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Hypoxia Induces Vascular Endothelial Growth Factor in Cultured Human Endothelial Cells*

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Smooth muscle cells, macrophages, glial cells, keratinocytes, and transformed cells have been established as synthesis sites for vascular endothelial growth factor (VEGF). The modulating effects of VEGF are essentially limited to endothelial cells (ECs), the only cell type consistently shown to express VEGF receptors. VEGF has thus been considered to act exclusively via a paracrine pathway. We sought to determine whether the role of human ECs might, under selected conditions, extend beyond that of a target to involve contingency synthesis of VEGF. In both unstimulated human umbilical vein ECs (HUVECs) and human derma-derived microvascular ECs (HMECs), Northern analysis detected no VEGF transcripts. Phorbol-12-myristate 13-acetate (10^{-7} M) treatment, however, induced VEGF mRNA expression in both HUVECs and HMECs, peaking at 3 and 6 h, respectively, and returning to undetectable levels by 12 h. *In vitro* exposure of HUVECs to a hypoxic environment (pO_2 = 35 mm of mercury) for 12, 24, and 48 h and exposure of HMECs for 6, 12, 24, and 48 h induced VEGF mRNA in a time-dependent fashion. Re-exposure to normoxia (pO_2 = 150 mm of mercury) for 24 h after 24 h of hypoxia returned VEGF mRNA transcripts to undetectable levels in HUVECs. Cobalt chloride and nickel chloride treatment each induced VEGF mRNA in ECs. Cycloheximide treatment further augmented expression of VEGF mRNA induced by cobalt chloride, nickel chloride, and hypoxia in HUVECs. VEGF protein production in hypoxic HUVECs was demonstrated immunohistochemically. Conditioned media from hypoxic HUVECs caused a 2-fold increase in the incorporation of tritiated thymidine. Finally, immune precipitates of anti-KDR probed with anti-Tyr(P) antibodies demonstrated evidence of receptor autophosphorylation in hypoxic but not normoxic HUVECs. These findings thus establish the potential for an autocrine pathway that may augment and/or amplify the paracrine effects of VEGF in stimulating angiogenesis.

Vascular endothelial growth factor (VEGF)[1] (1), known also as vascular permeability factor (2) and vasculotropin (3), is an endothelial cell-specific mitogen *in vitro* (1, 2) and an angiogenic growth factor *in vivo* (4–7). VEGF is distinguished from most other angiogenic cytokines by the presence of a secretion signal at its amino terminus (8) that allows VEGF secretion by intact cells (1). Northern analysis and *in situ* hybridization have demonstrated VEGF expression in adult organs undergoing angiogenesis, including the female reproductive system (9, 10), the eyes of patients with diabetic retinopathy (11, 12), and certain tumors (1, 13–18). VEGF is thus considered to be an important regulator of both physiologic as well as pathologic angiogenesis.

Within the vascular wall, VEGF can be produced by vascular smooth muscle cells (SMCs) (8, 19) and can thereby modulate endothelial cell (EC) functions via a paracrine pathway. Recent reports (20–22), however, have suggested that ECs of some nonhuman species may also produce VEGF mRNA and/or protein and thus constitute the basis for an alternative, autocrine pathway.

In this study, we investigated whether hypoxia, a fundamental stimulus of neovascularization that is known to increase VEGF expression in neoplastic tissues (23) and in certain normal human cells (19, 23) could induce VEGF mRNA expression and protein production in human ECs.

We observed that human ECs express VEGF mRNA in response to hypoxia or cations such as cobalt or nickel that may simulate the hypoxic stimulus. VEGF expression was in all cases augmented by pretreatment with cycloheximide. Immunoreactive VEGF protein was detected in hypoxic ECs, and conditioned medium from hypoxic HUVECs was shown to augment incorporation of tritiated thymidine, comparable to that observed with recombinant VEGF. Moreover, autophosphorylation of the KDR receptor was demonstrated in hypoxic but not normoxic ECs. These findings thus demonstrate that under hypoxic conditions, human ECs include the requisite elements for an autocrine pathway that may serve to amplify the angiogenic effects of VEGF.

MATERIALS AND METHODS

Reagents—PMA (phorbol-12-myristate 13-acetate), staurosporine, $CoCl_2$, $NiCl_2$, and cycloheximide were purchased from Sigma. Human transforming growth factor-β_1 (TGF- β_1) was purchased from Genzyme Corp. (Cambridge, MA).

Cell Culture—HUVECs were isolated from human umbilical cords by collagenase dissociation (24) and grown onto 1% gelatin-coated plates in medium 199 (Life Technologies, Inc.) with 20% heat-inactivated fetal bovine serum (FBS) (Life Technologies, Inc.), 100 μg/ml endothelial cell growth supplement (Sigma), and 50 units/ml heparin (Sigma) (HUVEC medium). Cells were passaged at confluence following dissociation with 0.05% trypsin (Life Technologies, Inc.). Cultured HUVECs were used

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[1] The abbreviations used are: VEGF, vascular endothelial growth factor; ECs, endothelial cells; SMCs, smooth muscle cells; PMA, phorbol-12-myristate 13-acetate; TGF-β_1, transforming growth factor beta-1; HUVECs, human umbilical vein endothelial cells; HMECs, human microvascular endothelial cells; FBS, fetal bovine serum; PBS, phosphate-buffered saline; Epo, erythropoietin; mmHg, mm of mercury.

between passages 4 and 9.

HMECs derived from adult dermis were purchased from Clonetics Corp. (San Diego, CA) and grown on 1.5% gelatin-coated plates in EGM-MV complete medium (Clonetics) containing 10 ng/ml epidermal growth factor, 1 μg/ml hydrocortisone, 3 mg/ml bovine brain extract, 10 μg/ml heparin, and 5% heat-inactivated FBS. Either the EGM-MV medium or a 1:1 mixture of EGM-MV and HUVEC medium were used during the hypoxic stimulation of HMECs. HMECs were used between passages 5 and 8.

A human clonal endothelial cell line (ECV304) developed from spontaneously transformed ECs isolated from a normal human umbilical cord (25) was also purchased from Clonetics. These ECV304 cells were cultured in medium 199 supplemented with 10% heat-inactivated FBS.

Human SMCs were cultured by explant outgrowth from unused portions of internal mammary arteries obtained at coronary bypass surgery as described previously (26). Cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% heat-inactivated FBS. SMCs were growth-arrested in serum-free medium (IT medium) for 48 h before each experiment (27) to minimize constitutive expression of the VEGF gene (8). Extracts of SMCs treated with PMA (10^{-7} M) or simply grown in 10% FBS were used as a positive control for VEGF gene expression (8).

RNA Analysis—RNA was isolated using RNAzol B (Biotecx Laboratories Inc., Houston, TX). RNA concentration was calculated from absorbance at 260 nm. All RNA samples contained intact rRNA and were devoid of genomic DNA contamination as evaluated by ethidium bromide staining of agarose gels. 15 or 20 μg of RNA from each sample was separated by electrophoresis in 1% agarose gel containing formaldehyde. After capillary transfer to a nylon membrane (Hybond-N, Amersham Corp.), rRNA was visualized by ethidium bromide staining to verify that equal amounts of RNA had transferred to the gel. Hybridization probes were labeled with [α-^{32}P]dCTP (New England Nuclear) using a random priming labeling kit (Boehringer Mannheim) and purified of unincorporated nucleotides with Bio-Spin 6 minicolumns (Bio-Rad). Probe-specific activities ranged between 1×10^8 to 1×10^9 cpm/μg. The DNA probes were: 1) for human VEGF, a 675-base pair *Eco*RI–*Bgl*II fragment of plasmid pSVI.VEGF.21 (generous gift from Dr. N. Ferrara, Genentech Inc., South San Francisco, CA) and 2) for β-actin, a 800-base pair *Pst*I fragment of the mouse β-actin gene (28, 29). Membranes were prehybridized for 1 h and hybridized for 3 h at 65 °C in Rapid-hyb buffer (Amersham Corp). Nonspecifically bound probe was removed with a final stringency wash in 0.1 × SSC+ 0.1% SDS at 65 °C. Blots were then exposed for autoradiography at −80 °C with an intensifier screen. Hybridization with the β-actin probe (28–30) as well as analysis of the area of the gel corresponding to the 18 S ribosomal RNA (31) were used to confirm equal loading of RNA.

PMA Treatment—The capability of human ECs to express VEGF was investigated initially using PMA. HUVECs and HMECs were stimulated with PMA 10^{-7} M for 1, 3, 6, 12, or 24 h. Identically stimulated SMCs were used to validate the time course of VEGF expression in response to PMA as previously reported (19). In an additional series of experiments, HUVECs were stimulated with PMA 10^{-7} or 10^{-6} M for 3 h to assess dose dependence. In selected experiments, PMA treatment was preceded by a 3-h exposure to staurosporine (10^{-6} or 10^{-7} M), a potent inhibitor of Ca^{2+}-dependent protein kinases.

Hypoxia—To achieve hypoxia, a preanalyzed air mixture (5% CO_2/95% N_2; AIRCO, Hingham, MA) was infused into air-tight chambers (Billups-Rothenberg Inc., Del Mar, CA) (chamber 1) or a small desiccator cabinet (PGC Scientifics; Gaithersburg, MD) (chamber 2), constructed with inflow and outflow valves, at a flow rate of 3 liters/min for 15 min twice a day as described previously (19, 28, 29). The pO_2, measured with a gas analyzer (278 Blood Gas Systems, Ciba-Corning Diagnostics Corp., Medfield, MA), reached a nadir of 35 and 70 mmHg in the culture medium in chambers 1 and 2, respectively, 6 h after the infusion was completed and persisted at 24 and 48 h (19). HUVECs were exposed to pO_2 = 35 mmHg for 12, 24, and 48 h and were also exposed to pO_2 = 70 mmHg for 24 h. To test for hypoxia-specific regulation of VEGF gene expression, HUVECs exposed for 24 h to pO_2 = 35 mmHg were then returned to a normoxic environment (atmospheric air/5% CO_2; pO_2 = 150 mmHg) for 24 h more. In additional experiments, HUVECs pretreated with cycloheximide (5 μg/ml) for 4 h were then exposed to pO_2 = 35 mmHg for 24 h. HMECs were exposed to pO_2 = 35 mmHg for 6, 12, 24, and 48 h. Control HUVECs and HMECs were maintained in normoxia in the same incubator and harvested at time 0 and at 24 h.

ECV304 cells were exposed to pO_2 = 35 mmHg for 6, 12, and 24 h or returned to normoxia for 12 h following 12 h of hypoxia. Control ECV304 cells were maintained in normoxia in the same incubator and harvested at time 0 and at 24 h.

Cobalt and Nickel Treatment—We tested the effects of cations known to replace ferrous iron from heme, resulting in reduced oxygen-binding capability and thereby chemically simulating hypoxia (32). Normoxic HUVECs were exposed to $CoCl_2$ (100 μM) or $NiCl_2$ (300 μM) for 24 h. Treatment with divalent cations was in certain experiments preceded by exposure to cycloheximide (5 μg/ml) for 4 h.

Factor VIII and α-Actin Immunocytochemistry—To help to exclude the possibility that VEGF expression in human ECs following PMA and hypoxia might have resulted from contamination of EC cultures by SMCs and/or fibroblasts, we performed immunohistochemical analysis of cultured ECs isolated in our laboratory (ECV304 cells do not express Factor VIII (25)). Factor VIII related antigen, an EC marker, was identified using an anti-Factor VIII rabbit polyclonal antibody (Signet Laboratories, Dedham, MA). HHF-35, a mouse monoclonal antibody prepared against α-muscle actin (Enzo Diagnostics, New York, NY), was used to identify SMCs. Briefly, HUVECs, HMECs, and SMCs were grown onto chamber slides and fixed with acetone for 2 min. Endogenous peroxidase was inactivated with 3% hydrogen peroxide. Nonspecific binding was blocked by incubation with 10% normal serum of the same species of the secondary antibody. Primary antibodies were then applied. Negative controls were appropriate dilution of nonimmune rabbit serum or of MOPC-21, a purified nonimmune mouse IgG_1 (Sigma). Following two washes in PBS, a biotinylated goat anti-rabbit or horse anti-mouse secondary antibody was applied, followed by an avidin biotin complex (Elite Avidin-Biotin Detection System; Signet Laboratories). All incubation times were 20 min. Incubation with 3-amino-9-ethylcarbazole for 10 min was used to visualize the final reaction product.

TGF-β_1 Treatment—Previous work has established that TGF-β_1 induces VEGF mRNA in both SMCs (19) and fibroblasts (33). We therefore applied this observation in the current series of experiments to further exclude the possibility that VEGF expression in human ECs following exposure to PMA and/or hypoxia might be due to contamination of the EC cultures by SMCs or fibroblasts. Cells were harvested at time 0 and at 3 and 6 h of TGF-β_1 (1 ng/ml) stimulation. SMCs, cultured with HUVEC medium for 48 h, were simultaneously stimulated and used as a positive control for VEGF induction.

Immunocytochemical Staining for VEGF—HUVECs were grown onto chamber slides and fixed with acetone for 2 min after 0, 24, and 48 h exposure to hypoxia (pO_2 = 35 mmHg). Endogenous peroxidase activity was exhausted with 3% hydrogen peroxide for 10 min, and nonspecific protein binding was blocked with 10% goat serum for 20 min. HUVECs were incubated overnight at 4 °C with an affinity-purified rabbit polyclonal antibody raised against human VEGF amino peptide (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) diluted 1:200 in 10% goat serum/PBS. Identical concentrations of nonspecific rabbit serum and of the primary antibody preabsorbed for 1 h with a 10-fold molar concentration of the VEGF peptide (Santa Cruz Biotechnology, Inc.) were applied in place of the primary antibody as negative controls. Cells were washed twice in PBS, incubated with biotin-labeled goat anti-rabbit antibody (Kirkegaard and Perry Laboratories, Gaithersburg, MD) diluted 1:100 in 2% goat serum/PBS for 20 min, and washed again before applying an avidin-biotin-peroxidase conjugate for 30 min. Finally, 3-amino-9-ethylcarbazole substrate was applied for 10 min, and the cells were lightly counterstained with hematoxylin. Sections of human colon cancer were simultaneously immunostained as positive controls (17).

Incorporation of Tritiated Thymidine—Conditioned media of HUVECs exposed to hypoxia or normoxia for 48 h were harvested, filtered, and frozen. To test the effect of these conditioned media on uptake of tritiated thymidine, HUVECs were seeded in duplicate in 24-well plates at 3.10^4 cells/well in standard growth medium. The following day, media were removed, and cells were incubated in medium 199 + 2% FBS for 18 h. The cells were then incubated for 24 h with VEGF in medium 199 without serum (final concentration, 50 ng/ml) or with conditioned medium of HUVECs grown in mormoxia or hypoxia (final concentration, 1/4). Tritiated thymidine (New England Nuclear; 3 μCi/well) was added to cells during the last 6 h of incubation. Cells were washed, harvested, and processed for counting in a scintillation counter. Results are expressed as the mean of cpm counted in replicate wells.

Immunoprecipitation and Western Blotting—HUVECs were grown to 80% confluency and maintained in hypoxia or normoxia for 24 h prior to cell lysis. Cell lysates from growth factor-stimulated HUVECs were prepared after inducing quiescence for 24 h in 1% BSA or following activation with VEGF for 10 min. For immunoprecipitation, whole cell lysates were incubated with anti-KDR antibodies for 2 h at 4 °C fol-

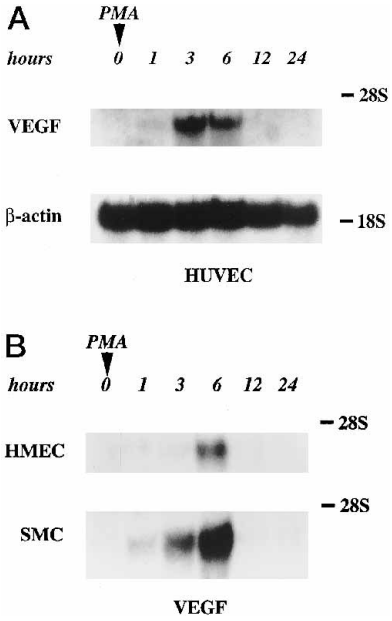

FIG. 1. *A*, PMA induces VEGF gene expression in HUVECs. HUVECs were treated with PMA (10^{-7} M) for the indicated times, and Northern analysis was performed after loading 20 μg of RNA of HUVECs. Unstimulated HUVECs contained no detectable VEGF mRNA. PMA-induced VEGF mRNA expression was evident at 1 h, peaked at 3 h, and declined at 6 h with return to undetectable level at 12 h. For comparison of RNA loading, β-actin probe was hybridized to the same blot. The locations of 28 S and 18 S rRNAs are indicated. *B*, PMA induces VEGF gene expression in HMECs. HMECs and SMCs were treated with PMA (10^{-7} M) as indicated. Northern analysis was performed after loading 20 μg of RNA. Unstimulated HMECs and SMCs contained no detectable VEGF mRNA. PMA stimulation induced VEGF mRNA expression at 6 h in HMECs and from 1 to 6 h in SMCs. VEGF mRNA returned to undetectable levels by 12 h in both HMECs and SMCs.

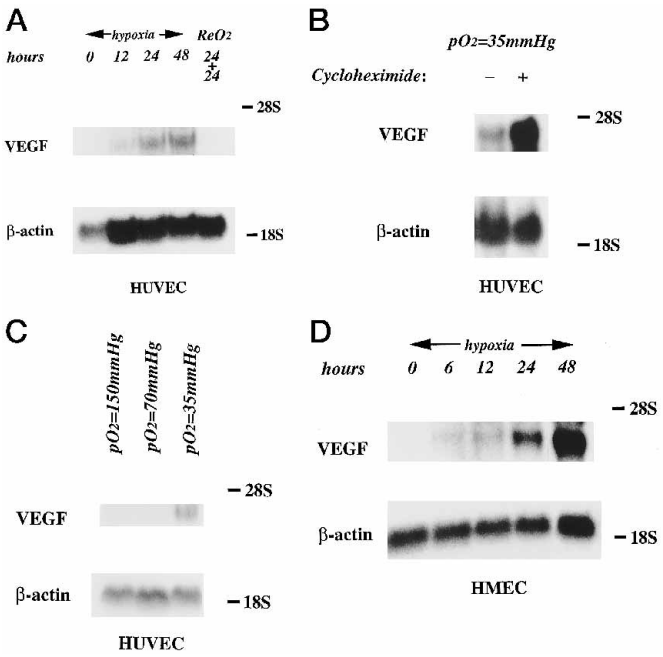

FIG. 2. *A*, hypoxia induces VEGF gene expression in HUVECs. HUVECs were exposed to hypoxic condition (pO_2 = 35 mmHg) for the indicated times. HUVECs exposed for 24 h to pO_2 = 35 mmHg were then returned to a normoxic condition (pO_2 = 150 mmHg) for 24 additional h (*ReO_2*, reoxygenation). Unstimulated HUVECs at time 0 contained no detectable VEGF mRNA. VEGF mRNA induction by hypoxia was time-dependent with maximal levels observed at 48 h. VEGF mRNA levels were undetectable after reoxygenation for 24 h. *B*, cycloheximide potentiates hypoxia-induced VEGF gene expression in HUVECs. HUVECs were exposed to hypoxia (pO_2 = 35 mmHg) for 24 h with or without pretreatment of cycloheximide (5 μg/ml) for 4 h. Northern analysis was performed after loading 20 μg of RNA. Cycloheximide markedly increased VEGF mRNA induction by hypoxia. For comparison of RNA loading, β-actin probe was hybridized to the same blot. The locations of 28 S and 18 S rRNAs are indicated. *C*, dose response of VEGF gene expression in HUVECs by hypoxia. The induction of VEGF mRNA in HUVECs was dependent on the degree of hypoxic stimulation, as it occurred upon exposure to pO_2 = 35 mmHg for 24 h, but not to pO_2 = 70 mmHg and normoxia (pO_2 = 150 mmHg) for the same time. Hybridization with β-actin probe is shown for comparison. *D*, hypoxia induces VEGF mRNA expression in HMECs. HMECs were treated for 6, 12, 24, and 48 h in hypoxia (pO_2 = 35 mmHg). Control HMECs maintained in normoxic environment were harvested at time 0. VEGF mRNA was detectable at 6 h of hypoxic stimulation and progressively increased with peak at 48 h. For comparison of similar loading of all lanes, the β-actin mRNA band and the area of the gel corresponding to the 18 S ribosomal RNA are shown.

lowed by addition of 40 μl of protein A-Agarose (Sigma) for 45 min. Immune complexes were collected by centrifugation and washed 4 times with 2 × PBS, 1 × TNE (10 mM Tris/HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA), and 1 × PBS. Immune precipitates were denatured by boiling for 3 min in gel-loading buffer and resolved by electrophoresis on 10% SDS-polyacrylamide gels. Proteins resolved on SDS-polyacrylamide gel electrophoresis were electrophoretically transferred on to polyvinylidene difluoride (QIAGEN) membrane. The membranes were blocked in PBS-T (PBS with Tween-20, 0.2%)/7% nonfat dry milk and 2% bovine serum albumin for 1 h and probed with anti-phosphotyrosine antibodies (UBI) followed by rabbit anti-mouse HRP-conjugated secondary antibodies (Sigma). Immunoreactive bands were visualized using ECL reagent (Amersham Corp.).

RESULTS

PMA Treatment Induces VEGF mRNA in HUVECs—Unstimulated HUVECs contained no VEGF mRNA detectable by Northern analysis (Fig. 1*A*). We observed a time-dependent induction of VEGF mRNA upon stimulation with PMA 10^{-7} M. VEGF mRNA transcripts were evident in HUVECs as early as 1 h, peaked at 3 h, declined at 6 h, and returned to undetectable levels by 12 h following administration of PMA (Fig. 1*A*). PMA 10^{-6} M for 3 h induced a greater increment in VEGF expression (data not shown). Pretreatment with staurosporine (10^{-7} or 10^{-6} M) for 3 h abrogated the induction of VEGF mRNA upon stimulation with PMA 10^{-7} or 10^{-6} M, respectively (data not shown).

PMA Treatment Induces VEGF mRNA in HMECs—PMA treatment of HMECs induced VEGF mRNA, which was maximal at 6 h. Accumulation was transient and was undetectable by 12 h post-PMA (Fig. 1*B*). In simultaneously stimulated SMCs, VEGF mRNA was detectable at 1 h of treatment, peaked at 6 h, and returned to undetectable levels by 12 h (Fig. 1*B*).

Hypoxia Induces VEGF mRNA in HUVECs—Hypoxic stimulation (pO_2 = 35 mmHg) of HUVECs induced VEGF mRNA at 12 h of treatment (Fig. 2*A*). VEGF mRNA induction by hypoxia was time-dependent, with maximal levels observed at 48 h. No later time points were checked. The induction of VEGF mRNA was hypoxia-specific and fully reversible, as demonstrated by the decrease in VEGF mRNA to undetectable levels upon 24 h of re-exposure to normoxia (Fig. 2*A*). Unstimulated HUVECs incubated in normoxia for 48 h showed no detectable VEGF mRNA (data not shown). These findings were consistently obtained in four separate experiments. Cycloheximide treatment potentiated the increase in VEGF mRNA levels induced by hypoxia (Fig. 2*B*).

The induction of VEGF mRNA in HUVECs was dependent on the degree of hypoxic stimulation, as it was detected upon exposure to pO_2 = 35 mmHg for 24 h but not to pO_2 = 70 mmHg for the same time (Fig. 2*C*).

Hypoxia Induces VEGF mRNA in HMECs—HMECs maintained in a normoxic environment showed no VEGF mRNA expression during 48 h of incubation. However, hypoxia ($p0_2$ =

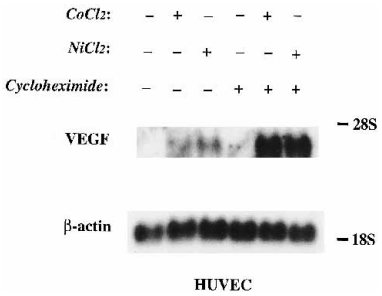

FIG. 3. **Co^{2+} and Ni^{2+} induce VEGF gene expression in HUVECs.** HUVECs were treated with $CoCl_2$ (100 μM) and $NiCl_2$ (300 μM) for 24 h. Cycloheximide (5 μg/ml) pretreatment was administered for 4 h. Northern analysis was performed after loading 15 μg RNA of HUVECs. Both Co^{2+} and Ni^{2+} induced VEGF transcripts. Cycloheximide markedly increased the induction of VEGF mRNA by Co^{2+} and Ni^{2+}. For comparison of RNA loading, β-actin probe was hybridized to the same blot.

35 mmHg) induced VEGF transcripts at 6, 12, 24, and 48 h. These results were obtained in four separate experiments (Fig. 2*D*).

Co^{2+} and Ni^{2+} Induce VEGF mRNA—To study the possibility that hypoxic induction of VEGF transcripts might involve a heme-containing sensor for oxygen concentration, similar to that reported for induction of erythropoietin (Epo) mRNA (32), we incubated HUVECs with Co^{2+} or Ni^{2+}. Both divalent cations have been used previously to chemically simulate hypoxia by displacing oxygen from a putative heme-containing sensor (32). Both $CoCl_2$ (100 μM) and $NiCl_2$ (300 μM) treatment for 24 h induced VEGF transcripts in HUVECs. Cycloheximide markedly increased the magnitude of VEGF mRNA induced by Co^{2+} and Ni^{2+} (Fig. 3).

Hypoxia Induces VEGF Protein in HUVECs—To evaluate whether hypoxic ECs could produce VEGF protein, we performed immunohistochemical staining of HUVECs, at times 0, 24, and 48 h of hypoxia. As shown in Fig. 4, VEGF was detected by immunocytochemical staining with anti-VEGF polyclonal antiserum in HUVECs incubated under hypoxic conditions for 24 and 48 h. The immunoreactivity of VEGF was distributed abundantly in the cytoplasm. Negative controls prepared with nonimmune rabbit serum or with the antiserum preabsorbed with a 10-fold excess of VEGF showed no staining.

VEGF Expression in Human ECs Is Not Due to Contamination by SMCs or Fibroblasts—We employed three independent strategies to exclude the possibility that induction of VEGF mRNA in human ECs was due to contaminating SMCs or fibroblasts.

First, experiments involving hypoxia, performed initially with HUVECs or HMECs, were repeated using ECV304 cells; this human endothelial cell line was cloned from transformed ECs isolated initially from human umbilical cord (25). These ECs also were successfully induced to express VEGF mRNA upon exposure to hypoxia (pO_2 = 35 mmHg), similar in magnitude to that observed in HUVECs and HMECs (data not shown).

Second, EC identity of HUVECs and HMECs was confirmed by uniform positive immunoreactivity to anti-Factor VIII antibody (Fig. 5). SMC contamination was excluded by the absence of immunostaining of HUVEC and HMEC cultures following application of an anti-α-muscle actin specific antibody (Fig. 5).

Finally, previous experiments have documented that VEGF expression is induced in cultures of SMCs (19) and fibroblasts (33) in response to TGF-β_1. In the current study, up-regulation of VEGF mRNA was reproduced in SMCs (cultured with HUVEC-conditioned media) stimulated with TGF-β_1, as a positive control. Treatment of HUVEC cultures with TGF-β_1, however, failed to generate detectable VEGF mRNA (Fig. 6).

Hypoxic Conditioned Media Induce Uptake of Tritiated Thymidine in HUVECs—As shown in Fig. 7, EC incorporation of the tritiated thymidine was similar for control (nonconditioned media) ECs and ECs treated with media conditioned by normoxic HUVECs. In contrast, conditioned media from hypoxia HUVECs induced in a 2.5-fold increase in uptake of tritiated thymidine, comparable to the increase observed in response to VEGF alone.

Hypoxia Induces Tyrosine Phosphorylation of VEGF Receptor in HUVECs—To determine whether VEGF expression by hypoxic HUVECs is sufficient to produce an autocrine effect in these cells, we immunoprecipitated KDR from hypoxia-stimulated and control (normoxic) HUVECs. Immune precipitates of anti-KDR probed with anti-Tyr(P) antibodies demonstrated a tyrosine phosphorylated band that comigrates with the activated VEGF receptor (Fig. 8).

DISCUSSION

VEGF is distinguished from certain other angiogenic cytokines by the presence at its amino terminus of a signal sequence that can direct secretion via the endoplasmic reticulum-Golgi system (1, 8). All four alternatively spliced isoforms of VEGF include this signal sequence. At least two tyrosine kinase receptors, Flt (34) and KDR (35, 36), have been shown to bind VEGF at high affinity and appear to be exclusively limited in their distribution to ECs. VEGF has thus been conventionally viewed as an EC-specific mitogen, secreted by a variety of cell types (8, 11–13, 17, 18, 37–43), including smooth muscle cells (SMCs) (8, 19), and operating via a paracrine pathway.

In contrast, certain other angiogenic cytokines, notably acidic and basic fibroblast growth factors, lack such a secretory signal sequence (44, 45). This has prompted previous investigators to study the possibility that these cytokines act via an alternative pathway. Indeed, several groups have established evidence to support the notion that acidic and basic fibroblast growth factors can indeed modulate EC behavior via an autocrine pathway (38, 46–48).

Evidence for a similar autocrine pathway in the case of VEGF has been contradictory. Substantive expression of VEGF transcripts and/or protein in ECs has been reported in nonhuman species. Ladoux and Frelin (20) identified transcripts for VEGF in rat brain capillary endothelium (but not in bovine aortic ECs). Uchida *et al.* (21) and Simorre-Pinatel *et al.* (22) identified VEGF transcripts in quiescent cultures of bovine glomerular ECs and bovine retinal ECs, respectively. Uchida *et al.* also performed immunoblot analyses of culture media conditioned by bovine glomerular ECs; upon stimulation with PMA, VEGF protein concentration increased markedly. Simorre-Pinatel *et al.* used Western blot analysis to identify VEGF protein in media conditioned by quiescent bovine retinal ECs but not in media from bovine aortic ECs; they concluded that "only ECs from retina or brain might express" VEGF.

At least three laboratories have previously investigated VEGF mRNA expression in HUVECs. Minchenko *et al.* (41) observed very low constitutive levels of VEGF mRNA that were unmodified by hypoxic stimulation; they concluded "that an autocrine loop is not involved in the vascular response to hypoxia." Harada *et al.* (49) reported that VEGF mRNA was "barely detectable" in HUVECs, "suggesting that VEGF may act as a paracrine rather than an autocrine factor." More recently, Shifren *et al.* (37) performed immunohistochemical staining of human fetal and adult tissues, using a murine monoclonal antibody prepared against recombinant human $VEGF_{165}$. Positive staining was localized primarily to epithelial cells and myocytes but not to ECs in either the fetus or the adult. Comparison of sites of VEGF production to previously

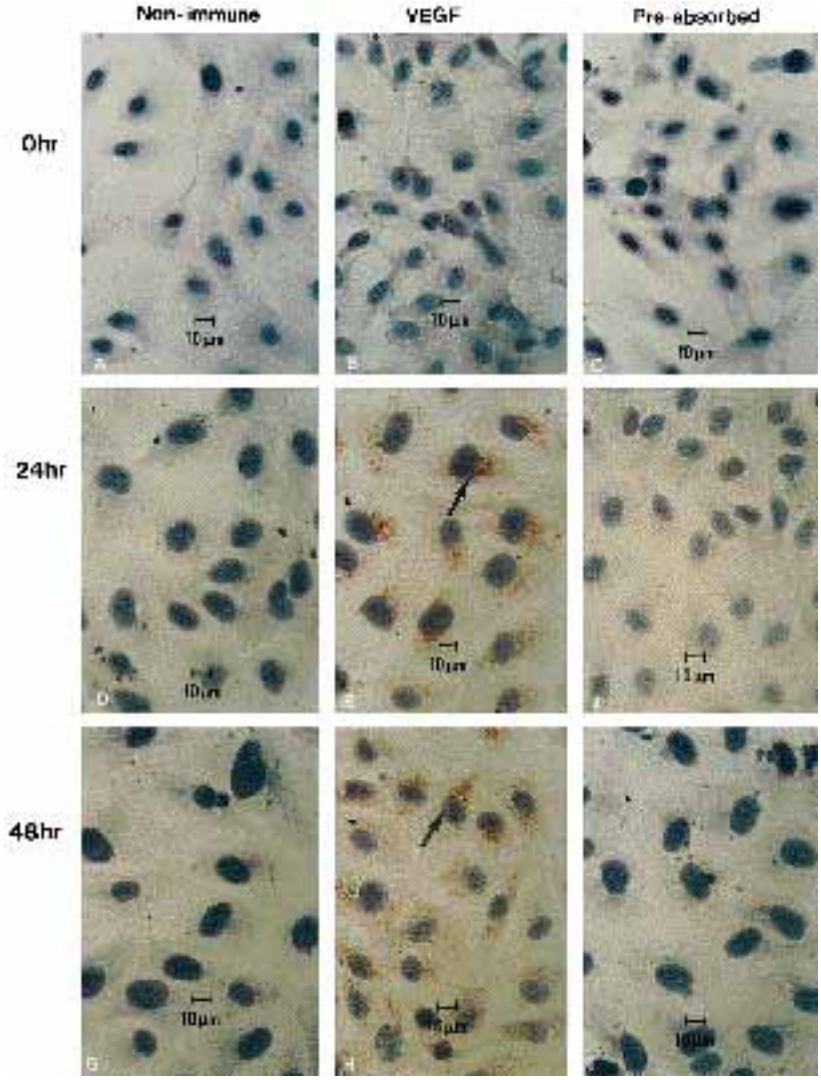

FIG. 4. **Hypoxic HUVECs express VEGF protein.** HUVECs were maintained in normoxic environment (*A–C*) or exposed to hypoxia for 24 (*D–F*) or 48 h (*G–I*). Immunoreactive VEGF protein was detected using an affinity purified anti-VEGF peptide rabbit polyclonal serum (VEGF). Unstimulated HUVECs showed faint immunopositivity (*B*). Increase in VEGF protein was clearly evident at 24 (*E*) and 48 (*H*) h of hypoxia. Specificity of VEGF immunostaining was demonstrated by absent immunostaining when a nonimmune rabbit serum (*A*, *D*, and *G*) or the peptide-preabsorbed anti-VEGF antibody (*C*, *F*, and *I*) were used as primary antibodies.

established sites for VEGF binding and expression of VEGF receptor mRNA were interpreted to be most consistent with the classical view of a paracrine mechanism of action.

Our findings are consistent with previous studies of human ECs: we were unable to detect VEGF mRNA in either HUVECs or HMECs under quiescent conditions. To determine whether human ECs could under any circumstances express VEGF, we administered a potent albeit nonphysiologic stimulus, PMA, previously shown to induce angiogenesis *in vitro* (50) and VEGF expression in vascular SMCs (8). PMA stimulated VEGF mRNA in both HUVECs and HMECs. Induction of VEGF mRNA was dose-dependent and was abrogated by pretreatment with staurosporine, suggesting that VEGF expression may be regulated by protein kinase C, which is activated by PMA (51, 52).

In contrast to PMA, hypoxia may constitute a physiologic stimulus for VEGF expression (6, 12, 43, 53–55). Previous studies have established that hypoxia may induce VEGF expression in both normal (19, 41) and transformed cells (23, 41, 43). We observed that in addition to PMA, hypoxia did indeed provoke VEGF expression in both HUVECs and HMECs. The absence of mRNA at time 0 suggests that VEGF mRNA observed following the onset of hypoxia is due to enhanced transcription (56, 57); recent work from other laboratories (58, 59), however, indicates that accumulation of VEGF may result not only from increased transcription but also from increased stability of mRNA, possibly involving sequence motifs in the 3′-untranslated region.

HUVECs used in this study were grown from initial isolates obtained by enzymatic dissociation of human vessels. To exclude the possibility that our observations represented spurious results due to contamination of EC cultures with non-ECs, specifically SMCs, or fibroblasts, we used three approaches. First, we performed identical experiments using a clonal human EC line and again observed induction of VEGF transcripts in response to hypoxia. Second, we demonstrated that the cultures of HUVECs and HMECs we employed showed no immunoreactivity with an anti-α-actin specific antibody; in contrast, both HUVECs and HMECs contained immunoreactive Factor VIII antigen. Third, we evaluated the response of both cultured SMCs and cultured ECs to TGF-β_1. Previous experiments in our laboratory and others established that TFG-β_1 induces VEGF mRNA in SMCs (19) and fibroblasts (33). In the present study, human SMCs, cultured in HUVEC media, expressed VEGF transcripts upon stimulation with TGF-β, whereas HUVECs did not. These experiments thus suggest that hypoxic-induced VEGF expression in cultured HUVECs and HMECs was not the spurious result of contamination by SMCs or fibroblasts.

Experiments in the current study demonstrate that the response of human ECs to hypoxia is not limited to expression of VEGF mRNA but involves production of VEGF protein as well. Immunoreactive VEGF was distributed abundantly in the cytosol of hypoxic ECs. Because the VEGF antibody that we used

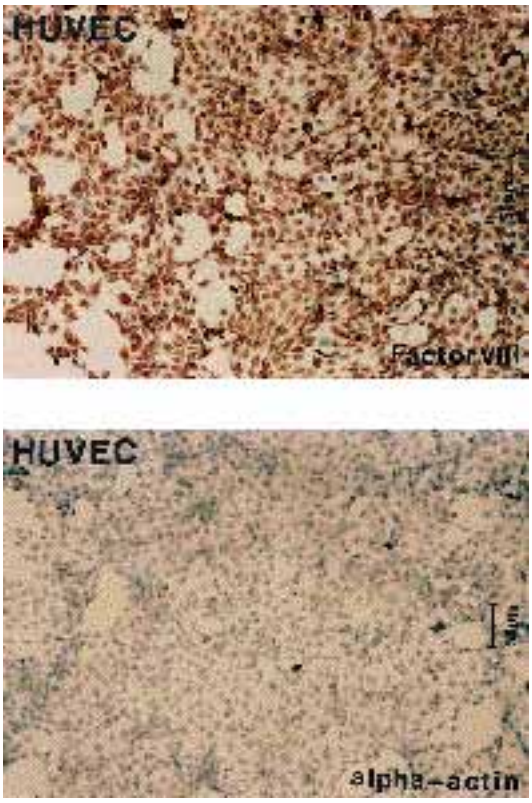

FIG. 5. **Identification of HUVECs.** EC identity of HUVECs was assessed by positive immunoreactivity with an anti-Factor VIII antibody and absent α-actin immunostaining.

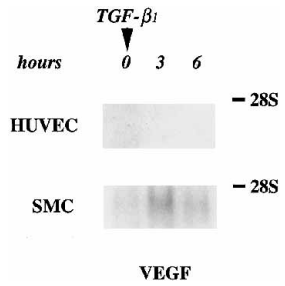

FIG. 6. **TGF-β_1 induces VEGF gene expression in SMCs but not in HUVECs.** HUVECs and SMCs cultured with HUVEC medium since 48 h before experiments were treated with TGF-β_1 (1 ng/ml) for the indicated times, and Northern analysis was performed after loading 15 μg of RNA. HUVECs expressed no detectable VEGF mRNA. TGF-β_1 induced expression of VEGF mRNA at 3 h, followed by a decrease at 6 h in SMCs but not in ECs.

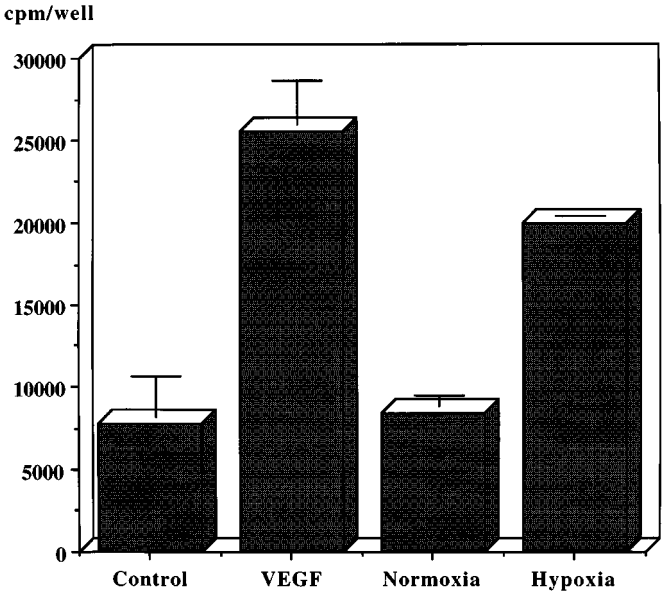

FIG. 7. **Uptake of tritiated thymidine was not increased in ECs treated with normoxic HUVEC-conditioned media compared with control (nonconditioned media) HUVECs.** In contrast, incorporation of tritiated thymidine was increased by 2.5-fold following treatment of ECs with hypoxic HUVEC-conditioned media, comparable to results observed with VEGF alone.

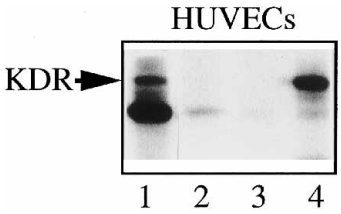

FIG. 8. **Hypoxia induces KDR phosphorylation.** HUVECs were grown to 80% confluence for 48 h in hypoxia (*lane 1*) or normoxia (*lane 2*). Immune precipitates of anti-KDR were probed with anti-Tyr(P) antibodies. Control and VGEF-stimulated HUVECs from confluent cultures are shown in *lanes 3* and *4*, respectively. The migration position for KDR is indicated by the *arrow*.

recognizes all alternatively spliced VEGF isoforms (43, 60), these experiments do not allow us to identify which VEGF species are produced by HUVECs in response to hypoxia. The fact that media conditioned by hypoxic HUVECs augmented uptake of tritiated thymidine, however, implies a freely or relatively freely secreted (121 or 165) VEGF isoform.

The precise mechanism by which hypoxia induces VEGF expression, whether in ECs or other cell types, remains uncertain. Goldberg *et al.* (32) have previously proposed the existence of an oxygen-sensing mechanism for Epo involving a heme protein; binding to this putative oxygen sensor was suggested to influence Epo production and secretion. Experimental evidence for this concept included the observation that Co^{2+}, Ni^{2+}, and hypoxia augmented Epo production, presumably by displacing ferrous iron in the porphyrin ring of heme, thereby locking heme in the deoxy conformation. Similar findings were observed for ECs in the present series of experiments. It is intriguing to note in this regard that homology exists between a sequence in the VEGF promoter (8) and a nucleotide sequence in the erythropoietin promoter identified as a binding site for hypoxia-specific transcription factor (HIF-1) (61).

Previous studies have indicated a variable requirement for new protein synthesis prior to hypoxic induction of Epo and VEGF mRNA (20, 23, 32, 62). We observed that cycloheximide pretreatment augmented VEGF expression induced by hypoxia as well as Co^{2+}, suggesting that a factor induced by hypoxia may modulate stability of Epo and VEGF message, in part by inhibition of translation of ribonucleases responsible for degradation of mRNA. Similar regulatory influences have been previously demonstrated for growth factors (63) and oncoproteins (64).

The findings in this study thus demonstrate that macrovascular and microvascular ECs under hypoxic conditions are capable of expressing VEGF mRNA and protein. The increase in thymidine incorporation observed in ECs following application of media conditioned by hypoxic HUVECs indicates that the magnitude of protein secreted is sufficient to drive mitogenesis. Because ECs also express the high affinity VEGF receptors, Flt and KDR, these cells in humans appear to include the requisite elements for an autocrine pathway. The finding that hypoxia induces activation of KDR in HUVECs is consistent with an autocrine pathway, specifically an external autocrine pathway (65) given that all VEGF isoforms include a

secretory signal sequence.

The extent to which such an autocrine pathway may complement endogenous production of VEGF from SMCs, macrophages, and tumor cells or facilitate the response to exogenous administration of VEGF (6, 7) remains to be determined. With regard to the latter, it has been recognized that single bolus administration of VEGF may stimulate development of new collateral vessels over a period of several days, despite the fact that the circulating half-life of VEGF is <3 min. One explanation for this observation is that as VEGF is rapidly cleared from the circulation; it binds avidly to heparan sulfate proteoglycans present on the luminal surface of the vascular endothelium. Experimental evidence to support this concept in the case of basic fibroblast growth factor has been previously reported by Fuks *et al.* (66). Alternatively, an autocrine loop activated under hypoxic conditions known to stimulate angiogenesis might also serve to amplify and thereby protract the response in ECs stimulated by exogenously administered VEGF.

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