEGF levels in BALF were increased in asthma in direct proportion to vascularity [19, 20]. In addition, through its permeability activity, VEGF contributes to oedema since it favours microvascular leakage. VEGF is secreted by several inflammatory cells, such as macrophages, mast cells and eosinophils, which intervene in the composition of the inflammatory infiltrate in allergic rhinitis and asthma [12, 31–34]. It has been demonstrated [35] that airway smooth muscle cells from patients with mild or moderate asthma, but not from healthy (control) subjects, promote angiogenesis *in vitro*. This pro-angiogenic capacity resides in elevated VEGF release and suggests that airway smooth muscle cells regulate airway neovascularisation in asthma.

In view of the present findings, new clinical strategies aimed at downregulation of VEGF and its receptors and signalling might be of benefit in the treatment of allergic diseases. Tentatively, the present data providing the first evidence of the anti-angiogenic activity of ebastine suggest its potential use as an anti-angiogenic molecule, besides its antihistamine activity in the treatment of allergic diseases in which angiogenesis occurs.

SUPPORT STATEMENT

STATEMENT OF INTEREST

Statements of interest for all authors, and for the study itself can be found at www.erj.ersjournals.com/misc/statements.dtl

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