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Nitric Oxide Is an Upstream Signal of Vascular Endothelial Growth Factor-induced Extracellular Signal-regulated Kinase$_{1/2}$ Activation in Postcapillary Endothelium*

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We recently demonstrated that nitric oxide (NO) significantly contributes to the mitogenic effect of vascular endothelial growth factor (VEGF), suggesting a role for the NO pathway in the signaling cascade following kinase-derivative receptor activation in vascular endothelium. The aim of this study was to investigate the intracellular pathways linked to VEGF/NO-induced endothelial cell proliferation. We assessed the activity of the mitogen-activated protein kinase (MAPK) that is specifically activated by growth factors, extracellular-regulated kinase (ERK$_{1/2}$), on cultured microvascular endothelium isolated from coronary postcapillary venules. ERK$_{1/2}$ was immunoprecipitated, and its activity was assessed with an immunocomplex kinase assay. In endothelial cells exposed for 5 min to the NO donor drug sodium nitroprusside at a concentration of 100 μM, ERK$_{1/2}$ activity significantly increased. VEGF produced a time- and concentration-dependent activation of ERK$_{1/2}$. Maximal activity was obtained after 5 min of stimulation at a concentration of 10 ng/ml. The specific MAPK kinase inhibitor PD 98059 abolished ERK$_{1/2}$ activation and endothelial cell proliferation in a concentration-dependent manner in response to VEGF and sodium nitroprusside. The NO synthase inhibitor N^{ω}-monomethyl-L-arginine, as well as the guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one, blocked the activation of ERK$_{1/2}$ induced by VEGF, suggesting that NO and cGMP contributed to the VEGF-dependent ERK$_{1/2}$ activation. These results demonstrate for the first time that kinase-derivative receptor activation triggers the NO synthase/guanylate cyclase pathway to activate the MAPK cascade and substantiates the hypothesis that the activation of ERK$_{1/2}$ is necessary for VEGF-induced endothelial cell proliferation.

Vascular endothelial growth factor (VEGF)[1] is a secreted protein that is a specific growth factor for endothelial cells, and it has been shown to increase vascular permeability (1, 2). It is angiogenic *in vivo* and in *in vitro* assays (3, 4), and its physiological importance in vasculogenesis is well documented (5, 6). The action of VEGF is regulated by two receptors belonging to the tyrosine kinase family, Flt-1 and KDR (or Flk) (7, 8). Flt-1, which has higher affinity for VEGF than KDR, is required for endothelial cell morphogenesis, whereas KDR is involved primarily in mitogenesis (5, 6, 9, 10).

The postreceptor signaling pathways underlying VEGF actions on endothelial cells are still unclear. VEGF has been shown to elevate intracellular inositol 1,4,5-trisphosphate and calcium levels and to stimulate tyrosine phosphorylation and von Willebrand factor release in cultured human umbilical vein endothelial cells (11). VEGF effects on permeability (12) and vascular tone (13) are coupled to nitric oxide (NO) production. Consistent with this observation, we have recently demonstrated that NO production and cGMP elevation contribute to the angiogenic effect of VEGF (14, 15). The activation of mitogen-activated protein kinase (MAPK) cascade by VEGF has been recently demonstrated (16).

MAPKs are important intermediates in signal transduction pathways that are stimulated by a variety of agents, such as growth factors, hormones, neurotransmitters, and physical and chemical stressing agents (17). Many receptor tyrosine kinase and G protein-coupled receptors have been shown to activate the MAPKs. The 44- and 42-kDa MAPK (ERK$_{1/2}$) isoforms are ubiquitously expressed and have been shown to be activated by dual specificity MAPK kinases (MEK$_1$/MEK$_2$) in response to diverse stimuli (18, 19).

This study was designed to characterize the transducing pathways underlying VEGF-activated endothelial cell proliferation. Recently, we have shown that NO is a downstream signal in VEGF effects (15). Here, we have investigated the role of NO on the intracellular pathway linked to VEGF receptor activation in postcapillary endothelium. We assessed MAPK activity specifically activated by a growth factor, *i.e.* ERK$_{1/2}$, on cultured endothelium isolated from coronary postcapillary venules.

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[1] The abbreviations used are: VEGF, vascular endothelial growth factor; NO, nitric oxide; NOS, NO synthase; ecNOS, calcium/calmodulin-dependent NOS; iNOS, inducible NOS; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; ERK, extracellular-regulated kinase; SNP, sodium nitroprusside; L-NMMA, N^{ω}-monomethyl-L-arginine; CVEC, coronary venular endothelial cell; DMEM, Dulbecco's modified Eagle's medium; MBP, myelin basic protein; TEMED, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; bFGF, basic fibroblast growth factor; ANOVA, analysis of variance; KDR, kinase-derivative receptor; MEK, MAPK/ERK kinase.

MATERIALS AND METHODS

Cell Line Culture Conditions and Proliferation Assay—Coronary venular endothelial cells (CVECs) were obtained and maintained in culture as described previously and characterized for their endothelial morphology by immunofluorescent staining for factor VIII antigen and uptake of acetylated low density lipoproteins (20). Cells between passages 15 and 25 were used in these experiments.

Cell proliferation was quantified by total cell number after 48 h of stimulation with test substances (14). To evaluate the effect of the MAPKK, NO synthase (NOS), and guanylate cyclase inhibitors, the drugs were added to the cells 30 min before the test substances. Proliferation is expressed as mean ± S.E. of total cells counted in each well.

Immunoprecipitation and Immunocomplex Kinase Assay of ERK½—CVECs were serum starved overnight. Following treatment, cells were washed twice with ice-cold Dulbecco's phosphate-buffered saline and lysed by adding 0.3 ml of buffer containing 50 mM Tris (pH 7.4), 1% Triton X-100, 1 mM EGTA, 100 mM NaCl, 1 mM Na_3VO_4, 0.2 M phenylmethylsulfonyl fluoride, 25 μg/ml leupeptin, 10 μg/ml aprotinin, and 10 mM NaF. To assess the calcium dependence of $ERK_{½}$ activation, CVECs were stimulated in the presence of 3 mM EGTA. Cell lysate containing 100 μg of protein in a total volume of 800 μl were precleared with nonimmune rabbit IgG and 30 μl of goat anti-rabbit IgG agarose beads on a rotating plate for 1 h at 4 °C and then centrifuged at 10,000 × *g* for 10 min. 1 μg of anti-ERK_1 polyclonal antibody, which is reactive with ERK_1 and to a lesser extent with ERK_2, and 25 μl of goat anti-rabbit IgG agarose beads were added to the supernatant, and the mixture was placed on a rotating plate overnight at 4 °C. Following a centrifugation at 10,000 × *g* for 5 min, the pellet was recovered and washed twice with the lysis buffer and once with the kinase buffer containing 20 mM Hepes (pH 7.6), 20 mM $MgCl_2$, and 2 mM dithiothreitol. The kinase assay was carried out at 30 °C for 10 min in 30 μl of assay buffer containing 5 μg of myelin basic protein (MBP) as specific substrate for $ERK_{½}$ (21), 20 μM ATP, and 3 μCi of [γ^{32}P]ATP. The reaction was stopped by the addition of Laemmli's sample buffer and boiled for 5 min. The samples were resolved by 12% SDS-polyacrilamide gel electrophoresis, stained with Coomassie Brilliant Blue, and exhaustively destained. The gel was dried, and the incorporation of [γ^{32}P]ATP was visualized by autoradiography. Gel slices of the 20-kDa MBP bands were also cut out in most of the experiments, and their radioactivity was measured by liquid scintillation counting.

Inositol Phosphate Activation—CVECs seeded onto 6-well plates (3×10^4 cells/well) after overnight incubation were labeled with [^{3}H]myoinositol (2 μCi/ml) in DMEM without cold inositol for 48 h. Excess of tritiated myoinositol was removed by three washes with cold DMEM followed by 4 h of incubation with cold DMEM at 37 °C. After one wash, cells were incubated for 10 min with 20 mM LiCl to block myoinositol-1-phosphatase and then with test compounds for the designed times. Reaction was stopped by the addition of ice-cold methanol for 30 min. Cells were scraped, and cell-associated inositols were extracted by chloroform-methanol (1:1). Water-soluble fractions were applied to anion-exchange columns (Resin AG-X8, 200–400 mesh, formate form), and water-soluble inositols were eluted by successive washes with 60 mM CH_5NO_2 + 5 mM $B_4Na_2O_7{\cdot}10\,H_2O$, 200 mM CH_5NO_2 + 0.1 M CH_2O_2, and 1.2 M CH_5NO_2 + 0.1 M CH_2O_2. Inositol phosphate 1 levels were measured as recovered radioactivity.

Calcium Measurements—Cytosolic calcium measurements were made using the calcium-sensitive fluorescence indicator indo-1 (Molecular Probes, Eugene, OR) (22). Cells were incubated with the acetoxymethyl ester for the dye in DMEM containing 1% Me_2SO for 40 min. At that time, the cells were rinsed with Dulbecco's phosphate-buffered saline and reincubated in DMEM without Me_2SO, dye, or phenol red for 20 min to allow for de-esterification. The experiments were performed on a stage-scanning photometric imaging system (ACAS 570, Meridian Instruments, Okemos, MI) at 37 °C. The cells were illuminated with the 355-nm line of an argon laser that was attenuated with neutral density filters and an acuosto-optic modulator to minimize bleaching. Fluorescence emission was monitored at 405 and 485 nm using narrow-band pass filters and dual photomultipliers. The fluorescent images of a suitable field of cells were captured before and after addition of VEGF, and the 405/485 nm ratio was analyzed. This ratio is a direct index to the calcium concentration within the cell at the time of image capture.

Differential Reverse Transcription PCR Analysis—Subconfluent and serum-starved cells were stimulated for 4–24 h with VEGF in the presence of 1% serum. At the end of incubation, total RNA was isolated by the standard guanidine thiocyanate-phenol-chloroform extraction (23). cDNA was synthetized as described (24). Differential reverse transcription PCR for NOS isoforms was carried out by using 5 μl of cDNA and specific primers for bovine calcium/calmodulin-dependent NOS (ecNOS) and iNOS with sequences as follows: ecNOS sense, 5′-GCTTGAGACCCTCAGTCAGG-3′; ecNOS antisense, 5′-GGTCTCCAGTCTTGAGCTGG-3′ (25); iNOS sense, 5′-TAGAGGAACATCTGGCCAGG-3′; iNOS antisense, 5′-TGGCAGGGTCCCCTCTGATG-3′ (26). Calibration was performed by co-amplification of the same cDNA samples with primers for glyceraldehyde-3-phosphate dehydrogenase as an internal standard, with sequences as described (24). For PCR amplification a Perkin-Elmer GeneAmp PCR System 2400 was used. The PCR cycles were as follows: 30 s at 94 °C, 1 min at 55 °C, and 1 min at 72 °C. After 30 cycles of amplification, aliquots of each sample product (20 μl) were electrophoresed on a 3% agarose gel and stained with ethidium bromide. The size of the amplification products was 296 for ecNOS and 372 for iNOS. Image processing and analysis of the intensity of the bands were performed as described (24). Results were evaluated as ratio between the target genes (ecNOS and iNOS) and glyceraldehyde-3-phosphate dehydrogenase amplification analysis.

Determination of NOS Activity—Subconfluent CVECs in 60 -mm culture dishes were serum starved overnight and then washed and equilibrated for 20 min at 37 °C with Hepes buffer containing: 145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 10 mM Hepes, 1 mM $CaCl_2$, 1 mM glucose, pH 7.4. Cells were incubated for 20 min with 1 μCi of [^{3}H]arginine plus 10 μM L-arginine. To assess the calcium dependence of NOS activation, experiments were performed in calcium-free Hepes buffer containing 1 mM EGTA. Test substances were added for 5 min at 37 °C. The reaction was stopped by cold Hepes buffer containing 4 mM EDTA, and the supernatant was removed. 0.5 ml of ethanol added to each monolayer was allowed to evaporate, and 2 ml of 10 mM Hepes-Na, pH 5.5, was added for 20 min. The supernatant was collected and applied to 0.8 ml of Dowex AG50WX-8 (Na-form) and vigorously shaken for 45 min. Then, 0.5 ml was collected and added to 3 ml of liquid scintillation counting mixture. NOS activity was expressed as pmol/mg of protein.

Measurement of cGMP Levels—cGMP levels were measured on cell extracts from confluent cell monolayers by radioimmunoassay using iodinated tracer (14). Cell monolayers were treated with 1 mM 3-isobutyl-5-methyl-xanthine for 15 min before stimulation. After stimulation, cells were rinsed with phosphate-buffered saline and removed by scraping in ice-cold 10% trichloroacetic acid. Following centrifugation, cGMP levels were assayed in the supernatant, and proteins were measured in the pellet by the Bradford's procedure. Data are expressed as fmol/mg of protein.

Reagents—Fetal calf serum was from Hyclone (Logan, UT). Purified rabbit IgG, anti-rabbit IgG agarose beads, myelin basic protein, phenylmethylsulfonyl fluoride, aprotinin, leupeptin, sodium nitroprusside (SNP), L-NMMA, dithiothreitol, BCA kit, and all reagents for cell culture were purchased from Sigma. Diff-Quik was from Mertz-Dade AG (Dudingen, Switzerland). Human recombinant VEGF and PD 98059 ([2-(2′-amino-3′-methoxyphenyl)-oxanaphthalen-4-one]) were from Calbiochem-Novabiochem International (San Diego, CA). [γ^{32}P]ATP was from NEN Life Science Products. Rabbit anti-$ERK_{½}$ polyclonal antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Acrylamide, TEMED, ammonium persulfate, and Coomassie Brilliant Blue were from Bio-Rad. 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) was purchased from Tocris Cookson Ltd., (Langford, United Kingdom). Mononucleotides dATP, dCTP, dGTP, and dTTP and the DNA marker VIII were from Boehringer Mannheim. Reverse transcriptase, oligo(dT)16, AmpliTaq DNA polymerase, and primers for glyceraldehyde-3-phosphate dehydrogenase and NOS isoforms were from Perkin-Elmer.

RESULTS

$ERK_{½}$ Activation Induced by VEGF Is Time-dependent, Concentration-dependent and Sensitive to Inhibition of the MAPKK—The ability of VEGF to stimulate the MAPK cascade was assessed. After 24 h of starvation, CVECs were stimulated with 10 ng/ml VEGF over a range of times from 2 to 15 min, and the activity of the immunoprecipitated $ERK_{½}$ was measured. The activation of $ERK_{½}$ was statistically higher than the unstimulated control within 2 min and reached the maximum at 5 min (Fig. 1*a*). The activation of $ERK_{½}$ by VEGF was concentration-dependent with maximal activity at 10 ng/ml, which doubled the basal activity of $ERK_{½}$ (Fig. 1, *b* and *d*). In the same experiments, bFGF (10 ng/ml) did not significantly induce $ERK_{½}$ activation. When the endothelial cells were preincubated for 30 min with increasing concentrations of the MAPKK

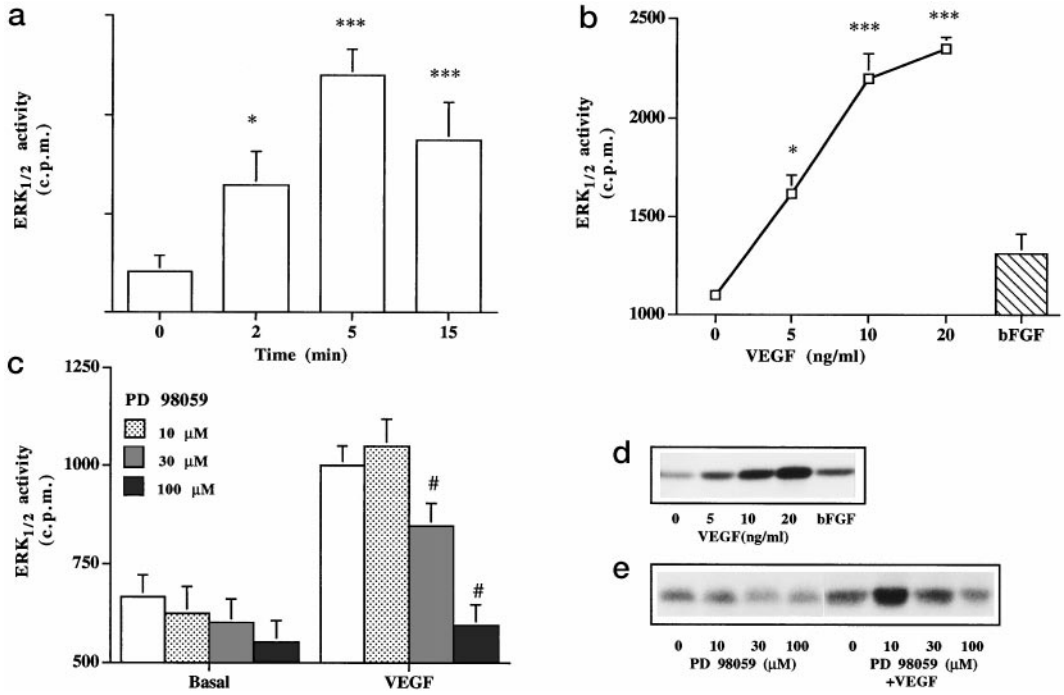

FIG. 1. **Time- and dose-dependent activation of ERK$_{1/2}$ exposed to VEGF.** *a*, CVECs were exposed for different times (2–5 min) to 10 ng/ml VEGF. *b*, cells were stimulated for 5 min with increasing concentrations (5–20 ng/ml) of VEGF and 10 ng/ml bFGF. *c*, effect of the MAPKK inhibitor PD 98995 (10–100 μM) on 10 ng/ml VEGF-induced ERK$_{1/2}$ activation. *d* and *e* are representative autoradiographies related to *b* and *c*, respectively. ERK$_{1/2}$ was immunoprecipitated, and its activity was measured with an *in vitro* kinase assay by using [γ^{32}P]ATP and MBP as substrate. The samples were resolved by 12% SDS-polyacrilamide gel electrophoresis followed by autoradiography. Gel slices of the 20 K_D MBP bands were cut out, and the radioactivity was measured by liquid scintillation counting. $n = 5$; mean ± S.E. *, $p < 0.05$; ***, $p < 0.001$ *versus* unstimulated control; #, $p < 0.001$ *versus* VEGF alone (ANOVA followed by Fisher's test).

inhibitor PD 98059 (27), VEGF-induced ERK$_{1/2}$ activation was inhibited (IC_{50} = 30 μM). At 100 μM, PD 98059 completely abolished the ERK$_{1/2}$ activation produced by 10 ng/ml of VEGF but did not modify the unstimulated control (Fig. 1, *c* and *e*).

NO Activates ERK$_{1/2}$—In previous reports, we demonstrated that the NO pathway is necessary for the proliferative effects of VEGF on microvascular endothelial cells (14). We therefore investigated whether NO contributed to VEGF mitogenic activity by activating the MAPK cascade. For this purpose, starved and subconfluent CVECs were treated with the NO donor SNP, and the activity of ERK$_{1/2}$ was measured. After 5 min of stimulation with 100 μM SNP, the MAPK activity was increased by 2-fold (Fig. 2*a*). NO-induced ERK$_{1/2}$ activation and the proliferative effect of NO were abolished by PD 98059 (Fig. 2, *a* and *b*), indicating that ERK$_{1/2}$ was specifically and directly activated by NO and that this phosphorylation cascade was involved in signaling mitogenesis in postcapillary endothelial cells.

Calcium-dependent Activation of NOS and ERK$_{1/2}$ by VEGF—We then characterized the NOS isoform mediating VEGF effect in CVECs. The rapid activation of ERK$_{1/2}$ in response to VEGF suggested that the acute activation of NO production in CVECs by ecNOS. Differential reverse transcription PCR of total RNA indicated that this isoform was predominantly expressed in CVECs (Fig. 3*a*). After 4 h from VEGF administration, ecNOS expression was not modified, indicating the absence of a transcriptional event between VEGF administration and NO production. iNOS expression was not detected at any time point between 4 and 24 h of exposure to the growth factor.

CVECs preloaded with the ratiometric fluorescent indicator indo-1 exhibited a rapid calcium transient upon exposure to VEGF (Fig. 3*b*). The upward stroke of the calcium transient began 3 min after addition of VEGF, the peak concentration of cytosolic calcium occurred at approximately 7 min, and recovery occurred over the next 20 min. After continued exposure of CVECs to VEGF for 70 min, cytosolic calcium had recovered only 65%, suggesting a continuing signal for calcium-calmodulin NO production beyond the rapid peak.

Consistent with the rapid cytosolic calcium elevation, within 5 min after VEGF exposure, NOS activity increased, and EGTA abolished its elevation (Fig. 3*c*). EGTA also abolished ERK$_{1/2}$ activation by VEGF, suggesting that calcium was required to trigger the MAPK cascade, as well as the NOS activity (Fig. 3*d*).

MAPKK and NOS/Guanylate Cyclase Inhibitors Block VEGF-induced Endothelial Cell Proliferation—The role of ERK$_{1/2}$ phosphorylation and of the NOS/cyclic GMP cascade in the VEGF/NO-induced endothelial cell proliferation was assessed on endothelial cells treated with either the MAPKK inhibitor PD 98059 (1–100 μM), the NOS inhibitor L-NMMA (1–200 μM) (28), or the guanylate cyclase inhibitor ODQ (0.1–10 μM) (29).

The MAPKK inhibitor specifically reduced the proliferative effect of VEGF in a concentration-dependent manner, whereas it did not inhibit the growth-promoting effect of bFGF (Fig. 4*a*). The IC_{50} for growth inhibition (10 μM) was in the same range of concentration as that for ERK$_{1/2}$ inhibition. At the highest concentration, PD 98059 slightly reduced the number of cells recovered under control conditions. The effect was independent from cytotoxicity, as indicated by trypan blue exclusion assays (data not shown).

The addition of VEGF doubled cGMP levels in CVECs, an effect specifically blocked by NOS inhibitors (Table I) as previously reported (14). L-NMMA inhibited VEGF-induced growth in a concentration-dependent manner; maximal growth inhibition was obtained at 200 μM (IC_{50} = 10 μM) (Fig. 4*b*). Conversely, no inhibition in cell growth was produced when bFGF was used as a mitogen (Fig. 4*b*).

ODQ produced concentration-dependent inhibition of the

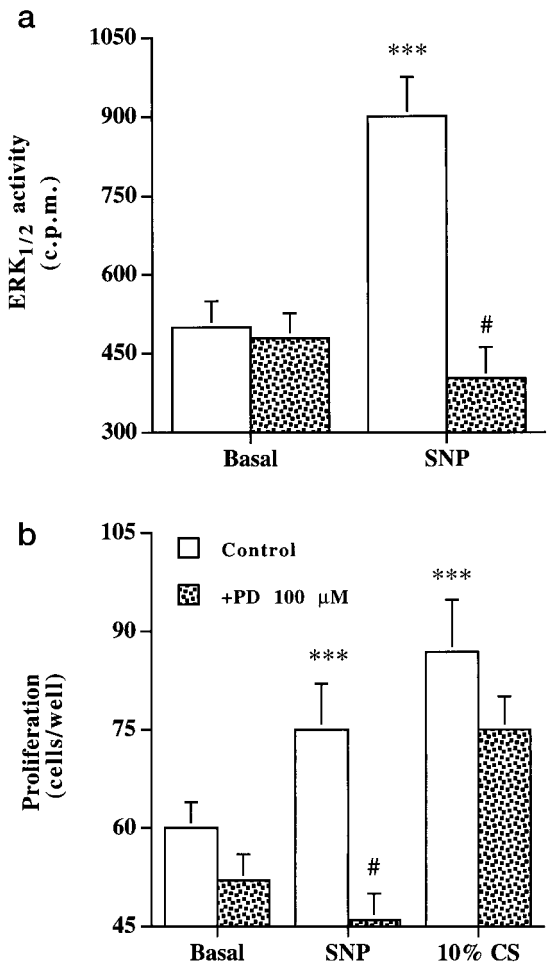

FIG. 2. **Effect of NO on ERK$_{1/2}$ activation and cell proliferation.** *a*, ERK$_{1/2}$ activation. CVECs were stimulated with 100 μM SNP. ERK$_{1/2}$ was immunoprecipitated, and its activity was measured with an *in vitro* kinase assay by using [γ^{32}P]ATP and MBP as substrate. The samples were resolved by 12% SDS-polyacrilamide gel electrophoresis followed by autoradiography. Gel slices of the 20-kDa MBP bands were cut out, and the radioactivity was measured by liquid scintillation counting. *b*, cell proliferation: 1.5×10^3 cells resuspended in 10% calf serum were seeded in each well of 96-well plates. After adherence (3–4 h), the medium was replaced with 1% calf serum DMEM containing test substances and incubated for 48 h. After fixation and staining of cells with hematoxylin-eosin with Diff-Quik, the number of cells was counted in seven random fields of each well at $\times$ 100 magnification with the aid of a 21-mm^2 ocular grid. Data are expressed as total number of cells counted/well. PD 98059 was given at 100 μM for 30 min before cell stimulation. $n = 3$; ***, $p < 0.001$ *versus* unstimulated control; #, $p < 0.05$ *versus* SNP alone (ANOVA followed by Fisher's test).

guanylate cyclase activation and cGMP levels elevation induced by VEGF as well as by the NO donor SNP (maximal effect at 10 μM; $IC_{50} = 0.5$ μM) (Table I). The minimal effective concentration of ODQ that inhibited cGMP formation was sufficient to block the proliferative effect of VEGF, and lower concentrations gave the same effect (Fig. 4*c*). Conversely, proliferation and cGMP elevation produced by the NO donor SNP were reduced by ODQ in a concentration-dependent manner (Fig. 4*c* and Table I). Maximal inhibition was obtained at 10 μM, and the IC_{50} was 0.5 μM for both the effects. The guanylate cyclase inhibitor did not produce significant reduction of bFGF-induced growth (Fig. 4*c*).

MAPKK Inhibitor Does Not Affect the Biochemical Cascade of NOS/Guanylate Cyclase Elicited by VEGF and NO—To demonstrate the exact biochemical location of the MAPK in the NO/NOS pathway in our system, the MAPKK inhibitor was tested on guanylate cyclase activation. PD 98059 did not affect the NOS/cGMP pathway activation stimulated by either VEGF or SNP on CVECs at any of the concentrations tested (Table I). Similar results were obtained when PD 98095 was assessed on NOS activity. The VEGF-induced NOS activity (223 $\pm$ 11 pmol/mg of protein *versus* a basal value of 169 $\pm$ 15 pmol/mg of protein) could be selectively blocked by 3 μM L-NMMA (131 $\pm$ 10 pmol/mg of protein; $IC_{50} = 50$ μM) but not by 100 μM PD 98059 (210 $\pm$ 22 pmol/mg of protein; $n = 3$).

The possibility that PD 98059 could affect other transducing pathways required for proliferation in our system was ruled out in parallel experiments in which inositol phosphate metabolism was assessed. PD 98059 at the concentration producing 100% reduction of the specific biochemical target (ERK$_{1/2}$ activation) failed to affect the metabolism of inositol phosphate. VEGF induced inositol phosphate 1 accumulation (448 $\pm$ 37 cpm/well over basal control), which was not affected by 100 μM PD 98059 pretreatment (642 $\pm$ 98 cpm/well; $n = 3$).

NOS/Guanylate Cyclase Inhibitors Prevent the VEGF-induced ERK$_{1/2}$ Activation—To investigate the exact location of the NOS/cGMP pathway in the phosphorylation cascade triggered by VEGF in our system, experiments were done to assess the involvement of NO pathway in VEGF-induced ERK$_{1/2}$ activation. CVECs were pretreated for 30 min with 200 μM L-NMMA and then stimulated for 5 min with 10 ng/ml VEGF. Data obtained showed that the pretreatment with L-NMMA abolished the increased ERK$_{1/2}$ activity elicited by VEGF (Fig. 5*a*). This effect was selective for the VEGF effect because no inhibition was found for cells stimulated with 10% calf serum (1680 $\pm$ 70 cpm and 1725 $\pm$ 150 cpm, with and without L-NMMA, respectively; $n = 3$).

Consistent with the observation that NO is the transducing molecule between the VEGF receptor and ERK$_{1/2}$, ODQ significantly inhibited the VEGF- and SNP-induced increase of ERK$_{1/2}$ activity (Fig. 5*b*).

DISCUSSION

The data presented here demonstrate that the mitogenic activity of VEGF on postcapillary endothelial cells requires the activation of the MAPK cascade and that NO/cGMP production mediates the MAPK activation following VEGF receptor interaction, ultimately leading to endothelial cell growth. These conclusions are based on the following observations: 1) VEGF stimulated the MAPK specifically linked to proliferation, *i.e.* ERK$_{1/2}$, as did the NO-donor drug SNP; 2) blockade of the NO pathway by L-NMMA and by ODQ prevented the ERK$_{1/2}$ activation by VEGF and SNP; and 3) inhibition of MAPK kinase activation, of NO synthase activity, and of cGMP production specifically blocked VEGF/NO-induced proliferation.

In rat liver sinusoidal endothelial cells, it was reported that VEGF stimulated phosphorylation of the MAPK (16). Postcapillary venular endothelium has the ability to respond promptly to mitogenic peptides. Using cultured endothelium from coronary postcapillary venules, we demonstrated that ERK$_{1/2}$ activation lies upstream of the proliferative effect of VEGF. The specificity of ERK$_{1/2}$ activation is confirmed by the use of the MAPKK (or MEK) inhibitor PD 98059 (27). This compound has been demonstrated to be a selective and noncompetitive MEK inhibitor in *in vitro* assay (30, 31) without any effect on ERK. PD 98059 at concentrations above 50 μM has been shown both to inhibit MEK$_{1/2}$ by binding a regulatory site on the enzyme and to prevent activation by c-Raf and MEK$_{1/2}$ kinase (30). Our data show that in this concentration range, PD 98059 prevented the ERK$_{1/2}$ activation and the proliferative effect induced by VEGF, demonstrating that the activation of ERK$_{1/2}$ is a necessary step for endothelial cell proliferation.

In previous work, we have shown that molecules able to increase NO levels induced endothelial cells proliferation and

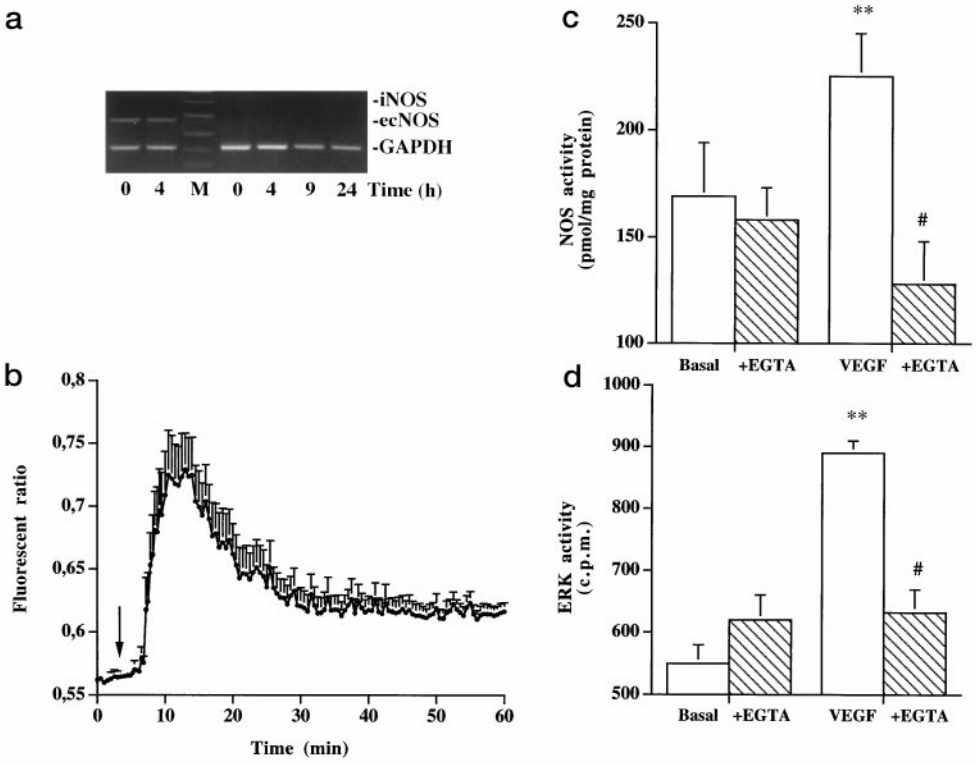

FIG. 3. **Calcium-dependent ecNOS and ERK$_{1/2}$ activity in CVECs treated with VEGF.** *a*, differential reverse transcription PCR for ecNOS and iNOS mRNA expression in CVECs stimulated with 10 ng/ml VEGF for the indicated times. The graph is representative of three experiments with similar results. *Left two lanes* (*0* and *4*), ecNOS expression at 0 and 4 h of stimulation, respectively; *M*, molecular weight standard; *right four lanes* (*0, 4, 9,* and *24*), iNOS expression at 0, 4, 9, 24 h of stimulation, respectively. *b*, effect of VEGF on cytosolic calcium mobilization in individual adherent CVECs. Addition of 10 ng/ml VEGF induced a synchronized rapid increase in cytosolic calcium followed by a long-lasting decline to levels above prestimulation values. Data are the means of tracing recorded from 22 individual cells. *c*, effect of calcium on NOS activity in CVECs. NOS activity (pmol/mg of protein) was evaluated by [^{3}H]L-arginine conversion in cells exposed to 10 ng/ml VEGF for 5 min (*open columns*). EGTA was used at 1 mM in calcium-free buffer (*hatched columns*) ($n = 3$). *d*, effect of calcium on ERK$_{1/2}$ activation. Unstimulated and VEGF-treated cells were lysed, ERK$_{1/2}$ was immunoprecipitated, and its activity was detected as MBP phosphorylation. Cells were stimulated with 10 ng/ml VEGF in the absence (*open columns*) and in presence (*hatched columns*) of 3 mM EGTA. ERK$_{1/2}$ activity is expressed as radioactivity of gel slides of phosphorylated MBP. $n = 2$; **, $p < 0.01$ *versus* unstimulated control; #, $p < 0.05$ *versus* VEGF (ANOVA followed by Fisher's test).

migration *in vivo* and *in vitro* (32, 33) and also that the activation of the NO pathway following VEGF stimulation significantly contributed to the mitogenic effect of VEGF (14). Here, we demonstrate that under the same experimental conditions, NO directly triggers the activation of the MAPK cascade. ERK$_{1/2}$ activation and endothelial cell proliferation promoted by NO are selectively blocked by the MAPKK inhibitor.

ODQ, a selective and specific inhibitor of the soluble guanylate cyclase (29), blocked in a concentration-dependent manner cGMP elevation in venular endothelial cells exposed to the NO donor and to VEGF. Consistent with cGMP being required to transduce the NO-dependent proliferation signal, neither VEGF or SNP promoted ERK$_{1/2}$ phosphorylation and growth in the presence of ODQ. The IC_{50} for proliferation and cGMP formation overlapped when SNP was the mitogen. Interestingly, minimal reduction of cGMP levels was sufficient to completely block the proliferation signal produced by VEGF. Because in our experimental model, production of cGMP is required for VEGF-induced cell adhesion (15), the effect of ODQ on VEGF proliferation might be related to the specific requirement of cell adhesion to fulfill the growth program encoded by VEGF in postcapillary venular endothelium.

The link between VEGF stimulation of CVECs, NO release, and the rapid activation of ERK appears to be ecNOS, the calcium/calmodulin-dependent enzyme found in endothelial cells. ecNOS is the predominant isoform expressed in CVECs, and its expression is not affected by the growth factor. We show that VEGF causes a rise in cytoplasmic calcium that peaks at 7 min and triggers NOS activity within 5–10 min. Consistently, ERK$_{1/2}$ activity peaks between 5 and 10 min. Thus, the time frame for increases in cytosolic calcium, NO production, and ERK$_{1/2}$ activity support a KDR/calcium/ecNOS/NO/soluble guanylate cyclase/cGMP/ERK$_{1/2}$ cascade activated by VEGF. The mechanism responsible for the calcium transient is not completely clear. However, as indicated by the elevation of inositol phosphate levels recovered in CVECs, release of calcium from the endoplasmic reticulum could occur by KDR-mediated activation of phospholipase C gamma 1 (34). Alternatively, VEGF may activate processes that accelerate calcium entry via plasmalemmal ion channels (35).

We recently demonstrated that NO synthase lies downstream of the angiogenesis induced by VEGF but not of that induced by bFGF (15). The present data provide a new insight on the mechanism underlying the role of NO in mediating VEGF effect by demonstrating that the NO pathway is upstream of the MAPK cascade activated by VEGF. In fact, the ERK$_{1/2}$ activation and the endothelial proliferation following VEGF/receptor activation are prevented in culture conditions in which NO production and cGMP elevation are impaired by the use of NOS/cGMP selective inhibitors. Conversely, blockade of the MAPKK does not affect the NOS/guanylate cyclase. Thus, the NO pathway activation is intermediate between the VEGF receptor activation and the MAPK phosphorylation in endothelial cells.

Other observations support a link between the NO and the MAPK cascade. Sing *et al.* (36) recently described that ERK activation is necessary for the induction of the inducible NOS by interleukin-1β in myocytes and cardiac microvascular endo-

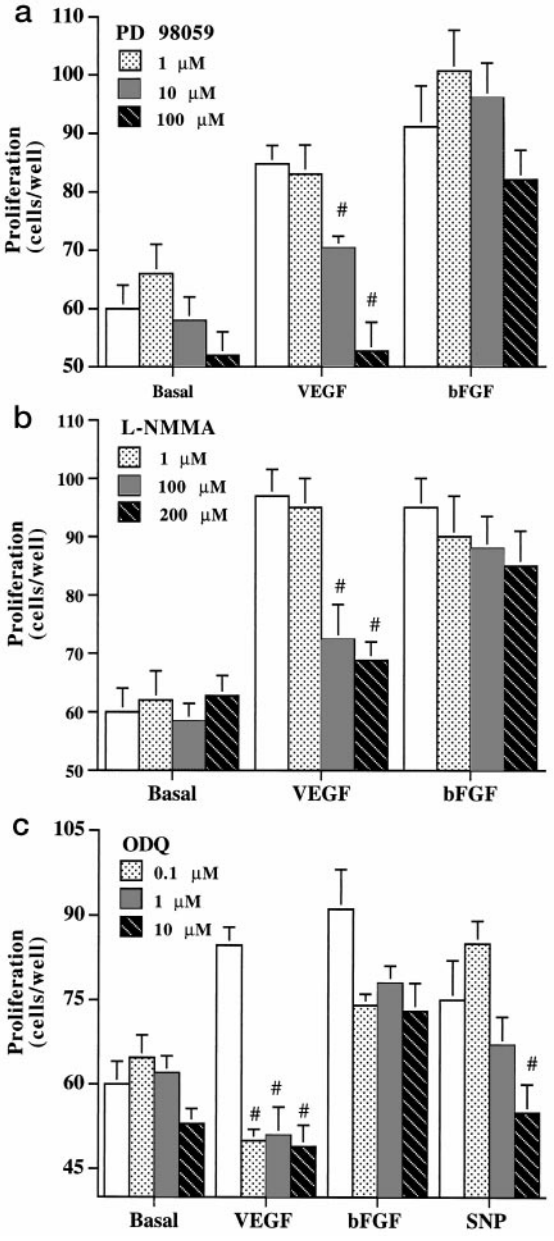

FIG. 4. **Effect of MAPKK (PD 98059) (*a*), NOS (L-NMMA) (*b*), and guanylate cyclase (ODQ) (*c*) inhibitors on VEGF-, bFGF- and SNP-induced proliferation of CVECs.** Cellular proliferation was evaluated following 48 h of exposure to 10 ng/ml VEGF, 10 ng/ml bFGF, and 100 μM SNP. The inhibitors were given at the indicated doses 30 min before growth factors. Fixed and stained cells were microscopically counted, and data are expressed as the total number of cells counted/well. $n = 3$; #, $p < 0.05$ *versus* VEGF- or SNP-induced proliferation (ANOVA followed by Fisher's test).

TABLE I
Effect of the NOS, guanylate cyclase, and MAPKK inhibitors on cGMP levels

cGMP levels were measured by radioimmunoassay in subconfluent CVECs exposed to VEGF (10 ng/ml) and SNP (100 μM) for 10 min. Cells were pretreated with inhibitors for 30 min before stimulation. Data are expressed as fmol/mg of protein of at least three experiments run in duplicate.

Treatment	cGMP levels		
	Basal	VEGF	SNP
None	44 ± 8	85 ± 14	90 ± 10
L-NMMA, 1 μM	50 ± 8	80 ± 10	ND[a]
L-NMMA, 100 μM	48 ± 6	51 ± 5[b]	ND
L-NMMA, 200 μM	42 ± 5	46 ± 3[b]	ND
+L-Arg, 500 μM	ND	84 ± 15	ND
ODQ, 0.1 μM	51 ± 11	74 ± 3	110 ± 2
ODQ, 1 μM	49 ± 13	48 ± 16[b]	45 ± 5[b]
ODQ, 10 μM	39 ± 5	40 ± 12[b]	30 ± 14[b]
PD 98059, 1 μM	56 ± 15	92 ± 12	82 ± 12
PD 98059, 10 μM	48 ± 10	84 ± 15	88 ± 10
PD 98059, 100 μM	60 ± 8	80 ± 8	78 ± 12

[a] ND, not done.
[b] $P < 0.05$ *versus* VEGF or SNP alone.

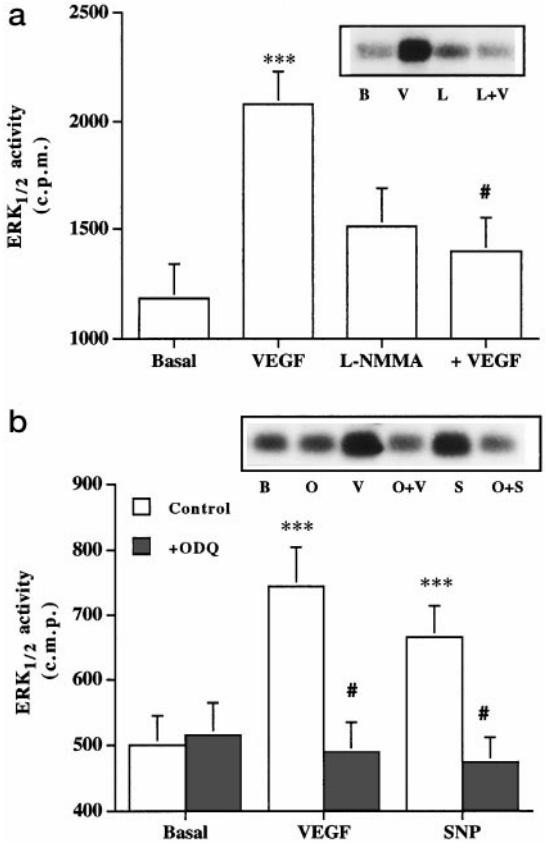

FIG. 5. **Effect of NOS (*a*) and guanylate cyclase (*b*) inhibition on VEGF- and SNP-induced ERK$_{1/2}$ activation.** CVECs were pretreated with 200 μM L-NMMA (*a*) and 10 μM ODQ (*b*) for 30 min and then stimulated with 10 ng/ml VEGF or 100 μM SNP for 5 min. Unstimulated and VEGF-treated cells were lysed, ERK$_{1/2}$ was immunoprecipitated, and its activity was detected as MBP phosphorylation. ERK$_{1/2}$ activity is expressed as radioactivity of gel slides of phosphorylated MBP. $n = 3$; ***, $p < 0.001$ *versus* unstimulated control; #, $p < 0.05$ *versus* VEGF or SNP alone (ANOVA followed by Fisher's test). *Insets:* representative autoradiographies of MBP phosphorylation. *C*, basal control; *V*, 10 ng/ml VEGF; *L*, 200 μM L-NMMA; *L* + *V*, L-NMMA + VEGF; *S*, 100 μM SNP; *O*, 10 μM ODQ; *O* + *V*, ODQ + VEGF; *O* + *S*, ODQ + SNP.

thelial cells. Elevated shear rate caused increased production of NO and activated the MAPK cascade in endothelial cells (37, 38). Whereas in the above mentioned reports, MAPK activation anticipates and/or parallels NO production, our data indicate an upstream role for NO.

The role of NO in promoting cell growth and differentiation is controversial. In angiogenesis, NO elevation has been shown to be positively correlated with neovascularization and tumor growth (15, 39, 40) in adult rodent models. Conversely, in the chorionallantoic membrane of the chick embryo and during the developmental maturation of *Drosophila*, NO acts as an antiproliferative agent (41, 42). ERK$_{1/2}$ is thought to be directly involved in transmitting signals from growth factor receptors to the nucleus to regulate gene transcription and protein synthesis, leading to proliferation or differentiation and apoptosis (17, 43, 44, 45). Recently, a difference in the actions of the ERK

and p38/JNK pathway has been demonstrated in PC12 cells; the activation of JNK-p38 cascade leads to apoptosis of PC12 cells, whereas the activation of ERK$_{1/2}$ seems to be necessary for survival and/or antiapoptosis of PC12 cells (45). The data here reported support the hypothesis of NO as a "prosurvival" or antiapoptotic effector for endothelial cells. Although it is presently difficult to speculate on whether the opposing effects of NO in controlling cell growth are due to species or to differentiation diversity, nevertheless our results using venular endothelial cells continue to emphasize the importance of NO as a balancing element in the molecular events between cell proliferation and differentiation.

REFERENCES

1. Ferrara, N., and Henzel, W. J. (1989) *Biochem. Biophys. Res. Commun.* **161,** 851–858
2. Keck, P. J., Hauser, S. D., Krivi, G., Sanzo, K., Warren, T., Feder, J., and Conolly, D. T. (1989) *Science* **246,** 1309–1312
3. Breier, G., Albrecht, U., Sterrer, S., and Risau, W. (1992) *Development* **114,** 521–532
4. Goto, F., Goto, K., Weindel, K., and Folkman, J. (1993) *Lab. Invest.* **69,** 508–517
5. Fong, G.-H., Rossant, J., Gertsenstein, M., and Breitman, M. L. (1995) *Nature* **376,** 66–70
6. Shalaby, F., Rossant, J., Yamaguchi, T. P., Gertsenstein, M., Wu, X.-F., Breitman, M. L., and Schuh, A. C.(1995) *Nature* **376,** 62–66
7. De Vries, C., Escobedo, J. A., Ueno, H., Houck, K., Ferrara, N., and Williams, L. T. (1992) *Science* **255,** 989–991
8. Terman, B. I., Dougher-Vermazen, M., Carrion, M. E., Dimitrov, D., Armellino, D. C., Gospodarowicz, D., and Bohlen, P. (1992) *Biochem. Biophys. Res. Commun.* **187,** 1579–1586
9. Waltenberger, J., Claesson-Welsh, L., Siegbahn, A., Shibuya, M., and Heldin, C.-H. (1994) *J. Biol. Chem.* **269,** 26988–26995
10. Keyt, B. A., Nguyen, H. V., Berleau, L. T., Duarte, C. M., Park, J., Chen, H., and Ferrara, N. (1996) *J. Biol. Chem.* **271,** 5638–5646
11. Brock, T. A., Dvorak, H. F., and Senger, D. R. (1991) *Am. J. Pathol.* **138,** 213–221
12. Wu, H. M., Qiaobing, H., Yuan, Y., and Granger, H. J. (1996) *Am. J. Physiol.* **271,** H2735-H2739
13. Ku, D. D., Zaleski, J. K., Liu, S., and Brock, T. A. (1993) *Am. J. Physiol.* **265,** H586-H592
14. Morbidelli, L., Chang, C.-H., Douglas, J. G., Granger, H. J., Ledda, F., and Ziche, M. (1996) *Am. J. Physiol.* **270,** H411–H415
15. Ziche, M., Morbidelli, L., Choudhuri, R., Zhang, H.-T., Donnini, S., Granger, H. J., and Bicknell, R. (1997) *J. Clin. Invest.* **99,** 2526–2534
16. D'Angelo, G., Struman, I., Martial, J., and Weiner, R. I. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 6374–6378
17. Seger, R., and Krebs, E. G. (1995) *FASEB J.* **9,** 726–735
18. Blenis, J. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 5889–5895
19. Davis, R. J. (1993) *J. Biol. Chem.* **268,** 14553–14556
20. Schelling, M. E., Meininger, C. J., Hawker, J. R., and Granger, H. J. (1988) *Am. J. Physiol.* **254,** H1211–H1217
21. Erickson, A. K., Payne, D. M., Martino, P. A., Rossomando, A. J., Shabanowitz, J., Weber, M. J., Hunt, D. F., and Sturgill, T. W. (1990) *J. Biol. Chem.* **265,** 19728–19735
22. Ziche. M., Zawieja, D., Hester, R. K., and Granger, H. J. (1993) *Am. J. Physiol.* **265,** H569–H580
23. Chomczynsky, P., and Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159
24. Ziche, M., Parenti, A., Ledda, F., Dell'Era, P., Granger, H. J., Maggi, C. A., and Presta, M. (1997) *Circ. Res.* **80,** 845–852
25. Ranjan, V., Xiao, Z., and Diamond, S. L. (1995) *Am. J. Physiol.* **269,** H550–H555
26. Adler, H., Frech, B., Thony, M., Pfister, H., Peterhans, E., and Jungi, T. W. (1995) *J. Immunol.* **154,** 4710–4718
27. Dudley, D. T., Pang, L., Decker, S. J., Bridges, A. J., and Saltiel, R. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 7686–7689
28. Moncada, S., and Higgs, A. (1993) *N. Engl. J. Med.* **329,** 2002–2012
29. Garthwaite, J., Southam, E., Boulton, C. L., Nielsen, E. B., Scmidt, K., and Mayer, B. (1995) *Mol. Pharmacol.* **48,** 184–188
30. Alessi, D. R., Cuenda, A., Cohen, P., Dudley, D. T., and Saltiel, A. R. (1995) *J. Biol. Chem.* **270,** 27489–27494
31. Cohen, P. (1997) *Trends Biol. Sci.* **7,** 353–361
32. Ziche, M., Morbidelli, L., Masini, E., Granger, H. J., Geppetti, P., and Ledda, F. (1993) *Biochem. Biophys. Res. Commun.* **192,** 1198–1203
33. Ziche, M., Morbidelli, L., Masini, E., Amerini, S., Granger, H. J., Maggi, C. A., Geppetti, P., and Ledda, F. (1994) *J. Clin. Invest.* **94,** 2036–2044
34. Guo, D., Jia, Q., Song, H.-Y., Warren, R. S., Donner, D. B. (1995) *J. Biol. Chem.* **270,** 6729–6733
35. Bates, D. O., Curry, F. E. (1996) *Am. J. Physiol.* **40,** H2520–H2528
36. Sing, K., Balligand, J.-L., Fisher, T. A., Smith, T. W., and Kelly, R. A. (1996) *J. Biol. Chem.* **271,** 1111–1117
37. Buga, G. M., Gold, M. E., Fukuto, J. M., and Ignarro, L. J. (1991) *Hypertension* **17,** 187–193
38. Pearce, M. J., McIntyre, T. M., Prescott, S. M., Zimmerman, G. A., and Whatley, R. E. (1996) *Biochem. Biophys. Res. Commun.* **218,** 500–504
39. Leibovich, S. J., Polverini, P. J., Fong, T. W., Harlow, L. A., and Koch, A. E. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 4190–4194
40. Jenkins, D. C., Charles, I. G., Thomsen, L. L., Moss, D. W., Holmes, L. S., Baylis, S. A., Rhodes, P., Westmore, K., Emson, P. C., and Moncada, S. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 4392–4396
41. Pipili-Synetos, E., Papageorgiou, A., Sakkoula, E., Sotiropoulou, G., Fotsis, T., Karakiulakis, G., and Maragoudakis, M. E. (1995) *Br. J. Pharmacol.* **116,** 1829–1834
42. Kuzin, B., Roberts, I., Peunova, N., and Enikolopov, G. (1996) *Cell* **87,** 639–649
43. Cowley, S., Paterson, H., Kemp, P., and Marshall. (1994) *Cell* **77,** 841–852
44. Alberola-Ila, J., Forbush, K. A., Seger, R., Krebs, E. G., and Perlmutter, R. M. (1995) *Nature* **373,** 620–623
45. Xia, Z., Dickens, M., Raingeaud, J., Davis, R. J., and Greenberg, M. E. (1995) *Science* **270,** 1326–1331

Nitric Oxide Is an Upstream Signal of Vascular Endothelial Growth Factor-induced Extracellular Signal-regulated Kinase½ Activation in Postcapillary Endothelium

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