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) 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). 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′ deletion analysis mapped the PGE₂ response element to the 135-bp sequence (−85/−50) of the human VEGF-A promoter. PGE₂-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. PGE₂ increased phosphorylation of Sp-1 and luciferase activity of a transfected Sp-1 reporter construct. PGE₅₂ receptor agonists EP₂ (ONO-AE1 259) and EP₄ (ONO-AE1 329) mimicked the effect of PGE₂, and reverse transcription-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.

Vascular endothelial growth factor (VEGF) 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 prostanooid production. Recent work suggests that this is also true of VEGF. Autocrine PGE₂ increases VEGF release in response to interleukin-1β in synovial fibroblasts and in response to bradykinin in HASMC (12, 15). Furthermore, exogenous PGE₂ 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 A₂, prostacyclin, and PGE₂ 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 PGE₂ 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-α, p53/Von Hippel Lindau, NFκB, and AP-1 as well as several potential transcription factor binding sites for Sp-1 and AP-2 (26). PGE₂ binds to G protein-coupled membrane receptors, the E prostanoid (EP) receptors. Four subtypes of EP receptors have been described, EP₁, EP₂,
EP₂ and EP₄, 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 PGE₂ to increase VEGF is unknown.

Here we determined the molecular mechanisms involved in the transcription regulation of the VEGF promoter by exogenous PGE₂ 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 PGE₂. PGE₂ caused phosphorylation of Sp-1 and electrophoretic mobility shift assay (EMSA), and chromatin immunoprecipitation (ChIP) demonstrated that PGE₂ increased Sp-1 binding to the VEGF promoter. Furthermore, studies with EP₂ and EP₄ receptor subtype agonists, the cAMP analogue 8-bromoadenosine-3',5'-monophosphate (8-Br-cAMP), forskolin, which increases adenylyl cyclase activity, and the β2 receptor agonist isoproterenol, showed that PGE₂-induced activation of Sp-1 was mediated by EP₂ and EP₄ 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% CO₂, 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 g/ml streptomycin, 4 mM l-glutamine, and 1.25 g/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 PGE₂ or ethanol vehicle (Sigma) was added. In time course experiments cells were incubated with 1 µM PGE₂ for 2–24 h. In the concentration response experiments cells were incubated for 24 h with 1 nM to 10 µM PGE₂. 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 EP₂ and EP₄ receptor subtype agonists ONO-AE1 259 and ONO-AE1 329, which were a gift from ONO Pharmaceuticals, Osaka, Japan, were used in the PGE₂ 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 (St. Louis, MO) and GL Biochem (Shanghai, China). M PGE₂ for 2–24 h. In subsequent concentration experiments the inhibitors were added to the culture medium 2 h prior to addition of PGE₂. Western blotting was performed as described previously using a specific polyclonal rabbit anti-human EP₂ receptor antibody (Cayman Chemical, Ann Arbor, MI). The cells were washed twice and incubated with polyclonal rabbit anti-human EP₂-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 FACScanCellSort flow cytometer (BD Biosciences) and logarithmic amplification of the green fluorescence channel (FL1), 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 EP₂ receptor antibody (Cayman Chemical, Ann Arbor, MI). The cells were lysed in Nu-Clear lysis buffer (Promega, Madison, WI) containing the Renilla luciferase triplicate value. This was then expressed as a percentage increase over unstimulated cells at each time point. Standard curves were generated using serial dilutions of KG1a DNA. Negative controls consisting of no template were included, and all reactions were set up in triplicate. VEGF-A expression was determined by the housekeeping gene β2-microglobulin. The β2-microglobulin triplicate value was divided by the mean of the VEGF-A triplicate value to give the fold expression of β2-microglobulin.

RNA Isolation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)—Cells in 6-well plates were treated with PGE₂ 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. The ultraviolet (UV)-illuminated gels were photographed, and the densitometry was analyzed using a GeneGnome gel documentation and analysis system (Syngene, Cambridge, UK). The ABI Prism 7700 detection system (Applied Biosystems, Warrington, Cheshire, UK) each reaction consisted of 1× Externa mastermix with SYBR green (Biogene, Cambridge, UK) and the ABI Prism 7700 detection system (Applied Biosystems, Warrington, UK). Each reaction consisted of 1× Externa mastermix, SYBR green (16,000 to final concentration), 40 nM of both sense and antisense primers, 1.6 µl of DNA (or dH₂O), and H₂O 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. Amplification 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 DNA. 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 β2-microglobulin triplicate value. This was then expressed as the fold increase over unstimulated cells at each time point. The concentration 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.

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Vascular Endothelial Growth Factor (VEGF) Assay—The human histiocytic lymphoma cell line U937, which is known to secrete high levels of VEGF (10), was cultured in a humidified, 95% air, 5% CO₂, 37 °C incubator using complete RPMI 1640 medium supplemented with 5% fetal calf serum and 1% penicillin, streptomycin, and l-glutamine. U937 cells were cultured to confluence in 24-well plates at a density of 3×10⁵ cells/well. The cells were incubated at 37 °C for 24 h prior to addition of PGE₂. Western blotting was performed as described previously using a specific polyclonal rabbit anti-human EP₂ receptor antibody (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 FACScanCellSort flow cytometer (BD Biosciences) and logarithmic amplification of the green fluorescence channel (FL1), 10,000 events were acquired and analyzed with CellQuest software (BD Biosciences).

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-diphenyltetrazolium bromide assay (29). At the end of the experiment the culture media was removed and 250 µl of media containing 1 mg/ml thiazolyl blue, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma) and incubated for 20 min in 37 °C. This medium was removed and 250 µl of Me₂SO 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 Lipofectamine LF2000 (Invitrogen) and 0.8 µl 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 PGE₂, 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 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 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).

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 to −50 binding region: sense 5′-CCCGGGGCCCCTGATCATG-3′ and antisense 5′-CTCGCCCCCGCCGGAGTGGCGGGCCCCCCCCTCCCC-3′ were purchased from Sigma.

All oligonucleotides were labeled using [γ-32P]ATP (Amersham Biosciences) and T4 polynucleotide kinase (Promega). Fifteen micrograms of nuclear protein, 32P-labeled double-stranded probe (40,000 counts per min/μg), and 2 μl of 5× binding buffer (Promega), 5 mM MgCl₂, 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 ng of specific goat antibody (Santa Cruz Biotechnology).

ChIP Assay—HASMC cells were cultured to confluence in 75-cm² flasks, grown arrested, and incubated with ethanol vehicle or 1 μM PGE₂ for 24 h. The ChIP assay was performed using the ChIP-IT kit (Active Motif, Rixensart, Belgium) 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 to −50 binding region: sense 5′-CCCGGGGCCCCTGATCATG-3′ and antisense 5′-CTCGCCCCCGCCGGAGTGGCGGGCCCCCCCCTCCCC-3′ were purchased from Sigma.

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ChIP Assay—HASMC cells were cultured to confluence in 75-cm² flasks, grown arrested, and incubated with ethanol vehicle or 1 μM PGE₂ 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′-GGTG-GAGCCTTCCCTTGA-3′, and reverse 5′-GATGCTCCCGGGCAGCAGC-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% Me₂SO and 1 μl 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 Me₂SO (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**

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

PGE₂ Increased VEGF Is Transcriptional—Real-time RT-PCR showed that PGE₂ 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. 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 1 μM PGE₂ compared with unstimulated cells (Fig. 2B).
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 $\mu$M 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. 5A) and a reduction in binding with the VEGF promoter-specific primers (Fig. 5F). 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. 5E). There was no transcription factor binding in experiments using mutated consensus Sp-1 oligonucleotides (Fig. 5D).

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 mM betaine and 6% Me$_2$SO using VEGF promoter-specific primers spanning −199 to +3 bp (Fig. 6A). 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. 6B).

EP$_2$ and EP$_3$ Receptor Agonists Mimic the Effect of PGE$_2$—Both EP$_2$ and EP$_3$ receptors, which positively couple to adenyl cyclase, are present on HASMC as demonstrated by flow cytometry and a specific antibody to the EP$_4$ receptor (Fig. 7, A and B). The EP$_2$ and EP$_3$ agonists, 1 $\mu$M of LF2000 and 0.8 $\mu$g 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).

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$_2$.

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. 3A. 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. 3B). 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. 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 $\mu$M PGE$_2$. 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).
and B), by Western blotting and a specific antibody to the EP₂ receptor (Fig. 7C) and RT-PCR (Fig. 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 (Figs. 7E and 8C). There was an additive effect when both agonists were used in suboptimal concentrations (Fig. 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 production in a concentration-dependent manner (Fig. 8A). Agents that increase cAMP also similarly increased VEGF release. Forskolin, a direct activator of adenyl cyclase (Fig. 8A), and the β-adrenoreceptor agonist isoproterenol (Fig. 8B), both increased VEGF and luciferase activity in cells transfected with the 2068 VEGF promoter construct (Fig. 8C).

**Mithramycin Inhibits PGE₂-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 PGE₂-stimulated VEGF protein release in a concentration-dependent manner. Maximal inhibition was seen using 1 μM mithramycin (Fig. 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**—Preincubation for 1 h with 10 μM H-89, an inhibitor of PKA, prior to a 24-h culture with 1 μM PGE₂, resulted in a significant reduction in secreted VEGF as measured by ELISA (Fig. 9A).

**Nuclear Sp-1 Protein Is Phosphorylated by PGE₂**—Western blotting demonstrated that Sp-1 protein expression was con-

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**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 min resulted in a supershift. B, 1 μM PGE₂ 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, 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 labeled VEGF promoter (hot VEGF) in the presence of a 50-fold excess of unlabeled VEGF promoter (cold VEGF) or unlabeled AP1 (cold AP1). F, antibody to Sp-1 diminishes Sp-1 binding to the VEGF promoter. The figures shown are representative of three experiments.
fined 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 proteins lamin A and C were used as controls (Fig. 9B).

**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 \( \beta \)-adrenoreceptor agonist salbutamol all increased the activity of a transiently transfected Sp-1 reporter luciferase construct. (Fig. 9C).

**DISCUSSION**

There are several key novel findings in this study. We found that PGE2 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 EP2 and EP4 receptors via cAMP and PKA. These studies are the first in any biological system to study the transcription factors involved in VEGF production by PGE2 and also delineate the upstream signaling cascade components.

We first determined whether PGE2 was acting via transcriptional or post-transcriptional mechanisms. Stimulation with PGE2 resulted in increased VEGF-A protein release after 2 h as measured by ELISA. Quantitative real-time RT-PCR also showed that PGE2 increased VEGF-A mRNA after 1 h. Pre-treatment 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 PGE2 treatment. Collectively these studies suggest that VEGF was regulated transcriptionally by PGE2 and this was confirmed by studies using VEGF promoter luciferase constructs. A few previous studies have looked at whether PGE2 increases VEGF transcriptionally or post-transcriptionally. PGE2-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 155-bp construct. However, all luciferase activity was lost using the 102-bp construct, suggesting that the main regulatory sites were contained within the 102–155-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 PGE2. These observations were also supported by EMSA results, which showed that PGE2 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 PGE2.

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 tumor proliferation and the induction of VEGF in response to some other stimuli (11, 34, 44, 45). For example, VEGF induction by interleukin-1$\beta$ (H9252) in cardiac myocytes and by tumor necrosis factor-$\alpha$ (H9251) in glioma cells is mediated through Sp-1 sites (13, 46). In contrast, transforming growth factor-$\beta$ (H9251) 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 for 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...
PGE₂ Induces VEGF via Sp₁

in HASM cells was mediated by EP₂ and EP₄ receptors. EP₂ and EP₄ receptors couple to Gₛ protein, which stimulates adenyl 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 adenyl cyclase, had similar effects to PGE₂ 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 Sp₁ 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 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 Sp₁-mediated gene transcription may provide a new therapeutic approach to influence remodeling processes. In conclusion, our studies provide evidence that PGE₂ induces VEGF via Sp₁-binding sites on the VEGF promoter via EP₂ and EP₄ receptors in a cAMP- and PKA-dependent mechanism involving phosphorylation of Sp₁.

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