

Nitric oxide mediates the mitogenic effects of insulin and vascular endothelial growth factor but not of leptin in endothelial cells[Ⓞ]

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The regulation of vascular wall homeostasis by nitric oxide (NO) generated by endothelium is being intensively studied. In the present paper, the involvement of NO in the vascular endothelial growth factor (VEGF), insulin or leptin-stimulated proliferation of human endothelial cells (HUVEC) was measured by [³H]thymidine or bromodeoxyuridine incorporation. VEGF and insulin, but not leptin, increased NO generation in HUVEC, as detected with ISO-NO electrode. Proliferation of HUVEC induced by leptin was not changed or was higher in the presence of *N*^m-nitro-L-arginine methyl ester (L-NAME) a nitric oxide synthase (NOS) inhibitor. In contrast, L-NAME blunted the proliferative effect of VEGF and insulin. Furthermore, we demonstrated that,

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Abbreviations: BrdU, bromodeoxyuridine; DAN, 2,3-diaminonaphthalene; EC, endothelial cells; ECGFS, endothelial cell growth factor supplement; eNOS, endothelial NOS; Epo, erythropoietin; ERK_{1/2}, extracellular signal regulated kinase 1/2; FCS, foetal calf serum; hASMC, human aortal smooth muscle cells; HUVEC, human umbilical vein endothelial cells; IGF-1, insulin-like growth factor-1; L-NAME, *N*^m-nitro-L-arginine methyl ester; MAP, mitogen-activated protein; NO, nitric oxide; NOS, nitric oxide synthase; PI-3K, phosphatidylinositol 3-kinase; PLC- γ , phospholipase C- γ ; VEGF, vascular endothelial growth factor; VEGFR-1, VEGF receptor-1; VEGFR-2, VEGF receptor-2; VSMC, vascular smooth muscle cells.

in human arterial smooth muscle cells (hASMC) transfected with endothelial NOS (eNOS) gene, the generation of biologically active VEGF protein was NO-dependent. Inhibition of NO generation by L-NAME decreased the synthesis of VEGF protein and attenuated HUVEC proliferation induced by conditioned media from transfected hASMC. Endothelium-derived NO seems to participate in VEGF and insulin, but not leptin, mitogenic activity. Additionally, the small amounts of NO released from endothelial cells, as mimicked by eNOS transfection into hASMC, may activate generation of VEGF in sub-endothelial smooth muscle cells, leading to increased synthesis of VEGF protein necessary for turnover and restitution of endothelial cells.

Angiogenesis, the formation of blood vessels from preexisting microvascular network, occurs during embryonic and adult life. It is initiated by the activation, migration and proliferation of endothelial cells (EC) under the regulatory control of growth factors, as well as physico-chemical factors such as hypoxia, shear or stretch stress. The next steps of angiogenesis include tube formation, connection of tubes, restoration of the vessel layers etc. [1-3]. Although EC in the normal adult organism have a very low turnover, they resume proliferation in diseases that are associated with neovascularization, including tumor growth, prolonged inflammation, wound healing, retinopathy or atherosclerosis. On the other hand, there is a growing body of evidence confirming the protective role of the endothelium-derived factors in preventing arterial wall remodeling in hypertension and arterial injury. Accelerated reendothelialisation has been suggested to protect the vessel wall against development of restenosis after angioplasty or development of cardiomyopathy [2, 4]. Administration of EC mitogens or its genes to promote therapeutic angiogenesis has been proposed for the treatment of patients with peripheral and/or myocardial ischemia or angioplasty [2, 5].

Vascular endothelial growth factor (VEGF) displaying high specificity for endothelium is produced during tissue ischemia by many tumor and normal cells, including vascular smooth muscle cells (VSMC) and macrophages [6]. VEGF is a 46-kDa dimeric glycoprotein similar to platelet-derived growth factor (PDGF) and placental growth factor (PlGF). It is generated in five (VEGF₁₂₁₋₂₀₆) different isoforms that arise

from alternative splicing of the VEGF primary transcript. VEGF₁₆₅ is the most abundant isoform *in vivo*, widely used in the research on angiogenesis. VEGF selectively stimulates EC by binding to cell surface receptors that possess intrinsic tyrosine kinase activity. Two VEGF receptors: VEGFR-1 (also known as Flt-1) and VEGFR-2 (also known as Flk-1/KDR) have been reported to be induced on endothelial cells by hypoxia [6]. The binding of VEGF to its receptors increases vascular permeability, vasodilatation, EC proliferation and migration and decreases blood pressure and tachycardia [6, 7]. Recently VEGF has been shown to increase its angiogenic properties by augmentation of nitric oxide (NO) release from bovine, rabbit or human endothelium [8, 9].

Nitric oxide, identified as the endothelium-derived relaxing factor (EDRF) is a molecule with diverse regulatory properties in the cardiovascular system [10]. The activities of NO include inhibition of platelet and leukocyte activation and adhesion, as well as inhibition of vascular smooth muscle cell proliferation and improvement of vasodilatation [10]. NO is generated by three isoforms of NO-synthase (NOS) which convert L-arginine to L-citrulline yielding free NO. The calmodulin/calcium complex-dependent constitutive enzymes, endothelial (eNOS) and neuronal (nNOS) synthases, generate picomolar amounts of NO. Inducible NOS (iNOS), induced by bacterial lipopolysaccharides (LPS) and inflammatory cytokines (interleukin-1 (IL-1 β), interferon- (IFN- β)), is expressed in pathological processes (inflammation, atherosclerosis, mechanical stress etc.) in most cells of the body. This isoform generates higher,

nanomolar amounts of NO [11]. NO exerts its biological activity by stimulation of guanylate cyclase, leading to increased intracellular cGMP synthesis. High amounts of NO may be toxic for surrounding cells by induction of apoptosis due to p53 expression [12, 13]. On the other hand, the anti-apoptotic effect of low concentrations of NO was also reported [14, 15].

Evidence that NO may induce angiogenesis in *in vitro* and *in vivo* models has been recently provided [8, 9, 16, 17] and the inhibition of angiogenesis by the non-selective NOS inhibitor *N*^ω-nitro-L-arginine methyl ester (L-NAME) was also demonstrated. On the other hand, Pilipi-Synetos and coworkers [18], using the chicken embryo chorioallantoic membrane (CAM) model, observed the inhibition of angiogenesis by L-arginine or by NO-donor (sodium nitroprusside, SNP) and the augmentation of angiogenesis by L-NAME.

Abdominal obesity, the risk factor for development of diabetes type II, is associated in humans with an elevated insulin plasma level due to insulin resistance, as well as with the increased plasma concentration of leptin produced by adipocytes [19, 20]. Both insulin and leptin have been shown to have mitogenic activity for EC. Rapid formation of capillary endothelial cells and increased endothelial mitosis were induced in rat skeletal muscle after long-lasting (from 24 h to 3 days) infusion of insulin [21], when leptin was demonstrated to exert the angiogenic properties, activating EC proliferation and tube formation [19]. The insulin evoked vasodilatation was also found to be related to endothelium-derived NO [22]. The leptin/NO relation has not been studied until now.

Our aim was to investigate the NO-dependency of endothelial cell proliferation induced by VEGF in comparison to those induced by insulin and leptin. Additionally, arterial smooth muscle cells transfected with eNOS were used to check the possibility, that NO

mediates expression of VEGF gene and synthesis of biologically active VEGF protein.

MATERIALS AND METHODS

The endothelial cell and smooth muscle cell cultures. Primary cultures of human endothelial cells (HUVEC) were isolated according to Jaffe's method [24]. Cells were grown in M199 medium containing streptomycin (200 µg/ml), penicillin (200 U/ml), 20% foetal calf serum (FCS), heparin (500 U/ml), L-glutamine (2 mM) and Endothelial Cell Growth Factor Supplement (ECGFS, 150 µg/ml) (Sigma, St. Louis, U.S.A.). Cells were cultured in vitronectin-coated T-25 cm² flasks (Corning, Warsaw, Poland). After reaching confluence, cells were trypsinized with 0.05% trypsin (Gibco BRL, Warsaw, Poland) solution and used for experiments within the 4th and 6th passage.

Human aortal smooth muscle cells (hASMC) were purchased from Clonetics (CellSystems, St. Katharine, Germany) and cultured according to the vendor's protocol. All experiments were performed on the cells incubated in DMEM F-12 medium with 5% FCS.

Preparation of plasmid DNA. The pKecNOS expression plasmid, with kanamycin resistance gene and containing 4.2 kb of bovine endothelial eNOS cDNA, was kindly provided by Dr. Thomas Michel (Boston, U.S.A.). Commercially available pSVβgal expression plasmid (Promega, Madison, U.S.A.) containing bacterial β-galactosidase gene driven by SV40 promoter was used as a control construct. The plasmids were amplified in *Escherichia coli* HB-101 strain and isolated with Qiagen Endo-Free Plasmid Kit. The purity of DNA was confirmed spectrophotometrically and by agarose electrophoresis.

Transfection of VSMC with Tfx-50 liposomes. Transfection was performed in 24-well plates, on hASMC cultures at 70–80% confluency. Plasmid DNA (0.5 µg) was mixed

with 200 μ l of DMEM F-12 medium without serum, and Tfx-50 liposome (Promega) was added in a proportion of 3:1 according to the manufacturer's protocol. After 15 min at room temperature the transfection mixture was poured onto the cells and left for one hour at 37°C. Then the transfection mixture was replaced by the regular culture medium containing 5% FCS. Conditioned media were harvested 3 days later.

Measurement of VEGF and NO synthesis. Concentrations of VEGF in media collected from intact cells, pSGgal or pKecNOS transfected cells were measured by ELISA kit (R&D, Abingdon, U.K.), according to the vendor's protocol.

Measurement of NO release from HUVEC was performed with ISO-NO selective electrode (World Precision Instruments, U.S.A.), which allows to measure electrochemically the concentration of crude NO gas in aqueous solutions [26]. The calibration of electrode was carried out in 0.1 M KJ and 0.1 M H₂SO₄ after addition of KNO₂ as a generator of NO.

The sensor probe was inserted vertically into confluent HUVEC culture growing in 96-well plates. All measurements were performed at room temperature. Just before the experiment medium was replaced with M199 without serum and growth factors, but supplemented with 10 ng/ml superoxide dismutase (Sigma) and 2 mM of L-arginine (Sigma). A stabilized amperometric NO probe was then introduced into the incubation medium, and the basal release of NO was continuously registered on a chart recorder. After steady state was attained, acetylcholine (3 μ M), insulin (10–100 μ U), VEGF (1–15 ng/ml), or leptin (1–30 nM) were added and NO release was measured. In some experiments the samples were additionally supplemented with N^w-nitro-L-arginine methyl (L-NAME, 2 mM) or genistein (20 μ g/ml) for 15 min before HUVEC stimulation. The results are expressed as nM of released NO by converting the current data to the concentration of NO received from the calibration curve. The pre-

sented data are means of three independent experiments performed in triplicates.

In some experiments, accumulation of nitrites in culture medium was measured by the fluorimetric method [27]. Briefly: 2,3-diaminonaphthalene (DAN) was reacted with nitrite under acidic conditions to form 1-(H)-naphthotriazol, a fluorescent product. Then 25 μ l of DAN (0.05 mg/ml in 0.62 M HCl) was added to 250 μ l of culture medium. After a 10 min incubation at room temperature, the reaction was terminated with 12.5 μ l of 2.8 M NaOH and fluorescence was measured with excitation at 365 nm and emission at 450 nm. The sensitivity of this method ranged from 70 nM to over 10 μ M nitrite concentration.

Measurement of HUVEC proliferation by [³H]-thymidine incorporation assay. HUVEC were seeded at a density of 21000 cells/1000 mm² in fibronectin-coated 6-well culture plates in M199 medium with 20% FCS. The cells at 70% confluency were growth-arrested by 24 h incubation in M199 medium with 0.5% FCS, and then the growth factors (insulin, leptin or VEGF) were added.

After 24 h incubation with growth factors, the cells were labeled with [³H]thymidine (1.62 μ Ci) for another 24 h. Then they were washed twice with ice-cold PBS, and fixed with 10% trichloroacetic acid and 96% ethanol at 37°C for 30 min. Cells were lysed with 10% NaOH (500 μ l) at 65°C for 1 h and neutralized by 12.5 M acetic acid (125 μ l). Relative [³H]-thymidine incorporation was determined by liquid scintillation spectrometry.

Measurement of HUVEC proliferation by bromodeoxyuridine (BrdU) incorporation assay. HUVEC (5000 cells per well) were seeded in a 96 well plate in M199 medium containing ECGFS and 10% FCS. After 3 h, the cells were washed and covered with 100 μ l of M199 medium with 5% FCS, without ECGFS, for 24 h. Then, 100 μ l of conditioned medium from intact or transfected hASMC was added. Part of each tested medium was preincubated with anti-VEGF antibodies (100 ng/ml, R&D) for 1 h at room temperature. Eight hours after

stimulation, the cells were treated with $10 \mu\text{M}$ BrdU (Boehringer-Manheim) for 10 h. BrdU incorporation was measured by ELISA method, according to the vendor's protocol.

Statistical analysis. Data are presented as means \pm S.D. Statistical evaluation was done with Student's *t*-test or ANOVA followed by Tukey test. $P < 0.05$ was accepted as statistically significant.

RESULTS

Influence of exogenous VEGF₁₆₅, insulin or leptin on NO release from HUVEC

VEGF (5–15 ng/ml) increased the release of NO from HUVEC in a concentration-dependent manner, as shown by the ISO-NO electrode (Fig. 1). This effect was significantly di-

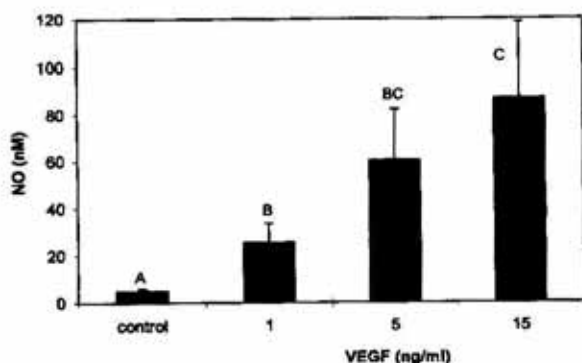


Figure 1. Dose-dependent NO release by HUVEC incubated with VEGF.

NO release was measured with ISO-NO electrode. Different letters indicate the statistically significant differences ($P < 0.05$; ANOVA followed by Tukey test).

minished by addition of NOS-inhibitor L-NAME (2 mM) or a tyrosine kinase inhibitor genistein (20 $\mu\text{g}/\text{ml}$) (Fig. 2). Similar results were obtained for recombinant insulin (10–90 μU) which, in a concentration-dependent manner, augmented the generation of NO (Fig. 3). The release of NO was also inhibited by preincubation of HUVEC with L-NAME (2 mM) (not shown). In contrast, in the presence

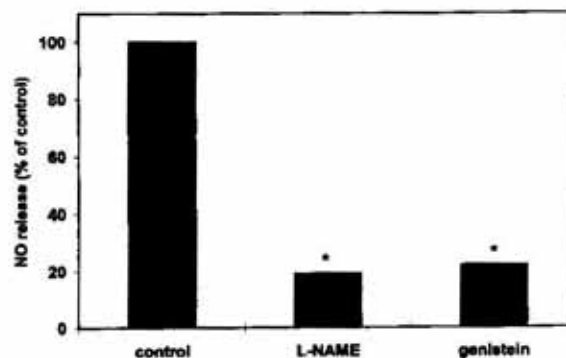


Figure 2. Inhibition of VEGF-induced NO release by HUVEC after preincubation with L-NAME (2 mM) or genistein (20 $\mu\text{g}/\text{ml}$).

Samples treated with VEGF (5 ng/ml) without inhibitors were used as a control. Release of NO was measured with ISO-NO electrode. Asterisks indicate the differences statistically significant in comparison to control value ($P < 0.001$, Student's *t*-test)

of leptin (1–30 nM) no activation of NO release from HUVEC was observed (not shown).

Proliferation of HUVEC measured as [³H]thymidine incorporation assay

The influence of VEGF, insulin or leptin on endothelial cell proliferation was examined in a series of 3 experiments performed in triplicates. The increase by VEGF (0.3 to 30 ng/ml) of incorporation of [³H]thymidine by HUVEC was concentration-dependent. The stimulating effect of VEGF was abolished by pretreatment of cells with L-NAME (2 mM) (Fig. 4).

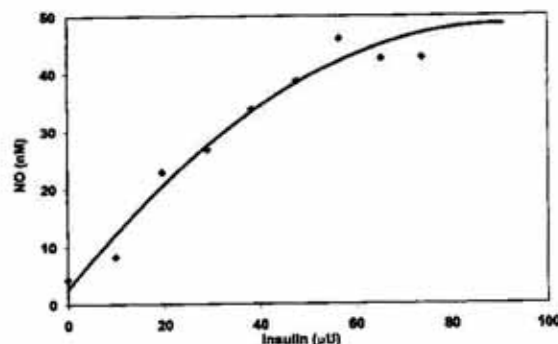


Figure 3. Dose-dependent NO release by HUVEC incubated with insulin.

NO release was measured with ISO-NO electrode.

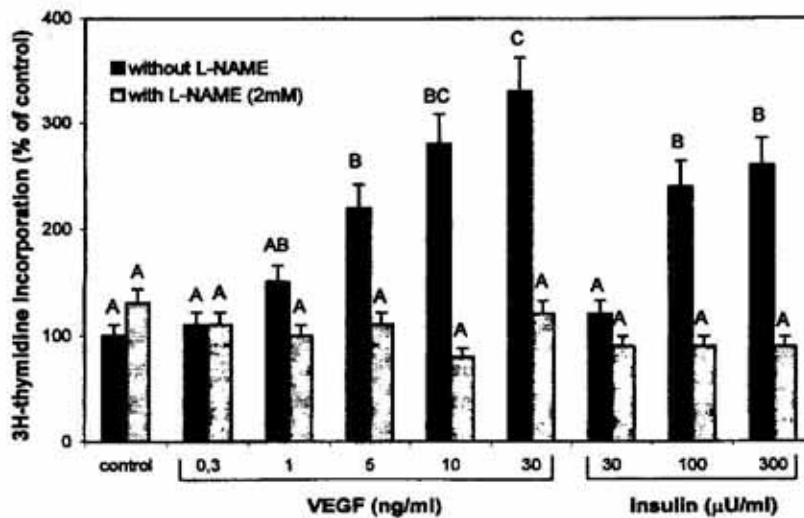


Figure 4. HUVEC proliferation induced by VEGF or insulin, measured by [^3H]thymidine incorporation.

Note that proliferation is inhibited by L-NAME. Different letters indicate statistically significant differences ($P < 0.001$ for VEGF and $P < 0.004$ for insulin; ANOVA followed by Tukey test).

Insulin also induced proliferation of HUVEC in a concentration-dependent manner although this effect was less pronounced compared with that of VEGF. Significant induction of proliferation was found when 30–300 $\mu\text{U/ml}$ of insulin was present, and this effect was inhibited by preincubation of cells with L-NAME (2 mM) (Fig. 4).

Leptin (1–10 nM) was found to activate HUVEC proliferation, however at a lower rate compared with VEGF₁₆₅ or insulin. The preincubation of cells with L-NAME did not prevent the mitogenic effect of leptin, and even an increase in thymidine incorporation in the presence of L-NAME was observed (Fig. 5). This effect, however, was not significant. A similar effect of L-NAME was sometimes found when high concentrations of VEGF (30 ng/ml) were used (not shown).

Generation of NO by pKecNOS-transfected hASMC results in VEGF synthesis

The concentrations of nitrites in media from pKecNOS transfected hASMC were significantly higher than in controls and ranged around 1.0 μM (Fig. 6a). These cells generated also significantly higher amounts of VEGF protein than intact cells or hASMC transfected with pSVgal control plasmid (Fig. 6b). L-NAME significantly inhibited NO generation by pKecNOS transfected hASMC

(Fig. 6a) and simultaneously reduced VEGF synthesis (Figs. 6a,b).

Media from pKecNOS transfected hASMC induce proliferation of HUVEC

Conditioned media from pKecNOS transfected cells induced proliferation of HUVEC as measured by BrdU incorporation. This effect was partially but considerably blocked by preincubation of media with anti-VEGF antibodies. Induction of HUVEC proliferation was significantly lower in the case of media harvested from intact cells or from cells transfected with pSV β gal control plasmid (Fig. 6).

DISCUSSION

The results of the present study indicate that increased generation of NO is involved in endothelial cell proliferation induced by VEGF or insulin, whereas the leptin-stimulated EC proliferation is NO-independent. Moreover, we have observed that NO may increase VEGF gene expression and synthesis of biologically active VEGF protein in eNOS-transfected vascular smooth muscle cells.

The role of NO in endothelial cell proliferation is not definitely recognized, and may be related to the proliferative status of cells.

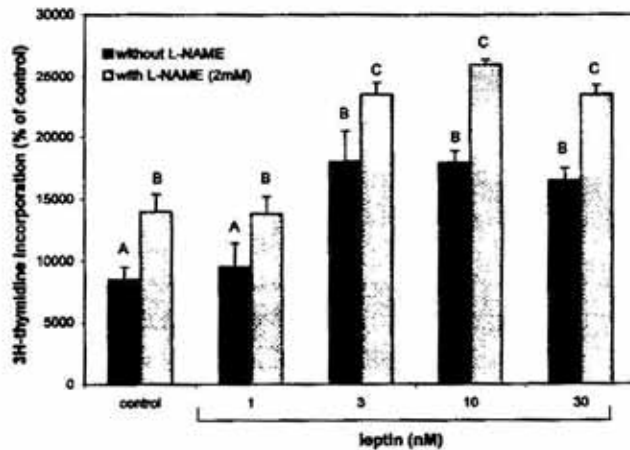


Figure 5. HUVEC proliferation induced by leptin, measured by [^3H]thymidine incorporation.

Note that proliferation is not inhibited, but even enhanced by L-NAME. Different letters indicate statistically significant differences ($P < 0.001$; ANOVA followed by Tukey test).

Lopez-Farre *et al.* [28] have observed that inhibition of NO biosynthesis in subconfluent culture of bovine endothelial cells induces DNA replication and promotes the transition from prereplicative (G_0/G_1) to replicative (S) phases. On the contrary, in confluent EC culture, the inhibition of NO generation or addi-

tion of VEGF resulted in augmented cell detachment and apoptosis manifested by increase in *c-myc* and *c-fos* oncogene-encoded protein expression and DNA fragmentation [28]. Surprisingly, the increased thymidine incorporation was observed in detached, but not in adherent cells. These effects were reversed by addition of non-specific NO-donor, 3-morpholinosydnonimine hydrochloride (SIN-1) [28]. The same authors have also demonstrated that subconfluent cells expressed more eNOS mRNA and generated more NO in the presence of mitogenic FCS, as compared to confluent EC culture. Thus they have con-

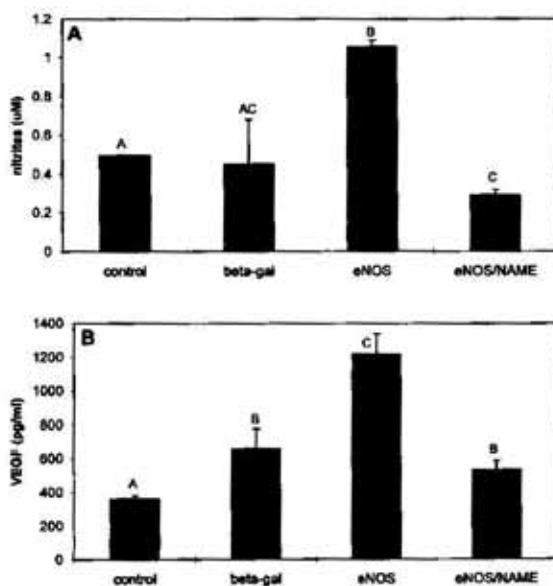


Figure 6. Generation of NO (A) and VEGF (B) by hASMC transfected with control β -galactosidase (beta-gal) gene or endothelial eNOS gene.

Note that inhibition of NO synthesis by L-NAME results in a decrease in VEGF protein synthesis. Generation of NO was detected as nitrite accumulation and measured by fluorimetric method. Synthesis of VEGF was measured by ELISA. Different letters indicate statistically significant differences ($P < 0.05$; ANOVA followed by Tukey test).

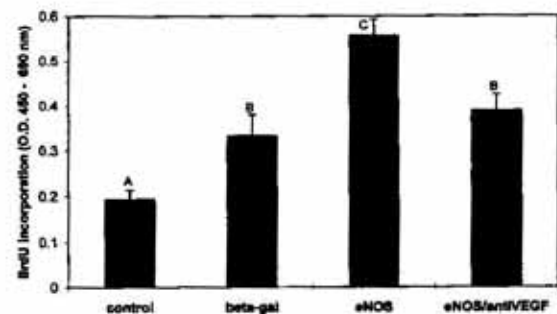


Figure 7. HUVEC proliferation induced by media from hASMC transfected with control β -galactosidase (beta-gal) or endothelial eNOS genes.

Control cells were incubated with medium from intact hASMC. Note that increased proliferation induced by medium from eNOS transfected cells is inhibited by anti-VEGF antibodies. Proliferation was measured by BrdU incorporation. Different letters indicate the statistically significant differences ($P < 0.05$; ANOVA followed by Tukey test).

cluded that eNOS gene expression and NO generation protects endothelial cell against detachment and apoptosis and maintains them in quiescent, confluent state by decreasing susceptibility of EC to growth factors [28]. These results are inconsistent with those reported by others [8, 17, 18, 29] and with our observations in which the role of NO in mediation of EC proliferation and its involvement in angiogenesis has been documented.

VEGF was also found to inhibit EC apoptosis [5, 6]. Furthermore, VEGF induces endothelial permeability and fenestration as well as podokine formation, an event necessary for the capillary outgrowth [34, 35]. Additionally it upregulates the release of endothelial urokinase (uPA) and tissue plasminogen activator (tPA), increases the generation of connexin-43, integrins $\alpha_v\beta_3$ (important for angiogenesis mediated by basic fibroblast growth factor (bFGF) or tumor necrosis factor- α (TNF- α)) and $\alpha_v\beta_5$ (important for angiogenesis induced by VEGF, transforming growth factor- β (TGF- β), or phorbol esters). VEGF augments the biosynthesis of metalloproteinases and matrix proteins, including osteopontin, fibronectin, or vitronectin [5, 6, 36, 37]. Some of these effects, such as increased permeability, podokine formation or mitogenesis have been described to be related to VEGF-induced release of NO by endothelial NOS [16–18, 35].

The increase in NO generation induced by VEGF is probably mediated by VEGFR-2 (KDR/flk-1) receptor, which is expressed by regenerating endothelial cell in arteries after balloon-injury or ischemia [23]. The same receptor is also critical for VEGF-mediated increase in endothelial permeability and in induction of EC mitosis and migration [38, 39].

In cultured endothelial cells, binding of VEGF to its receptors causes receptor dimerisation and autophosphorylation leading to phospholipase C (PLC γ) activation. Binding of VEGF to VEGFR-2 receptor promotes also mitogen-activated protein (MAP) kinase activation [6]. PLC γ promotes an in-

crease in intracellular Ca^{2+} concentration, and activation of calcium/calmodulin-dependent enzymes [16]. The increase in intracellular Ca^{2+} upregulates eNOS activity and NO generation [16]. Additionally, the prolonged, several hours lasting incubation of EC with VEGF results in the upregulation of eNOS gene, and increased eNOS protein generation [16]. The specific MAP kinase inhibitor (PD 98059), NOS-inhibitor (L-NMMA) as well as guanylate cyclase inhibitor (LY 83583) abolish the extracellular signal regulated kinase 1/2 (ERK $_{1/2}$) activation and endothelial cell proliferation suggesting that NO and cGMP contributes to the VEGF-dependent ERK $_{1/2}$ activation [49]. All above mentioned events are also supposed to be involved in generation of metalloproteinases that are required for the breakdown of the basement membrane of blood vessels in the first steps of angiogenesis, as well as in the expression of specific integrins required for EC migration and proliferation [6]. That is why the tyrosine kinase inhibitors (genistein or geldanamycin), prevented the VEGF induced generation of NO and angiogenesis, but did not influence the bradykinin-induced generation of NO and Ca^{2+} mobilization [16].

Wortmannin, a phosphatidylinositol-3-kinase (PI-3K) inhibitor, attenuated not only the VEGF- but also the insulin- and insulin-like growth factor-1 (IGF-1) induced NO release from HUVEC, pointing to the existence of a second, PI-3K-dependent release of NO from EC. Wortmannin was also found to inhibit angiogenesis in the chicken chorioallantoic membrane, once more stressing the participation of NO in the angiogenic activity of VEGF, insulin and IGF-1 in EC [47, 48].

Both the inhibition of NO generation and inhibition of guanylate cyclase activity blocked VEGF-, but not bFGF-induced angiogenesis as well as EC migration and proliferation [8, 9, 16–18, 41]. Prenatal inhibition of nitric oxide synthase by feeding of pregnant rats with NOS inhibitors resulted in teratogenic limb reduction [42]. Therefore VEGF, as well as in-

sulin or IGF-1 may promote angiogenesis by NO-mediated EC proliferation. However, it is not the only mechanism of induction of EC proliferation, since some other factors like epithelial growth factor, TGF β , or – as we have observed – leptin also promote DNA synthesis by EC, but this effect is not related to the increased NO generation. Interestingly, leptin was found to promote EC differentiation and augments the new vessel-like tube formation [19]. Our results suggests that the decrease in NO generation due to pretreatment of EC with NO-synthase inhibitor does not inhibit, but even increases leptin-induced incorporation of [³H]thymidine, resembling the effect described previously by Lopez-Farre *et al.* [28].

Thus we have confirmed the observations that VEGF and insulin the receptors for which are coupled with tyrosine kinase, may release NO from EC. In non-differentiated EC this effect may be important for EC growth promoting angiogenic properties of these mitogens [21, 22]. A similar mechanism, i.e. NO-mediated proliferation of HUVEC has been also suggested for the endothelin-mediated endothelin receptor-B activation [43].

Tsurumi *et al.* [23] demonstrated the inhibitory activity of high doses of NO-donors on VEGF release from the rat arterial wall. The inhibition of binding of transcription factor AP-1 to the VEGF promoter by NO was suggested as the mechanism of down-regulation of VEGF gene expression [23]. However, our recent results indicate that NO may induce VEGF gene expression and/or VEGF protein synthesis in vascular smooth muscle cells. Similarly, the NO-related increase in tumor vasculogenesis and blood flow [50] was confirmed by Chin and co-workers [51], who demonstrated that NO-donors induce mRNA of the VEGF gene in human A-172 glioblastoma and human Hep-G2 hepatoma cells. This NO-mediated effect on VEGF gene expression is probably mediated by cGMP dependent synthesis of protein [51]. Additionally it was found that NO-donors prolong the half-life of

VEGF mRNA [51]. It has been also reported that inducible NOS, generating NO, as well as peroxynitrite and free radicals, increase VEGF synthesis in the colon cancer carcinoma [52].

The hypoxia-induced increase in VEGF gene expression is mediated by the hypoxia inducible factor-1 (HIF-1) binding site [53], a regulatory element recognized by protein (proteins) which also binds to the regulatory element of erythropoietin (Epo) promoter [54]. Ohigashi *et al.* [55] demonstrated that inhibition of NOS by L-NAME prevents cGMP accumulation and hypoxia-induced Epo gene expression in rodents, arguing for the importance of NO in hypoxia-induced gene expression.

In summary: we suggest that NO generated by the endothelial cells activated by physical factors (such as shear stress, cyclic stretching, circulating autacoids) as well as by VEGF or insulin, is necessary for maintenance of the adherent, quiescent endothelial layer. Additionally, we postulate that NO may stimulate expression of VEGF gene in subendothelial vascular smooth muscle cells leading to increased generation of VEGF protein necessary for EC turnover and restitution. The decrease of NO availability results in impairment of pleiotropic endothelial function and promotes remodeling of arterial wall. The understanding of NO/VEGF reciprocal regulation is crucial for atherosclerosis, hypertension, or tissue ischemia treatment [56]. Our observations argue for the possible importance of the local VEGF or/and NOS gene delivery [57, 58] for restoration of the functional endothelial layer.

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