Human airway smooth muscle cells secrete vascular endothelial growth factor: up-regulation by bradykinin via a protein kinase C and prostanoid-dependent mechanism

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ABSTRACT **Bronchial vascular remodeling is an important feature of the pathology of chronic asthma, but the responsible mechanisms and main sources of angiogenic factors are unclear. Here we report that human airway smooth muscle cells express vascular endothelial growth factor $(VEGF)_{121,\ 165,\ 189,\ 206}$ splice variants and secrete VEGF protein constitutively. VEGF protein secretion was increased by the proinflammatory asthma mediator bradykinin through post-transcriptional mechanisms. Bradykinin-induced VEGF secretion was dependent on the B_2 bradykinin receptor, activation of protein kinase C, and generation of endogenous prostanoids. This is the first report that bradykinin can increase VEGF secretion in any biological system and the first to show that airway smooth muscle cells produce VEGF. Our results suggest a novel role for human airway smooth muscle in contributing to bronchial mucosal angiogenesis in chronic asthma by secretion of VEGF and suggest a wider role for mesenchymal cell products in mediating angiogenesis in inflammatory and allergic diseases.—Knox, A. J., Corbett, L., Stocks, J., Holland, E., Zhu, Y. M., Pang, L. Human airway smooth muscle cells secrete vascular endothelial growth factor: up-regulation by bradykinin via a protein kinase C and prostanoid-dependent mechanism.** ***FASEB J.*** **15, 2480–2488 (2001)**

Key Words: VEGF · BK · cyclooxygenase · PKC

HYPEREMIA OF THE bronchial vasculature is a prominent feature of asthmatic airways with an increase in the size and number of blood vessels both inside and outside the smooth muscle layer, suggesting that vascular dilatation and proliferation are important components of airway remodeling in chronic asthma (1–4). Bronchial vascular changes are likely to contribute to airway narrowing and bronchial hyperresponsiveness in several ways: *1)* through the exudation and transudation of proinflammatory mediators, cytokines, and growth factors, *2)* by causing edema of the airway wall, *3)* by facilitating the trafficking of inflammatory cells into the airways down chemokine gradients, and *4)* by supporting the increased airway smooth muscle mass (5) characteristic of the airways in chronic asthma, thereby allowing increased airway smooth muscle force generation. The key bronchial angiogenic factors, the site of their production in the airway, and the mediators responsible for angiogenesis have not been studied extensively.

Even though the mechanisms responsible for these changes in vascular density have not been fully studied in the airway, there are a number of candidate proangiogenic factors. Recent evidence suggests that vascular endothelial growth factor (VEGF), a homodimeric glycoprotein of 34–45 kDa, plays a key role in regulating physiological and pathological angiogenesis in other biological systems (6–8); a recent biopsy study of asthma has shown an increase in submucosal VEGF (9). VEGF is mitogenic for endothelial cells, increases endothelial permeability, and induces endothelial cell expression of proteolytic enzymes that promote extra cellular matrix (ECM) degradation, thereby enhancing endothelial cell migration (8). There are at least five VEGF gene products, termed A through E; VEGF-A appears to be particularly important in angiogenesis. VEGF-A exists in several isoforms (110, 121, 145, 165, 189, 206), which are produced by alternative splicing of the primary VEGF transcript or by limited proteolysis. These isoforms differ in their mitogenicity and ECM binding due to the presence or absence of heparin binding regions, thus potentially serving different functions.

We and others have recently shown that human airway smooth muscle cells (HASM) have important synthetic functions in asthmatic airways (10). These include the production of prostanoids (11, 12) and chemokines (13–18), which influence airway inflammation and growth factors (19), and matrix-degrading enzymes (20), which modify airway remodeling. We hypothesized that the cells might also function as a source of angiogenic factors and therefore contribute to bronchial vascular remodeling. To test this hypoth-

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esis, we measured VEGF mRNA and protein production by HASM cells and studied the signaling mechanisms involved in VEGF production. Bradykinin (BK) is a potent proinflammatory mediator of asthma and other allergic diseases (21–24) that we have previously shown can alter the synthetic functions of HASM cells, particularly the generation of prostanoids (12) and the chemokine interleukin 8 (IL-8; 13). As BK is known to be a vasodilator in several tissues (24), we wanted to ascertain whether it might also contribute to angiogenesis.

Here we report that cultured HASM cells express the $VEGF_{121,\ 165,\ 189,\ 206}$ splice variants and secrete VEGF protein constitutively. Furthermore, we report for the first time in any cell type that VEGF secretion can be up-regulated by bradykinin. Our studies suggest that the generation of endogenous prostanoids and activation of protein kinase C (PKC) are key signaling events in bradykinin-induced VEGF secretion. These studies identify a novel role for HASM cells as a source of VEGF in the airways and suggest that kinins such as bradykinin may contribute to inflammatory angiogenesis.

MATERIALS AND METHODS

Cell culture

Human tracheas were obtained from postmortem individuals (one male aged 44 and one female aged 52, with no evidence of airway diseases) within 12 h of death. Primary cultures of human ASM cells were prepared from explants of ASM according to methods previously reported (11, 12). Cells at passages 5–6 were used for all experiments. We have already shown that the cells grown in this manner depict the immunohistochemical and light microscopic characteristics of typical ASM cells (11).

Experimental protocols

The cells were cultured to confluence in 10% fetal calf serum (Seralab, Crowly Down, Sussex, UK)-Dulbecco's modified Eagle's medium (DMEM, Sigma, Poole, Dorset, UK) in humidified 5% CO_2/95% air at 37°C in 24-well culture plates and growth-arrested in serum-deprived medium for 24 h before experiments. Immediately before each experiment, fresh serum-free medium containing BK (Sigma) was added. In the time course experiments, the cells were incubated with BK (0.1 μM) for 2–24 h whereas in the concentration response experiments cells were incubated for 24 h with 0.01–100 μM BK. In most experiments thereafter, the cells were incubated with 0.1 μM BK for 24 h. At the times indicated, culture media were harvested and stored at °20°C. Radioimmunoassay was carried out for PGE_2 content as a representative of prostanoid generation (11) and/or the enzyme-linked immunosorbent assay (ELISA) for VEGF. The anti-PGE_2 antiserum (Sigma) had negligible cross-reactivity in our hands (11). To test the inhibition by various drugs on the effect of BK, the nonselective COX inhibitor indomethacin, the B_1 receptor antagonist desArg^9,[Leu^8]-BK (dAL-BK) (Sigma), the selective COX-2 inhibitor NS-398 (N-(2-cyclohexyloxy-4-nitrophenyl)-methanesulfonamide; Cayman Chemical, Ann Arbor, MI), the B_2 receptor antagonist DArg[Hyp^3,Thi^5,Dtic^7,Oic^8]-BK (HOE-140, kind gift from Prof. Dr. R. N. Zahlten and Prof. Dr. B. A. Scholkens, Hoechst Aktiengesellschaft, Frankfurt, Germany), and the PKC inhibitors Go6983, calphostin C, and bis-indolyl maleimide (all from Calbiochem, Nottingham, Notts, UK) were added 60 min before the addition of BK. Where drugs were dissolved in organic vehicles (e.g., dimethyl sulfoxide or ethanol), controls were treated with vehicles. Experiments with the COX substrate arachidonic acid (AA), exogenous PGE_2, forskolin, isoproterenol, and phorbol 12-myristate 13-acetate (PMA) (all from Sigma) were conducted in the same manner as BK.

VEGF assay

The concentration of VEGF in the culture medium was determined by ELISA (R&D Systems, Abingdon, Oxon, UK) according to the manufacturer's instructions. A monoclonal antibody specific for VEGF had been precoated onto a microplate. For cell culture supernatant samples, 50 μl of assay diluent was added to each well. Standards and samples (200 μl) were pipetted into the wells and incubated for 2 h at room temperature. Any VEGF present was bound by the immobilized antibody. Plates were washed three times to remove any unbound substances and 200 μl of an enzyme-linked polyclonal antibody specific for VEGF was added to the wells and incubated for an additional 2 h at room temperature. Plates were washed again to remove any unbound antibody-enzyme reagent, and 200 μl of a substrate solution was added for 20 min and color developed in proportion to the amount of VEGF bound in the initial step. The reaction was stopped by adding 50 μl of stop solution (2 N sulfuric acid); the degree of color that had been generated was determined by measuring the OD at 450 nm (reference Filter 570 nm) within 30 min in a Dynatech MR500 microplate reader (Billinghurst, West Sussex, UK). The standard curve was linearized and subjected to regression analysis. The VEGF concentration of unknown samples were calculated using the standard curve. The results were expressed as picogram/milliliter. The sensitivity of the ELISA kit in our study was at least 7 pg/ml, which was consistent with the manufacturer's specifications.

RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR)

Cells in 6-well plates were treated with BK at a final concentration of 1 μM and collected at 0, 0.5 h, 2 h, 4 h, and 8 h, respectively. Total RNA was isolated by using the RNeasy mini kit (Qiagen, West Sussex, UK) following the manufacturer's protocol; 1 μg of total RNA was reverse transcribed in a total volume of 20 μl including 200 units of M-MLV reverse transcriptase (Promega, Madison, WI), 25 units of RNase inhibitor (Promega), 0.5 μg of oligo$(dT)_{15}$ primer, 0.5 mM of each dNTPs, and 1× first-strand buffer provided by Promega. The reaction was incubated at 42°C for 90 min.

Aliquots of the RT products were subsequently used for PCR amplification. 10 μl of RT products was brought to a volume of 50 μl containing 1 mM $MgCl_2$, 0.12 mM of each dTNPs, 1 unit of *Taq* polymerase (Sigma), 0.5 μM of both the upstream and downstream PCR primers, and 1× PCR buffer provided by Sigma. Two pairs of primers were used for this study. The primer sequences were VEGF sense: 5′-CGAAGTGGTGAAGTTCATGGATG-3′; VEGF antisense: 5′-TTCTGTATCAGTCTTTCCTGGTGA-3′; GAPDH sense: 5′-CCACCCATGGCAAATTCCATGGCA-3′ GAPDH antisense 5′-TCTAGACGGCAGGTCAGGTCCACC-3′. Amplification was carried out in Techne PHC-3 thermal cycler (Cambridge, UK) after an initial denaturation at 94°C for 3 min. This was

followed by 35 cycles of PCR of PCR using the following temperature and time profile: denaturation at 94°C for 1 min, primer annealling at 56°C for 1 min, primer extension at 72°C for 1 min, and a final extension of 72°C for 10 min. The PCR products were visualized by electrophoresis on 1% agarose gel in 0.5× TBE buffer after staining with 0.5 μ g/ml ethidium bromide. The UV illuminated gels were photographed, and the densitometry was analyzed using a GeneGenius gel documentation and analysis system (Syngene, Cambridge, UK).

Cell viability

The toxicity of all chemicals used in this study and their vehicles to human ASM cells was determined by MTT assay (11, 12). After 24 h incubation, 20 μl of 5 mg/ml MTT [thiazolyl blue, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltertrazolium bromide, Sigma] was added to the culture medium in 96-well plates and incubated for 1 h at 37°C. After removing the medium, 200 μl DMSO was added to solubilize the blue-colored tetrazolium, the plates were shaken for 5 min, and the OD_{550} values were read in a Dynatech MR5000 microplate reader. Viability was set as 100% in control cells.

Statistical analysis

Data were expressed as mean ± SE from *n* determinations. Statistical analysis was performed by using the statistical software from SPSS (Chicago, IL) (25). Student's unpaired two-tailed *t* test was used to determine the significant differences between the means. *P* values of less than 0.05 were accepted as statistically significant.

RESULTS

$VEGF_{121, 165, 189, 206}$ are expressed constitutively by HASM cells

RT-PCR in untreated cells showed four bands with molecular weights of 121, 165, 189, and 206 consistent with constitutive expression of the $VEGF_{121,}$ $VEGF_{165,}$ $VEGF_{189}$, and $VEGF_{206}$ isoforms (**Fig. 1**). $VEGF_{121}$ was the most prominent band seen. Direct sequencing of the PCR products by a Dye Terminator sequencing kit (Perkin-Elmer, Foster City, CA) on a 377 automated DNA sequencer (Perkin-Elmer) confirmed their molecular identity (data not shown).

BK increases VEGF protein production

To investigate the kinetics of VEGF production, HASM cells were cultured in the presence or absence of BK (0.1 μM). Cell culture supernatants from BK-treated cells were collected at 0, 2, 4, 8, 16, and 24 h. VEGF protein was released constitutively by HASM cells. There was a time-dependent increase in VEGF release after stimulation with BK: a significant difference was observed beginning 4 h after stimulation compared with VEGF production from untreated cells ($P<0.05$); the highest VEGF concentration was achieved after 24 h stimulation ($P<0.01$) (**Fig. 2*A***). When the cells were cultured with BK at concentrations of 0.01, 0.1, 1.0, 10, and 100 μM for 24 h, a concentration-dependent increase in VEGF production was also observed, significant from 0.01 μM ($P<0.001$) (Fig. 2*B*). However, the mRNA expression of VEGF isoforms was not increased by BK (**Fig. 3**), suggesting that BK was regulating VEGF release by post-transcriptional mechanisms. To avoid the saturation of PCR after the reverse transcriptase, different cycles of PCR were performed and the results were consistent.

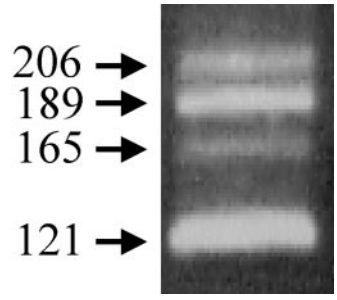

Figure 1. Representative gel from RT-PCR experiment showing 4 VEGF isoforms ($VEGF_{121, 165, 189, and 206}$) expressed by HASM cells. The experiment was repeated 3 times with similar results.

Endogenous prostanoids are involved in BK-induced VEGF production

To determine the role of prostanoids and the COX isoforms responsible for their production, we studied the effect of the nonselective COX inhibitor indomethacin (IND) and the selective COX-2 inhibitor NS-398 on BK-induced VEGF production. BK (0.1 μM) increased VEGF release as before. The increase was inhibited by IND, suggesting that prostanoids partly mediated the response (**Fig. 4**). The COX-2 selective inhibitor NS-398 had a similar effect, suggesting that products of COX-2 were involved (Fig. 4). Both IND and NS398 caused a concomitant reduction in PGE_2 synthesis (Fig. 4). To further investigate the role of COX products in BK-stimulated VEGF production, we determined whether PGE_2 (the principal HASM COX product) could cause VEGF production and whether this could be mimicked by exogenously applied COX substrate AA, which we have previously shown liberates PGE_2 from these cells. Consistent with our hypothesis, we found that PGE_2 caused a concentration-dependent increase in VEGF release (**Fig. 5**). Furthermore, arachidonic acid increased both VEGF and PGE_2 production, and VEGF and PGE_2 release in response to AA were both inhibited by IND (**Fig. 6**).

Effect of PGE_2 on VEGF release is mediated by cAMP

PGE_2 can act on the EP_2 and EP_4 receptors coupled to adenylyl cyclase and increase cAMP. To determine whether elevation of cAMP was involved in the effect of PGE_2 on VEGF production, we assumed that the direct adenylyl cyclase activator forskolin and the β_2 adrenoreceptor agonist isoproterenol would also increase VEGF. We found that both agents increased VEGF production consistent with the effect of PGE_2 being cAMP mediated (**Fig. 7**).

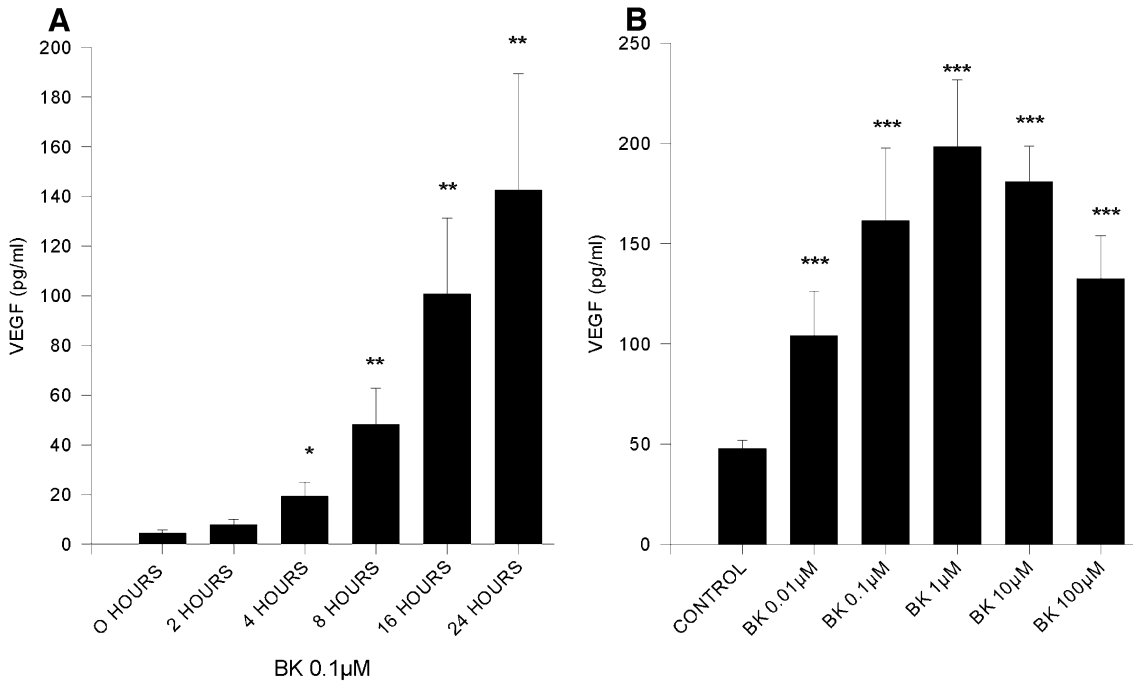

Figure 2. Kinetics (*A*) and concentration response (*B*) of bradykinin (BK) on VEGF production. Human ASM cells were incubated with 0.1 μM BK for the times indicated or with increasing concentrations of BK for 24 h for the concentration response. VEGF accumulation in the medium was measured by ELISA as described in Materials and Methods. Each point represents the mean ± SE of 9 determinations from 3 independent experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. controls.

The B_2 BK receptor mediates the effect of BK on VEGF release

To characterize the BK receptor(s) involved in BK-induced VEGF production, we examined the effect of the pretreatment of cells with the selective B_2 receptor antagonist HOE-140 and the B_1 receptor antagonist desArg^9,[Leu^8]-BK (both 100 μM) (12). We found that HOE-140 abolished BK-induced VEGF production whereas (**Fig. 8**) desArg^9,[Leu^8]-BK was without effect, suggesting that B_2 receptors are responsible for mediating BK-induced VEGF production from HASM cells.

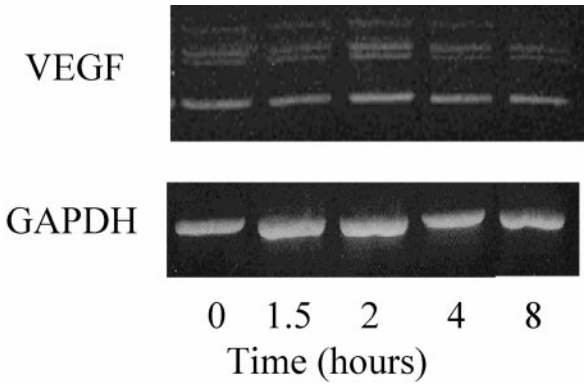

Figure 3. Time course of VEGF mRNA expression after stimulation with BK (1 μM) for 0, 1/2, 2, 4, 8 h and corresponding GAPDH mRNA levels. The experiment was repeated 3 times with similar results. Densitometry of the results (not shown) showed no significant increase of any VEGF isoform at any time point when expressed as a ratio of GAPDH expression.

PKC is involved in BK-induced VEGF release

To determine the role of PKC in BK-induced VEGF production, we pretreated cells for 60 min with the selective PKC inhibitors Go6983 (1 μM), calphostin C (1 μM), and bis-indolyl maleimide (1 μM). We also determined whether the PKC activator PMA could increase VEGF release. We found that all 3 PKC inhibitors inhibited VEGF production (**Fig. 9**). Furthermore, PMA increased VEGF release, consistent with a role for PKC in BK-induced VEGF production (Fig. 7).

Cell viability

Cell viability after treatment with the chemicals used in this study was consistently >95% vs. cells treated with the vehicles (data not shown).

DISCUSSION

There are several novel findings in our study, which is the first to suggest that BK can increase VEGF production in any biological system and to probe the mechanisms involved. It is also the first study to show that HASM cells can synthesize VEGF. We found that the generation of COX products and PKC play key signal-

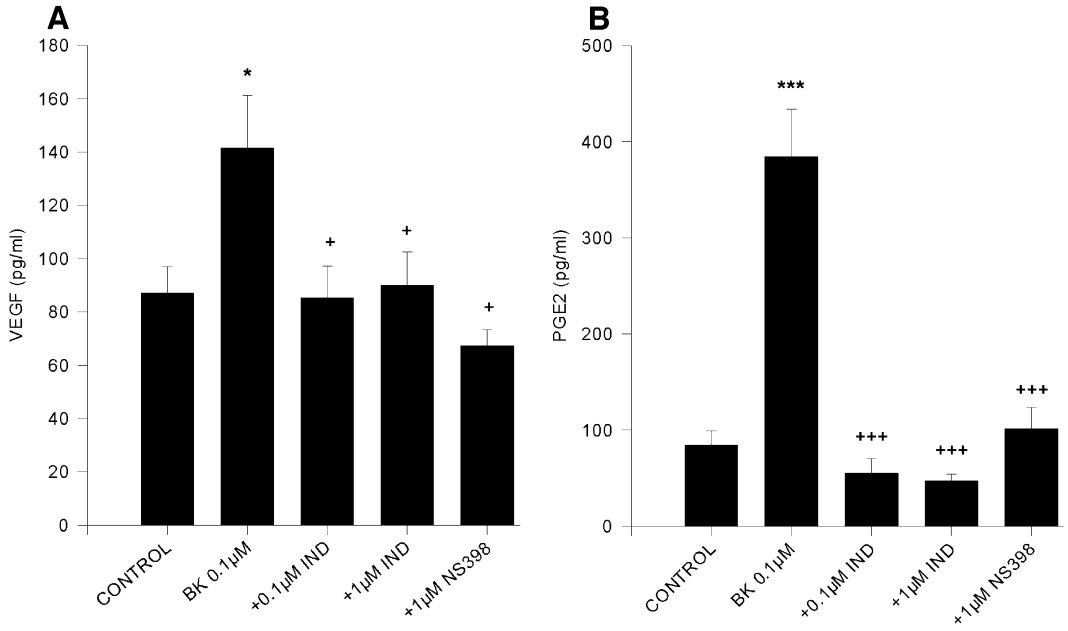

Figure 4. Effect of COX inhibitors on BK-induced and VEGF (*A*) and PGE_2 (*B*) generation. Human ASM cells were pretreated with or without various concentrations of indomethacin (IND) or NS-398 for 1 h before incubation with BK (0.1 μM) for 24 h. PGE_2 and VEGF concentrations were measured by RIA and ELISA, respectively, as described in Materials and Methods. Each point represents the mean ± SE of 6 determinations from 2 independent experiments. $*P < 0.05$, $***P < 0.001$ vs. control, $+P < 0.05$, $+++P < 0.001$ vs. BK-stimulated cells.

ing roles in BK-induced VEGF release and that the B_2 bradykinin receptor was responsible for the effect of BK. Our findings implicate a role for HASM cell-derived VEGF in mediating angiogenesis of bronchial vessels in chronic asthma in a paracrine manner. We suggest that BK may be an important player in inflammatory angiogenesis in the airway and possibly in other sites.

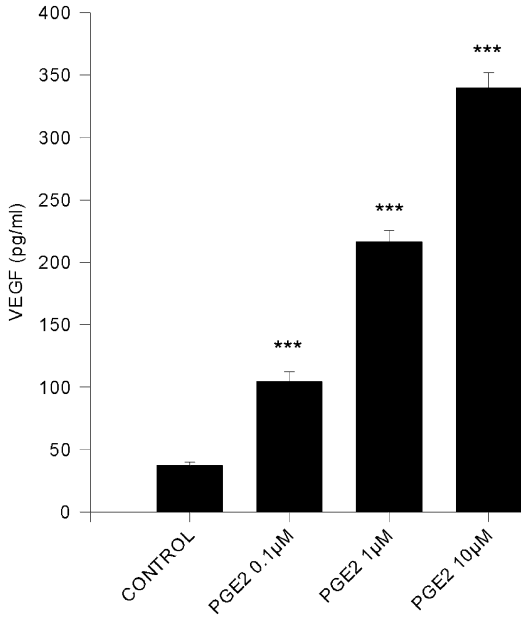

Figure 5. Effect of exogenously applied PGE_2 release on VEGF production. Human ASM cells were incubated with 0, 0.1, 1.0, or 10 μM PGE_2 for 24 h. VEGF accumulation in the medium was measured by ELISA as described in Materials and Methods. Each point represents the mean ± SE of 7 determinations from 2 independent experiments. $***P < 0.001$ vs. control cells.

Airway smooth muscle cells have long been studied largely from the perspective of target cells whose contractile and proliferation state are altered by local inflammatory events underlying the pathogenesis of asthma. Recently, however, a growing body of data has emerged to support the notion that HASM cells have the potential to act as effector cells in perpetuating airway inflammation by expressing and secreting inflammatory products, including prostanoids (11, 12) and cytokines such as IL-11, IL-6 (16), RANTES (17), and eotaxin (14, 18). Airway smooth muscle can also produce autocrine growth factors that may influence the remodeling changes (hyperplasia, hypertrophy) they undergo in chronic asthma (19). Autocrine secretion of matrix-degrading metalloproteinases (20) may also influence remodeling. Our present observations that BK causes VEGF production from HASM cells further support the concept of ASM as an inflammatory cell that can contribute to airway remodeling. Our demonstration that cultured HASM produce VEGF is consistent with immunohistochemical studies of rat lung showing that ASM cells stain positively for VEGF (26). Other cells that may act as a source of VEGF in the airway include mast cells (27) and epithelial cells (28). A recent bronchial biopsy study showed increased submucosal VEGF expression in asthma, although biopsies are not deep enough to capture the smooth muscle layer.

We used RT-PCR to characterize the VEGF-A isoforms produced by HASM cells using primers designed

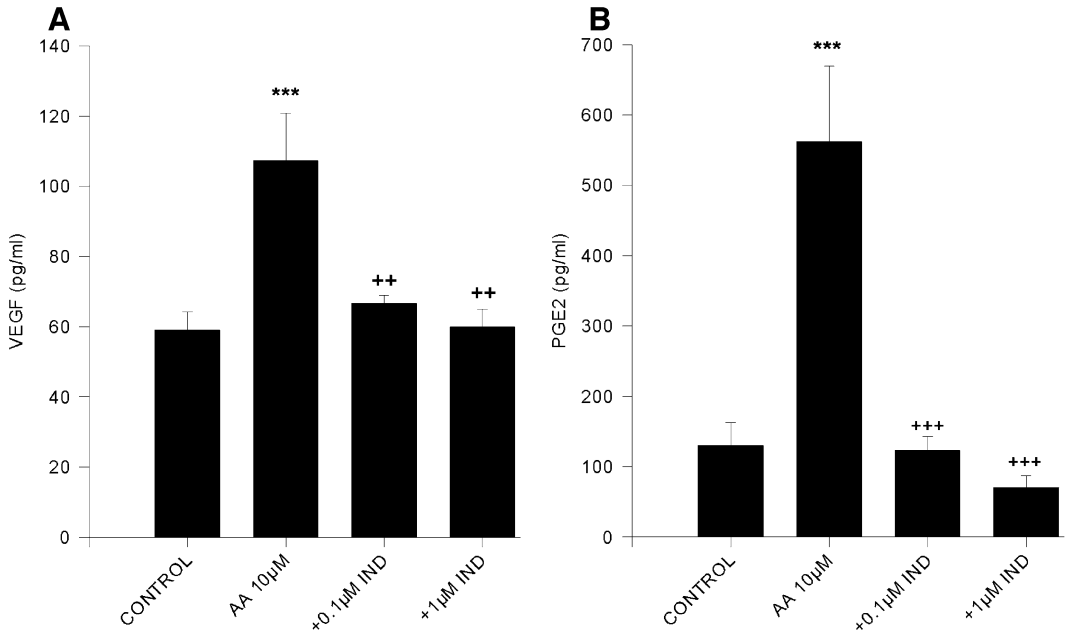

Figure 6. Effect of arachidonic acid (AA) on VEGF (*A*) and PGE_2 (*B*) production from human ASM cells. Human ASM cells were incubated with 10 μM AA for 24 h with increasing concentrations of indomethacin preincubated for 1 h. VEGF and PGE_2 accumulation in the medium was measured by ELISA and RIA, respectively, as described in Materials and Methods. Each point represents the mean ± SE of 8–11 determinations from 2–3 independent experiments. ***$P < 0.001$ vs. controls, ++$P < 0.01$, +++$P < 0.001$ vs. AA.

to transcribe all six known isoforms. We found that four isoforms were expressed by HASM cells namely $VEGF_{121, 165, 189, and 206.}$ $VEGF_{145}$ was not expressed, consistent with previous studies, suggesting its expression is confined to reproductive tissues (8). The largest band in HASM cells was $VEGF_{121}$.

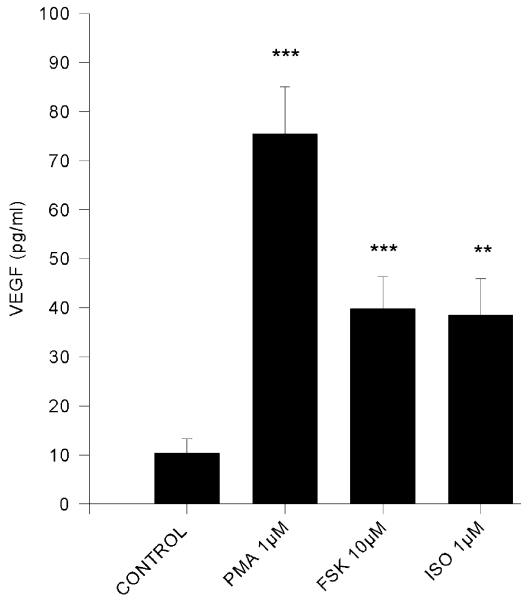

Figure 7. Effect of adenylyl cyclase activator forskolin (FSK), β_2 adrenoreceptor agonist isoproterenol (**ISO**) and the protein kinase C activator phorbol 12 myristate 13 acetate (PMA) on the production of VEGF as measured by ELISA. Cells were incubated for 4 h with the stimulants. Each point represents mean ± SE of 9 determinations from 3 independent experiments. **$P < 0.01$, ***$P < 0.001$, vs. control cells.

Several agents have been shown to increase VEGF production in diverse cell systems. VEGF expression by accessory cells outside the lung can be up-regulated by

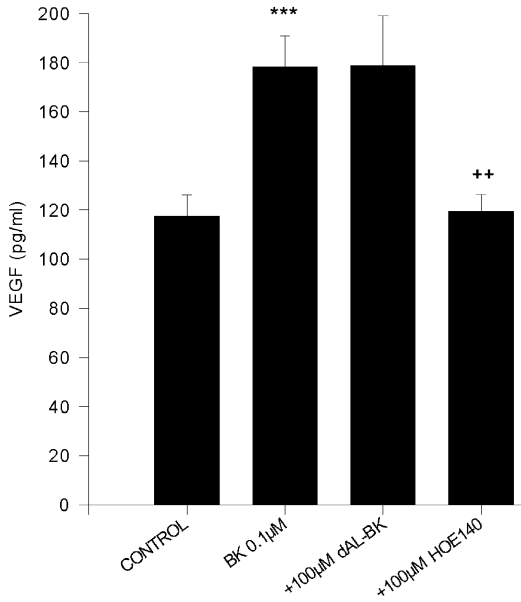

Figure 8. Effect of selective BK receptor antagonists on BK-induced VEGF production. Human ASM cells were pretreated with or without the selective B_1 receptor antagonist $desArg^9$,$[Leu^8]$-BK or the selective B_2 receptor antagonist HOE-140 (both 100 μM) for 1 h before incubation with 0.1 μM BK for 24 h. VEGF accumulation in the medium was measured by ELISA as described in Materials and Methods. Each point represents the mean ± SE of 12 determinations from 3 independent experiments. ***$P < 0.001$ vs. controls, ++$P < 0.01$ vs. the effect of BK.

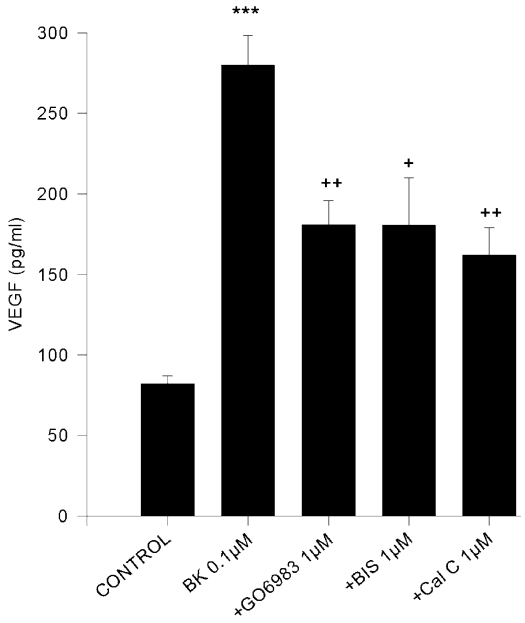

Figure 9. Effect of protein kinase C inhibitors on BK-induced VEGF production. Cells were preincubated with inhibitors Go6983, calphostin C and bis-indolyl maleimide (all 1 μM) for 1 h before the addition of 0.1 μm BK for a further 24 h Each point represents the mean ± SE of 6 determinations from 2 experiments ***$P < 0.001$ vs. controls +$P < 0.05$, ++$P < 0.01$ vs. the effect of BK.

hypoxia, PDGF, transforming growth factor β (29), and phorbol esters (30). We found that stimulation of HASM cells with BK resulted in a time- and concentration-dependent increase in release of VEGF protein. This has not been previously reported in any cell system and may be relevant to the mechanism of action of BK in inflammatory diseases, including asthma. We characterized the bradykinin receptor involved in these effects using selective B_1 and B_2 receptor antagonists (12) and showed that the B_2 receptor was implicated. This is consistent with the report that BK-stimulated synthesis of IL-1β, IL-2, and IL-6 from isolated lung strips can be blocked by a B_2 receptor antagonist (31) and our previous studies showing that BK-mediated induction of COX-2 and the release of prostanoids and IL-8 from human ASM cells were mediated by the B_2 receptor (12, 13). B_2 receptor antagonists may therefore have a role to play in controlling asthmatic airway inflammation and remodeling. This is supported by a clinical study of HOE 140 showing an improvement in airflow in patients with chronic severe asthma (32). Bradykinin is an inflammatory mediator that plays a role in inflammatory joint (33) and bowel disease (34), both of which are characterized by angiogenesis. It will be interesting to determine whether BK is involved in angiogenesis in these other diseases. We were unable to demonstrate any increase in mRNA to any of the VEGF isoforms after BK stimulation, suggesting that BK is acting to up-regulate VEGF post-transcriptionally. There was some variability in baseline results between experiments that is likely to reflect differences between cell lines and experimental variability produced by variables such as the exact degree of cell confluence, assay variability, etc. Although our experiments focused on bradykinin, it will be interesting to determine in future studies whether any other asthma mediators and cytokines can also up-regulate VEGF production.

We have previously shown that autocrine production of endogenous prostanoids regulates secretory functions of HASM cells, particularly the release of the CXC chemokine IL-8 (13). We were therefore interested to determine whether prostanoids were involved in BK mediated VEGF production. Our previous studies showed that BK causes early PGE_2 release by arachidonic acid mobilization from constitutive COX-1, followed by a later PGE_2 release due to COX-2 induction (12). Since we have previously reported the details on BK-stimulated PGE_2 release and COX-2 induction in human ASM cells (12), the Western blot results were not shown here. Our studies with indomethacin, a nonselective COX inhibitor that inhibited VEGF release, suggest a role for endogenous COX products in VEGF production. The fact that the nonselective COX-2 inhibitor NS-398 had a similar effect on BK-induced VEGF generation suggests that the products of inducible COX-2 are involved. We cannot, however, exclude a contribution from COX-1-derived PGE_2, as highly selective COX-1 inhibitors for whole cell systems are not available. The suggestion that endogenous COX products regulate VEGF release is further strengthened by our experiments showing that exogenous PGE_2 (the major COX metabolite in these cells) (11) or generation of PGE_2 using the COX substrate arachidonic acid both increased VEGF. Furthermore, VEGF and PGE_2 release in response to arachidonic acid were reduced by indomethacin. We measured PGE_2 as the sole prostanoid in our studies, as we have previously shown it is the dominant prostanoid produced by HASM cells (11). PGE_2 exerts some of its effects by activating prostaglandin EP2 and EP4 receptors, which are coupled to adenylyl cyclase, to increase intracellular cyclic AMP production (35). To determine whether cAMP pathways were involved in the effects of PGE_2 on VEGF, we compared the effects of PGE_2 with forskolin, a direct adenylyl cyclase activator, and isoprenaline, which activates adenylyl cyclase via β_2 adrenoreceptors. Both of these agents stimulated VEGF production, consistent with the effects of PGE_2 being mediated by cAMP. These results are similar to a study in a human monocytic cell line where PGE_2 increased VEGF through a cAMP related mechanism (36).

PKC exists as a family of isoenzymes that transduce a wide range of biological signals in diverse cell systems (37). To probe the role of PKC in BK-induced VEGF release, we studied the effect of three PKC inhibitors: calphostin C, Go 6983, and bis-indolyl maleimide (37–39). We found that all three inhibited BK-induced VEGF production and that the PKC activator PMA also increased VEGF release, providing evidence of a role for PKC in VEGF production. Our results are consistent with studies showing a role for PKC in VEGF production in response to cytokines in other biological systems

(40, 29). The fact that COX-2 inhibitors block the entire bradykinin response (but that PKC inhibitors have a partial effect) would be consistent with studies showing that bradykinin can activate PKC (41) and that phorbol esters can induce COX-2 (42), and would suggest that the role of PKC is probably upstream of COX-2 induction. The cascade we propose is that bradykinin activates PKC, which then induces COX-2. The major COX product PGE_2 then activates VEGF via cAMP pathways.

In conclusion, we have shown that HASM cells are a source of several isoforms of VEGF-A and that VEGF production is increased by bradykinin via B_2 receptors, possibly through a post-transcriptional mechanism. Prostanoid generation and PKC activation appear to be critical signaling events in this process. We suggest that paracrine production of VEGF by HASM cells may contribute to bronchial vascular remodeling in chronic asthma. Our findings may be relevant as well to other chronic inflammatory diseases where angiogenesis contributes to the remodeling process. FJ

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