VASCULAR ENDOTHELIAL GROWTH FACTOR INDUCTION BY PROSTAGLANDIN E₂ IN HUMAN AIRWAY SMOOTH MUSCLE CELLS IS MEDIATED BY E PROSTANOID EP₂/EP₄ RECEPTORS AND SP-1 TRANSCRIPTION FACTOR BINDING SITES.

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Prostaglandin E₂ (PGE₂) has been shown to increase vascular endothelial growth factor A (VEGF-A) production but the mechanisms involved are poorly defined. Here we characterised the transcriptional mechanisms involved in human airway smooth muscle cells (HASMC). PGE₂ increased VEGF-A mRNA and protein but not mRNA stability. PGE₂ stimulated the activity of a transiently transfected 2068 bp (-2018 to + 50) VEGF-A promoter driven luciferase construct. Functional 5′ deletional analysis mapped the PGE₂ response element to the 135 bp sequence (-85/+50) of the human VEGF-A promoter. There was no reduction in PGE₂ induced luciferase activity in cells transfected with a 135 bp VEGF promoter construct containing mutated EGR-1 binding sites. In contrast luciferase activity was reduced in cells transfected with a 135 bp VEGF promoter fragment containing mutated Sp-1 binding sites suggesting that Sp-1 binding is critical. Electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP) assay confirmed binding of Sp-1 to the VEGF promoter. PGE₂ increased phosphorylation of Sp-1 and luciferase activity of a transfected Sp-1 reporter construct. PGE₂ receptor agonists EP₂ (ONO-AE1 259) and EP₄ (ONO-AE1 329) mimicked the effect of PGE₂ and RT-PCR, Western blotting and flow cytometry confirmed the presence of EP₂ and EP₄ receptors. VEGF protein release and Sp-1 reporter activity were increased by forskolin and isoproterenol, which increase cytosolic cAMP, and the cAMP analogue, 8 – bromoadenosine - 3’, -5’ cyclophosphoric acid.

These studies suggest that PGE₂ increases VEGF transcriptionally and involves the Sp-1 binding site via a cAMP dependent mechanism involving EP₂ and EP₄ receptors.

VEGF is a 45 kDa heparin-binding homodimeric glycoprotein which is an important growth and survival factor for endothelial cells. ¹ VEGF plays a critical role in physiological and pathological angiogenesis in most biological systems. ² VEGF is implicated in tumour neovascularisation and in angiogenesis associated with a number of chronic inflammatory diseases such as asthma, chronic obstructive pulmonary disease, inflammatory bowel disease, rheumatoid and osteoarthritis. ³ ⁴ ⁵ ⁶ ⁷ VEGF is secreted by a variety of cell types but not by endothelial cells themselves and mesenchymal cells serve as an important source of VEGF in a number of inflammatory and neoplastic processes. ⁸ There are at least five members of the VEGF family including placental growth factor, VEGF-A, VEGF-B, VEGF-C and VEGF-D. ⁹ The most potent angiogenic factor in vivo is VEGF-A which has six splice variants – 121, 145, 165, 183, 189, and 204 amino acids. ¹⁰ A number of stimuli are capable of increasing VEGF release in different biological systems. Inflammatory cytokines such as IL-1β and TGFβ increased VEGF release in human cholangiocellular carcinoma cells, synovial fibroblasts, cardiac myocytes and airway smooth muscle cells. ¹¹-¹⁴ We have previously shown that the pro-inflammatory asthma mediator, bradykinin (BK) increased VEGF production in human airway smooth muscle cells (HASMC). ¹⁵ A number of studies have shown that the products of COX-2, the inducible form of cyclooxygenase, may mediate the effect of cytokines.
and mediators on the release of chemokines and cytokines in an autocrine manner through a mechanism involving endogenous prostanoid production. Recent work suggests that this is also true of VEGF. Autocrine PGE\textsubscript{2} increases VEGF release in response to IL-1β in synovial fibroblasts and in response to bradykinin in HASMC. \textsuperscript{12,15} Furthermore exogenous PGE\textsubscript{2} increases VEGF expression in fibroblasts and osteoblasts. \textsuperscript{16,17,18} These studies are consistent with the known role of COX products in angiogenesis \textsuperscript{19,20}: COX-2 derived thromboxane A\textsubscript{2}, prostacyclin and PGE\textsubscript{2} stimulate endothelial cell migration and angiogenesis \textsuperscript{21} whereas COX inhibitors have protective effects on angiogenesis in experimental models. \textsuperscript{22,23} In asthma both COX-2 and VEGF are increased but the two have not been firmly linked. \textsuperscript{24,25} Collectively these studies suggest that PGE\textsubscript{2} can contribute to angiogenesis via increased VEGF production but the molecular mechanisms involved have not been studied in detail, particularly the balance between transcriptional and post-transcriptional events.

The VEGF promoter contains the hypoxia response element, hypoxia inducible factor-1α (HIF-1α), p53/Von Hippel Lindau (VHL), NFκB and AP-1 as well as several potential transcription factor binding sites for Sp-1 and AP-2. \textsuperscript{26} PGE\textsubscript{2} binds to G-protein coupled membrane receptors, the E prostanoid (EP) receptors. Four subtypes of EP receptors have been described EP\textsubscript{1}, EP\textsubscript{2}, EP\textsubscript{3} and EP\textsubscript{4} encoded by different genes. \textsuperscript{27} Each subtype is tissue specific and uses different intracellular signalling mechanisms suggesting potentially different inflammatory responses depending on receptor subtype binding. \textsuperscript{28} The receptor used by PGE\textsubscript{2} to increase VEGF is unknown.

Here we determined the molecular mechanisms involved in the transcriptional regulation of the VEGF promoter by exogenous PGE\textsubscript{2} in human airway smooth muscle cells. Mutational and deletional analysis of the VEGF promoter showed that Sp-1 transcription factor binding was essential for the increase in VEGF promoter activity produced by PGE\textsubscript{2}. PGE\textsubscript{2} caused phosphorylation of Sp-1 and EMSA and ChIP demonstrated that PGE\textsubscript{2} increased Sp-1 binding to the VEGF promoter. Furthermore studies with EP\textsubscript{2} and EP\textsubscript{4} receptor subtype agonists, the cAMP analogue 8 – bromoadenosine - 3’, 5’-cyclophosphoric acid (8-Br-cAMP), forskolin which increases adenylyl cyclase activity and the β-2 receptor agonist isoproterenol, showed that PGE\textsubscript{2} induced activation of Sp-1 was mediated by EP\textsubscript{2} and EP\textsubscript{4} receptors via cAMP.

**MATERIALS AND METHODS**

**Cell culture** Human tracheas were obtained from three post-mortem individuals. Primary cultures of human ASM cells were prepared from explants of ASM according to methods previously reported. \textsuperscript{29,30} Cells at passage 6 were used for all experiments. We have previously shown that cells grown in this manner depict the immunohistochemical and light microscopic characteristics of typical ASM cells. \textsuperscript{30}

**Experimental protocols** The cells were cultured to confluence in 24-well culture plates in a humidified, 5% CO\textsubscript{2}, 37°C incubator using Dulbecco’s modified Eagle’s medium (DMEM, Sigma, Poole, Dorset, UK) supplemented with 10% fetal calf serum (FCS, Seralab, Crawly Down, Sussex, UK), 100U/ml penicillin, 100µg/ml streptomycin, 4mM L-glutamine and 2.5µg/ml Amphotericin B (Sigma, Poole, Dorset, UK). The cells were growth-arrested in serum-free medium for 24 h prior to experiments. Immediately before each experiment, fresh serum-free medium containing PGE\textsubscript{2} or ethanol vehicle (Sigma, Poole, Dorset, UK) was added. In time course experiments cells were incubated with 1 µM PGE\textsubscript{2} for 2-24 h. In the concentration response experiments cells were incubated for 24h with 1 nM to 10 µM PGE\textsubscript{2}. In subsequent experiments 24h incubation times were used. At the indicated times, the culture media were harvested and stored at -20°C.

The highly selective EP\textsubscript{2} and EP\textsubscript{4} receptor subtype agonists ONO-AE1 259 and ONO-AE1 329, which were a gift from ONO Pharmaceuticals, Osaka, Japan, were used in the PGE\textsubscript{2} receptor studies. \textsuperscript{31,32} The cAMP analogue 8-Br-cAMP, the PKA inhibitor H-89, and forskolin and isoproterenol that increase cytosolic cAMP were purchased from Sigma, (Poole, Dorset, UK). Mithramycin was purchased from Tocris Cookson Ltd.,...
Aliquots of the RT products were subsequently amplified. Amplification was carried out with a PTC-100 programmable thermal controller (MJ Research Inc., Watertown, Massachusetts, USA) after an initial denaturation at 94 °C for 3 min. This was followed by 35 cycles of PCR of RT-PCR using the following temperature and time profile: denaturation at 94°C for 1 min, primer annealing 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 visualised by electrophoresis on 2% agarose gel in 0.5X TBE buffer after staining with 0.5µg/ml ethidium bromide. The ultraviolet (UV)-illuminated gels were photographed, and the densitometry was analysed using a GeneGenius gel documentation and analysis system (Syngene, Cambridge, UK).

RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR) Cells in 6-well plates were treated with PGE2 and collected at time 0, 1h, 2h, 4h, 8h and 24h, respectively. Total RNA was isolated by using the RNeasy mini kit (Qiagen, West Sussex, UK) following the manufacturer’s protocol with on-column DNase digestion. 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, USA), 25 units of RNase inhibitor (Promega), 0.5µg of oligo(dT)15 primer, 0.5mM of each dNTPs, 1 X 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 50µl containing 1mM MgCl2, 0.12 mM of each dNTPs, 1 unit of Taq polymerase (Promega), 0.5 µM of both the upstream and downstream PCR primers, 1 X PCR buffer provided by Promega.

Amplification was carried out with a PTC-100 programmable thermal controller (MJ Research Inc., Watertown, Mass., USA) after an initial denaturation at 94 °C for 3 min. This was followed by 35 cycles of PCR of RT-PCR using the following temperature and time profile:

- Denaturation at 94°C for 1 min, primer annealing 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 following primers were used: EP2-R sense 5’ – TCC AAT GAC TCC CAG TCT GAG GA – 3’, antisense 5’ – TCA AAG TGC AGC CTG TTT AC-3’.

The PCR products were visualised by electrophoresis on 2% agarose gel in 0.5X TBE buffer after staining with 0.5µg/ml ethidium bromide. The ultraviolet (UV)-illuminated gels were photographed, and the densitometry was analysed using a GeneGenius gel documentation and analysis system (Syngene, Cambridge, UK).

Quantitative Real time RT-PCR. VEGF-A expression was determined using primer sequences: - sense 5’ – CTTGCCCTTGCTCTTACC - 3’ and antisense 5’ – CACACAGAGTGGGCTTGAG - 3’. β-2-microglobulin was used as the housekeeping gene. 1 ng of reverse transcribed cDNA was subjected to real time PCR using Excite Real Time Mastermix with sybr green (Biogene, Cambridge, UK) and the ABI Prism 7700 detection system (Applied Biosystems, Warrington, Cheshire, UK). Each reaction consisted of 1x Excite mastermix, sybr green (1:60000 final concentration), 40nM of both sense and antisense primers, 1.6µl DNA (or dH2O) and H2O to a final volume of 20µl. Thermal cycler conditions included incubation at 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Integration of the fluorescent SYBR green into the PCR product was monitored after each annealing step. Amplification of one specific product was confirmed by melt curve analysis where a single melting peak eliminated the possibility of primer-dimer association. For melting curve analysis to be performed the products were heated from 60°C to 95°C over 20 minutes after the 40 cycles.

To enable the levels of transcripts to be quantified standard curves were generated using serial dilutions of KG1a cDNA. Negative controls consisting of no template were included and all reactions were set up in triplicate. VEGF-A expression was normalised to the housekeeping gene by dividing the mean of the VEGF-A triplicate value by the mean of the β-2-microglobulin triplicate value. This was then expressed as fold increase over unstimulated cells at each time point.

Flow cytometric analysis of EP4 receptors. HASMC were detached using a sterile scraper, washed and incubated with polyclonal rabbit anti-human EP4-R (Cayman Chemical, Ann Arbor, MI). The cells were washed twice and incubated
with FITC conjugated goat anti rabbit secondary antibody (Sigma). Pre-immune rabbit serum was used as the negative control (Sigma). Using the FACSCalibur flow cytometer (Becton Dickinson, Cowley, Oxford) and logarithmic amplification of the green fluorescence channel (FL-1), 10,000 events were acquired and analysed with CellQuest software (Becton Dickinson, Cowley, Oxford).

**Western blotting.** The nuclear protein fractions were prepared using Nu-Clear extraction kit (Sigma, Poole, Dorset, UK) following the manufacturer’s protocol. Western blotting was performed as described previously using a specific polyclonal rabbit anti human EP₂ receptor antibody (Cayman Chemical, Ann Arbor, MI) or mouse monoclonal anti human Sp1 antibody (IC6 Santa Cruz Biotechnology, CA, USA) and HRPO conjugated secondary antibody (DakoCytomation, Ely, Cambridgeshire). The human histiocytic lymphoma cell line U937 which is known to express EP₂ receptors was used as the positive control. The human T lymphoblastic leukaemia cell line Jurkat was used as a positive Sp-1 control.

**Cell Viability** The toxicity of all the chemicals and vehicles used in this study was determined by MTT assay. At the end of the experiment the culture media was removed and replaced with 250 µl media containing 1 mg/ml thiazolyl blue, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltertrazolium bromide (MTT Sigma, Poole, Dorset, UK) and incubated for 20 minutes in 37°C. This medium was removed and 250 µl DMSO was added to solublize the blue coloured tetrazolium. The optical density was read at 550 nm in a TECAN GENios (Tecan UK Limited, Theale, Reading, UK) microplate reader. Viability was set as 100% in control cells.

**Transfection with VEGF promoter driven luciferase constructs and Sp-1 reporter luciferase construct.** Cells were cultured in 24 well plates to confluence, growth arrested for 24 hours and transfected using 1µl LF2000 (Lipofectamine LF2000, Gibco Life Technologies Ltd., Paisley) and 0.8µg DNA per well according to the company instructions. The cells were co-transfected with 1ng per well of the internal control plasmid pRL-SV40 (Promega UK, Southampton, UK) containing the Renilla luciferase gene. After 24 hours incubation with or without 1µM PGE₂ the cells were harvested and the firefly and Renilla luciferase activities measured using the Dual Luciferase Assay System Kit (Promega UK, Southampton, UK) and Microlumat Plus LB 96V luminometer (Berthold Technologies GmbH & Co. KG, Bad Wildbag, Germany). The VEGF promoter driven luciferase constructs were a kind gift from Professor Dieter Marmé, Institute of Molecular Oncology, Tumor Biology Center, Freiberg, Germany. The Sp-1 reporter construct containing 6 Sp-1 binding sites was a kind gift from Professor Jeffrey E Kudlow, School of Medicine, The University of Alabama at Birmingham.

**Electrophoretic mobility shift assay (EMSA)** The nuclear protein fractions for EMSA were prepared using Nu-Clear extraction kit (Sigma, Poole, Dorset, UK) following the manufacturer’s protocol. Protein concentrations were determined using the Bio-Rad protein assay. Consensus Sp-1, AP-2 and EGR-1 oligonucleotides were purchased from Santa Cruz Biotechnology, CA, USA. VEGF promoter specific oligonucleotides which recognised the -85/-50 binding region: sense 5′-CCCGGGGCGGGCGGGGCGGCGGGGTCCCGGCGGGGCGGAG 3′ and antisense 5′-CTCCGCCCCGCCCCGGCCGCCGCCGCCGCCCCGGC CCGCCCCGGG 3′ were purchased from Sigma, Poole, Dorset, UK.

All oligonucleotides were labelled using [γ³²P] ATP (Amersham, UK) and T4 polynucleotide kinase (Promega, UK, Southampton, UK). Fifteen micrograms of nuclear protein, ²⁵P labelled double-stranded probe (40,000 counts per minute/ng) and 2 µl of 5X binding buffer [20% glycerol, 5mM MgCl₂, 2.5mM EDTA, 2.5mM DTT, 250mM NaCl, 50mM Tris-HCl (pH 7.5), 0.25mg/ml poly(dI-dC).poly(dI-dC)] were mixed in a total volume of 10 µl. In competition assays, 50X unlabelled competitors were added at the same time of probe addition. The mixture was incubated at room temperature for 30 min, then loaded on a 5% polyacrylamide gel in 0.5X TBE buffer, and subjected to electrophoresis for 60 minutes. The gel was dried and exposed for autoradiography on Kodak XAR film at -70°C for 19-48 hours. Supershift were performed using 4 µg of specific goat polyclonal anti-human Sp-1.
Chromatin Immunoprecipitation (ChIP) assay. The HASM cells were cultured to confluence in 75 cm² flasks, growth arrested and incubated with ethanol vehicle or 1 μM PGE₂ for 30 minutes. The ChIP assay was performed using the ChIP-IT kit (Active Motif, Rixensart, Belgium) following the manufacturer’s protocol and using 4 μg goat anti-human polyclonal Sp-1 antibody (PEP 2) and AP-2 (C-18) or EGR-1 (588) antibody (Santa Cruz Biotechnology, CA, USA).

The VEGF primers used yielded a 202 bp product corresponding to −199 to +3 of the VEGF gene promoter and were: forward 5'-GGT CGT TCC CCT TCA- 3' and reverse 5'-GAT CCT TCC CCT TCA- 3'. 40 cycles of a two step PCR program 95°C for 1 minute and 60°C for 1 minute in the presence of 6% DMSO and 1M betaine using Red Taq and 2.5mM magnesium chloride (Sigma, Poole, Dorset, UK).

Potential problems with PCR resulting from high melting temperatures were reduced by addition of DMSO and the amplification of GC rich templates was enhanced by betaine. The PCR products were visualised by electrophoresis on 2% agarose gel in 0.5X TBE buffer after staining with 0.5ig/ml ethidium bromide. The ultraviolet-illuminated gels were photographed, and densitometry performed using GeneGenius gel documentation and analysis system (Syngene, Cambridge, Cambridgshire, UK).

Statistical analysis: VEGF ELISA and luciferase levels were expressed as the mean of the triplicate or quadruplicate wells for that experiment. The experiments were repeated at least three times and the results shown represent the mean and standard error of the mean (SEM). Analysis of variance (ANOVA) was used to determine significant differences. A p value of <0.05 (2-tailed) was regarded as statistically significant.

RESULTS

PGE₂ increases VEGF-A₁₆₅ protein production. There was a significant increase in VEGF release above control in cells cultured for 24 hours with concentrations of PGE₂ ranging from 1 nM to 1μM. (Figure 1A). Cells treated with 1μM PGE₂ for 2, 4, 8, 16 and 24 hours also showed significantly increased VEGF levels compared with unstimulated control cells (Figure 1B).

PGE₂ increased VEGF is transcriptional. Real time RT-PCR showed that PGE₂ increased VEGF-A mRNA levels with time with a six-fold increase at 60 minutes and a peak twelve-fold increase by 4 hours compared with controls at these times (Figure 2A). To confirm that this was due to VEGF-A gene transcription rather than stabilisation of mRNA the cells were cultured for 30 minutes with 5 μg/ml actinomycin D, an inhibitor of RNA polymerase II, followed by 1μM PGE₂. Pre-treatment with actinomycin D prevented the PGE₂ induced increase in VEGF mRNA (Figure 2A).

The cells were transfected with a 2068 bp VEGF promoter fragment (-2018 to +50) ligated to firefly luciferase. There was a 5.6 ± 0.48 fold increase in luciferase activity in cells treated with 1μM PGE₂ compared with unstimulated cells (Figure 2B). These results suggest that induction of VEGF by PGE₂ is transcriptional and not mediated by post-transcriptional stabilisation of PGE₂ mRNA.

Mutations in the Sp-1 binding sites in the VEGF promoter reduce PGE₂ stimulated luciferase activity. To determine which transcription factors are involved the cells were transfected with 2068 bp of the wild type VEGF promoter and a series of deletion constructs ligated to a firefly luciferase reporter plasmid. A diagram representing the VEGF promoter showing the key transcription factor binding sites and the positions where the restriction enzymes cleave the promoter to generate the series of deletions is shown in Figure 3A. 1 μM PGE₂ increased the luciferase levels 4.2 ± 0.65 in cells transfected with the 2068 construct. There was a significant PGE₂ mediated increase in luciferase activity with all of the deletions series except the smallest 102 bp fragment of the VEGF promoter (Figure 3B). However, deleting the sequences between -1286 and -789 bp resulted in a reduction in the stimulatory effect of the PGE₂. This suggests that the upstream AP-1, AP-2 or HIF-1α sequences may also be involved in PGE₂ mediated VEGF increase.

Transfection studies using the wild type construct and a construct containing mutations of the three Sp-1 (-88/-50) binding sites showed a significant
PGE₂ increases Sp-1 binding to the VEGF promoter

We used EMSA to determine whether PGE₂ treatment increased Sp-1 binding to the VEGF promoter. Incubation with 1 μM PGE₂ for 60 minutes induced binding activity with Sp-1 consensus and VEGF promoter oligonucleotides (Figure 5A and 5E). This was not seen with AP-1 and EGR-1 consensus (Figure 5B and 5C). Supershift studies using a monoclonal antibody to Sp-1 produced gel retardation with the consensus sequence (Figure 5A) and a reduction in binding with the VEGF promoter specific primers (Figure 5F). Competition with 50 fold excess unlabelled VEGF promoter oligonucleotides blocked transcription factor binding whereas excess irrelevant AP-1 oligonucleotides did not block Sp-1 transcription factor binding, demonstrating that the binding was specific (Figure 5E). There was no transcription factor binding in experiments using mutated consensus Sp-1 oligonucleotides (Figure 5D).

ChIP

The PGE₂ mediated increase in Sp-1 binding to the VEGF promoter demonstrated by EMSA was confirmed using the chromatin immunoprecipitation (ChIP) assay. Protein/DNA complexes were immunoprecipitated with antibody to Sp-1 and the DNA isolated and purified. An aliquot of non-immunoprecipitated chromatin was used as the Input control and no antibody control was included to show specificity. Input, control and immunoprecipitated DNA was subjected to 40 cycles of 2 step PCR in the presence of 1M betaine and 6% DMSO using VEGF promoter specific primers spanning –199 to +3 bp. (Figure 6A). Densitometry showed a significant increase in Sp-1 binding to the VEGF promoter following incubation with PGE₂. The results were normalised to the Input control (Figure 6B).

EP₂ and EP₄ receptor agonists mimic the effect of PGE₂

Both EP₂ and EP₄ receptors, which positively couple to adenylyl cyclase, are present on HASMCC as demonstrated by flow cytometry and a specific antibody to the EP₂ receptor (Figure 7A and 7B), by Western blotting and a specific antibody to the EP₂ receptor (Figure 7C) and RT-PCR (Figure 7D),

To determine which PGE₂ receptors were important we looked at the effect of PGE₂ receptor agonists on VEGF production together with luciferase activity in cells transfected with the 2068 VEGF promoter construct. We found that both EP₂ (ONO-AE1 259) and EP₄ (ONO-AE1 329) receptor agonists increased VEGF production and luciferase activity (Figure 7E and 8C) in the same way as PGE₂. There was an additive effect when both agonists were used in suboptimal concentrations (Figure 7E). This suggests that PGE₂ is acting via both EP₂ and EP₄ receptors.

Increasing intracellular cAMP mimics the effect of PGE₂

The cAMP analogue 8 - Br-cAMP increased VEGF protein production in a concentration dependent manner (Figure 8A). Agents which increase cAMP also similarly increased VEGF release. Forskolin, a direct activator of adenylyl cyclase (Figure 8A), and the β-adrenoceptor agonist isoproterenol (Figure 8B) both increased VEGF and luciferase activity in cells transfected with the 2068 VEGF promoter construct (Figure 8C).

Mithramycin inhibits PGE₂ induced activation of VEGF

The anti cancer antibiotic, mithramycin A (MTR) selectively binds to G-C rich regions of DNA preventing Sp-1 binding. Pre-incubation for 1 hour with 500nM and 1μM mithramycin significantly reduced PGE₂ stimulated VEGF protein release in a concentration dependent manner. Maximal inhibition was seen using 1μM mithramycin (Figure 9A). Basal levels of VEGF production were not changed significantly by mithramycin treatment (data not shown).

Inhibition of PKA abrogates PGE₂ induced activation of VEGF

Pre-incubation for 1 hour with 10μM H-89, an inhibitor of PKA, prior to 24 hours culture with 1μM PGE₂ resulted in a significant reduction in secreted VEGF as measured by ELISA (Figure 9A).

Nuclear Sp-1 protein is phosphorylated by PGE₂

Western blotting demonstrated that Sp-1 protein expression was confined to the nucleus and was phosphorylated by PGE₂. Previous studies have shown that the 106k Sp-1 band represents the phosphorylated protein. 41 GAPDH and the
nuclear specific protein lamin A and C were used as controls (Figure 9B). Increasing intracellular cAMP increases Sp-1 luciferase reporter activity. Agents which increase cAMP also increased a six repeat Sp-1/luciferase reporter construct. The cAMP analogue 8-bromo cAMP, forskolin, a direct activator of adenylyl cyclase and the β-adrenoceptor agonist isoproterenol all increased the activity of a transiently transfected Sp-1 reporter luciferase construct. (Figure 9C)

**DISCUSSION**

There are several key novel findings in this study. We found that PGE\(_2\) increases VEGF-A expression through transcriptional mechanisms involving the GC-rich Sp-1 transcription factor binding sites on the proximal (-88/-50) region of the VEGF promoter. Furthermore the effect was mediated by EP\(_2\) and EP\(_4\) receptors via cAMP and PKA. These studies are the first in any biological system to study the transcription factors involved in VEGF production by PGE\(_2\) and also delineate the upstream signalling cascade components.

We first determined whether PGE\(_2\) was acting via transcriptional or post-transcriptional mechanisms. Stimulation with PGE\(_2\) resulted in increased VEGF-A protein release after 2 hours as measured by ELISA. Quantitative real time RT-PCR also showed that PGE\(_2\) increased VEGF-A mRNA after 1 hour. Pre treatment of the cells with the RNA polymerase II inhibitor actinomycin D prevented this, suggesting that the increased VEGF mRNA was due to transcriptional rather than post-transcriptional mechanisms. Furthermore mRNA stability experiments showed no alteration in mRNA half-life after PGE\(_2\) treatment. Collectively these studies suggest that VEGF was regulated transcriptionally by PGE\(_2\) and this was confirmed by studies using VEGF promoter luciferase constructs. A few previous studies have looked at whether PGE\(_2\) increases VEGF transcriptionally or post-transcriptionally. PGE\(_2\) mediated VEGF upregulation was transcriptional in osteoblasts\(^{16}\) and overexpression of myc in B cells increased the initiation of VEGF mRNA translation.\(^{42}\)

To determine key transcription factor binding sites we used a series of deletions of the VEGF promoter ranging from 2068 to 102 bp. We found that promoter activity was maintained down to the 135 bp construct. However, all luciferase activity was lost using the 102 bp construct suggesting that the main regulatory sites were contained within the region 102-135bp. This region contains one AP-2, two EGR-1 and three Sp-1 transcription factor binding sites. To explore this further we used constructs with mutations in the Sp-1 or EGR-1 binding sites. We found no reduction in luciferase levels using constructs containing mutated EGR-1 sites whereas a construct with mutations in all three Sp-1 sites resulted in loss of luciferase activity suggesting that Sp-1 binding was crucial to VEGF induction by PGE\(_2\). These observations were also supported by EMSA results which showed that PGE\(_2\) increased Sp-1 but not AP-2 or EGR-1 binding.

Specificity of binding was demonstrated by experiments using excess unlabelled oligonucleotides and supershift with Sp-1 antibody. The EMSA results were confirmed by ChIP assay and specific antibody to Sp-1. Consistent with a role for Sp-1, VEGF production was inhibited by the Sp-1 inhibitor mithramycin.\(^{43}\) Using Western blotting we also showed that Sp-1 is a nuclear protein which is phosphorylated by PGE\(_2\).

Ours are the first studies to show that Sp-1 is involved in PGE\(_2\) induced VEGF production although Sp-1 is important in the activation of genes involved in tumour proliferation and the induction of VEGF in response to some other stimuli.\(^{11;34;44;45}\) For example, VEGF induction by IL-1β in cardiac myocytes and by TNF-α in glioma cells is mediated through Sp-1 sites.\(^{13}\)\(^{46}\) In contrast transforming growth factor-α induced VEGF via AP-2 transcription factor binding.\(^{47}\)

Having shown that VEGF production was transcriptionally mediated via Sp-1 we then went on to characterise the prostanoid receptor involved. PGE\(_2\) binds to a family of 7 transmembrane G-protein coupled membrane receptors, the E prostanoid (EP) receptors. Four subtypes of EP receptors have been described EP\(_1\), EP\(_2\), EP\(_3\) and EP\(_4\) encoded by different genes.\(^{27}\) Each subtype is tissue specific and uses different intracellular signalling mechanisms suggesting potentially different inflammatory responses depending on receptor subtype binding.\(^{28}\) We focussed on the two EP receptors, EP\(_3\) and EP\(_4\), which activate cAMP. We found that both
EP₂ and EP₄ receptors were expressed in HASMC. This contrasts to a previous study which reported EP₂ but not EP₄ receptor expression in HASM.²⁸ We confirmed our findings using both RT-PCR and either Western blotting or FACS for EP₂ and EP₄ respectively using antibodies designed for these methodologies. The fact that both mRNA and protein to EP₄ receptors was present suggests that these cells do indeed express EP₄ receptors. Furthermore experiments with EP₂ and EP₄ receptor agonists mirrored the effect of PGE₂ on VEGF protein production and reporter activity suggesting that both of these receptors are implicated in this process. This is similar to Clarke et al who suggested that positive regulation of G-CSF by E-Ring 8-isoprostanes in HASMC was mediated by EP₂ and EP₃ receptors.⁴⁹

EP₂ and EP₄ receptors couple to Gₛ protein which stimulates adenylyl cyclase activity increasing intracellular cAMP levels resulting in PKA signalling. We performed experiments using a variety of pharmacological tools to probe the role of different components of this pathway. Isoproterenol which elevates cAMP via beta adrenoceptors and forskolin, a direct activator of adenylyl cyclase, had similar effects to PGE₂ on VEGF protein production and VEGF promoter luciferase expression suggesting cAMP pathways regulate VEGF release. Further evidence in support of a role for cAMP was obtained from studies using 8-Br-cAMP, a cell permeable cAMP analogue. Increasing cAMP also increased the activity of a transfected Sp-1 luciferase reporter construct. To explore the main downstream target of cAMP, PKA, we studied the effect of the PKA inhibitor H-89. We found that H-89 markedly inhibited PGE₂ induced VEGF protein production suggesting that it was PKA mediated, although it is possible that other kinases mediated this effect.⁵⁰

Our studies have relevance for asthma where several immunohistochemical studies have shown that COX-2 and VEGF are both upregulated.²⁴²⁵ They are also of relevance to a wide range of inflammatory and malignant diseases where increased prostanoid production has been implicated in angiogenic processes mediated via VEGF release. Strategies targeting Sp-1 mediated gene transcription may provide a new therapeutic approach to influence remodelling processes. In conclusion our studies provide evidence that PGE₂ induces VEGF via Sp-1 binding sites on the VEGF promoter via EP₂ and EP₄ receptors in a cAMP and PKA dependent mechanism involving phosphorylation of Sp-1.

REFERENCES


FOOTNOTES

Thanks to Dieter Marmé, Institute of Molecular Oncology, Tumor Biology Center, Freiburg, Germany for providing the VEGF luciferase promoter constructs, to Jeffrey E Kudlow, School of Medicine at the University of Alabama at Birmingham for the Sp-1 reporter construct. Funded by Asthma UK.

The abbreviations used are: VEGF, vascular endothelial growth factor; HASMC, human airway smooth muscle cells; EMSA, Electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation; 8-BR-cAMP, 8 – bromoadenosine - 3’,-5’ cyclophosphoric acid; BK bradykinin; COX, cyclooxygenase; RTPCR, reverse transcriptase polymerase chain reaction; PGE2, prostaglandin E2; MTR, mithramycin; EP, E prostanoid: ELISA, enzyme-linked immunosorbent assay.

FIGURE LEGENDS

**Fig.1.** (A) VEGF production in response to 1 nM to 1 µM of PGE2. The VEGF released into the culture medium was measured by ELISA. (B) Time course of VEGF production in HASMC treated with 1 µM PGE2 for 0, 2, 4, 6, 8, 16 and 24 hours compared with unstimulated control cells. Each point represents the mean ± SEM of quadruple determinations from three independent experiments (* p<0.05, ** p<0.01 and *** p<0.001 by ANOVA).

**Fig. 2.** (A) Time-course of PGE2 mediated VEGF mRNA induction. ASMC were incubated with and without 1 µM PGE2 for 0, 1, 2, 4, 8 and 24 hours. The housekeeping gene β2 microglobulin and VEGF mRNA were measured by quantitative real time RT-PCR. Pre-incubation with 5 µg/ml actinomycin D (ACT D), an inhibitor of transcription, inhibits PGE2 mediated VEGF mRNA at all time points. The VEGF results were normalised by dividing the mean of the triplicate VEGF result by the mean of the triplicate β2 microglobulin result and are expressed as fold increase over control.
(B) Luciferase activity in ASMC transiently transfected for 24 hours with either a 2068 bp fragment of the VEGF promoter (-2018/+50) or control vector pGL3 basic, ligated to a luciferase reporter construct. Cells were cultured to confluence, growth arrested and transfected with 1 µl LF2000 and 0.8 µg DNA per well. There was a significant increase in promoter activity in cells stimulated for 24 hours with 1 nM up to 1 µM PGE₂ compared with unstimulated control cells (** p<0.01, *** p<0.001). The figure represents the mean and SEM of three experiments performed in triplicate.

**Fig. 3.** (A) Representation of the VEGF promoter driven luciferase constructs used in the transfection studies showing the positions of the transcription factor binding sites. (B) Increase in luciferase expression in ASMC transiently transfected with the deletion series of the VEGF promoter luciferase constructs after 24 hours incubation with 1 µM PGE₂. Cells were cultured to confluence, growth arrested and transfected using 1 µl LF2000 and 0.8 µg DNA per well. The figure represents the mean and SEM of three experiments performed in triplicate. (* p<0.05, ** p<0.01 and *** p<0.001 by ANOVA).

**Fig. 4.** (A) Position of the transcription factor binding sites present on the 135 bp VEGF promoter. (B) The effect of binding site mutations on VEGF promoter activity in response to 1 µM PGE₂. Three Sp-1 binding sites GGGCGG mutated to GTTCGG and both EGR-1 binding sites GCGGGGCG mutated to GCTAGGGCG. The graph shows fold increase of luciferase activity in cells treated with PGE₂ compared unstimulated cells. The figure represents the mean and SEM of three experiments performed in triplicate and was analysed by ANOVA, *** p<0.001.

**Fig. 5.** (A) PGE₂ increases consensus Sp-1 binding and addition of anti-Sp-1 antibody to the nuclear extracts from cells treated with 1 µM PGE₂ for 60 minutes resulted in a supershift. (B) 1 µM PGE₂ for 60 minutes did not increase consensus AP-2 binding or (C) EGR-1 binding. Addition of antibodies to AP-2 or EGR-1 to the nuclear extracts did not result in gel retardation. (D) Sp-1 binding is inhibited when a mutated consensus Sp-1 construct is used. (E) PGE₂ increases transcription factor binding to the VEGF promoter (-88/-50). Binding was specific as shown by competitive binding. Nuclear extracts from PGE₂ treated cells were incubated with labelled VEGF promoter (hot VEGF) in the presence of a 50 fold excess of unlabelled VEGF promoter (cold VEGF) or unlabelled AP-1 (cold AP-1). (F) Antibody to Sp-1 diminishes Sp-1 binding to the VEGF promoter. The figures shown are representative of three experiments.

**Fig. 6.** (A) Representative ChIP assay PCR showing PGE₂ increases Sp-1 binding to the VEGF promoter. Immunoprecipitation (IP) was carried out using antibody to Sp-1. The PCR primers amplified –199 to +3 region of the VEGF promoter. (B) Densitometry of ChIP PCR normalised to the Input. Duplicate experiments repeated in triplicate (*** p<0.001 ANOVA).

**Fig. 7.** (A) Flow cytometry histogram of unstimulated ASMC stained with pre-immune rabbit serum control and FITC conjugated secondary antibody. (B) Flow cytometry histogram of unstimulated ASMC stained with polyclonal rabbit anti-human EP₂ receptor and FITC conjugated secondary antibody. (C) Western blotting of ASMC showing EP₂ receptor protein. U937 cells were used as a positive control. (D) RT-PCR demonstrating mRNA for both EP₂ and EP₄ receptors. (E) Agonists to EP₂ and EP₄ receptors also increase VEGF protein production by ELISA over control. 1 µM PGE₂ is also shown (*** p<0.001 ANOVA).

**Fig. 8.** Agents which increase intracellular cAMP levels mimic 1 µM PGE₂ and increase VEGF production. (A) Concentration response to forskolin (FSK), which directly activates adenylyl cyclase and the cAMP analogue 8 – bromoadenosine - 3’,-5’ cyclophosphoric acid increases VEGF,
the β adrenergic agonists (B) Isoproterenol (ISO). (C) Agents which act via cAMP mimic PGE₂ and stimulate VEGF promoter driven luciferase activity. (* p<0.05, ** p<0.01 and *** p<0.001 by ANOVA).

**Fig. 9.** (A) Reduction of VEGF protein release by the Sp-1 inhibitor Mithramycin A (MTR) and PKA inhibitor H-89. Pre-incubation with 500nM and 1µM MTR or 1µM and 10µM H-89 prior to 24 hours culture with 1µM PGE₂ significantly reduced PGE₂ stimulated VEGF measured by ELISA. (* and # p<0.05, ** and ## p<0.01, *** and ### p<0.001 by ANOVA, NS not significant). (B) Western blotting showing the nuclear localisation of Sp-1 and increased phosphorylation of 106k Sp-1 by 15 minutes incubation with 1µM PGE₂. Lanes 1 and 2 are cytosolic fraction - control (1) and PGE₂ (2). Lanes 3 and 4 are nuclear fraction - control (3) and PGE₂ (4). GAPDH and nuclear lamin were used as housekeeping controls. (C) PGE₂, cAMP analogue and salbutamol and forskolin which increase cellular cAMP, increased luciferase activity of a transfected Sp-1 luciferase reporter construct (** p<0.01 and *** p<0.001 by ANOVA).
Figure 1
Figure 2

**Figure A**

Bar chart showing the expression of VEGF A/B2M over time in control, PGE$_2$, and PGE$_2$ ACTD groups.

**Figure B**

Bar chart showing the expression of Firefly luciferase with different concentrations of PGE$_2$ in pGL3 groups.
A

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B

![Figure 3](http://www.jbc.org/)

**Figure 3**
Figure 4

A

EGR-1

AP2

Sp1 (I)  Sp1 (II)  Sp1 (III)

CCC GGG GGG GGG GGG GGG GGG GGG GGG

GGGTCC GGG GGG GGG GGG GGG GGG AG

-50

B

Fold Increase

Control  2068  135 WT  Sp1m  EGR-1m

VEGF promoter

Fold Increase

0  1  2  3

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Figure 6
Figure 7
Figure 8

A

![Graph showing VEGF pg/ml vs FSK µM]

B

![Graph showing VEGF pg/ml vs ISO µM]

C

![Graph showing Firefly luciferase vs various treatments]

Firefly luciferase

Control, PGE2 1uM, EP-2 agonist 1uM, EP-4 agonist 1uM, FSK 100 uM, ISO 10 uM, 8-bromo 1mM
Figure 9

A

![Graph showing VEGF pg/ml levels with different treatments](image)

Control | PGE2 | PGE2 100nM MTR | PGE2 + 500 nM MTR | PGE2 + 1uM MTR | PGE2 + 10 uM H-89 | PGE2 + 10 uM H-89

*** | NS | ** | NS | NS

B

![Western blot showing protein levels](image)

Lane 1: Control
Lane 2: PGE2 1uM
Lane 3: 8 bromo cAMP 1mM
Lane 4: Salb 100 uM
Lane 5: FSK 1uM

106k phospho Sp-1
70k Lamin A
Lamin C
37k GAPDH

C

![Bar graph showing fold increase](image)

Control | PGE2 1uM | 8 bromo cAMP 1mM | Salb 100 uM | FSK 1uM

*** | ** | ** | ***

Fold Increase
Vascular endothelial growth factor induction by prostaglandin E2 in human airway smooth muscle cells is mediated by E prostanoid EP2/EP4 receptors and SP-1 transcription factor binding sites

Dawn A. Bradbury, Deborah L. Clarke, Claire Seedhouse, Lisa M. Corbett, Joanne Stocks and Alan J. Knox

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