# Cytokines upregulate vascular endothelial growth factor secretion by human airway smooth muscle cells: Role of endogenous prostanoids

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**Abstract Here, we report that vascular endothelial growth factor (VEGF)-A secretion by human airway smooth muscle cells was increased by interleukin 1 beta (IL-1β) and transforming growth factor beta (TGFβ). IL-1β and TGFβ induced cyclo-oxygenase (COX)-2 protein and increased prostaglandin $E_2$ ($PGE_2$). Both IL-1β and TGFβ increased VEGF-$A_{165}$ mRNA and VEGF promoter luciferase construct activity, in addition VEGF-A protein was inhibited by actinomycin D suggesting transcriptional regulation. The COX inhibitors indomethacin and NS398 inhibited IL-1β but not TGFβ mediated VEGF-A production. Furthermore, the effect of the COX inhibitors was overcome by adding exogenous $PGE_2$. In conclusion, IL-1β increases VEGF-A secretion by COX-2 derived $PGE_2$ production whereas TGFβ uses COX-independent pathways.**


*Keywords:* Vascular endothelial growth factor; Transforming growth factor beta; Interleukin 1 beta; Prostaglandin $E_2$; Cyclo-oxygenase

## 1. Introduction

Angiogenesis is a feature of several inflammatory and malignant diseases. Pro-inflammatory cytokines contribute by generating vascular endothelial growth factor (VEGF) and other pro-angiogenic molecules [1,2]. There are at least five VEGF gene products termed A–E. VEGF-A, which has several splice variants (110, 121, 145, 165, 189, and 206), is particularly important in angiogenesis.

Cytokines such as interleukin 1 beta (IL-1β) and transforming growth factor beta (TGFβ) can increase VEGF release in some cells [3–6]. These agents can exert their biological effects partly by autocrine generation of lipid mediators [7,8] particularly prostanoids. Arachidonic acid is converted into prostaglandin (PG) $H_2$, by cyclo-oxygenase (COX) then to prostanoids by specific synthases [9]. Three COX isoforms exist. COX-1 produces housekeeping prostanoids, COX-2 is induced by inflammatory cytokines [9] and COX-3 is a splice variant of COX-1 [10]. COX products regulate several inflammatory and remodelling processes including chemokine production [11], apoptosis [12] and matrix metalloproteinase production [12]. Although, COX products are involved in angiogenic processes [12] their involvement in cytokine induced VEGF production has not been clearly defined.

In addition to their contractile and proliferative properties, human airway smooth muscle (HASM) cells secrete lipid mediators, cytokines and chemokines, growth factors and matrix degrading metalloproteinases reviewed in [13]. We recently showed that HASM can produce VEGF-A [14] in response to bradykinin. Here, we determined the role of autocrine prostanoid secretion in cytokine mediated VEGF release by HASM cells whose main COX product is $PGE_2$.

## 2. Materials and methods

### 2.1. Cell culture

Human tracheas were obtained from five post-mortem individuals within 12 h of death. Primary cultures of HASM cells were prepared from explants [15] and used at passage 5–6. Cells were cultured to confluence in 10% fetal calf serum (Seralab, Crawly Down, Sussex, UK)–Dulbecco's modified Eagle's medium (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 prior to experiments.

### 2.2. VEGF-A assay

$VEGF_{165}$ was measured by enzyme linked immunosorbent assay (ELISA, R&D Systems, Abingdon, Oxon, UK) [15].

### 2.3. $PGE_2$ assay

$PGE_2$ levels were measured by radioimmunoassay [15].

### 2.4. Western blot analysis

Western blotting for COX-2 was performed as described [15].

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

Cells were treated with IL-1β (1 ng/ml) or TGFβ (10 ng/ml) and collected at 0, 1, 2, 4, and 8 h. Total RNA was isolated (RNeasy kit, Qiagen, West Sussex, UK) and reverse transcriptase polymerase chain reaction (RT-PCR) performed using primers/methods we have described [14]. Densitometry was analysed using a GeneGenius gel documentation and analysis system (Syngene, Cambridge, UK).

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*Abbreviations:* VEGF, vascular endothelial growth factor; TGFβ, transforming growth factor beta; IL-1β, interleukin 1 beta; $PGE_2$, prostaglandin $E_2$; COX, cyclo-oxygenase; mRNA, messenger RNA; RT-PCR, reverse transcriptase polymerase chain reaction; HASM, human airway smooth muscle; ELISA, enzyme linked immunosorbent assay; NS-398, *N*-(2-cyclohexyloxy-4-nitrophenyl)-methanesulfonamide; MTT, thiazolyl blue, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

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### 2.6. VEGF reporter construct

The VEGF reporter constructs used in transient transfection contained sequences derived from the human VEGF promoter driving firefly luciferase. A 2.6-kb (bp −2361 to +298) VEGF promoter fragment was used (provided by D. Mukhopadhyay, Harvard, Boston, MA) [16]. HASM cells were co-transfected using FuGENE 6 (Roche) with the vector (pRL-TK, Promega) 0.5 μg/ml DNA for 8 h, then cultured with IL-1β or TGFβ for 16 h. Transfection efficiency was 30%. Cells were then collected and lysed. Firefly and *Renilla* luciferase activities were measured by luminometer using the dual-luciferase reporter assay system (Promega).

### 2.7. Drugs/chemicals

The broad spectrum COX inhibitor indomethacin (Sigma), the selective COX-2 inhibitor *N*-(2-cyclohexyloxy-4-nitrophenyl)-methanesulfonamide (NS-398), from (Cayman Chemical, Ann Arbor, MI, USA), actinomycin D, (Sigma) were added 1 h prior to IL-1β or TGFβ. Where drugs were dissolved in organic vehicles (e.g., dimethyl sulfoxide), controls were treated with vehicles.

### 2.8. Cell viability

The toxicity of the chemicals and their vehicles was determined by MTT (thiazolyl blue, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay [14].

### 2.9. Statistical analysis

Data were expressed as means ± S.E.M. Statistical analysis was performed using ANOVA followed by student's unpaired two-tailed *t* test as a post hoc test to determine the differences between means. $P < 0.05$ was statistically significant.

## 3. Results

### 3.1. IL-1β and TGFβ increase VEGF-A protein production

IL-1β (0, 0.001, 0.01, 0.1 and 1.0 ng/ml for 24 h) concentration-dependently increased VEGF-A, significant from 0.1 ng/ml ($P < 0.001$) (Fig. 1A). TGFβ (0.1, 1, 10, and 20 ng/ml) caused a concentration related increase significant from 1 ng/ml ($P < 0.05$) (Fig. 2A). A time-dependent increase in VEGF-A release occurred after IL-1β significant after 2 h ($P < 0.001$), maximal at 24 h ($P < 0.001$) (Fig. 1B) and with TGFβ significant from 2 h ($P < 0.05$), maximal at 24 h ($P < 0.001$) (Fig. 2B).

### 3.2. IL-1β and TGFβ increase VEGF-A mRNA

VEGF-$A_{165}$ messenger RNA (mRNA) increased over time after IL-1β and TGFβ treatment (Fig. 3A). Actinomycin D (5 μg/ml) a transcription inhibitor, markedly reduced IL-1β and TGFβ induced mRNA (Fig. 3B) and VEGF-A protein release (data not shown) suggesting that regulation was transcriptional. Transcriptional arrest studies with actinomycin D showed no evidence of mRNA stabilisation (data not shown).

### 3.3. IL-1β and TGFβ act transcriptionally

To confirm that IL-1β and TGFβ were acting transcriptionally we transiently transfected cells with a VEGF promoter

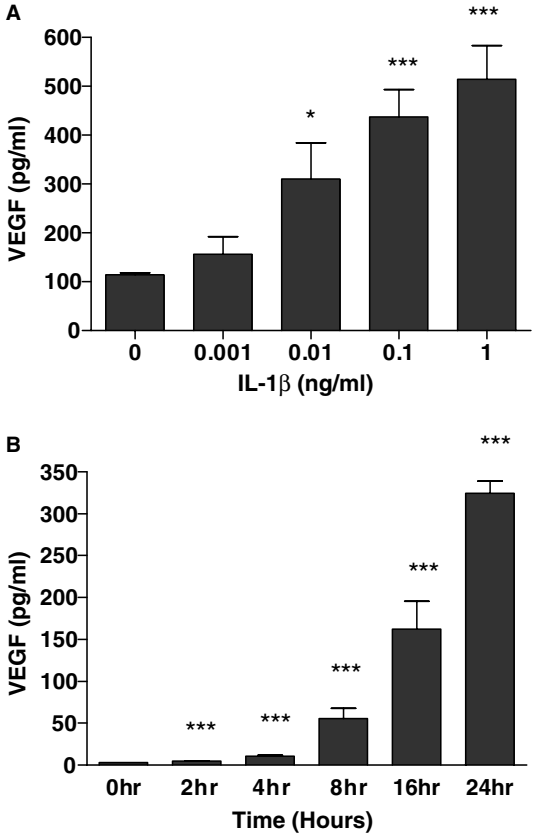


Fig. 1. Concentration response (A) and kinetics (B) of the effect of IL-1β on VEGF-A release. HASM cells were incubated with 1 ng/ml of IL-1β in kinetic studies or increasing IL-1β concentrations for 24 h for the concentration response. Mean ± S.E.M., $n = 6$ from two experiments, experiments in a further two cell lines showed similar results. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to controls by *t* test after ANOVA showed significant difference ($P < 0.001$).

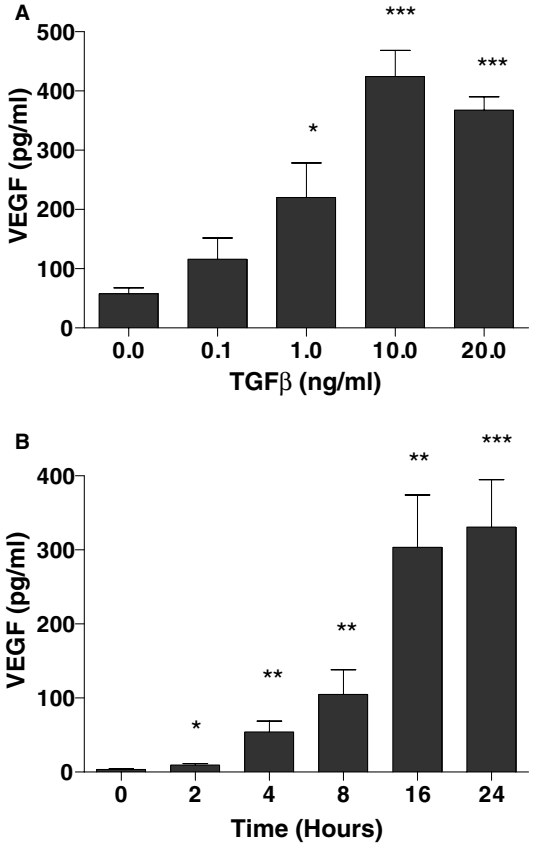


Fig. 2. Concentration response (A) and kinetics (B) of the effect of TGFβ on VEGF-A release. HASM cells were incubated with increasing TGFβ concentrations for 24 h in concentration response or 1 ng/ml TGFβ in kinetic experiments. Mean ± S.E.M., $n = 6$ from two experiments, experiments in a further two cell lines showed similar results. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to controls by *t* test after ANOVA showed significant difference ($P < 0.001$).

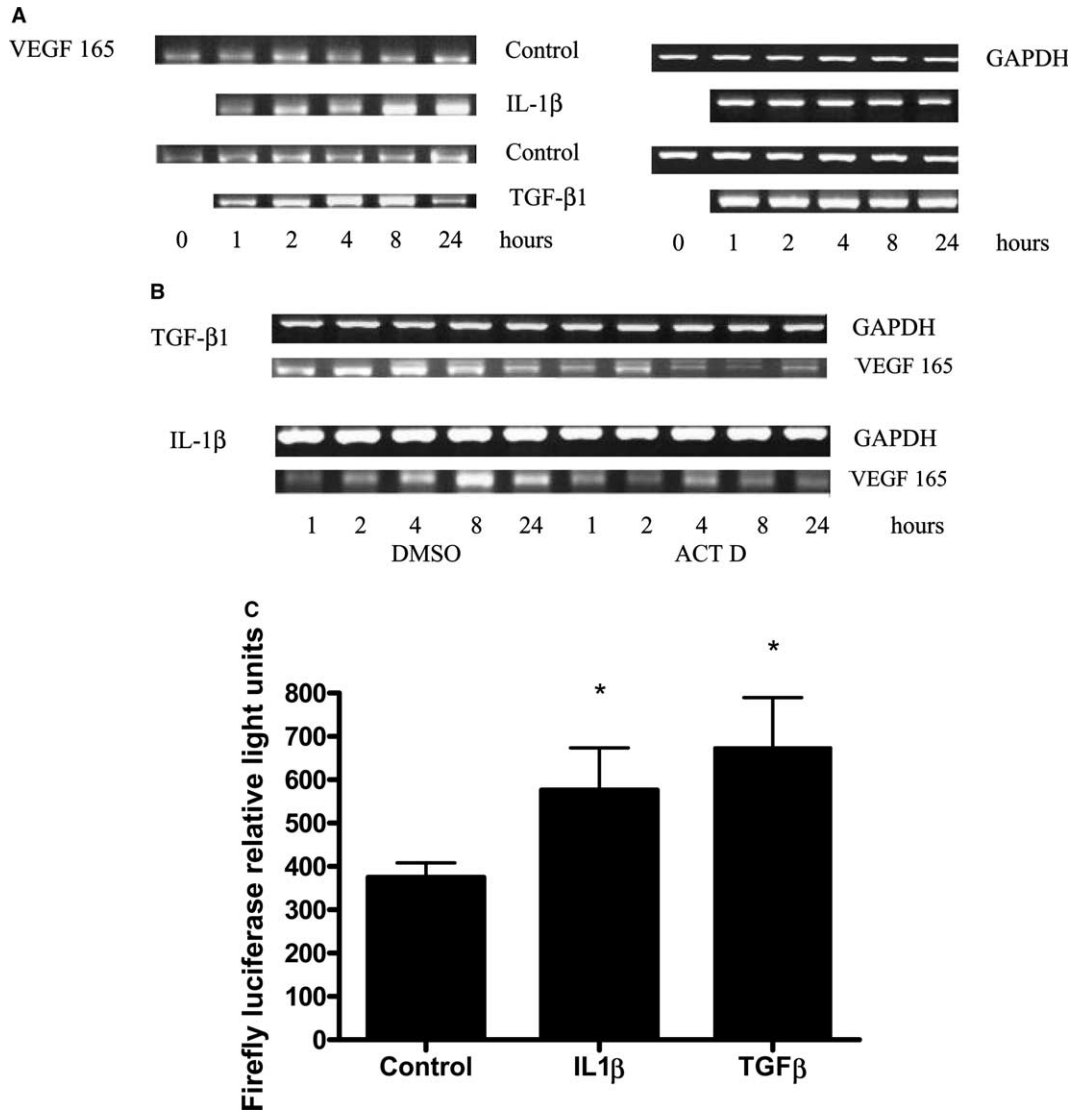


Fig. 3. (A) VEGF-$A_{165}$ mRNA after IL-1β or TGFβ for 0, 1, 2, 4, 8 and 24 h and corresponding GAPDH mRNA levels. Densitometry showed an increase in VEGF-$A_{165}$/GAPDH ratio maximal at 4 h for both IL-1β and TGF β (not shown). Representative of three experiments, experiments in a further 1–2 cell lines showed similar results. (B) Effect of actinomycin D on VEGF-A mRNA expression. Cells were pretreated with actinomycin D (5 μg/ml) for 30 min before IL-1β (1 ng/ml) or TGFβ (10 ng/ml). (C) IL-1β and TGFβ increase VEGF promoter luciferase reporter construct activity. $^{*}P < 0.05$ compared to controls. Representative of five experiments.

luciferase reporter construct. Both IL-1β and TGFβ increased luciferase activity (Fig. 3C).

### 3.4. COX inhibitors inhibit IL-1β but not TGFβ induced VEGF-A production

VEGF-A release by IL-1β (24 h) was markedly inhibited by indomethacin (1 μM) (Fig. 4). NS398 (1 μM) had a similar effect suggesting COX-2 products were involved. The effects of both COX inhibitors were overcome by adding exogenous $PGE_2$ (0.01 μM), the concentration released by HASM cells in response to IL-1β. Both indomethacin (1 μM) and NS398 (1 μM) reduced IL-1β induced $PGE_2$ synthesis (Fig. 5). In contrast neither indomethacin (1 μM) nor NS398 (1 μM) had an effect on TGFβ induced VEGF release (24 h) (Fig. 4).

The relative potency of IL-1β and TGFβ (1 ng/ml, 24 h) at inducing COX-2 and releasing $PGE_2$ were compared. $PGE_2$ levels were much greater with IL-1β than TGFβ (Fig. 5). Consistent with this, IL-1β induced alterations in COX-2 protein levels were sustained longer than TGFβ treated cells.

### 3.5. Cell viability

Cell viability was consistently >95% (data not shown).

## 4. Discussion

The main finding here is that endogenous $PGE_2$ is a major autocrine factor in increasing VEGF-A release in response to IL-1β but not TGFβ in HASM cells. We confirmed the role of COX-derived $PGE_2$ in IL-1β induced VEGF using broad spectrum and COX-2 selective COX inhibitors and $PGE_2$ rescue experiments. RT-PCR, actinomycin D and studies with a VEGF reporter construct suggested that both IL-1β and TGFβ were acting transcriptionally.

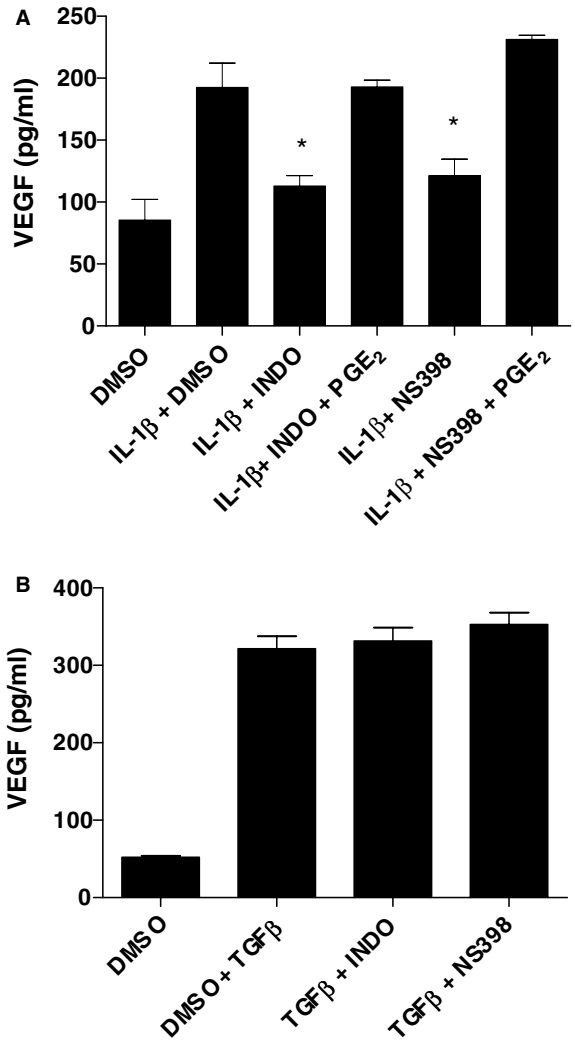


Fig. 4. Effect of COX inhibitors on (A): IL-1β (1 ng/ml) or (B) TGFβ (10 ng/ml) induced VEGF-A generation, $PGE_2$ was used at 0.01 μM in rescue experiments. Mean ± S.E.M., $n = 6$ from two experiments, experiments in a further two cell lines showed similar results. $^{*}P < 0.05$, compared with control.

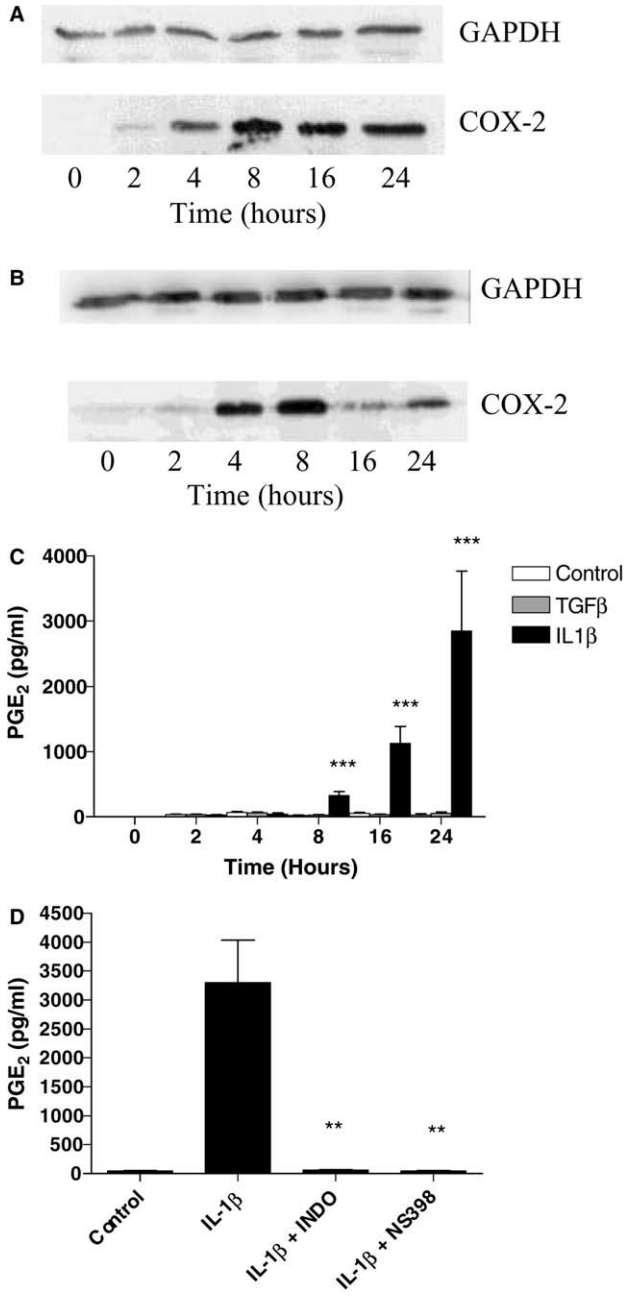


Fig. 5. (A) 1 ng/ml IL-1β induces COX-2 protein production (35 μg protein loaded). (B) 1 ng/ml TGFβ induces COX-2 protein production (35 μg protein loaded). (C) 24 h $PGE_2$ release after 1 ng/ml IL1β or TGFβ. $N = 12$ from three experiments. $^{**}P < 0.01$, $^{***}P < 0.001$ by $t$ test after ANOVA showed significant difference ($P < 0.001$). (D) Effect of indomethacin (1 μM) or NS398 (1 μM) on $PGE_2$ production by IL-1β (1 ng/ml). Mean ± S.E.M., $n = 6$ from two experiments, experiments in a further cell line showed similar results.

The finding that cytokines increase VEGF production by HASM cells is relevant to the bronchial vascular angiogenic changes in asthma [17]. Bronchial biopsies in asthma shown increased staining for submucosal VEGF-A [18], VEGF-A is increased in induced sputum in stable asthma [19,20] and in bronchoalveolar lavage fluid in acute asthma [21]. Furthermore, VEGF-A receptor antagonists inhibited airway inflammation and hyperresponsiveness in a murine asthma model [22]. COX-2 expression is also increased in asthmatic airways reviewed in [23]. Our studies suggest that stimulation of angiogenesis via VEGF-A may be an important function of COX-2.

We found that stimulation of HASM cells with IL-1β or TGFβ resulted in a time- and concentration-dependent increase in release of VEGF-A protein. Studies measuring mRNA showed that both IL-1β and TGFβ increased $VEGF\text{-}A_{165}$ mRNA. We focused on this isoform as it is the isoform measured in the ELISA assay and is the most biologically active. Actinomycin D inhibited mRNA induction and VEGF protein release suggesting that IL-1β and TGFβ were acting transcriptionally. Furthermore, transcriptional arrest studies showed no alteration in mRNA stability. It has previously been reported that IL-1β and TGFβ can release VEGF from HASM cells [24] but that study did not study the role of endogenous prostanoids by using pharmacological inhibitors and did not study whether regulation was transcriptional or post-transcriptional. As our RT-PCR studies with actinomycin D suggested that regulation was transcriptional we went on to do studies using VEGF promoter luciferase constructs.

Both IL-1β and TGFβ increased the activity of a VEGF promoter luciferase construct indicating that VEGF is regulated transcriptionally by both of these agents. The fact that the changes in luciferase activity were modest is consistent with the fact that primary cells such as HASM cells are difficult to transfect. Studies in some other cell types have shown that IL-1β and TGFβ can activate VEGF transcriptionally [3,4,6], although have not probed the role of endogenous prostanoids.

We characterised the role of prostanoids using broad spectrum (indomethacin) and COX-2 selective (NS398) inhibitors. The concentrations chosen inhibit COX-2 derived $PGE_2$ generation by over 90% [25]. The fact that NS-398 had a similar effect to indomethacin on IL-1β induced VEGF-A generation suggests COX-2 involvement. This concentration of NS398 does not inhibit COX-1 mediated $PGE_2$ generation from unstimulated HASM cells where COX-1 is the only COX isoform [25] expressed. The suggestion that endogenous COX products regulate IL-1β induced VEGF-A release is further strengthened by our experiments showing that exogenous $PGE_2$ (the major COX metabolite in these cells) [17] could overcome the COX inhibitor effects at concentrations comparable to those produced by IL-1β. These studies also exclude any potential non-COX mediated effect of the COX inhibitors such as activation of PPAR's [26]. Unlike the case with IL-1β, inhibitor studies suggested that TGFβ's effects were COX independent. Consistent with this TGFβ was a weaker inducer of $PGE_2$ release than IL-1β, causing only a transient COX-2 induction. One study has reported that IL-1β induced VEGF-A generation in synovial fibroblasts was COX-2 dependent but did not study the mechanism of regulation [5]. No previous studies in any cell type have looked at the role of COX products in TGFβ's effects.

In conclusion, VEGF-A production is increased in HASM cells by IL-1β and TGFβ suggesting that cytokine-mediated paracrine VEGF-A production by HASM cells may contribute to bronchial vascular remodelling in chronic asthma. The fact that COX-2 derived $PGE_2$ was an intermediatory in the response to IL-1β suggests that COX-2 induction may promote angiogenesis in asthmatic airways. However, TGFβ released VEGF-A predominantly by prostanoid independent pathways. Our findings may be relevant to other chronic inflammatory diseases where angiogenesis contributes to remodelling.

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## References

[1] Yancopoulos, G.D., Davis, S., Gale, N.W., Rudge, J.S., Wiegand, S.J. and Holash, J. (2000) Vascular-specific growth factors and blood vessel formation. Nature 407, 242–248.
[2] Thurston, G. and Gale, N.W. (2004) Vascular endothelial growth factor and other signaling pathways in developmental and pathologic angiogenesis. Int. J. Hematol. 80, 7–20.
[3] Jung, Y.D., Liu, W., Reinmuth, N., Ahmad, S.A., Fan, F., Gallick, G.E. and Ellis, L.M. (2001) Vascular endothelial growth factor is upregulated by interleukin-1 beta in human vascular smooth muscle cells via the P38 mitogen-activated protein kinase pathway. Angiogenesis 4, 155–162.
[4] Tanaka, T., Kanai, H., Sekiguchi, K., Aihara, Y., Yokoyama, T., Arai, M., Kanda, T., Nagai, R. and Kurabayashi, M. (2000) Induction of VEGF gene transcription by IL-1 beta is mediated through stress-activated MAP kinases and Sp1 sites in cardiac myocytes. J. Mol. Cell Cardiol. 32, 1955–1967.
[5] Inoue, H., Takamori, M., Shimoyama, Y., Ishibashi, H., Yamamoto, S. and Koshihara, Y. (2002) Regulation by PGE2 of the production of interleukin-6, macrophage colony stimulating factor, and vascular endothelial growth factor in human synovial fibroblasts. Br. J. Pharmacol. 136, 287–295.
[6] Benckert, C., Jonas, S., Cramer, T., Von Marschall, Z., Schafer, G., Peters, M., Wagner, K., Radke, C., Wiedenmann, B., Neuhaus, P., Hocker, M. and Rosewicz, S. (2003) Transforming growth factor beta 1 stimulates vascular endothelial growth factor gene transcription in human cholangiocellular carcinoma cells. Cancer Res. 63, 1083–1092.
[7] El Haroun, H., Bradbury, D., Clayton, A. and Knox, A.J. (2004) Interleukin-1beta, transforming growth factor-beta1, and bradykinin attenuate cyclic AMP production by human pulmonary artery smooth muscle cells in response to prostacyclin analogues and prostaglandin E2 by cyclooxygenase-2 induction and down-regulation of adenylyl cyclase isoforms 1, 2, and 4. Circ. Res. 20 (94), 353–361.
[8] Pang, L., Holland, E. and Knox, A.J. (1998) Role of cyclooxygenase-2 induction in interleukin-1beta induced attenuation of cultured human airway smooth muscle cell cyclic AMP generation in response to isoprenaline. Br. J. Pharmacol. 125, 1320–1328.
[9] Pang, L., Pitt, A., Petkova, D. and Knox, A.J. (1998) The COX-1/COX-2 balance in asthma. Clin. Exp. Allergy 28, 1050–1058.
[10] Davies, N.M., Good, R.L., Roupe, K.A. and Yanez, J.A. (2004) Cyclooxygenase-3: axiom, dogma, anomaly, enigma or splice error? – Not as easy as 1, 2, 3. J. Pharm. Pharm. Sci. 7, 217–226.
[11] Pang, L. and Knox, A.J. (1998) Bradykinin stimulates IL-8 production in cultured human airway smooth muscle cells: role of cyclooxygenase products. J. Immunol. 161, 2509–2515.
[12] Subbaramaiah, K. and Dannenberg, A.J. (2003) Cyclooxygenase 2: a molecular target for cancer prevention and treatment. Trends Pharmacol. Sci. 24, 96–102.
[13] Howarth, P.H., Knox, A.J., Amrani, Y., Tliba, O., Panettieri Jr., R.A. and Johnson, M. (2004) Synthetic responses in airway smooth muscle. J. Allergy Clin. Immunol. 114, S32–S50.
[14] Knox, A.J., Corbett, L., Stocks, J., Holland, E., Zhu, Y.M. and Pang, L. (2001) 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.
[15] Pang, L. and Knox, A.J. (1997) Effect of interleukin-1 beta, tumour necrosis factor-alpha and interferon-gamma on the induction of cyclo-oxygenase-2 in cultured human airway smooth muscle cells. Br. J. Pharmacol. 121, 579–587.
[16] Mukhopadhyay, D., Knebelmann, B., Cohen, H.T., Ananth, S. and Sukhatme, V.P. (1997) The von Hippel-Lindau tumor suppressor gene product interacts with Sp1 to repress vascular endothelial growth factor promoter activity. Mol. Cell Biol. 17, 5629–5639.
[17] Orsida, B.E., Li, X., Hickey, B., Thien, F., Wilson, J.W. and Walters, E.H. (1999) Vascularity in asthmatic airways: relation to inhaled steroid dose. Thorax 54, 289–295.
[18] Hoshino, M., Takahashi, M. and Aoike, N. (2001) Expression of vascular endothelial growth factor, basic fibroblast growth factor, and angiogenin immunoreactivity in asthmatic airways and its relationship to angiogenesis. J. Allergy Clin. Immunol. 107, 295–301.
[19] Asai, K., Kanazawa, H., Otani, K., Shiraishi, S., Hirata, K. and Yoshikawa, J. (2002) Imbalance between vascular endothelial growth factor and endostatin levels in induced sputum from asthmatic subjects. J. Allergy Clin. Immunol. 110, 571–575.
[20] Kanazawa, H., Hirata, K. and Yoshikawa, J. (2002) Involvement of vascular endothelial growth factor in exercise induced bronchoconstriction in asthmatic patients. Thorax 57, 885–888.
[21] Lee, Y.C. and Lee, H.K. (2001) Vascular endothelial growth factor in patients with acute asthma. J. Allergy Clin. Immunol. 107, 1106.
[22] Lee, Y.C., Kwak, Y.G. and Song, C.H. (2002) Contribution of vascular endothelial growth factor to airway hyperresponsiveness and inflammation in a murine model of toluene diisocyanate-induced asthma. J. Immunol. 168, 3595–3600.

[23] Pang, L. (2001) COX-2 expression in asthmatic airways: the story so far. Thorax 56, 335–336.
[24] Wen, F.Q., Liu, X., Manda, W., Terasaki, Y., Kobayashi, T., Abe, S., Fang, Q., Ertl, R., Manouilova, L. and Rennard, S.I. (2003) TH2 Cytokine-enhanced and TGF-beta-enhanced vascular endothelial growth factor production by cultured human airway smooth muscle cells is attenuated by IFN-gamma and corticosteroids. J. Allergy Clin. Immunol. 111, 1307–1318.
[25] Range, S.P., Pang, L., Holland, E. and Knox, A.J. (2000) Selectivity of cyclo-oxygenase inhibitors in human pulmonary epithelial and smooth muscle cells. Eur. Respir. J. 15, 751–756.
[26] Pang, L., Nie, M., Corbett, L. and Knox, A.J. (2003) Cyclooxygenase-2 expression by nonsteroidal anti-inflammatory drugs in human airway smooth muscle cells: role of peroxisome proliferator-activated receptors. J. Immunol. 170, 1043–1051.