

Selective Inhibition of Vascular Endothelial Growth Factor–mediated Angiogenesis by Cyclosporin A: Roles of the Nuclear Factor of Activated T Cells and Cyclooxygenase 2

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Abstract

Cyclosporin A (CsA) is an immunosuppressive drug that inhibits the activity of transcription factors of the nuclear factor of activated T cells (NFAT) family, interfering with the induction of cytokines and other inducible genes required for the immune response. Here we show that CsA inhibits migration of primary endothelial cells and angiogenesis induced by vascular endothelial growth factor (VEGF); this effect appears to be mediated through the inhibition of cyclooxygenase (Cox)-2, the transcription of which is activated by VEGF in primary endothelial cells. Consistent with this, we show that the induction of Cox-2 gene expression by VEGF requires NFAT activation. Most important, the CsA-mediated inhibition of angiogenesis both *in vitro* and *in vivo* was comparable to the Cox-2 inhibitor NS-398, and reversed by prostaglandin E₂. Furthermore, the *in vivo* corneal angiogenesis induced by VEGF, but not by basic fibroblast growth factor, was selectively inhibited in mice treated with CsA systemically. These findings involve NFAT in the regulation of Cox-2 in endothelial cells, point to a role for this transcription factor in angiogenesis, and may provide a novel mechanism underlying the beneficial effects of CsA in angiogenesis-related diseases such as rheumatoid arthritis and psoriasis.

Key words: NFAT • cyclosporin A • VEGF • cyclooxygenase • angiogenesis

Introduction

Angiogenesis, the formation of new blood vessels from pre-existing vascular beds, occurs normally during organ development and differentiation in embryogenesis, and is required for wound healing and reproductive functions in the adult. Angiogenesis is also involved in the pathogenesis of several disorders including chronic inflammatory diseases and cancer. Vascular endothelial growth factor (VEGF)¹ is a

potent endothelial cell mitogen and exerts a pivotal role in angiogenesis under physiological and pathological conditions (1, 2). Thus, VEGF is crucial for embryonic development, as targeted inactivation of even a single VEGF allele results in embryonic lethality (3), and it is required for survival in early postnatal life when the endothelium is still proliferating (4). On the other hand, enhanced expression of VEGF has been detected in physiological processes associated with the menstrual cycle, pregnancy, wound healing, and pathological conditions including rheumatoid arthritis, psoriasis, diabetic retinopathy, and cardiovascular and cerebral ischemia (5, 6). In addition, a large body of evidence supports a key role for VEGF in tumorigenesis related to the requirement of neovascularization for the delivery of nutrients and oxygen to tumor cells. In fact, VEGF expression is induced under hypoxic conditions and contributes to the tumor neovascularization and dissemination (2, 7, 8). Accordingly, VEGF expression has been found to be in-

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¹Abbreviations used in this paper: AA, arachidonic acid; bFGF, basic fibroblast growth factor; Cox, cyclooxygenase; CRE, cAMP response element; CsA, cyclosporin A; EMSA, electrophoretic mobility shift assay; HUVEC, human umbilical vein endothelial cell; NFAT, nuclear factor of activated T cells; NSAID, nonsteroidal antiinflammatory drug; PG, prostaglandin; PA, plasminogen activator; VEGF, vascular endothelial growth factor.

creased in nearly every type of cancer analyzed, and anti-VEGF neutralizing antibodies inhibit tumor growth in vivo (9–11).

VEGF belongs to a family that also includes the placenta growth factor (PGF), VEGF-B, VEGF-C, and VEGF-D, each of which interacts with a different tyrosine kinase receptor (12, 13). Signaling through these receptors results in the activation of phospholipase C γ , leading to an increase in intracellular levels of inositol phosphate and calcium (14, 15). As in other cell types, calcium induces the activation of calcineurin and nuclear factor of activated T cells (NFAT) in endothelial cells, a signaling pathway that is blocked by cyclosporin A (CsA) (16, 17). NFAT is a family of transcription factors (18) that is composed of at least four structurally related members that are expressed in the cytoplasm of resting cells: NFATc, NFATp, NFAT3, and NFAT4, also termed NFAT2/NFATc1, NFAT1/NFATc2, NFATc4, and NFATc3, respectively (19–24), as well as the constitutively nuclear NFAT5/TonE-BP (25, 26). We have recently shown that in human umbilical vein endothelial cells (HUVECs), VEGF triggers dephosphorylation, translocation, and activation of NFAT, processes involved in the regulation of tissue factor gene expression by VEGF (16). In addition, VEGF has been shown to induce other genes involved in the degradation of matrix proteins, endothelial cell migration, and vascular sprouting. Among these genes are the plasminogen activators (PAs) uPA and PA, the urokinase receptor (uPAR), and metalloproteinase interstitial collagenase (1, 2, 5).

Cyclooxygenases have been recently implicated in the regulation of angiogenesis, and Cox inhibition by nonsteroidal antiinflammatory drugs (NSAIDs) has been shown to interfere with the production of angiogenic factors (27), and to inhibit PG synthesis and angiogenesis in colon-26 cells and endothelial cells, respectively (28, 29). Coxs, also known as prostaglandin endoperoxide synthases, catalyze the conversion of arachidonic acid (AA) to PGH₂, the first step in the biosynthesis of PGs, thromboxane and prostacyclin (30). The two known Cox isoforms differ in their pattern of expression and biology: Cox-1 is constitutively expressed in most tissues, whereas Cox-2 is inducible in many cell types in response to a wide variety of stimuli including antigens, mitogens, hormones, and inflammatory mediators (31–35). The human Cox-2 gene promoter contains multiple cis-acting regulatory elements including a TATA box, an E box, and binding sites for important transcription factors, including a cAMP response element (CRE), NF-IL6 (C/EBP), and NF- κ B (36–39). In endothelial cells, Cox-2 is induced in response to multiple stimuli such as TNF- α (40), phorbol esters (41, 42), hypoxia (43), basic fibroblast growth factor (bFGF; reference 44), IL-1 α (45), and lipopolysaccharide (36).

To provide new insights into the molecular mechanisms by which VEGF exerts its angiogenic activity, we have searched for novel genes regulated by this molecule in HUVECs and found that Cox-2 is induced by VEGF. A detailed study of the Cox-2 gene promoter also revealed an essential role for NFAT in the induction of this gene by

VEGF. Because of the ability of CsA to inhibit NFATs and the role of Cox-2 in angiogenesis, we have tested the effect of CsA on Cox-2 gene expression and angiogenesis, and found that CsA blocks both the activation of Cox-2 and angiogenic effects induced by VEGF. These results may contribute to a better understanding of the mechanisms underlying the antiangiogenic actions of NSAIDs, and may also provide explanations for the beneficial effects of CsA in diseases where pathological neovascularization is associated with an enhanced expression of VEGF.

Materials and Methods

Cell Culture and Reagents. HUVECs were isolated from umbilical veins as described previously (46). Cells were serially passaged and cultured using 199 medium (Biowhittaker) supplemented with 20% FCS, bovine brain extract, and heparin in tissue culture flasks precoated with gelatin. Cells were used between passages 6 and 10. The recombinant human VEGF₁₆₅ was purchased from PeproTech. CsA was from Sandoz and Sigma-Aldrich, and TNF- α (3.2×10^7 U/mg) was from Wicheim.

Western Blot Analysis. Confluent HUVEC cultures grown in 35-mm tissue culture dishes were maintained before treatment for 12 h in OPTI-MEM (Life Technologies), supplemented with 0.5% FCS. After the different treatments, cells were washed with cold PBS and lysed in 80 μ l Laemmli buffer. Cells extracts were boiled, separated by SDS-PAGE, and transferred onto nitrocellulose membranes that were then incubated in blocking solution (5% fat-free milk solution in PBS) for 2 h at room temperature. Membranes were then washed three times in PBS-T (PBS, 0.05% Tween 20), incubated with monoclonal antibodies directed against human Cox-2 or Cox-1 (Alexis Biochemicals), washed three times in PBS-T, and incubated with peroxidase-labeled goat anti-mouse IgG (Pierce Chemical Co.). Membrane-bound antibody was visualized with the Amersham Pharmacia Biotech enhanced chemiluminescence (ECL) detection reagent.

Northern Blot and Nuclear Run-on Assays. For both assays, confluent HUVECs were grown to confluence on 150-mm dishes and maintained for 12 h in OPTI-MEM (Life Technologies), supplemented with 0.5% FCS, then pretreated with or without CsA for 2 h and subsequently stimulated with VEGF for the times indicated. For Northern blot analysis, total RNA was isolated from treated and control HUVEC cultures using TRIzol reagent (Life Technologies) as recommended by the manufacturer. A total of 20 μ g was separated on formaldehyde gels and blotted onto nylon membranes (Nytran; Schleicher & Schuell) according to standard protocols (47). Human Cox-1, Cox-2, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA probes were labeled by the random priming method (47), and hybridizations were performed overnight at 65°C in 7% SDS, 500 mM sodium phosphate buffer, pH 7.2, and 1 mM EDTA according to Church and Gilbert (48). Filters were then washed twice for 30 min each in 1% SDS and 40 mM sodium phosphate buffer, pH 7.2, at 65°C, and blots were exposed to x-ray film for autoradiography. Nuclear run-on reactions were performed essentially as described previously (49). In brief, cells were disrupted with NP-40 buffer (1% [vol/vol] NP-40, 50 mM Tris-HCl, pH 7.5) and nuclei were collected by centrifugation. Nuclear transcription assays were performed and [³²P]UTP (3,000 Ci/mmol; Amersham Pharmacia Biotech) labeled RNA was isolated by the TRIzol reagent and then hybridized to nylon membranes containing 5 μ g of dot-blotted linearized cDNAs for

Cox-2 and GAPDH, or Bluescript plasmid. Filters were hybridized with 2×10^6 cpm/ml, washed, and exposed as described above.

Determination of Cyclooxygenase Activity. Confluent HUVECs were maintained for 12 h in OPTI-MEM (Life Technologies), supplemented with 0.5% FCS, then pretreated with or without 200 ng/ml CsA or 1 μ M NS-398 for 2 h, and further stimulated with VEGF for an additional 8 h in culture medium. Levels of the stable metabolite of prostacyclin, 6-keto-PGF_{1 α} , in the culture supernatants, were determined using a commercial enzyme immunoassay (Amersham Pharmacia Biotech). To evaluate cyclooxygenase activity in intact cells, the media were aspirated after treatment, the cells were rinsed twice with in HBS, pH 7.4, supplemented with 0.1% BSA, and incubated for 30 min in the same buffer plus 10 μ M AA. The stable metabolite of prostacyclin, 6-keto-PGF_{1 α} , was determined in the supernatants using the same enzyme immunoassay described above. Results are expressed as picograms per 5×10^5 cells, as direct counts showed no major variation in cell number during the experiments. All samples were tested in triplicate.

Plasmid Constructs, Site-directed Mutagenesis, and Transient Transfection Assays. Human Cox-2 promoter constructs were created by cloning BamHI-BglII-flanked PCR products derived from Jurkat genomic DNA into the BglII-digested pXP2Luc reporter plasmid as described (50). For transient transfection experiments, HUVECs were plated in 100-mm tissue culture dishes (1.5×10^6 cells/plate) the day before. Cells were transfected with 10 μ g of the indicated luciferase reporter plasmid in 4 ml DMEM plus 10% FCS using a calcium phosphate procedure as described previously (16). In brief, HUVECs were incubated with DNA for 4.5 h and washed twice with PBS. Cells were then trypsinized, centrifuged, resuspended in OPTI-MEM supplemented with 0.5% FCS, and split among 24-well tissue culture plates (nine wells were from one 100-mm dish) precoated with 0.5% gelatin. After 16 h, transfected cells were exposed to different stimuli for 6 h. Luciferase activity was measured according to the instructions of the Luciferase system kit (Promega). Transfection efficiency was normalized by cotransfection with 0.1 μ g of the Renilla luciferase expression vector pRLCMV (Promega), measured by the Dual luciferase assays kit (Promega). The results presented are expressed as the means and standard deviations of determinations performed in triplicate. A representative experiment is shown of the reporter assays in all cases.

In vitro-directed mutagenesis was performed with the Quick-Change site-directed mutagenesis kit (Stratagene) as described previously (50), with the following synthetic oligonucleotide primers (lowercase letters indicate the mutated positions): GGG-GAGAGGAGttAAcAATTTGTGGGGGTACG (-115 to -83 dNFAT mutant primer); GGGGTACGAAAAGCGTctA-GAAACAGTCATTTTCG (-89 to -55 pNFAT mutant primer); and GCCCCACCGGCaTACcCAtTTTTTTTAA-GGG (-144 to -113 NFIL6 mutant primer). The nucleotide sequence of the mutants was confirmed by automatic DNA sequencing.

Electrophoretic Mobility Shift Assays. Nuclear extracts were prepared from HUVECs as described previously (16). In brief, 2 μ g of nuclear protein was incubated with 1 μ g of poly(dI-dC) DNA carrier in DNA-binding buffer (10% [wt/vol] polyvinyl-ethanol, 12.5% [vol/vol] glycerol, 50 mM Tris, pH 8, 2.5 mM EDTA, 2.5 mM DTT) in a final volume of 10.5 μ l on ice for 10 min. Then, 10^5 cpm (10^8 cpm/ μ g) of the ³²P-labeled double-stranded oligonucleotide (2 μ l) were added, and the reaction was incubated at room temperature for 30 min. For competition ex-

periments, a 20-fold molar excess of unlabeled oligonucleotide was added before the addition of the probe. When indicated, nuclear extracts were incubated with 0.5 μ l of either a preimmune serum or the indicated antisera for 15 min at 4°C before the addition of the probe. DNA-protein complexes were resolved by electrophoresis in 4% nondenaturing polyacrylamide gels.

The following synthetic oligonucleotides (5' to 3') were used as probes/competitors in electrophoretic mobility shift assays (EMSA) were as follows (factor binding sites are underlined): gatcGGAGGAAAACTGTTTCATACAGAAGGCGT (distal NFAT site of the human IL-2 promoter); tcgaCAAGGG-GAGAGGAGGGAAAAATTTGTGGC (nucleotides -117 to -91 containing the putative distal NFAT site of the human Cox-2 promoter); tcgaCAAAAGGCGGAAAGAAACAGTCA-TTTC (nucleotides -82 to -58 containing the putative proximal NFAT-AP1 site of the human Cox-2 promoter); gatcAGTGGGGACTACCCCT (nucleotides -277 to 211 containing the NF- κ B site of the human Cox-2 promoter); and gatcGGCTTACGCAATTTTT (-135 to -123 containing the NF-IL6 site of the human Cox-2 promoter).

In Vitro Angiogenesis Assay. HUVECs were trypsinized, counted, and resuspended (2.0×10^4 cells/well) in OPTI-MEM supplemented with 1% FCS. Cells were treated with 200 ng/ml CsA, 50 ng/ml VEGF, 10 μ M NS-398, neutralizing goat antibodies against VEGF and bFGF (rhVEGF₁₆₅, rhVEGF₁₂₁), and recombinant human bFGF from R&D Systems), or vehicle as indicated. Matrigel basement membrane matrix (Becton Dickinson) was diluted 1:2 in cold serum-free RPMI 1640, without growth factors and heparin. 50 μ l diluted Matrigel was plated into flat-bottomed 96-well tissue culture plates and allowed to gel for 1–2 h at 37°C, before cell seeding. Then, the cell suspension was plated (80 μ l/well) onto the surface of the Matrigel and incubated at 37°C. After 12 h, cells were photographed using a ZEISS inverted phase-contrast photomicroscope. Capillary tubes were defined as cellular extensions linking cell masses or branch points, and tube formation was quantified from photographs of standardized fields from triplicate wells.

Corneal Neovascularization Assay. The assay was performed as described previously (48). In brief, hydroneutral pellets of ~ 1 μ l containing 50 ng/pellet bFGF or 100 ng/pellet VEGF alone or in combination with a 20 μ g/ml anti-TGF- β antibody were implanted into a pocket surgically created in avascular corneas of anesthetized C57/Bl6 mice (National Cancer Institute, Bethesda, MD) 0.5–1 mm from the limbus. CsA (20 mg/kg/d), PGE₂ (0.1 mg/kg/d), and NS-398 (5 mg/kg/d) were administered by intraperitoneal injections. Neovascularization was observed on day 5 after implantation. Vigorous ingrowth of the blood vessels in the direction of the pellet was scored as a positive response.

Collagen Gel Assays. HUVECs were trypsinized, counted, and resuspended (10^5 cells/well) in serum-free endothelial basal growth medium (EBM; Life Technologies). Cells were pretreated with 200 ng/ml CsA for 2 h and further stimulated with 50 ng/ml VEGF, 10 ng/ml bFGF, or 1 ng/ml PGE₂. Three-dimensional collagen gels were prepared by diluting type I collagen (ICN Biomedicals) in serum-free DMEM (GIBCO BRL) at a final concentration of 500 μ g/ml. 500 μ l diluted collagen was placed into flat-bottomed 24-well tissue culture plates and allowed to gel for 1–2 h at 37°C, before cell seeding. Cell suspensions were plated (500 μ l/well) onto the surface of the gels and incubated at 37°C. The gels were photographed under a phase-contrast microscope and migrating cells (dendritic-shaped cells, whose plane of focus was beneath the surface monolayer) were

counted in four random fields per well (original magnifications: $\times 20$). All experiments were performed in triplicate.

Cell Migration Assays. Migration assays were performed using dermal microvascular endothelial cells as described previously (51). Cells were incubated overnight in EBM containing 0.1% BSA, collected, resuspended in EBM plus 0.1% BSA, at $1.0\text{--}1.5 \times 10^6$ cells/ml containing CsA where indicated and plated on the lower surface of gelatinized 5.0- μm pore filter (Nucleopore) in an inverted, modified Boyden chamber. Cells were allowed to adhere for 2 h at 37°C, the chambers were reinverted, test samples were added to the top well, and the chamber was incubated at 37°C to allow migration. Chambers were then disassembled, membranes were fixed and stained, and the number of cells that had migrated to the top of the filter in 10 high power fields were counted (1 high power field $\times 1,000$). EBM supplemented with 0.1% BSA was used as a negative control to measure background resulting from random migration. All samples were tested in quadruplicate.

Results

VEGF Induces Cox-2 Gene Expression in HUVECs. It has been reported that Cox-2 regulates angiogenesis by modulating the production of angiogenic factors (27), and that Cox-2 inhibitors prevent in vitro angiogenesis induced by VEGF and bFGF (29). To test whether VEGF regulates Cox-2 expression in endothelial cells, we carried out Western blot experiments in HUVEC extracts obtained at different times after treatment with VEGF. These experiments showed that Cox-2 protein, expressed at very low levels in unstimulated cells, was already induced 4 h after VEGF treatment, reached higher levels of expression at 8–12 h, and markedly declined to baseline levels by 24 h (Fig. 1 A). By contrast, Western blot analysis experiments showed that the levels of Cox-1 remained unchanged after the treatment with VEGF (Fig. 1 B).

We have shown previously that in HUVECs, VEGF activates the calcium/calcineurin signaling pathway and the

translocation of NFAT to the nucleus, leading to the induction of expression of tissue factor in a CsA-sensitive fashion (16). In addition, Cox-2 gene expression has been shown to be induced early during T cell activation, a process that is also dependent on NFAT and inhibited by CsA (18, 31, 50). Therefore, we analyzed the potential involvement of this pathway in the regulation of Cox-2 gene expression in HUVECs. As shown in Fig. 1 A, pretreatment of cells with CsA completely abrogated the induction of Cox-2 by VEGF. This inhibitory effect was dose dependent and occurred at the same concentration range that was inhibitory for NFAT in T lymphocytes or HUVECs (16, 17, 24, 52). Thus, concentrations of CsA in the range of 50–200 ng/ml efficiently inhibited the induction of Cox-2 by VEGF in HUVECs and by PMA plus calcium ionophore in Jurkat T cells (data not shown). Exposure of HUVECs to TNF- α also resulted in Cox-2 protein induction. However, this induction was refractory to inhibition by CsA, and displayed a different kinetic of expression to that induced by VEGF. As shown in Fig. 1 C, Cox-2 induction by TNF- α occurred 8 h later and protein levels remained high even 24 h after treatment. Another angiogenic growth factor, bFGF, induced early onset of CsA-resistant expression of Cox-2 in HUVECs. This induction, more transient than that induced by VEGF or TNF- α , was observed by 4–8 h and decreased to basal levels 12 h after stimulation (Fig. 1 D).

To determine the level at which VEGF regulates the expression of Cox-2 protein, we initially performed Northern blot analysis using RNA isolated from HUVECs activated with VEGF in the presence or absence of CsA. In these experiments, very low levels of Cox-2 mRNA found in unstimulated cells were clearly upregulated by VEGF in a CsA-sensitive manner (Fig. 2 A). In accordance with the results obtained by Western blotting, constitutive expressions of Cox-1 and GAPDH mRNAs were not modified by VEGF or CsA (Fig. 2 A). Nuclear run-on studies showed that transcription of Cox-2 gene, undetectable in

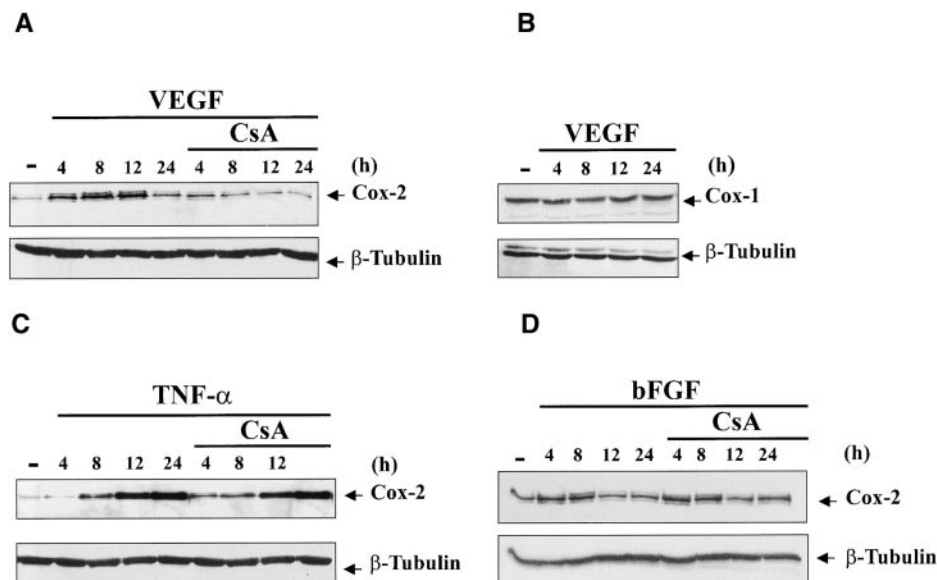


Figure 1. VEGF induces Cox-2 in HUVECs in a CsA-sensitive manner. HUVECs, either pretreated with or without CsA (200 ng/ml) for 2 h were left untreated (–) or were treated with 50 ng/ml VEGF (A and B), 50 ng/ml TNF- α (C), or 10 ng/ml bFGF (D), for the indicated periods of time. Cell extracts were analyzed by Western blot probed with anti-Cox-2, anti-Cox-1, or anti- β -tubulin antibodies to ensure equal loading. The data are representative of at least three independent experiments with similar results.

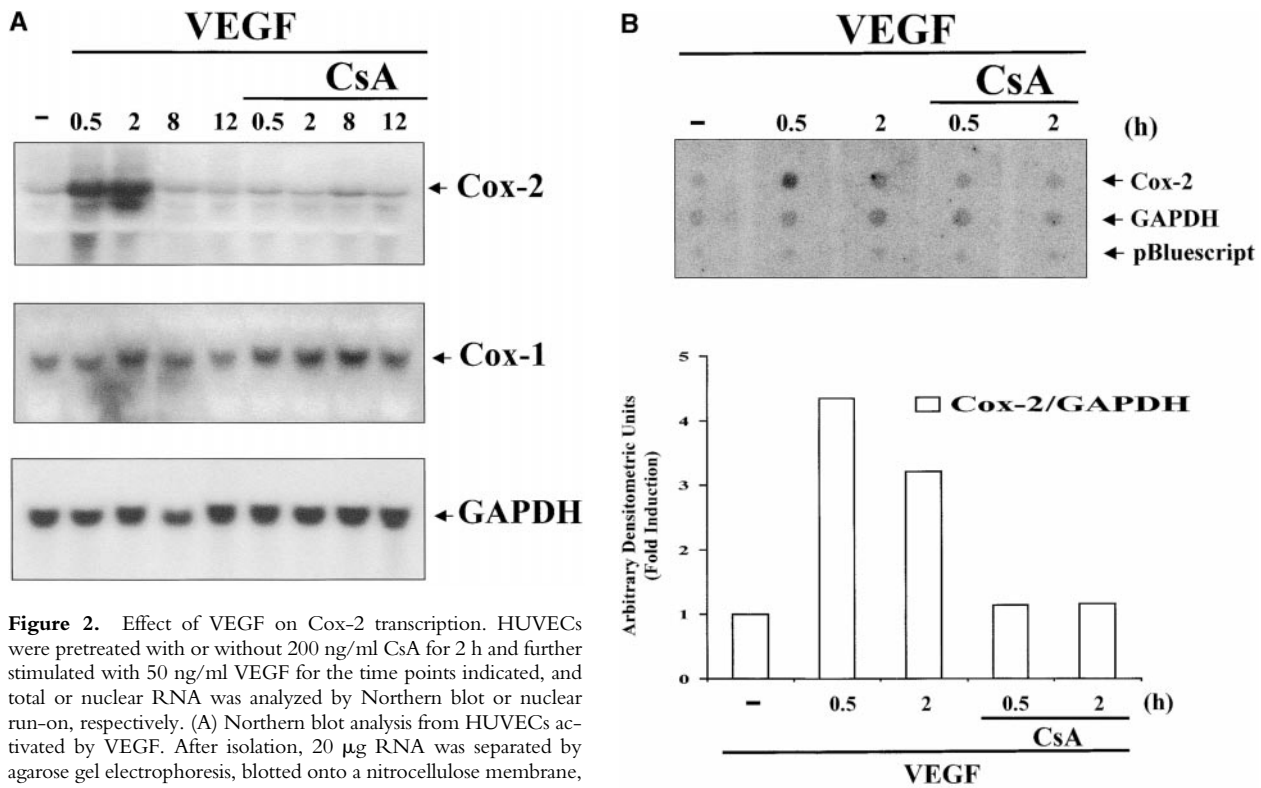


Figure 2. Effect of VEGF on Cox-2 transcription. HUVECs were pretreated with or without 200 ng/ml CsA for 2 h and further stimulated with 50 ng/ml VEGF for the time points indicated, and total or nuclear RNA was analyzed by Northern blot or nuclear run-on, respectively. (A) Northern blot analysis from HUVECs activated by VEGF. After isolation, 20 μ g RNA was separated by agarose gel electrophoresis, blotted onto a nitrocellulose membrane, and hybridized with a human Cox-2 cDNA probe, then sequentially rehybridized with Cox-1 and GAPDH-specific cDNA probes. (B) Nuclear run-on analysis using nuclear RNA from HUVECs activated by VEGF. Nuclear RNA samples, labeled with [32 P]UTP, were hybridized to nylon membrane containing 5 μ g of dot-blotted linearized cDNAs for Cox-2, GAPDH, or Bluescript plasmid. The results shown in the autoradiographs (top) are represented (in arbitrary densitometric units of Cox-2/GAPDH) as fold induction over the baseline levels of unstimulated cells (1) (bottom).

control untreated HUVECs, was significantly increased by 0.5–2 h of treatment with VEGF. Furthermore, this VEGF-driven stimulation of the Cox-2 transcription rate was also sensitive to CsA (Fig. 2 B). As expected, the induction of expression of Cox-2 by VEGF resulted in the upregulation of cyclooxygenase activity, as was evidenced by the increased production of prostacyclin, determined as 6-keto-PGF $_{1\alpha}$ accumulation in the supernatants of VEGF-treated endothelial cells (Fig. 3 A). Moreover, VEGF also induced an increase in prostacyclin synthesis in a direct assay of Cox activity in intact cells in the presence of AA (Fig. 3 B). In each case, the stimulation of prostacyclin by VEGF was sensitive to the specific Cox-2 inhibitor NS-398 and was efficiently inhibited by CsA (Fig. 3, A and B).

Transcriptional Regulation of the Cox-2 Promoter by VEGF and TNF- α . The 5' flanking region of the Cox-2 gene contains an E box, a CRE, and functional binding sites for NF-IL6 (C/EBP- β) and NF- κ B. In addition, we have recently identified two NFAT sites involved in the regulation of Cox-2 gene expression during T cell activation (50). These sites contain the GGAAA NFAT core sequence and are located at positions -71 and -101 relative to the transcription start site of the gene. They are designated as proximal and distal NFAT (pNFAT and dNFAT) sites, respectively (Fig. 4, top).

To analyze whether the VEGF-mediated upregulation of Cox-2 expression could be reflected at the promoter level,

and to evaluate the potential contribution of the NFAT sites, we conducted transfection experiments in HUVECs with luciferase reporter plasmids driven by a series of deletion fragments spanning from positions -1796 bp down to -46 bp upstream of the Cox-2 gene transcription start site. We analyzed the transcriptional inducibility of these constructs by VEGF. These experiments showed that transcription driven by constructs with deletions spanning from -1796 to -88 of the Cox-2 promoter region (P2-1900 to P2-192 deletion constructs) was efficiently induced by VEGF, but that the -88 to -46 bp promoter region contained sequences essential for VEGF-dependent induction of the Cox-2 promoter (Fig. 4). In accordance with Western blot and reverse transcription PCR experiments, CsA inhibited the activation of all reporter constructs by VEGF (Fig. 4). Therefore, cis-acting elements mapping between the nucleotides at positions -88 to -46 appear to be required for the VEGF-mediated activation of the Cox-2 promoter. In addition, the CsA-sensitive inducibility of Cox-2 expression strongly suggested the implication of the Ca $^{2+}$ /calcineurin pathway in the VEGF-mediated induction of the Cox-2 gene.

VEGF Induces NFAT-binding Activity to the Cox-2 Promoter. Two motifs including the NFAT core and an AP-1-like site were located within the -46 to -170 bp region of the Cox-2 promoter. Because of the critical role of this region for the inducibility of the promoter by VEGF, we

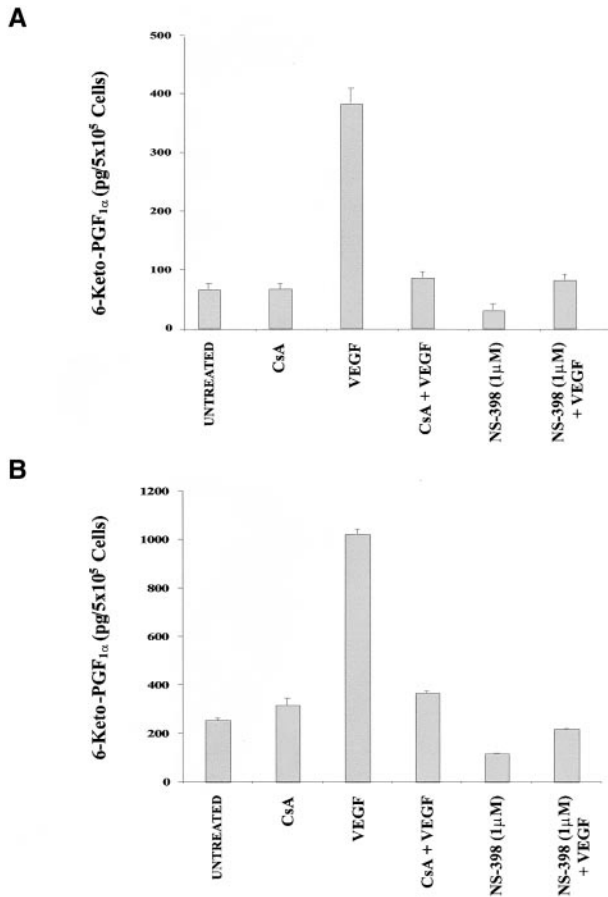


Figure 3. Induction of prostacyclin synthesis by VEGF. HUVECs were pretreated or not with 200 ng/ml CsA or 1 μM NS-398 for 2 h and subsequently treated with VEGF (50 ng/ml) for an additional 8-h period, and the levels of 6-keto-PGF_{1α} were determined in the culture medium supernatants (A) or in the supernatants of intact cells after addition of 10 μM AA (B). Prostacyclin (determined as 6-keto-PGF_{1α}) was quantified by using an enzyme immunoassay. Two independent experiments yielding similar results were performed in triplicate. Results are expressed as picogram per 5 × 10⁵ cells.

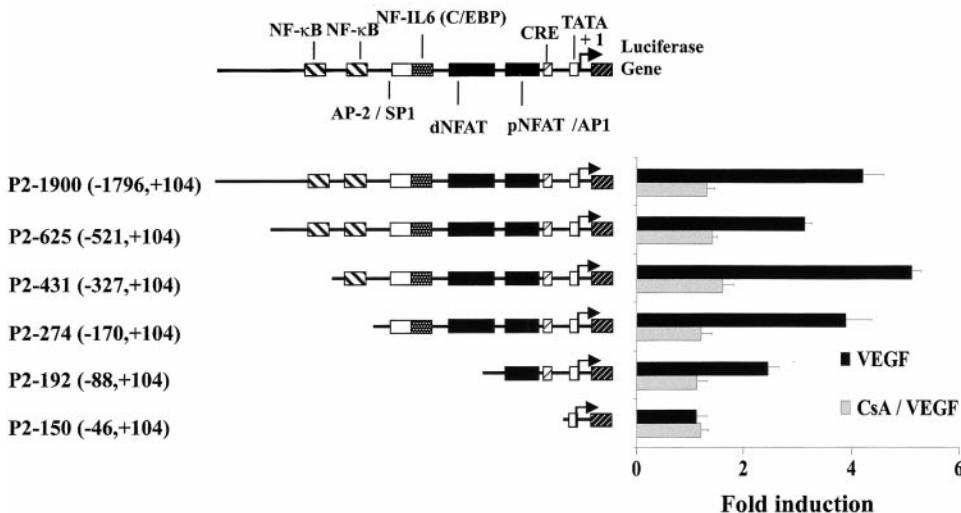


Figure 4. Activation of Cox-2 gene promoter by VEGF. HUVECs were transfected with the different Cox-2 promoter reporter plasmids for 4.5 h. 16 h later, the cells were pretreated with 200 ng/ml CsA for 2 h where indicated, and stimulated with 50 ng/ml VEGF for an additional 6 h. Experiments were performed in triplicate. Luciferase activity is expressed as fold induction over the baseline levels of transfected unstimulated cells. Results of a representative experiment out of three performed are shown. Deletions ranging from -1796 to -46 relative to the transcription start site of the Cox-2 promoter were used. The extent of the 5' truncations is shown with numbers indicating positions relative to the transcription start site. Consensus sequences are denoted by boxes.

analyzed the ability of these sites to bind NFAT. For this purpose, we designed synthetic oligonucleotides that included the pNFAT and dNFAT sites, and used them as probes in EMSAs. Both the dNFAT and the pNFAT Cox-2 probes efficiently and specifically bound to nuclear proteins from VEGF-treated HUVECs. This was evidenced by competition of the major retarded band with a 20-fold molar excess of an unlabeled NFAT consensus probe. Furthermore, CsA blocked the formation of the specific complexes generated with both probes (Fig. 5, A and B). The involvement of NFAT in this binding was further confirmed using an anti-all-NFAT antiserum directed against a common epitope of the DNA-binding domain of all NFAT members, which completely abolished the formation of the specific complexes (Fig. 5, A and B). In agreement with our previous work (16), TNF-α was unable to induce specific binding to both NFAT probes. Similarly, VEGF failed to induce binding to the NF-κB or NF-IL6 sites of the Cox-2 promoter (data not shown). These results indicate that VEGF induced binding to the NFAT motifs within the Cox-2 gene promoter in a CsA-sensitive manner.

Essential Role of NFAT for the Transcriptional Regulation of Cox-2 by VEGF. Given the relevance of the region spanning from nucleotides -88 to -46 for the inducibility of the Cox-2 promoter by VEGF, as well as the presence of functional NFAT binding sites within this region (Figs. 4 and 5), we next determined the contribution of these sites to the overall transcriptional regulation of the Cox-2 promoter by VEGF. To this end, we performed site-directed mutagenesis experiments using the parental P2-274 (-170, +104) wild-type construct as a template to generate specific mutations within the dNFAT site, the pNFAT site, or both. We also introduced a 3-bp substitution into the core region of the NF-IL6 site of the P2-431 (-327, +104) construct for comparison (Fig. 6 A). Transfection experiments with these plasmids indicated that mutation of the dNFAT site resulted in a 20–30% loss in the VEGF-

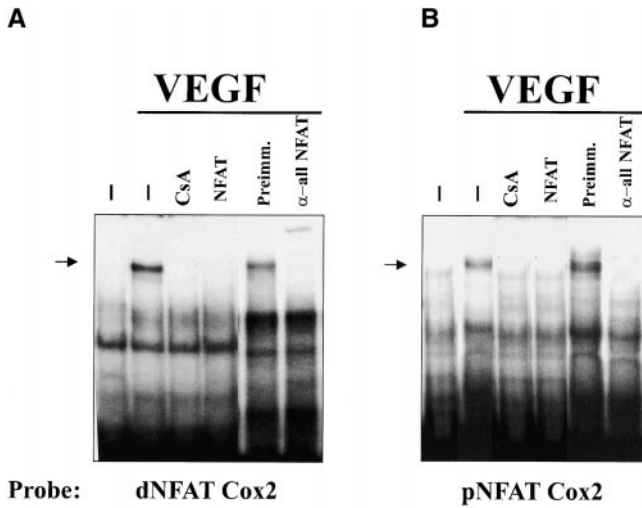


Figure 5. VEGF induces NFAT–DNA binding activity to the *Cox-2* promoter. Nuclear extracts from HUVECs stimulated for 20 min with 50 ng/ml VEGF with or without 200 ng/ml CsA were analyzed by EMSA with probes including the distal NFAT site of the *Cox-2* human promoter (nucleotides –117 to –91) (A) or the proximal NFAT site of the *Cox-2* human promoter (nucleotides –82 to –58) (B). A 20-fold molar excess of the unlabeled NFAT consensus site oligonucleotide of the IL-2 promoter was added to the binding reaction mixtures to determine the specificity of the binding. EMSAs were performed in the presence or absence of either preimmune serum (Preimm.) or the 674 anti-all-NFAT (α -all) antiserum. The specific DNA–NFAT complexes are indicated by an arrow.

induced reporter activity, whereas mutation of pNFAT alone, or double mutation of the dNFAT and pNFAT (P2-274 d&pNFAT mut construct) caused >90% reduction in the inducibility of the promoter by VEGF (Fig. 6 B). By contrast, the mutation of the NF-IL6 site hardly affected the inducibility of the promoter by VEGF (Fig. 6 B). Therefore, the pNFAT site is required for the inducibility of the *Cox-2* promoter by VEGF.

In Vitro Inhibition of Endothelial Cell Migration and Angiogenesis by CsA. Recently, NSAIDs have been shown to inhibit angiogenesis through direct effects on endothelial cells (29). In view of our findings that VEGF induces *Cox-2* gene expression, as well as the requirement of NFAT and the sensitivity to CsA of this process, we proceeded to test whether CsA was an inhibitor of VEGF-induced angiogenesis. We evaluated the effect of CsA on the ability of HUVECs to form capillary-like structures when seeded on Matrigel. We initially found that CsA alone significantly inhibited tube formation of HUVECs in Matrigel (Fig. 7 A). VEGF, and also PGE₂, induced a significant increase in the number of capillary-like structures compared with that observed with Matrigel alone. The specific contribution of endogenously secreted VEGF to the overall in vitro angiogenesis was assessed by adding saturating amounts of an anti-VEGF neutralizing antibody to the Matrigel. It is noteworthy that the inhibition displayed by the anti-VEGF antibody was very similar to that obtained with CsA (Fig. 7 A). Furthermore, the inhibition displayed by CsA was as potent as that exerted by the selective *Cox-2* inhibitor NS-398 (Fig. 7 A). Since CsA has been shown to induce TGF- β in other experimental models (53), we also analyzed the

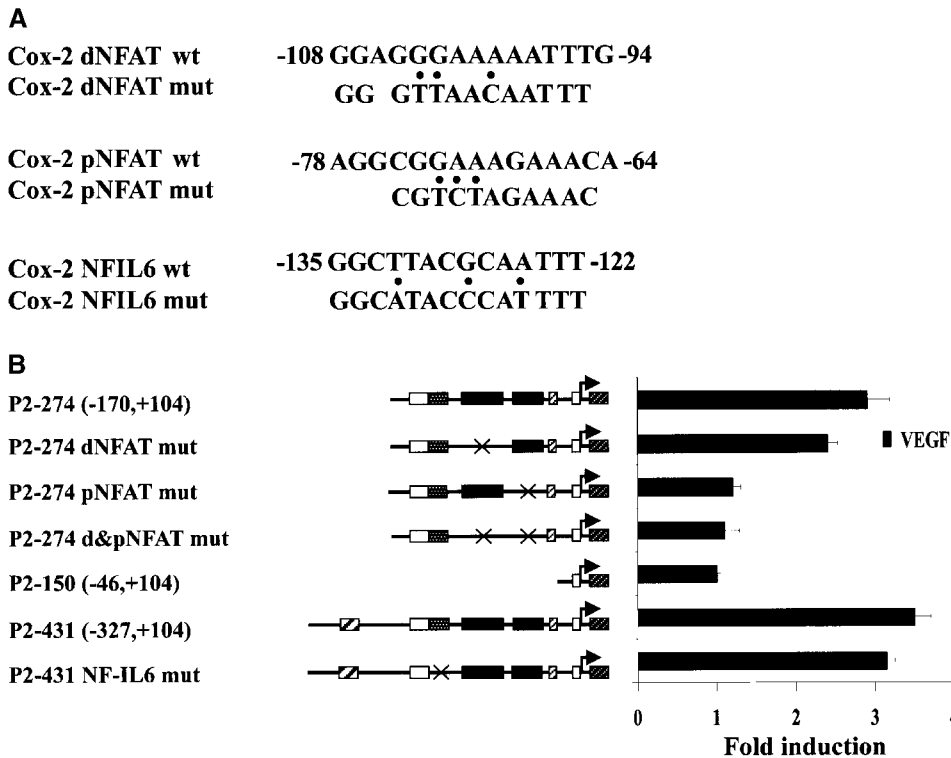


Figure 6. Role of NFAT for the *Cox-2* promoter activity induced by VEGF. (A) Sequences of the wild-type and mutated NFAT and NF-IL6 sites of the *Cox-2* gene promoter. The consensus sequences are marked in bold and the substitutions introduced are indicated by dots. (B) Reporter vectors harboring mutations in the NFAT or NF-IL6 sites were transfected into HUVECs for 4.5 h. 16 h after transfection, the cells were stimulated with 50 ng/ml VEGF for 6 h and luciferase activity was determined. The activity is expressed as fold induction over the baseline levels of transfected unstimulated cells. One out of three independent experiments performed is shown. The P2-274 (–170, +104) and P2-431 (–327, +104) constructs were used as templates for site-directed mutagenesis. Mutated sites are indicated by X.

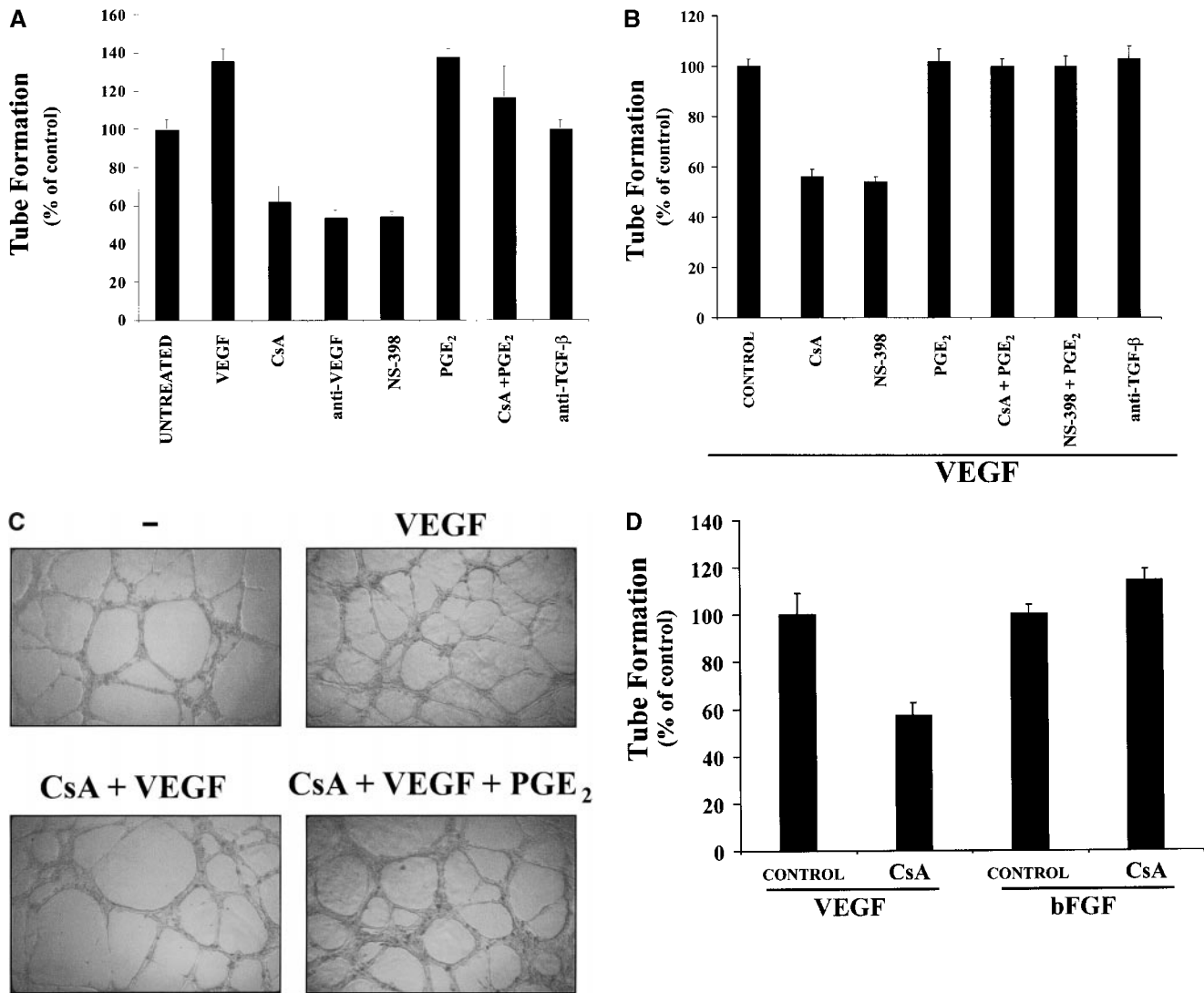


Figure 7. Inhibition of in vitro endothelial cell morphogenesis by CsA. HUVECs (2×10^4 cells/well) were seeded into 96-well plates precoated with 50 μ l of Matrigel. HUVECs were untreated (control) or treated with CsA, a neutralizing anti-VEGF antibody, NS-398, PGE₂, a neutralizing anti-TGF- β antibody alone (A), or in the presence of exogenous VEGF (B). (C) Representative photographs (original magnifications: $\times 100$) of five different fields corresponding to the experiments showed in A and B. (D) HUVECs either pretreated with or without CsA were treated with VEGF or bFGF and tube formation was quantified. Results are expressed as the percentage of tubes formed in the presence of CsA relative to the tubes formed in the presence of VEGF (100%) or bFGF (100%). For the different treatments shown in A, B, and C, 200 ng/ml CsA, 50 ng/ml VEGF, 10 ng/ml bFGF, 1 ng/ml PGE₂, 10 μ g/ml of anti-VEGF antibody, 4 μ g/ml of anti-TGF- β antibody, and 10 μ M NS-398 were used alone or in the indicated combinations. When used, CsA was added 1 h before the cells were plated on Matrigel, and then its concentration was maintained during the treatment. Tube formation was quantified 12 h after cells were plated in Matrigel by counting the number of tubular structures in four to six fields. Results are representative of at least four independent experiments performed.

effect of a neutralizing anti-TGF- β antibody on tube formation in Matrigel. However, anti-TGF- β did not interfere with the inhibitory effect of CsA or with the proangiogenic properties of exogenous VEGF. Because bFGF, which induced Cox-2 gene expression in a CsA-insensitive fashion (Fig. 1 D), was present in Matrigel, we also tested the ability of CsA and NS-398 to interfere with tube formation in the presence of exogenous recombinant VEGF (Fig. 7 B). Under these conditions both CsA and NS-398 inhibited tube formation, and this effect was completely abolished by the addition of exogenous PGE₂ (Fig. 7, A and B). Conversely, the addition of exogenous bFGF

caused an increase in the basal levels of tube formation in Matrigel that was not affected by CsA (Fig. 7 D).

To identify possible specific functions of endothelial cells that could be targeted by CsA, we next analyzed the effect of 200 ng/ml CsA on VEGF-induced incorporation of [³H]thymidine and proliferation of HUVECs. However, CsA failed to interfere with these processes. At higher doses of CsA, we observed inhibition of VEGF- and fetal bovine serum-induced proliferation (data not shown). Since 200 ng/ml of CsA completely blocks the VEGF-mediated activation of NFAT and Cox-2 expression in HUVECs, these effects of high doses of CsA did not appear to be mediated

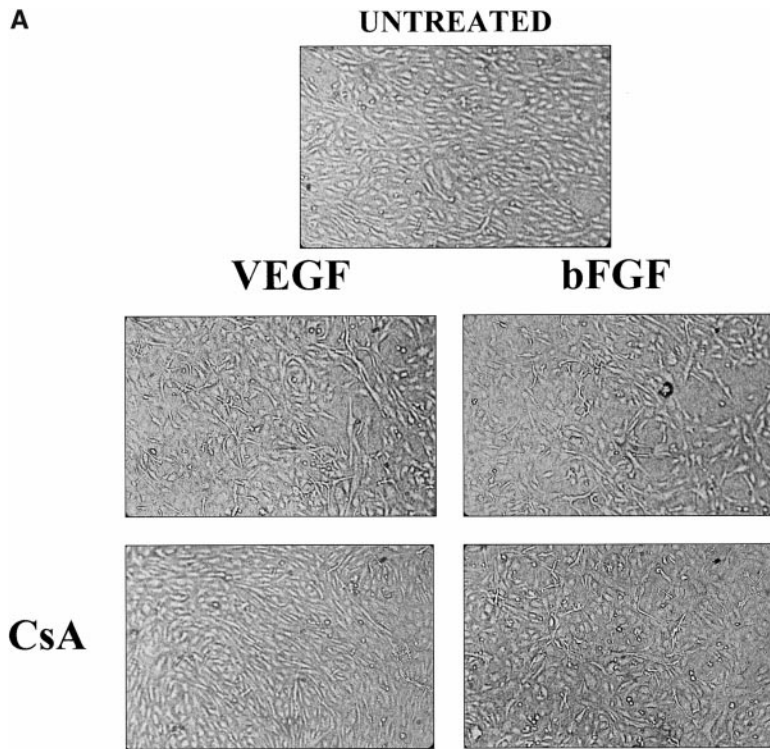
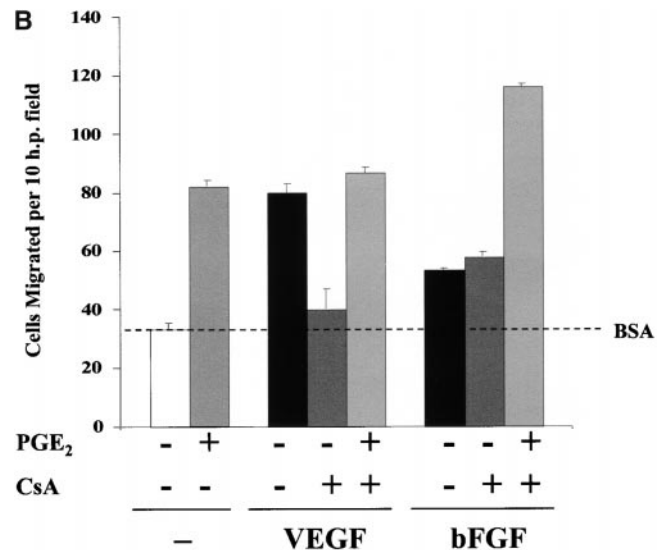
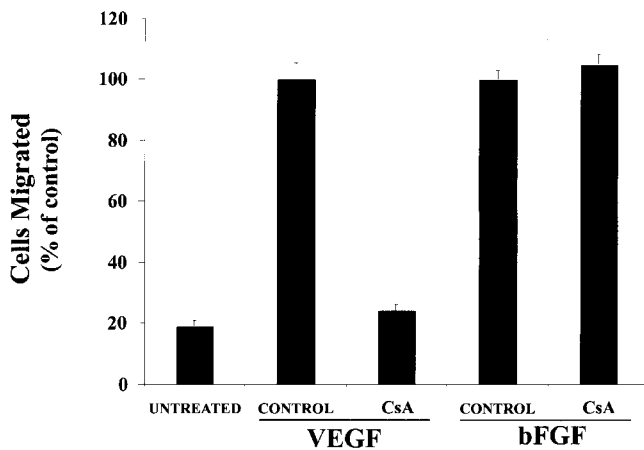


Figure 8. Effect of CsA on endothelial cell migration. (A) Phase-contrast micrographs of migrating cells invading collagen gels and forming tubelike structures. HUVECs were seeded onto the surface of collagen gels, in the presence or absence of 50 ng/ml VEGF or 10 ng/ml bFGF, and were tested alone or in combination with 200 ng/ml CsA for 24 h (top). Migrating cells were quantified under the different conditions with respect to control cells in the presence of VEGF (100%) or bFGF (100%) (bottom). (B) Migration of dermal microvascular endothelial cells. 200 ng/ml CsA was tested for its ability to inhibit endothelial cell migration induced by 100 pg/ml VEGF or 20 ng/ml bFGF. Endothelial cells in EBM plus 0.1% BSA were plated onto the lower surface of a gelatinized 5.0- μ m filter in an inverted, modified Boyden chamber. Results are expressed as the number of cells migrated by 10 high power fields. EBM supplemented with 0.1% BSA was used as a negative control (background resulting from random migration). One representative experiment out of three independent performed is shown.



by NFAT or Cox-2. Likewise, we did not detect any significant effect of CsA on the levels of KDR/Flk-1 VEGF receptor in HUVECs exposed to VEGF (data not shown). However, the migration of HUVECs into a type I collagen gel in response to VEGF was severely impaired by CsA (Fig. 8 A). In a Boyden chamber assay, primary microvascular endothelial cells were unable to migrate up the gradient of VEGF in the presence of CsA (Fig. 8 B), while the bFGF-induced migration was unaffected by CsA in each type of migration assays (Fig. 8, A and B).

In Vivo Inhibition of Angiogenesis by CsA. To determine whether the inhibitory effect of CsA on in vitro angiogenesis of HUVECs was also operative in vivo, we examined

the effect of CsA on corneal angiogenesis. For this purpose, we implanted pellets containing VEGF or bFGF in corneas of mice systemically treated with 20 mg/kg CsA once every 2 d or vehicle alone. As shown in Table I, after 5 d of pellet implantation both bFGF and VEGF effectively induced vigorous neovascularization in 4/4 and 11/12 corneas, respectively. However, CsA-treated mice yielded a differential response depending on the angiogenic stimulus used. Thus, while in mice implanted with pellets containing bFGF the systemic treatment with CsA had no effect on corneal neovascularization, CsA markedly inhibited that mediated by VEGF (Fig. 9 A and Table I). In these experiments 100% of mice receiving bFGF implants displayed a

Table I. Inhibition of Corneal Neovascularization by CsA

Sample	No antibody			Anti-TGF- β		
	PBS	bFGF	VEGF	PBS	bFGF	VEGF
Olive oil	0/4	4/4*	11/12*	1/4	5/5	7/8
CsA	0/3	4/4	2/10*	0/4	6/6	2/8*

VEGF (100 ng/pellet) or bFGF (50 ng/pellet), alone or in combination with anti-TGF- β (20 μ g/ml), were incorporated into hydron-sucralfate slow-release pellets that were implanted into the avascular corneas of both eyes in C57/Bl6 mice. The animals were injected with CsA (20 mg/kg once every 2 d) or control vehicle (olive oil, 10% ethanol). Vigorous ingrowth of new capillaries from the limbus towards the pellets was scored as a positive response. Maximum response was observed on day 5 after implantation. Ratios indicate the number of positive corneas/total implanted. Photographs of the representative corneas are shown in Fig. 9 A.

*One cornea gave a mild response with a few sprouting vessels that did not reach the pellet.

positive angiogenic response regardless of the treatment with CsA, whereas in response to VEGF only 2/10 corneas displayed a significant ingrowth of vessels (Table I). The presence of an anti-TGF- β neutralizing antibody did not interfere with the inhibitory effects of CsA on corneal angiogenesis, and similar results were obtained with VEGF and bFGF in the presence or absence of anti-TGF- β antibody (Fig. 9 A and Table I). As in the case of migration and

Matrigel assays, the inhibition of the VEGF-mediated corneal angiogenesis by CsA was significantly restored by the systemic treatment of mice with PGE₂ (Fig. 9 B and Table II). Furthermore, as also observed in the Matrigel assays, the effect of NS-398 on corneal neovascularization was similar to that of CsA. In this set of experiments, while 100% corneas displayed a positive angiogenic response to VEGF, only 1/8 corneas (12.5%) from mice receiving systemic treatment with NS-398 or CsA displayed a significant capillary ingrowth in response to VEGF (Fig. 9 B and Table II).

Discussion

VEGF is a potent mitogen for endothelial cells involved in physiological and pathological angiogenesis. VEGF switches on a multistep program that leads to the activation, proliferation, migration, and differentiation of endothelial cells. Because of the complexity of this program, a high number of genes are expected to be involved in the regulation of these processes, but a rather limited number of VEGF-induced genes has been described so far. In this study, we have identified Cox-2 as one of the genes induced by VEGF, and characterized the molecular mechanisms involved in the regulation of this gene by VEGF in HUVECs. We have shown that activation of Cox-2 gene expression by VEGF requires the activation of the calcium/calcineurin-regulated NFAT transcription factor, and that both the VEGF-mediated angiogenesis and the induction of Cox-2 gene expression are CsA-sensitive processes.

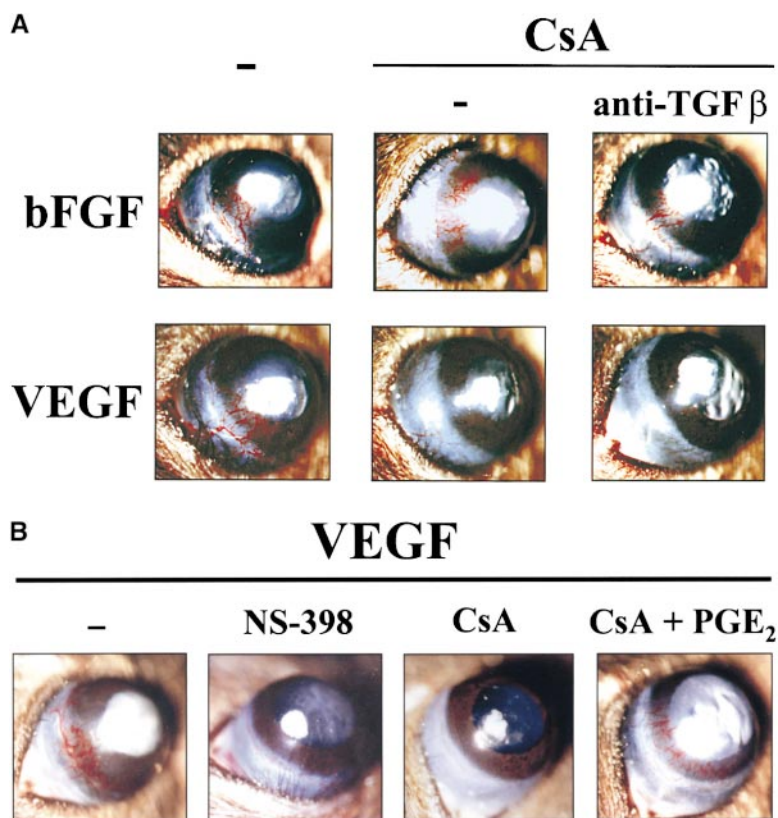


Figure 9. Inhibition of corneal neovascularization by CsA and NS-398. VEGF or bFGF, alone or in combination with anti-TGF- β , were incorporated into pellets and implanted in corneas of mice that were treated systemically with CsA or control vehicle (A), with CsA in combination with PGE₂, or with NS-398 alone (B) as indicated in Tables I and II. Vigorous ingrowth of new capillaries from the limbus towards the pellets was scored as a positive response. Maximal response was observed at day 5 after implantation. (A) Photographs of representative corneas from the experiment summarized in Table I. (B) Representative corneas from the experiment summarized in Table II.

Table II. *Effects of NS-398 and PGE₂ on Corneal Neovascularization*

VEGF	Systemic treatment	Positive corneas/total implanted
+	Olive oil	6/6
+	CsA	1/8*
+	CsA + PGE ₂	5/8‡
+	NS-398	1/8§
-	Olive oil	0/4
-	CsA	0/3

Animals were injected with CsA (20 mg/kg once every 2 d) alone or in combination with PGE₂ (0.1 mg/kg/d) or with NS-398 (5 mg/kg/d) alone, and the corneal response to VEGF (100 ng/pellet) was analyzed. "Weak response" describes corneas in which a few sprouting vessels were observed that did not reach the pellet. Photographs of the representative corneas are shown in Fig. 9 B.

*One weak positive response.

‡Two weak positive responses.

§Three weak positive responses.

We have found that Cox-2 is differentially regulated by TNF- α and VEGF in HUVECs. Previous studies have revealed the functional roles of NF- κ B, NF-IL6, ATF/CRE, and E box motifs in Cox-2 promoter regulation depending on the cell type and the stimuli used. In HUVECs, TNF- α but not VEGF required NF-IL6 for the transcriptional activation of Cox-2 promoter (Hernandez, G.L., and J.M. Redondo, unpublished data). On the other hand, VEGF efficiently induces NFAT binding activity to two functional (pNFAT and dNFAT) sites within the Cox-2 gene promoter, one of which was essential for the transcriptional response to VEGF. By contrast, the double mutation of NFAT sites or the treatment with CsA failed to affect the promoter inducibility by TNF- α (data not shown). Consistent with this, reverse transcription PCR experiments indicated that TNF- α upregulated the expression of Cox-2 mRNA, but CsA failed to inhibit this induction (data not shown).

We have recently reported the relevance of these NFAT sites in the regulation of the Cox-2 promoter upon T cell activation (50), a process where the activation of the Ca²⁺/calcineurin pathway is essential for the activation of several important genes (18, 24). Since NFAT activation is blocked by CsA, the requirement for this transcription factor in the VEGF-mediated expression of the Cox-2 is consistent with the efficient inhibition exerted by CsA on the activation of Cox-2 gene promoter, transcription, protein expression, and prostacyclin synthesis induced by VEGF. Using different experimental approaches, we have clearly shown a selective upregulation of Cox-2 gene expression and activity by VEGF. These results are in disagreement with the previous work by Bryant et al., where VEGF was found to induce Cox-1 but not Cox-2 in endothelial cells (54). The reasons for these discrepancies are at present unknown.

Several epidemiological and clinical studies have documented reduction of cancer risk in patients treated with NSAIDs (55–57). Because of the ability of these drugs to

inhibit cyclooxygenase enzymes, a number of studies have been focused on the role of these enzymes in cancer. Thus, a markedly increased expression of Cox-2, but not Cox-1, was found in >80% of colon adenocarcinomas (58). In animal models of colorectal neoplasia both the increased levels of Cox-2 in tumors and the beneficial effects of Cox inhibitors in preventing polyp formation and carcinogenesis were found (for a review, see reference 57). Cox-2-deficient mice are also protected in a genetic model of intestinal polyposis (59). Besides the involvement of Cox-2 in colorectal neoplasia, recent studies have also provided evidence for a role for cyclooxygenase enzymes regulating angiogenesis (27–29), thus establishing a potential new mechanism linking Cox-2 induction and tumor progression. In vitro angiogenesis experiments using cocultures of endothelial cells and colon carcinoma cells showed the ability of NSAIDs to downregulate the production of proangiogenic factors by colon carcinoma cells, leading to the inhibition of angiogenesis (27). More recently, NSAIDs have been reported to inhibit endothelial tube formation through a direct effect on endothelial cells (29). In view of the ability of VEGF to induce Cox-2 in HUVECs that we reported here, the inhibition of angiogenesis by NSAIDs would appear to be mediated not only by their effect on the production of VEGF and other angiogenic factors (27), but also through interference with the VEGF-mediated activation of Cox-2 in endothelial cells. Similarly, the direct inhibition displayed by Cox-2 inhibitors on angiogenesis of endothelial cells in Matrigel (29) may be explained, at least in part, by our results identifying the Cox-2 gene as a target induced by VEGF in endothelial cells.

CsA is an immunosuppressive drug whose effects on the immune system appear to be mediated through the inhibition of NFAT (18, 24). Our results indicate that CsA can also inhibit angiogenesis induced by VEGF in vivo and in vitro. The inhibitory effect of CsA on angiogenesis in Matrigel assay becomes discernible at doses similar to those required to inhibit lymphocyte activation, is coincidental with the inhibition of Cox-2 expression, and is consistent with the requirement of NFAT for the expression of this gene. Furthermore, the extent of inhibition of capillary morphogenesis by CsA was similar to that displayed by both NS-398, a selective inhibitor of Cox-2, and a neutralizing anti-VEGF antibody. In addition, the inhibition by CsA of endothelial migration and in vivo and in vitro angiogenesis was reversed by PGE₂, further eliminating possible nonspecific toxic effects of this drug. Taken together, our data strongly suggest an important role for NFAT in VEGF-induced angiogenesis that would be mediated, and least in part, through the regulation of Cox-2 gene expression. However, our data do not demonstrate a direct involvement of NFAT in angiogenesis. Given the functional redundancy displayed by different NFAT family members, as well as the embryonic lethality seen in NFATc null mice (60, 61), an approach based on the analysis of angiogenesis in NFAT deficient mice presents considerable difficulties. In this regard, other alternative approaches such as the study of angiogenesis in transgenic mice expressing either

dominant negative versions of NFAT or inhibitory calcineurin binding peptides (62) in a conditional fashion, will be very useful in addressing the actual role of NFATs in angiogenesis in the adult. Despite all these considerations, and taking into account the major role VEGF plays in physiological and pathological angiogenesis, our results also suggest that CsA could be used for antiangiogenic purposes. In fact, CsA has been shown previously to retard neovascularization in the cornea in a rat xenotransplantation model (63), and to inhibit matrix metalloprotease-2 secretion, chemotaxis, proliferation, and morphogenesis in a transformed endothelial cell line in vitro (64). However, a dose-dependent inhibitory effect of CsA reported by Iurlaro et al. (64) was observed at concentrations 10–80-fold higher than those required for either the NFAT inhibition, or the blockade of in vitro angiogenesis in HUVECs. In this regard, we have also found that although CsA at doses of 200 ng/ml (or lower) failed to interfere with the proliferation of HUVECs, at higher doses it inhibited proliferation of HUVECs. However, we have previously shown that doses of 200 ng/ml completely block the translocation, DNA binding, and transactivation of NFAT in HUVECs (16), and we have shown in the current study that the same doses block the VEGF-mediated expression of the Cox-2 gene. Thus, it appears that the inhibitory effects observed at higher doses of CsA are not mediated through NFAT inhibition or related to the inhibition of Cox-2. In fact, we have compared the ability of different doses of CsA to inhibit Cox-2 activation in HUVECs and T lymphocytes activated with PMA plus Ca²⁺ ionophore. These experiments revealed that the doses of CsA (50–200 ng/ml) previously shown to inhibit cell activation and NFAT in T lymphocytes (24, 52) were similarly effective at inhibiting Cox-2 expression in HUVECs (data not shown). These results also suggest that calcineurin levels in HUVECs and T lymphocytes are similar.

CsA has been reported to induce the synthesis of TGF- β (53). However, we have found that neutralizing anti-TGF- β antibodies had an effect on neither the CsA-mediated inhibition of in vitro angiogenesis nor in vivo corneal neovascularization induced by VEGF. Thus, despite the potential side effects of CsA via its ability to induce TGF- β , CsA clearly displays antiangiogenic activity. This property of CsA may mediate some of the beneficial effects of the drug observed in nonneoplastic pathologies such as rheumatoid arthritis and psoriasis, where angiogenesis is associated with an enhanced expression of VEGF and its receptors (65, 66). In addition, non-immune-mediated situations in which VEGF-mediated neovascularization occurs, such as diabetic retinopathy (67, 68), could also be potential therapeutic targets of CsA. Our results encourage studies to evaluate the therapeutic potential of this drug in animal models of retinopathy that, if successful, could easily be expanded to clinical trials.

We are very grateful to Drs. R. González-Amaro, B. Jiménez, A. Rodríguez, F. Sánchez-Madrid, and J. Vázquez for their critical reading of the manuscript. We would also like to thank Drs. A. Al-

franca, A. García-Arroyo, M. Yáñez-Mo, and C. Domínguez for helping us with in vitro angiogenesis and migration assays.

This work was supported by grant PM99-0116 from Ministerio de Educación y Cultura (MEC-DGES) of Spain (to J.M. Redondo) and grants FEDER 1FD97-0514-CO2-01 and FEDER FD97-0275 from MEC-DGES and the European Community to J.M. Redondo and M. Fresno, respectively. G.L. Hernández was supported by grants from Consejo Superior de Investigaciones Científicas y Tecnológicas (CONICET) of Argentina and Comunidad Autónoma de Madrid grant 8.3/0024/2000, and M. Fresno by grant PM97-0130, O. Volpert by American Heart Association grant AHA SDG 0030023N, and S. Martínez-Martínez by grant 8.3/19/1998 from the Comunidad Autónoma de Madrid. The Centro de Biología Molecular “Severo Ochoa” is supported by a grant from the Fundación Ramón Areces.

Submitted: 1 August 2000

Revised: 6 December 2000

Accepted: 23 January 2001

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