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

(Accepted for publication, April 7, 1997, and in revised form, October 20, 1997)

Astrid Parenti, Lucia Morbidelli, Xiao-Lan Cui, Janice G. Douglas, John D. Hood, Harris J. Granger, Fabrizio Ledda, and Marina Ziche

From the Department of Preclinical and Clinical Pharmacology, University of Florence, Viale Morgagni 65, 50134 Florence, Italy, the Division of Hypertension, School of Medicine, Case Western Reserve University, Cleveland, Ohio 44106-4982, and the Microcirculation Research Institute and Department of Medical Physiology, Texas A & M University System Health Science Center, College Station, Texas 77843-1144

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) $\frac{1}{2}$, on cultured microvascular endothelium isolated from coronary postcapillary venules. ERK $\frac{1}{2}$ 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 $\mu$M, ERK $\frac{1}{2}$ activity significantly increased. VEGF produced a time- and concentration-dependent activation of ERK $\frac{1}{2}$. 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 $\frac{1}{2}$ activation and endothelial cell proliferation.

Vascular endothelial growth factor (VEGF) 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 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) $\frac{1}{2}$ isoforms are ubiquitously expressed and have been shown to be activated by dual specificity MAPK kinases (MEK/MEK) 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 $\frac{1}{2}$, on cultured endothelium isolated from coronary postcapillary venules.

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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 ERK2—CVECs were serum starved overnight. Following treatment, cells were washed twice in 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 EDTA, 100 mM NaCl, 1 mM Na3VO4, 0.2 mM phenylmethylsulfonyl fluoride, 25 μg/ml leupeptin, 10 μg/ml aprotinin, and 10 mM NaF. To assess the calcium dependence of ERK2 activation, CVECs were stimulated in the presence of 3 mM EDTA. 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 x g for 10 min. 1 μg of anti-ERK2 polyclonal antibody, which is reactive with ERK2 and to a lesser extent with ERK1, 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 x g for 10 min, the pellet was recovered and washed twice with the same buffer for 1 h and then incubated at 4 °C with the kinase buffer containing 20 mM Na3VO4, 1 mM dithiothreitol, (pH 7.6), 20 mM MgCl2, 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 ERK2 (21), 20 μM ATP, and 3 μCi of [γ-32P]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 resolved by 12% SDS-polyacrilamide gel electrophoresis, stained with Coomassie Brilliant Blue, and exhaustively destained. The gel 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 × 104 cells/well) after overnight incubation were labeled with [3H]myo-inositol (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-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 the addition of ice-cold methanol for 30 min. The cells were then 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 EDTA, 100 mM NaCl, 1 mM NaN3, 0.2 mM phenylmethylsulfonyl fluoride, 25 μg/ml leupeptin, 10 μg/ml aprotinin, and 10 mM NaF. To assess the calcium dependence of ERK2 activation, CVECs were stimulated in the presence of 3 mM EDTA. 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 x g for 10 min. 1 μg of anti-ERK2 polyclonal antibody, which is reactive with ERK2 and to a lesser extent with ERK1, 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 x g for 5 min, the pellet was recovered and washed twice with the same buffer for 1 h and then incubated at 4 °C with the kinase buffer containing 20 mM Na3VO4, 1 mM dithiothreitol, (pH 7.6), 20 mM MgCl2, 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 ERK2 (21), 20 μM ATP, and 3 μCi of [γ-32P]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 resolved by 12% SDS-polyacrilamide gel electrophoresis, stained with Coomassie Brilliant Blue, and exhaustively destained. The gel 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.

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increased by 2-fold (Fig. 2a). NO-induced ERK1/2 activation and min of stimulation with 100 inhibitor PD 98995 (10–100 μM) on 10 ng/ml VEGF-induced ERK1/2 activation. d and e are representative autoradiographies related to b and c, respectively. ERK1/2 was immunoprecipitated, and its activity was measured with an in vitro kinase assay by using [γ-32P]ATP and MBP as substrate. The samples were resolved by 12% SDS-polyacrylamide gel electrophoresis followed by autoradiography. Gel slices of the 20 Kd, 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).

VEGF-induced ERK1/2 activation produced by 10 ng/ml of VEGF but did not modify the unstimulated control (Fig. 1, c and e). NO Activates ERK1/2—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 ERK1/2 was measured. After 5 min of stimulation with 100 μM SNP, the MAPK activity was increased by 2-fold (Fig. 2a). NO-induced ERK1/2 activation and the proliferative effect of NO were abolished by PD 98995 (10–100 μM) (Fig. 2, a and b), indicating that ERK1/2 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 ERK1/2 by VEGF—We then characterized the NOS isoform mediating VEGF effect in CVECs. The rapid activation of ERK1/2 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. 3a). 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. 3b). 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 over 85%, 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. 3c). EGTA also abolished ERK1/2 activation by VEGF, suggesting that calcium was required to trigger the MAPK cascade, as well as the NOS activity (Fig. 3d).

MAPKK and NOS/Guanylate Cyclase Inhibitors Block VEGF-induced Endothelial Cell Proliferation—The role of ERK1/2 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 98995 (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. 4a). The IC50 for growth inhibition (10 μM) was in the same range of concentration as that for ERK1/2 inhibition. At the highest concentration, PD 98995 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 (IC50 = 10 μM) (Fig. 4b). Conversely, no inhibition in cell growth was produced when bFGF was used as a mitogen (Fig. 4b).

ODQ produced concentration-dependent inhibition of the...
guanylate cyclase activation and cGMP levels elevation induced by VEGF as well as by the NO donor SNP (maximal effect at 10 \( \mu \)M; IC\(_{50}\) = 0.5 \( \mu \)M) (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. 4c). Conversely, proliferation and cGMP elevation produced by the NO donor SNP were reduced by ODQ in a concentration-dependent manner (Fig. 4c and Table I). Maximal inhibition was obtained at 10 \( \mu \)M, and the IC\(_{50}\) was 0.5 \( \mu \)M for both the effects. The guanylate cyclase inhibitor did not produce significant reduction of bFGF-induced growth (Fig. 4c).

**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 ± 11 pmol/mg of protein versus a basal value of 169 ± 15 pmol/mg of protein) could be selectively blocked by 3 \( \mu \)M 1-L-NMMA (131 ± 10 pmol/mg of protein; IC\(_{50}\) = 50 \( \mu \)M) but not by 100 \( \mu \)M PD 98059 (210 ± 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 ± 37 cpm/well over basal control), which was not affected by 100 \( \mu \)M PD 98059 pretreatment (642 ± 98 cpm/well; \( n \) = 3).

**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 1-L-NMMA and by ODQ prevented the ERK\(_{1/2}\) activation by VEGF (Fig. 5a). This effect was selective for the VEGF effect because no inhibition was found for cells stimulated with 10% calf serum (1680 ± 70 cpm and 1725 ± 150 cpm, with and without 1-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. 5b).

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 \( \mu \)M has been shown both to inhibit MEK and by binding a regulatory site on the enzyme and to prevent activation by c-Raf and MEK 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
VEGF or SNP promoted ERK1/2 phosphorylation and growth in venular endothelial cells. Consistent with cGMP being required to activate the NO-dependent proliferation signal, neither donor nor SNP promoted ERK1/2 activation and endothelial cell proliferation promoted by VEGF. 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 traces recorded from 22 individual cells. c, effect of calcium on NO activity in CVECs. NO activity (pmol/mg of protein) was evaluated by [%³H]-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 ERK1/2 activity. Unstimulated and VEGF-treated cells were lysed, ERK1/2 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. ERK1/2 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. ERK1/2 activation and endothelial cell proliferation promoted by NO are selectively blocked by the MAPKK inhibitor, ODQ.

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 ERK1/2 phosphorylation and growth in the presence of ODQ. The IC₅₀ 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, ERK1/2 activity peaks between 5 and 10 min. Thus, the time frame for increases in cytosolic calcium, NO production, and ERK1/2 activity support a KDR/calcium/ecNOS/NO/soluble guanylate cyclase/cGMP/ERK1/2 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 ERK1/2 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-
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 anti-proliferative 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

![Figure 4](http://www.jbc.org/)

**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](http://www.jbc.org/)

**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 | Basal cGMP | VEGF cGMP | SNP cGMP |
|-----------|------------|-----------|----------|
| None      | 44 ± 8     | 85 ± 14   | 90 ± 10  |
| L-NMMA, 1 μM | 50 ± 8     | 80 ± 10   | ND       |
| L-NMMA, 100 μM | 48 ± 6    | 51 ± 5  | ND       |
| L-NMMA, 200 μM | 42 ± 5    | 46 ± 3  | 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  | 45 ± 5  | ND       |
| ODQ, 10 μM | 39 ± 5     | 40 ± 12  | 30 ± 14 | ND       |
| 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  |

*ND, not done.

**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.
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\textsubscript{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.

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Astrid Parenti, Lucia Morbidelli, Xiao-Lan Cui, Janice G. Douglas, John D. Hood, Harris J. Granger, Fabrizio Ledda and Marina Ziche

J. Biol. Chem. 1998, 273:4220-4226.
doi: 10.1074/jbc.273.7.4220

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