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Vascular Endothelial Growth Factor Induction by Prostaglandin E_2 in Human Airway Smooth Muscle Cells Is Mediated by E Prostanoid EP_2/EP_4 Receptors and SP-1 Transcription Factor Binding Sites*

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Prostaglandin E_2 (PGE_2) can increase vascular endothelial growth factor A (VEGF-A) production but the mechanisms involved are unclear. Here we characterized the transcriptional mechanisms involved in human airway smooth muscle cells (HASMC). PGE_2 increased VEGF-A mRNA and protein but not mRNA stability. PGE_2 stimulated the activity of a transiently transfected 2068-bp (−2018 to +50) VEGF-A promoter-driven luciferase construct. Functional 5′ deletional analysis mapped the PGE_2 response element to the 135-bp sequence (−85/+50) of the human VEGF-A promoter. PGE_2-induced luciferase activity was reduced in cells transfected with a 135-bp VEGF promoter fragment containing mutated Sp-1 binding sites but not in cells transfected with a construct containing mutated EGR-1 binding sites. Electrophoretic mobility shift assay and chromatin immunoprecipitation assay confirmed binding of Sp-1 to the VEGF promoter. PGE_2 increased phosphorylation of Sp-1 and luciferase activity of a transfected Sp-1 reporter construct. PGE_2 receptor agonists EP_2 (ONO-AE1 259) and EP_4 (ONO-AE1 329) mimicked the effect of PGE_2, and reverse transcription-PCR, Western blotting, and flow cytometry confirmed the presence of EP_2 and EP_4 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 PGE_2 increases VEGF transcriptionally and involves the Sp-1 binding site via a cAMP-dependent mechanism involving EP_2 and EP_4 receptors.

Vascular endothelial growth factor (VEGF)[1] is a 45-kDa heparin-binding homodimeric glycoprotein that 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 tumor neovascularization and in angiogenesis associated with a number of chronic inflammatory diseases, such as asthma, chronic obstructive pulmonary disease, inflammatory bowel disease, rheumatoid and osteoarthritis (3–7). 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 interleukin-1β and transforming growth factor-β 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, 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_2 increases VEGF release in response to interleukin-1β in synovial fibroblasts and in response to bradykinin in HASMC (12, 15). Furthermore, exogenous PGE_2 increases VEGF expression in fibroblasts and osteoblasts (16–18). These studies are consistent with the known role of COX products in angiogenesis (19, 20): COX-2-derived thromboxane A_2, prostacyclin, and PGE_2 stimulate endothelial cell migration and angiogenesis (21), whereas COX inhibitors have protective effects on angiogenesis in experimental models (22, 23). In asthma both COX-2 and VEGF are increased but the two have not been firmly linked (24, 25).

Collectively these studies suggest that PGE_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, p53/Von Hippel Lindau, NFκB, and AP-1 as well as several potential transcription factor binding sites for Sp-1 and AP-2 (26). PGE_2 binds to G protein-coupled membrane receptors, the E prostanoid (EP) receptors. Four subtypes of EP receptors have been described, EP_1, EP_2,

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[1] 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; COX, cyclooxygenase: RT, reverse transcription; PGE_2, prostaglandin E_2; EP, E prostanoid; ELISA, enzyme-linked immunosorbent assay; PKA, protein kinase A; ANOVA, analysis of variance; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PKA, protein kinase A; AP, activator protein; EGR, early growth response.

EP_3, and EP_4, encoded by different genes (27). Each subtype is tissue-specific and uses different intracellular signaling mechanisms suggesting potentially different inflammatory responses depending on receptor subtype binding (28). The receptor used by PGE_2 to increase VEGF is unknown.

Here we determined the molecular mechanisms involved in the transcriptional regulation of the VEGF promoter by exogenous PGE_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_2. PGE_2 caused phosphorylation of Sp-1 and electrophoretic mobility shift assay (EMSA), and chromatin immunoprecipitation (ChIP) demonstrated that PGE_2 increased Sp-1 binding to the VEGF promoter. Furthermore, studies with EP_2 and EP_4 receptor subtype agonists, the cAMP analogue 8-bromoadenosine-3′,5′-cyclophosphoric acid (8-Br-cAMP), forskolin, which increases adenylyl cyclase activity, and the β2 receptor agonist isoproterenol, showed that PGE_2-induced activation of Sp-1 was mediated by EP_2 and EP_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 (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 (30).

Experimental Protocol—The cells were cultured to confluence in 24-well culture plates in a humidified, 5% CO_2, 37 °C incubator using Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal calf serum (Seralab, Crawly Down, Sussex, UK), 100 units/ml penicillin, 100 μg/ml streptomycin, 4 mM L-glutamine, and 2.5 μg/ml amphotericin B (Sigma). 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_2 or ethanol vehicle (Sigma) was added. In time course experiments cells were incubated with 1 μM PGE_2 for 2–24 h. In the concentration response experiments cells were incubated for 24 h with 1 nM to 10 μM PGE_2. In subsequent experiments, 24-h incubation times were used. At the indicated times, the culture media were harvested and stored at −20 °C. The highly selective EP_2 and EP_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_2 receptor studies (31, 32).

The cAMP analogue 8-Br-cAMP, the PKA inhibitor H-89, and forskolin and isoproterenol, which increase cytosolic cAMP, were purchased from Sigma. Mithramycin was purchased from Tocris Cookson Ltd. (Avonmouth, Bristol, UK). The cells were preincubated for 1 h with the inhibitors.

VEGF-A Assay—The enzyme-linked immunosorbent assay (ELISA) was used to measure VEGF-A (R&D Systems, Abingdon, Oxon, UK) according to the manufacturer's instructions and has been described by us in detail elsewhere (15).

RNA Isolation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)—Cells in 6-well plates were treated with PGE_2 and collected at time 0, 1, 2, 4, 8, and 24 h, 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 Moloney murine leukemia virus 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 (Promega), 0.5 μM of both the upstream and downstream PCR primers, and 1× PCR buffer, provided by Promega.

Amplification was carried out with a PTC-100 programmable thermal controller (MJ Research Inc., Watertown, MA) after an initial denaturation at 94 °C for 3 min. This was followed by 35 cycles of 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: EP_2-R sense 5′-TCCAATGACTCCCAGTCTGAGGA-3′, antisense 5′-TCAAAGGTCAGCCTGTTTAC-3′; EP_4-R sense 5′-TCTGACCTCGGTGTCCAAAAATCG-3′, antisense 5′-TGGGTACTGCAGCCGCGAGCTA-3′ (33), and GAPDH sense 5′-CCACCCATGGCAAATTCCATGGCA-3′, GAPDH antisense 5′-TCTAGACGGCAGGTCAGGTCCACC-3′.

The PCR products were visualized by electrophoresis on a 2% agarose gel in 0.5× TBE buffer after staining with 0.5 μg/ml ethidium bromide. The ultraviolet (UV)-illuminated gels were photographed, and the densitometry was analyzed 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′-CTTGCCTTGCTGCTCTACC-3′ and antisense 5′-CACACAGGATGGCTTGAAG-3′ (34). β_2-Microglobulin was used as the housekeeping gene (35). 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 1× Excite mastermix, SYBR green (1:60000 final concentration), 40 nM of both sense and antisense primers, 1.6 μl of DNA (or dH_2O), and H_2O to a final volume of 20 μl. Thermal cycler conditions included incubation at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Integration of the fluorescent SYBR green into the PCR product was monitored after each annealing step. Amplification of one specific product was confirmed by melting 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 to 95 °C over 20 min 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 normalized 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 EP_4 Receptors—HASMC were detached using a sterile scraper, washed, and incubated with polyclonal rabbit anti-human EP_4-R (Cayman Chemical, Ann Arbor, MI). The cells were washed twice and incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit secondary antibody (Sigma). Preimmune rabbit serum was used as the negative control (Sigma). Using the FACSCalibur flow cytometer (BD Biosciences) and logarithmic amplification of the green fluorescence channel (FL-1), 10,000 events were acquired and analyzed with CellQuest software (BD Biosciences).

Western Blotting—The nuclear protein fractions were prepared using Nu-Clear extraction kit (Sigma) following the manufacturer's protocol. Western blotting was performed as described previously using a specific polyclonal rabbit anti-human EP_2 receptor antibody (Cayman Chemical) or mouse monoclonal anti-human Sp1 antibody (1C6; Santa Cruz Biotechnology, Santa Cruz, CA) and horseradish peroxidase-conjugated secondary antibody (DakoCytomation, Ely, Cambridgeshire, UK) (33). The human histiocytic lymphoma cell line U937, which is known to express EP_2 receptors, was used as the positive control. The human T lymphoblastic leukemia 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 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltertrazolium bromide assay (29). At the end of the experiment the culture media was removed and replaced with 250 μl of media containing 1 mg/ml thiazolyl blue, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltertrazolium bromide (Sigma) and incubated for 20 min in 37 °C. This medium was removed and 250 μl of Me_2SO was added to solubilize the blue-colored 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 h, and transfected using 1 μl of LF2000 (Lipofectamine LF2000, Invitrogen) and 0.8 μg of DNA per well according to the manufacturer's instructions. The cells were co-transfected with 1 ng/well of the internal control plasmid pRL-SV40 (Promega UK, Southampton, UK) containing the Renilla luciferase gene. After 24 h incubation with or without 1 μM PGE_2, the cells were harvested and the firefly and Renilla luciferase activities were measured using the Dual Luciferase Assay System Kit (Promega) and Microlumat Plus LB 96V luminometer (Berthold Technologies GmbH &

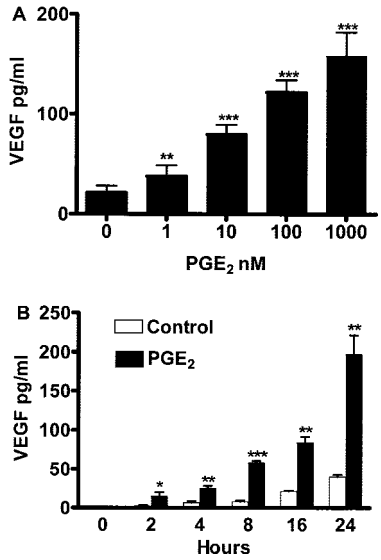

FIG. 1. *A*, VEGF production in response to 1 nM to 1 μM PGE_2. The VEGF released into the culture medium was measured by ELISA. *B*, time course of VEGF production in HASMC treated with 1 μM PGE_2 for 0, 2, 4, 6, 8, 16, and 24 h compared with unstimulated control cells. Each point represents the mean ± S.E. of quadruple determinations from three independent experiments (*, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ by ANOVA).

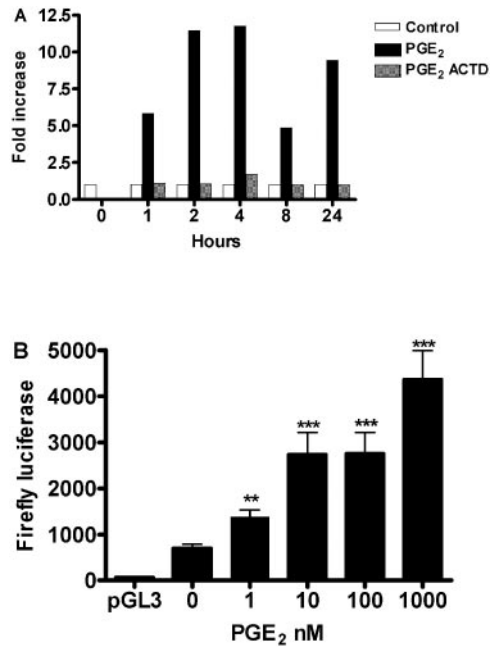

FIG. 2. *A*, time course of PGE_2-mediated VEGF mRNA induction. ASMC were incubated with and without 1 μM PGE_2 for 0, 1, 2, 4, 8, and 24 h. The housekeeping gene β_2-microglobulin and VEGF mRNA were measured by quantitative real-time RT-PCR. Preincubation with 5 μg/ml actinomycin D (*ACT D*), an inhibitor of transcription, inhibits PGE_2-mediated VEGF mRNA at all time points. The VEGF results were normalized by dividing the mean of the triplicate VEGF result by the mean of the triplicate β_2-microglobulin result and are expressed as -fold increase over control. *B*, luciferase activity in ASMC transiently transfected for 24 h 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 of LF2000 and 0.8 μg of DNA per well. There was a significant increase in promoter activity in cells stimulated for 24 h with 1 nM up to 1 μM PGE_2 compared with unstimulated control cells (**, $p < 0.01$; ***, $p < 0.001$). The figure represents the mean ± S.E. of three experiments performed in triplicate.

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, Freiburg, Germany (36). 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 (37).

EMSA—The nuclear protein fractions for EMSA were prepared using the Nu-Clear extraction kit (Sigma) 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. VEGF promoter-specific oligonucleotides that recognized the −85/−50 binding region: sense 5′-CCCGGGGCGGGCCGGGGGCGGGGTCCCGGCGGGGCGGAG-3′ and antisense 5′-CTCCGCCCCGCCGGGACCCCGCCCCCGGCCCGCCCCGGG-3′ were purchased from Sigma.

All oligonucleotides were labeled using [γ-^{32}P]ATP (Amersham Biosciences) and T4 polynucleotide kinase (Promega). Fifteen micrograms of nuclear protein, ^{32}P-labeled double-stranded probe (40,000 counts per min/ng), and 2 μl of 5× binding buffer (20% glycerol, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM dithiothreitol, 250 mM NaCl, 50 mM Tris-HCl (pH 7.5), 0.25 mg/ml poly(dI-dC)·poly(dI-dC)) were mixed in a total volume of 10 μl. In competition assays, 50× unlabeled 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 0.5× TBE buffer, and subjected to electrophoresis for 60 min. The gel was dried and exposed for autoradiography on Kodak XAR film at −70 °C for 19–48 h. Supershift were performed using 4 μg of specific goat polyclonal anti-human Sp-1 (PEP 2), AP-2 (C-18), or EGR-1 (588) antibody (Santa Cruz Biotechnology).

ChIP Assay—HASM cells were cultured to confluence in 75-cm^2 flasks, growth arrested, and incubated with ethanol vehicle or 1 μM PGE_2 for 30 min. The ChIP assay was performed using the ChIP-IT kit (Active Motif, Rixensart, Belgium) following the manufacturer's protocol and using 4 μg of goat anti-human polyclonal Sp-1 antibody (PEP 2) (Santa Cruz Biotechnology) for each immunoprecipitation.

The VEGF primers used yielded a 202-bp product corresponding to −199 to +3 of the VEGF gene promoter and were: forward 5′-GGTCGAGCTTCCCCTTCA-3′, and reverse 5′-GATCCTCCCCGCTACCAG-3′. Forty cycles of a two-step PCR program, 95 °C for 1 min and 60 °C for 1 min, in the presence of 6% Me_2SO and 1 M betaine using Red *Taq* and 2.5 mM magnesium chloride (Sigma, Poole, Dorset, UK) were used (38). Potential problems with PCR resulting from high melting temperatures were reduced by addition of Me_2SO (39), and the amplification of GC-rich templates was enhanced by betaine (40).

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

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

RESULTS

PGE_2 Increases VEGF-A_{165} Protein Production—There was a significant increase in VEGF release above control in cells cultured for 24 h with concentrations of PGE_2 ranging from 1 nM to 1 μM (Fig. 1*A*). Cells treated with 1 μM PGE_2 for 2, 4, 8, 16, and 24 h also showed significantly increased VEGF levels compared with unstimulated control cells (Fig. 1*B*).

PGE_2 Increased VEGF Is Transcriptional—Real-time RT-PCR showed that PGE_2 increased VEGF-A mRNA levels with time with a 6-fold increase at 60 min and a peak 12-fold increase by 4 h compared with controls at these times (Fig. 2*A*). To confirm that this was because of VEGF-A gene transcription rather than stabilization of mRNA, the cells were cultured for 30 min with 5 μg/ml actinomycin D, an inhibitor of RNA polymerase II, followed by 1 μM PGE_2. Pretreatment with actinomycin D prevented the PGE_2-induced increase in VEGF mRNA (Fig. 2*A*).

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 1 μM PGE_2 compared with unstimulated cells (Fig. 2*B*).

A

VEGF promoter	2068 (-2018/+50)	1340 (-1286/+50)	840 (-789/+50)	465 (-414/+50)	318 (-267/+50)	135 (-85/+50)
-1892/-1886	AP-1					
-1690/-1678	NF-κB					
-1227/-1220	AP-1	AP-1				
-985/-939	HIF-1α	HIF-1α				
-837/-830	AP-2	AP-2				
-620/-614	AP-1	AP-1	AP-1			
-518/-513	Sp-1	Sp-1	Sp-1			
-490/-484	AP-1	AP-1	AP-1			
-281/-276	NF-1	NF-1	NF-1	NF-1		
-239/-234	Sp-1	Sp-1	Sp-1	Sp-1	Sp-1	
-194/-48	p53/VHL	p53/VHL	p53/VHL	p53/VHL	p53/VHL	
-135/-128	AP-2	AP-2	AP-2	AP-2	AP-2	
-97/-87	Sp-1	Sp-1	Sp-1	Sp-1	Sp-1	
-85/-78	Sp-1	Sp-1	Sp-1	Sp-1	Sp-1	Sp-1
-77/-69	Egr-1	Egr-1	Egr-1	Egr-1	Egr-1	Egr-1
-74/-66	Sp-1	Sp-1	Sp-1	Sp-1	Sp-1	Sp-1
-65/-58	AP-2	AP-2	AP-2	AP-2	AP-2	AP-2
-60/-51	Egr-1	Egr-1	Egr-1	Egr-1	Egr-1	Egr-1
-58/-51	Sp-1	Sp-1	Sp-1	Sp-1	Sp-1	Sp-1

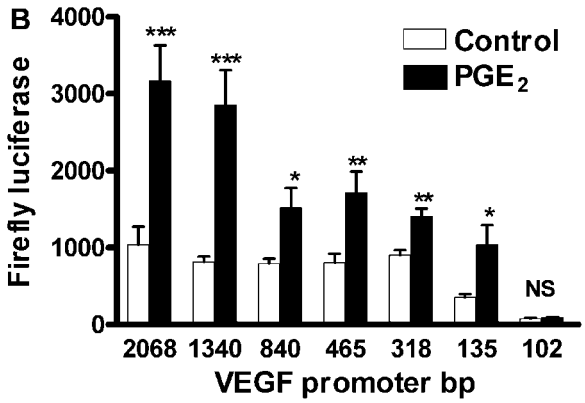

FIG. 3. *A*, representation of the VEGF promoter-driven luciferase constructs used in the transfection studies showing the positions of the transcription factor binding sites. *B*, increase in luciferase expression in ASMC transiently transfected with the deletion series of the VEGF promoter luciferase constructs after 24 h incubation with 1 μM PGE_2. Cells were cultured in 24-well plates to confluence, growth arrested, and transfected using 1 μl of LF2000 and 0.8 μg of DNA per well. The figure represents the mean ± S.E. of three experiments performed in triplicate (*, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ by ANOVA).

These results suggest that induction of VEGF by PGE_2 is transcriptional and not mediated by post-transcriptional stabilization of PGE_2 mRNA.

Mutations in the Sp-1 Binding Sites in the VEGF Promoter Reduce PGE$_2$-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 Fig. 3*A*. One micromolar PGE_2 increased the luciferase levels 4.2 ± 0.65 in cells transfected with the 2068 construct. There was a significant PGE_2-mediated increase in luciferase activity with all of the deletions series except the smallest 102-bp fragment of the VEGF promoter (Fig. 3*B*). However, deleting the sequences between −1286 and −789 bp resulted in a reduction in the stimulatory effect of the PGE_2. This suggests that the upstream AP-1, AP-2, or hypoxia-inducible factor-1α sequences may also be involved in PGE_2-mediated VEGF increase.

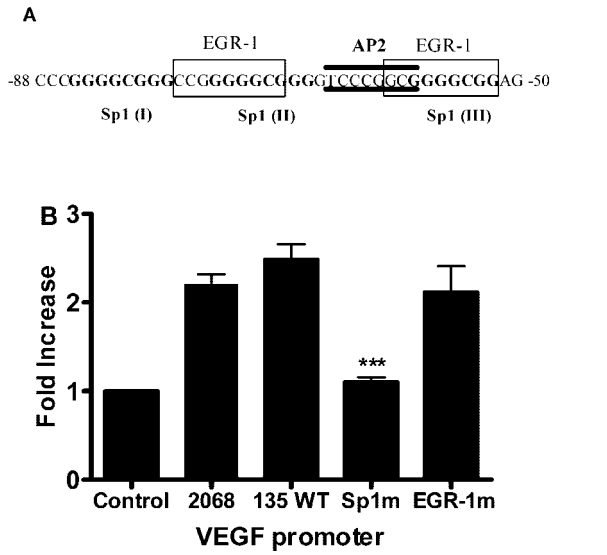

FIG. 4. *A*, position of the transcription factor binding sites present on the 135-bp VEGF promoter. *B*, the effect of binding site mutations on VEGF promoter activity in response to 1 μM PGE_2. Three Sp-1 binding sites, GGGCGG mutated to GTTCGG, and both EGR-1 binding sites, GCGGGGGCG, mutated to GCTAGGGCG. The graph shows -fold increase of luciferase activity in cells treated with PGE_2 compared unstimulated cells. The figure represents the mean ± S.E. of three experiments performed in triplicate and was analyzed by ANOVA (***, $p < 0.001$).

Transfection studies using the wild type construct and a construct containing mutations of the three Sp-1 (−88/−50) binding sites showed a significant reduction in luciferase activity (Fig. 4, *A* and *B*), suggesting that these factors were necessary for VEGF induction by PGE_2.

PGE$_2$ Increases Sp-1 Binding to the VEGF Promoter—We used EMSA to determine whether PGE_2 treatment increased Sp-1 binding to the VEGF promoter. Incubation with 1 μM PGE_2 for 60 min induced binding activity with Sp-1 consensus and VEGF promoter oligonucleotides (Fig. 5, *A* and *E*). This was not seen with AP-1 and EGR-1 consensus oligonucleotides (Fig. 5, *B* and *C*). Supershift studies using a monoclonal antibody to Sp-1 produced gel retardation with the consensus sequence (Fig. 5*A*) and a reduction in binding with the VEGF promoter-specific primers (Fig. 5*F*). Competition with 50-fold excess unlabeled 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 (Fig. 5*E*). There was no transcription factor binding in experiments using mutated consensus Sp-1 oligonucleotides (Fig. 5*D*).

ChIP—The PGE_2-mediated increase in Sp-1 binding to the VEGF promoter demonstrated by EMSA was confirmed using the 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 were subjected to 40 cycles of a two-step PCR in the presence of 1 M betaine and 6% Me_2SO using VEGF promoter-specific primers spanning −199 to +3 bp (Fig. 6*A*). Densitometry showed a significant increase in Sp-1 binding to the VEGF promoter following incubation with PGE_2. The results were normalized to the input control (Fig. 6*B*).

EP$_2$ and EP$_4$ Receptor Agonists Mimic the Effect of PGE$_2$—Both EP_2 and EP_4 receptors, which positively couple to adenylyl cyclase, are present on HASMC as demonstrated by flow cytometry and a specific antibody to the EP_4 receptor (Fig. 7, *A*

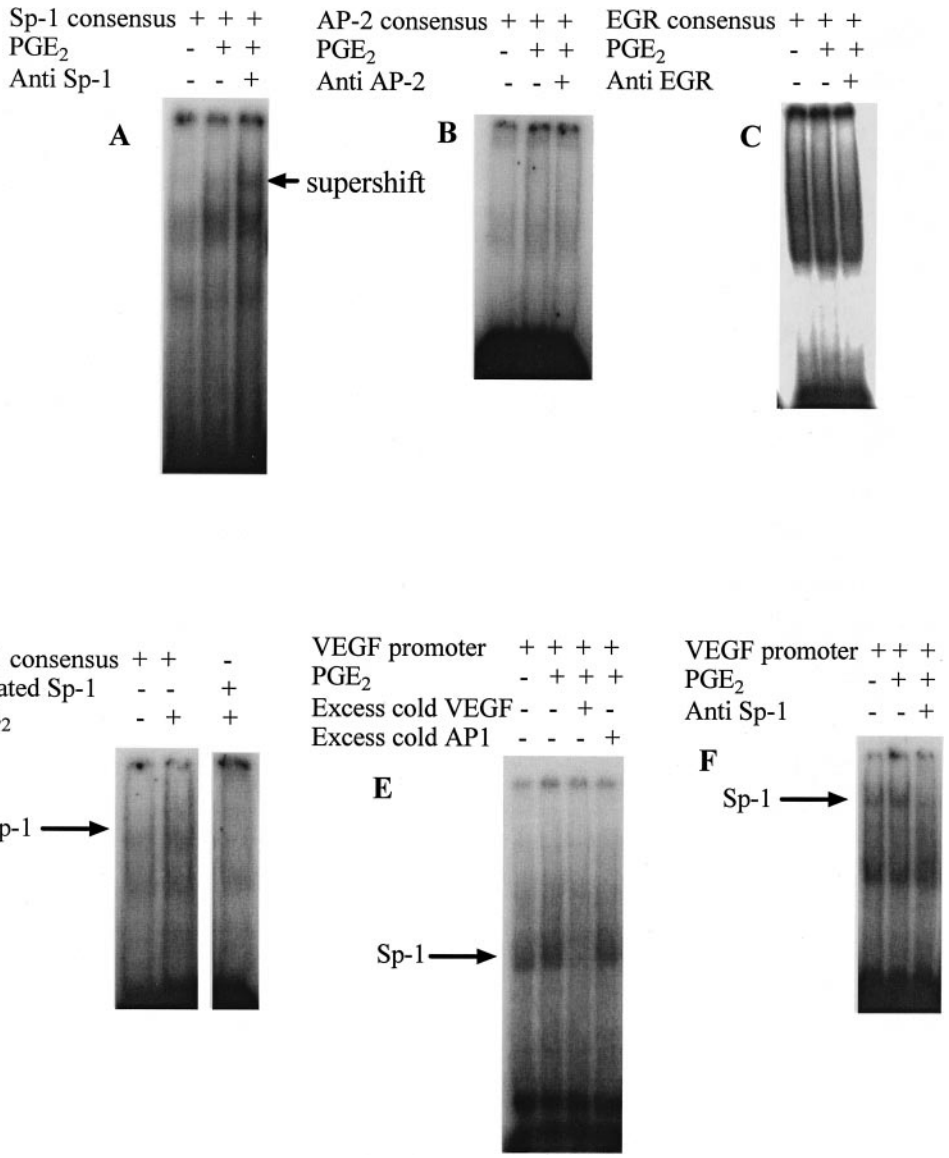

FIG. 5. *A*, PGE_2 increases consensus Sp-1 binding and addition of anti-Sp-1 antibody to the nuclear extracts from cells treated with 1 μM PGE_2 for 60 min resulted in a supershift. *B*, 1 μM PGE_2 for 60 min did not increase the consensus AP-2 binding or *C*, EGR-1 binding. Addition of antibodies to 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*, PGE_2 increases transcription factor binding to the VEGF promoter (−88/−50). Binding was specific as shown by competitive binding. Nuclear extracts from PGE_2-treated cells were incubated with labeled VEGF promoter (hot VEGF) in the presence of a 50-fold excess of unlabeled VEGF promoter (cold VEGF) or unlabeled 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.

and *B*), by Western blotting and a specific antibody to the EP_2 receptor (Fig. 7*C*) and RT-PCR (Fig. 7*D*).

To determine which PGE_2 receptors were important we looked at the effect of PGE_2 receptor agonists on VEGF production together with luciferase activity in cells transfected with the 2068 VEGF promoter construct. We found that both EP_2 (ONO-AE1 259) and EP_4 (ONO-AE1 329) receptor agonists increased VEGF production and luciferase activity (Figs. 7*E* and 8*C*) in the same way as PGE_2. There was an additive effect when both agonists were used in suboptimal concentrations (Fig. 7*E*). This suggests that PGE_2 is acting via both EP_2 and EP_4 receptors.

Increasing Intracellular cAMP Mimics the Effect of PGE_2—The cAMP analogue 8-Br-cAMP increased VEGF protein production in a concentration-dependent manner (Fig. 8*A*). Agents that increase cAMP also similarly increased VEGF release. Forskolin, a direct activator of adenylyl cyclase (Fig. 8*A*), and the β-adrenoreceptor agonist isoproterenol (Fig. 8*B*), both increased VEGF and luciferase activity in cells transfected with the 2068 VEGF promoter construct (Fig. 8*C*).

Mithramycin Inhibits PGE_2-induced Activation of VEGF—The anticancer antibiotic, mithramycin A selectively binds to GC-rich regions of DNA preventing Sp-1 binding. Preincubation for 1 h with 500 nM and 1 μM mithramycin significantly reduced PGE_2-stimulated VEGF protein release in a concentration-dependent manner. Maximal inhibition was seen using 1 μM mithramycin (Fig. 9*A*). Basal levels of VEGF production were not changed significantly by mithramycin treatment (data not shown).

Inhibition of PKA Abrogates PGE_2-induced Activation of VEGF—Preincubation for 1 h with 10 μM H-89, an inhibitor of PKA, prior to a 24-h culture with 1 μM PGE_2, resulted in a significant reduction in secreted VEGF as measured by ELISA (Fig. 9*A*).

Nuclear Sp-1 Protein Is Phosphorylated by PGE_2—Western blotting demonstrated that Sp-1 protein expression was con-

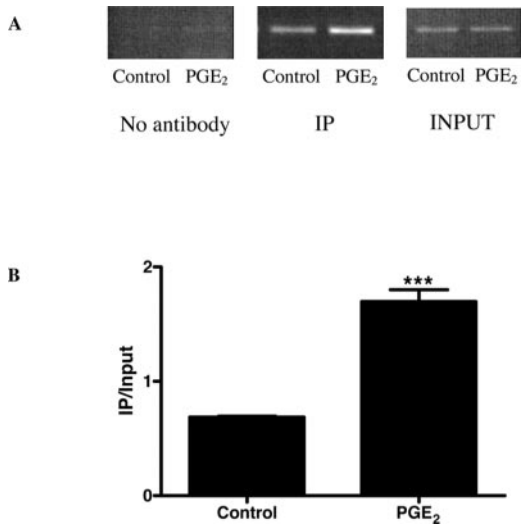

FIG. 6. *A*, representative ChIP assay PCR showing PGE_2 increases in Sp-1 binding to the VEGF promoter. Immunoprecipitation (*IP*) was carried out using antibody to Sp-1. The PCR primers were amplified in the −199 to +3 region of the VEGF promoter. *B*, densitometry of ChIP PCR normalized to the input. Duplicate experiments were repeated in triplicate (***, $p < 0.001$ by ANOVA).

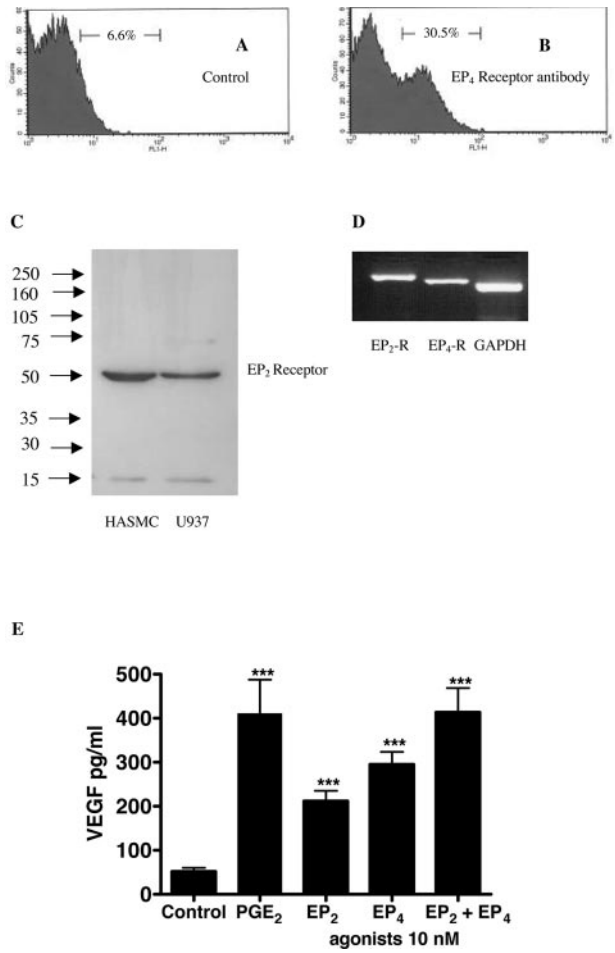

FIG. 7. *A*, flow cytometry histogram of unstimulated ASMC stained with preimmune rabbit serum control and fluorescein isothiocyanate-conjugated secondary antibody. *B*, flow cytometry histogram of unstimulated ASMC stained with polyclonal rabbit anti-human EP_4 receptor and fluorescein isothiocyanate-conjugated secondary antibody. *C*, Western blotting of ASMC showing EP_2 receptor protein. U937 cells were used as a positive control. *D*, RT-PCR demonstrating mRNA for both EP_2 and EP_4 receptors. *E*, agonists to EP_2 and EP_4 receptors also increase VEGF protein production by ELISA over control. 1 μM PGE_2 is also shown (***, $p < 0.001$ by ANOVA).

fined to the nucleus and was phosphorylated by PGE_2. Previous studies have shown that the 106k Sp-1 band represents the phosphorylated protein (41). GAPDH and the nuclear-specific proteins lamin A and C were used as controls (Fig. 9*B*).

Increasing Intracellular cAMP Increases Sp-1 Luciferase Reporter Activity—Agents that increase cAMP also increased a 6-repeat Sp-1/luciferase reporter construct. The cAMP analogue 8-Br-cAMP, forskolin, a direct activator of adenylyl cyclase, and the β-adrenoreceptor agonist salbutamol all increased the activity of a transiently transfected Sp-1 reporter luciferase construct. (Fig. 9*C*).

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 signaling 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 h as measured by ELISA. Quantitative real-time RT-PCR also showed that PGE_2 increased VEGF-A mRNA after 1 h. Pretreatment of the cells with the RNA polymerase II inhibitor actinomycin D prevented this, suggesting that the increased VEGF mRNA was because of 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 up-regulation 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 102–135-bp region. 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 unlabeled oligonucleotides and supershift with Sp-1 antibody. The EMSA results were confirmed by the 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 that is phosphorylated by PGE_2.

Ours are the first studies to show that Sp-1 is involved in

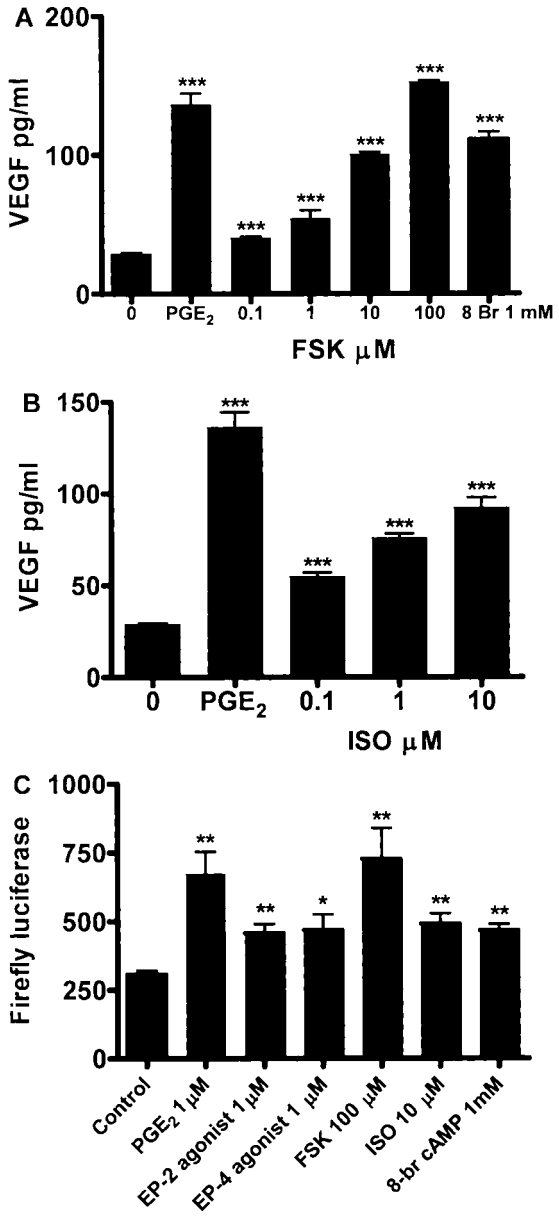

FIG. 8. **Agents that increase intracellular cAMP levels mimic 1 μM PGE_2 and increase VEGF production.** *A*, concentration response to forskolin (*FSK*), which directly activates adenylyl cyclase and the cAMP analogue, 8-Br-cAMP, increases VEGF, the β-adrenergic agonists. *B*, isoproterenol (*ISO*). *C*, agents that act via cAMP mimic PGE_2 and stimulate VEGF promoter-driven luciferase activity (*, $p < 0.05$, **, $p < 0.01$; and ***, $p < 0.001$ by ANOVA).

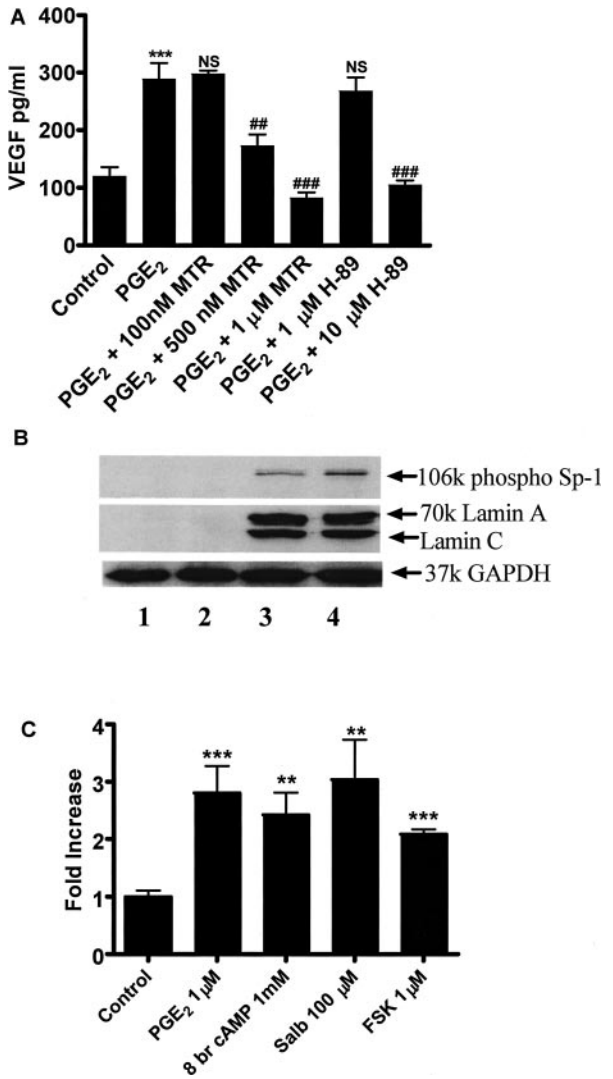

FIG. 9. *A*, reduction of VEGF protein release by the Sp-1 inhibitor mithramycin A (*MTR*) and PKA inhibitor H-89. Preincubation with 500 nM and 1 μM MTR or 10 μM H-89 prior to 24 h culture with 1 μM PGE_2 significantly reduced PGE_2 stimulated VEGF measured by ELISA (* and #, $p < 0.05$; ** and ##, $p < 0.01$; *** and ###, $p < 0.001$ by ANOVA). *NS*, not significant. *B*, Western blotting showing the nuclear localization of Sp-1 and increased phosphorylation of 106k Sp-1 by 15 min incubation with 1 μM PGE_2. *Lanes 1* and *2* are cytosolic fractions: control (*1*) and PGE_2 (*2*). *Lanes 3* and *4* are nuclear fractions: control (*3*) and PGE_2 (*4*). GAPDH and nuclear lamin were used as housekeeping controls. *C*, PGE_2, 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).

PGE_2-induced VEGF production, although Sp-1 is important in the activation of genes involved in tumor proliferation and the induction of VEGF in response to some other stimuli (11, 34, 44, 45). For example, VEGF induction by interleukin-1β in cardiac myocytes and by tumor necrosis factor-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 characterize the prostanoid receptor involved. PGE_2 binds to a family of 7 transmembrane G protein-coupled membrane receptors, the 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 signaling mechanisms, suggesting potentially different inflammatory responses depending on receptor subtype binding (28). We focused on the two EP receptors, EP_2 and EP_4, which activate cAMP. We found that both EP_2 and EP_4 receptors were expressed in HASMC. This contrasts to a previous study that reported EP_2 but not EP_4 receptor expression in HASM (48). We confirmed our findings using both RT-PCR and either Western blotting or fluorescence-activated cell sorter for EP_2 and EP_4, respectively, using antibodies designed for these methodologies. The fact that both mRNA and protein to EP_4 receptors was present suggests that these cells do indeed express EP_4 receptors. Furthermore, experiments with EP_2 and EP_4 receptor agonists mirrored the effect of PGE_2 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.* (49) who suggested that positive regulation of granulocyte colony-stimulating factor by E-Ring 8-isoprostanes

in HASMC was mediated by EP_2 and EP_4 receptors.

EP_2 and EP_4 receptors couple to G_S protein, which stimulates adenylyl cyclase activity increasing the intracellular cAMP levels resulting in PKA signaling. We performed experiments using a variety of pharmacological tools to probe the role of different components of this pathway. Isoproterenol, which elevates cAMP via β-adrenoceptors and forskolin, a direct activator of adenylyl cyclase, had similar effects to PGE_2 on VEGF protein production and VEGF promoter luciferase expression, suggesting that 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 PGE_2-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 up-regulated (24, 25). 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 remodeling processes. In conclusion, our studies provide evidence that PGE_2 induces VEGF via Sp-1 binding sites on the VEGF promoter via EP_2 and EP_4 receptors in a cAMP- and PKA-dependent mechanism involving phosphorylation of Sp-1.

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REFERENCES

1. Ferrara, N., and Henzel, W. J. (1989) *Biochem. Biophys. Res. Commun.* **161,** 851–858
2. Ferrara, N. (2000) *Recent Prog. Horm. Res.* **55,** 15–35
3. Kanazawa, H., Hirata, K., and Yoshikawa, J. (2002) *Thorax* **57,** 885–888
4. Kanazawa, H., Asai, K., Hirata, K., and Yoshikawa, J. (2003) *Am. J. Med.* **114,** 354–358
5. Clavel, G., Bessis, N., and Boissier, M. C. (2003) *Joint Bone Spine* **70,** 321–326
6. Ballara, S. C., Miotla, J. M., and Paleolog, E. M. (1999) *Int. J. Exp. Pathol.* **80,** 235–250
7. Beck, P. L., and Podolsky, D. K. (1999) *Inflamm. Bowel Dis.* **5,** 44–60
8. Berse, B., Brown, L. F., van de Water, L., Dvorak, H. F., and Senger, D. R. (1992) *Mol. Biol. Cell* **3,** 211–220
9. Achen, M. G., and Stacker, S. A. (1998) *Int. J. Exp. Pathol.* **79,** 255–265
10. Keyt, B. A., Berleau, L. T., Nguyen, H. V., Chen, H., Heinsohn, H., Vandlen, R., and Ferrara, N. (1996) *J. Biol. Chem.* **271,** 7788–7795.
11. 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) *Cancer Res.* **63,** 1083–1092
12. Inoue, H., Takamori, M., Shimoyama, Y., Ishibashi, H., Yamamoto, S., and Koshihara, Y. (2002) *Br. J. Pharmacol.* **136,** 287–295
13. Tanaka, T., Kanai, H., Sekiguchi, K., Aihara, Y., Yokoyama, T., Arai, M., Kanda, T., Nagai, R., and Kurabayashi, M. (2000) *J. Mol. Cell Cardiol.* **32,** 1955–1967
14. Wen, F. Q., Liu, X., Manda, W., Terasaki, Y., Kobayashi, T., Abe, S., Fang, Q., Ertl, R., Manouilova, L., and Rennard, S. I. (2003) *J. Allergy Clin. Immunol.* **111,** 1307–1318
15. Knox, A. J., Corbett, L., Stocks, J., Holland, E., Zhu, Y. M., and Pang, L. (2001) *FASEB J.* **15,** 2480–2488
16. Harada, S., Nagy, J. A., Sullivan, K. A., Thomas, K. A., Endo, N., Rodan, G. A., and Rodan, S. B. (1994) *J. Clin. Investig.* **93,** 2490–2496
17. Harada, S., Rodan, S. B., and Rodan, G. A. (1995) *Clin. Orthop. Relat. Res.* **313,** 76–80
18. Ben Av, P., Crofford, L. J., Wilder, R. L., and Hla, T. (1995) *FEBS Lett.* **372,** 83–87
19. Gately, S. (2000) *Cancer Metastasis Rev.* **19,** 19–27
20. Gately, S., and Li, W. W. (2004) *Semin. Oncol.* **31,** 2–11
21. Daniel, T. O., Liu, H., Morrow, J. D., Crews, B. C., and Marnett, L. J. (1999) *Cancer Res.* **59,** 4574–4577
22. Jones, M. K., Wang, H., Peskar, B. M., Levin, E., Itani, R. M., Sarfeh, I. J., and Tarnawski, A. S. (1999) *Nat. Med.* **5,** 1418–1423
23. Peterson, H. I. (1986) *Anticancer Res.* **6,** 251–253
24. Pang, L., Pitt, A., Petkova, D., and Knox, A. J. (1998) *Clin. Exp. Allergy* **28,** 1050–1058
25. Hoshino, M., Takahashi, M., and Aoike, N. (2001) *J. Allergy Clin. Immunol.* **107,** 295–301
26. Tischer, E., Mitchell, R., Hartman, T., Silva, M., Gospodarowicz, D., Fiddes, J. C., and Abraham, J. A. (1991) *J. Biol. Chem.* **266,** 11947–11954
27. Coleman, R. A., Smith, W. L., and Narumiya, S. (1994) *Pharmacol. Rev.* **46,** 205–229
28. Narumiya, S., Sugimoto, Y., and Ushikubi, F. (1999) *Physiol. Rev.* **79,** 1193–1226
29. Pang, L., and Knox, A. J. (1997) *Am. J. Physiol.* **273,** L1132–L1140
30. Pang, L., and Knox, A. J. (1997) *Br. J. Pharmacol.* **121,** 579–587
31. Maruyama, T., Asada, M., Shiraishi, T., Ishida, A., Egashira, H., Yoshida, H., Maruyama, T., Ohuchida, S., Nakai, H., Kondo, K., and Toda, M. (2001) *Bioorg. Med. Chem. Lett.* **11,** 2029–2031
32. Tani, K., Naganawa, A., Ishida, A., Egashira, H., Sagawa, K., Harada, H., Ogawa, M., Maruyama, T., Ohuchida, S., Nakai, H., Kondo, K., and Toda, M. (2001) *Bioorg. Med. Chem. Lett.* **11,** 2025–2028
33. Clarke, D. L., Belvisi, M. G., Smith, S. J., Hardaker, E., Yacoub, M. H., Meja, K. K., Newton, R., Slater, D. M., and Giembycz, M. A. (2005) *Am. J. Physiol.* **288,** L238–L250
34. Schafer, G., Cramer, T., Suske, G., Kemmner, W., Wiedenmann, B., and Hocker, M. (2003) *J. Biol. Chem.* **278,** 8190–8198
35. Pallisgaard, N., Clausen, N., Schroder, H., and Hokland, P. (1999) *Genes Chromosomes Cancer* **26,** 355–365
36. Finkenzeller, G., Technau, A., and Marme, D. (1995) *Biochem. Biophys. Res. Commun.* **208,** 432–439
37. Biggs, J. R., Kudlow, J. E., and Kraft, A. S. (1996) *J. Biol. Chem.* **271,** 901–906
38. Stoner, M., Wormke, M., Saville, B., Samudio, I., Qin, C., Abdelrahim, M., and Safe, S. (2004) *Oncogene* **23,** 1052–1063
39. Chakrabarti, R., and Schutt, C. E. (2002) *BioTechniques* **32,** 866, 868, 870–872, 874
40. Henke, W., Herdel, K., Jung, K., Schnorr, D., and Loening, S. A. (1997) *Nucleic Acids Res.* **25,** 3957–3958
41. Segura, J. A., Donadio, A. C., Lobo, C., Mates, J. M., Marquez, J., and Alonso, F. J. (2005) *Cancer Lett.* **218,** 91–98
42. Mezquita, P., Parghi, S. S., Brandvold, K. A., and Ruddell, A. (2005) *Oncogene* **24,** 889–901
43. Miller, D. M., Polansky, D. A., Thomas, S. D., Ray, R., Campbell, V. W., Sanchez, J., and Koller, C. A. (1987) *Am. J. Med. Sci.* **294,** 388–394
44. Milanini-Mongiat, J., Pouyssegur, J., and Pages, G. (2002) *J. Biol. Chem.* **277,** 20631–20639
45. Milanini, J., Vinals, F., Pouyssegur, J., and Pages, G. (1998) *J. Biol. Chem.* **273,** 18165–18172
46. Ryuto, M., Ono, M., Izumi, H., Yoshida, S., Weich, H. A., Kohno, K., and Kuwano, M. (1996) *J. Biol. Chem.* **271,** 28220–28228
47. Gille, J., Swerlick, R. A., and Caughman, S. W. (1997) *EMBO J.* **16,** 750–759
48. Burgess, J. K., Ge, Q., Boustany, S., Black, J. L., and Johnson, P. R. (2004) *J. Allergy Clin. Immunol.* **113,** 876–881
49. Clarke, D. L., Belvisi, M. G., Hardaker, E., Newton, R., and Giembycz, M. A. (2005) *Mol. Pharmacol.* **67,** 383–393
50. Davies, S. P., Reddy, H., Caivano, M., and Cohen, P. (2000) *Biochem. J.* **351,** 95–105

Vascular Endothelial Growth Factor Induction by Prostaglandin E_2 in Human Airway Smooth Muscle Cells Is Mediated by E Prostanoid EP_2/EP_4 Receptors and SP-1 Transcription Factor Binding Sites

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