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# Review

# Regulation of Vascular Endothelial Growth Factor Synthesis by Nitric Oxide: Facts and Controversies

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## ABSTRACT

**Vascular endothelial growth factor (VEGF) is the major molecule governing angiogenesis, defined as the growth of blood vessels from vascular structure. There is abundant evidence that nitric oxide (NO) is an effector molecule mediating the activity of VEGF. By binding to its receptors, VEGF initiates the signaling cascades leading to NO production and angiogenic activation of endothelial cells. Recent data show that NO induces VEGF synthesis in numerous cell types, including vascular smooth muscle cells, macrophages, keratinocytes, and tumor cells. NO enhances VEGF production by augmenting its expression through activation of Akt kinase, followed by induction of several transcription factors, of which stabilization of hypoxia-inducible factor (HIF-1) is the critical step. With respect to its effect on VEGF expression, NO mimics hypoxia, the classical activator of HIF-1 and VEGF synthesis. The effect of NO on VEGF production is also mediated by heme oxygenase, an enzyme generating carbon monoxide, which appears to stimulate VEGF release. In this review, we attempt to elucidate the molecular mechanisms underlying the effects of NO on VEGF synthesis. We also discuss some discrepant data and suggest explanations for various aspects of the NO–VEGF relationship.** *Antioxid. Redox Signal.* 5, 123–132.

## INTRODUCTION

IT IS INCREASINGLY RECOGNIZED that nitric oxide (NO) is associated with many physiological processes, including regulation of gene expression. In the cardiovascular system, NO appears to be a key mediator in vascular homeostasis. Interaction of NO with the heme iron of guanylyl cyclase enhances 3′,5′-cyclic guanosine monophosphate (cGMP) synthesis, causing vasorelaxation and induction of the production of new proteins. Decreased generation of NO by endothelial cells is responsible for vasoconstriction and the initiation of inflammatory events leading to the formation of atherosclerotic plaques (for review, see 15). Abundant evidence collected recently demonstrates that NO not only is crucial to the physiological functions of the cardiovascular system, but also is required for its development. In particular, the role of NO in the formation of new blood vessels has attracted broad attention.

## REGULATION OF VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) EXPRESSION

VEGF can be produced by numerous cells, including macrophages, vascular smooth muscle cells (VSMC), pericytes, fibroblasts, keratinocytes, tumor cells, lymphocytes, megakariocytes, neutrophils, basophiles, mast cells, and astrocytes (for review, see 66). Binding of VEGF to its receptors on endothelial cells activates multifactorial intracellular cascades, with NO being an effector molecule (for review, see 89).

In many cells cultured *in vitro,* VEGF is continuously synthesized and its expression is strongly enhanced by hypoxia, stimulation with certain cytokines [*e.g.,* interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), IL-6], growth factors [platelet-derived growth factor-β, basic fibroblast growth factor, transforming growth factor-β (TGF-β)], prosta-

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glandins ($PGE_1$, $PGE_2$, $PGJ_2$), or cyclic AMP (for review, see 66).

VEGF generation is regulated both at the transcriptional level and by modulation of mRNA stability (for review and references, see 66). VEGF mRNA is intrinsically labile, with a half-life estimated to be ~30 min. Hypoxia increases transcription of the VEGF gene, augments the stability of its mRNA, and enhances translation through a mechanism implicating protein interaction with intraribosomal entry site within the 5′ untranslated region (UTR) (for review, see 66).

Transcriptional regulation of VEGF synthesis is mediated by activation of several transcription factors. The consensus sequences for binding of HIF-1 (hypoxia-inducible factor), activator protein-1 (AP-1), AP-2, SP-1, CREB, and STAT-3 are present in the promoter region of VEGF (5, 31, 64, 80).

HIF-1 is the major mediator of hypoxic regulation of VEGF transcription. Under low oxygen tension, the VEGF promoter is activated by binding of HIF-1 to a hypoxia-responsive element (HRE) located at −985 to −939 bp upstream of the transcription start codon (27, 49, 50). HIF-1 is a heterodimer composed of α and β subunits, both of which are basic helix–loop–helix PAS domain proteins. The HIF-1α and HIF-1β subunits are constitutively produced in most, if not all, human and rodent tissues; however, formation of the HIF-1 heterodimer is prevented by degradation of HIF-1α under normal oxygen tension (for review, see 75).

The half-life of HIF-1α in posthypoxic cells is <5 min (35). Inactivation of HIF-1α is mediated by its ubiquitination and proteasomal degradation. This is dependent on association of HIF-1α with the von Hippel–Lindau (VHL) tumor suppressor protein, which functions as a component of the ubiquitin protein ligase (for review, see 75).

Very recently, it was demonstrated that the interaction between human VHL and a specific domain of the HIF-1α subunit is regulated through hydroxylation by the enzyme HIF-1α prolyl-hydroxylase of conservative proline residues (P402 and P564) within a polypeptide segment known as the oxygen-dependent degradation domain (38, 39). This reaction requires molecular oxygen and 2-oxoglutarate as cosubstrates and iron as a cofactor. Following modification, HIF-1α chains are captured by the VHL ubiquitin protein ligase E3 complex, ubiquitilated, and destroyed by the proteasome. Binding of p300/CBP transactivator additionally enhances HIF-1 activity. The latter step is also modulated by hydroxylation, but this time the asparagine residue of the HIF-1α carboxyl-terminal transactivation domain is modified (56).

Besides being affected by decreased oxygenation, HIF-1 activity is enhanced by hypoxia mimics, such as cobalt (63) and deferoxamine (7) and by a number of activators, such as insulin and insulin growth factor-1, TGF-β, and fibroblast growth factor, the organomercurial compound mersalyl, and the peptide antibiotic PR39 (for review, see 75). Recently, NO entered the scene as an important regulator of HIF-1α activity, and this relationship will be discussed later.

## NO AND VEGF SYNTHESIS

NO has been recognized as both a positive and a negative modulator of carcinogenesis, tumor growth, and metastases (for review, see 55). The effect of NO can be dependent on the amount of NO generated by tumor cells and/or host cells, as well as the genetic constitution of tumor cells, which may become resistant or sensitive to this molecule. Generated in high quantities by inducible NO synthase (iNOS) in tumor–associated macrophages, NO can induce apoptosis in tumor cells and prevent tumor growth and metastasis. On the other hand, NO can also promote tumor growth in some experimental models (40, 55).

NO can participate in tumor growth by stimulation of angiogenesis. Indeed, the first suggestion of the role of NO in blood vessel formation came from a study demonstrating that human colon adenocarcinoma DLD-1 cells transfected with iNOS gene grew faster and displayed higher blood vessel density in athymic mice than wild-type tumors did (41). The p53 protein regulates iNOS-dependent angiogenic effects (3). Thus, cancer cells expressing iNOS and wild-type p53 showed reduced tumor growth, whereas tumors with mutated p53 had accelerated growth associated with increased neovascularizations (3). In turn, VEGF expression was enhanced in tumor cells expressing iNOS in the presence of mutated p53 (3).

Enhancement of VEGF production has been observed in numerous cell types treated with NO donors, including tumor cells (14, 49), mesangial cells (30), human articular chondrocytes (83), and rat hepatic stellate cells (4). VEGF expression increased concomitantly with iNOS expression in keratinocytes during cutaneous wound repair (28, 29, 79) and in macrophages treated with cytokines (57, 86).

In our studies, we have determined the effect of NO on VEGF synthesis in VSMC, augmenting NO generation in several ways. First, we demonstrated that enhancement of VEGF synthesis in VSMC by IL-1β or a cytokine combination is dependent on endogenous NO generation induced by the cytokine mixture (21). When NO synthesis by iNOS was blocked with $N^{\omega}$-nitro-L-arginine methyl ester (L-NAME), a NO synthase (NOS) inhibitor, VEGF generation was significantly attenuated. Next, we showed that transfection of VSMC with plasmid containing either endothelial NOS (eNOS) (20, 21, 43) or iNOS (43) genes, resulting in generation of NO by the transfected cells, led to enhancement of VEGF production. Inhibition of NO synthesis by such engineered cells attenuated VEGF generation. The degree of stimulation of VEGF synthesis depended on the amount of NO produced; cells stably transfected with eNOS and generating more NO than transiently transfected VSMC made more VEGF than transient transfectants did (43).

VEGF mRNA expression and protein synthesis were enhanced in VSMC treated with NO donors such as *S*-nitroso-*N*-acetylpenicillamine (SNAP) and *S*-nitrosoglutathione (GSNO). VEGF production was also increased in the presence of 3-morpholinosydnonimine (SIN-1), a compound simultaneously releasing NO and superoxide anion ($O_2^-$), but not after treatment with sodium nitroprusside (SNP) (17, 21, 43) (Fig. 1). In the presence of SNP, the production of VEGF was attenuated, but this effect also occurred when the cells were treated with sodium ferro- and ferricyanide, compounds related to SNP but not releasing NO (21, 43).

More support for the stimulatory role of NO in VEGF expression comes from studies of transgenic mice. VEGF production induced by cytokines was significantly attenuated in

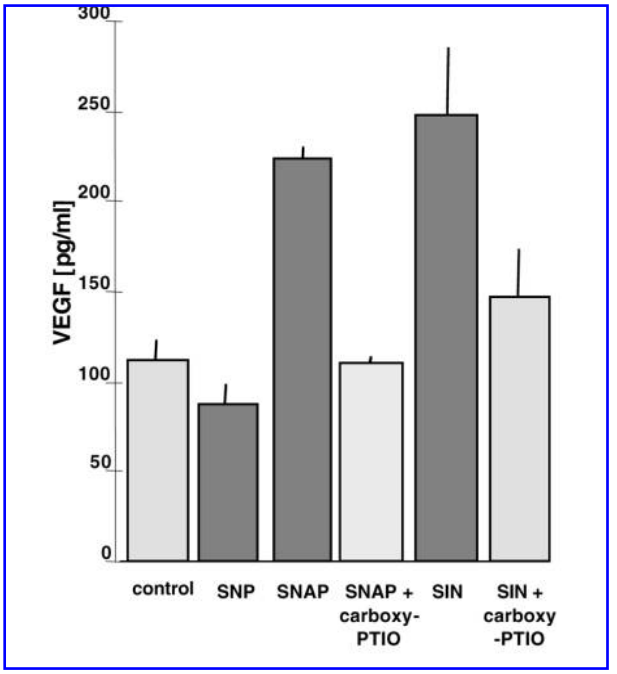


**FIG. 1. The effect of NO on VEGF synthesis.** Rat VSMC were treated with three chemically distinct NO donors (100 μ*M* each). After 24 h, VEGF production was determined by ELISA measurement of VEGF protein in cell culture media. Note that both SNAP (releases NO) and SIN-1 (concomitantly releases NO and $O_2^-$) enhanced VEGF synthesis, an effect abolished by carboxy-PTIO (100 μ*M*), a NO scavenger. SNP, a complex molecule (releases NO, iron ions, and cyanides), did not enhance VEGF production.

macrophages from iNOS knockouts (57). Tumors growing in iNOS−/− mice displayed lower VEGF synthesis (52). The sources of VEGF in those tumors were mast cells, which in wild-type animals express iNOS in response to cytokine stimulation. Wound healing was impaired in iNOS−/− animals, whose mast cells did not express VEGF in response to cytokine stimulation (45).

Angiogenesis is one of the adaptations developed in muscles subjected to exercise training. Benoit *et al.* (8) demonstrated that an acute exercise leads to the release of NO, prostacyclin ($PGI_2$), and adenosine (A) in skeletal muscles. They also showed that levels of VEGF mRNA in rat skeletal muscles were increased 50% by SNP and acetylcholine, were unaffected by $PGE_1$ and $PGE_2$, and were reduced 40% by $PGI_2$ (8). Those experiments suggest that NO derived from eNOS activated by acetylcholine may enhance VEGF production. Accordingly, treatment with NOS blockers attenuated the skeletal muscle VEGF mRNA response to exercise (32).

## MECHANISMS OF INDUCTION OF VEGF SYNTHESIS BY NO

### *The role of cGMP production*

NO regulates a variety of cellular functions through binding to the heme group of soluble guanylyl cyclase and production of cGMP. NO or related molecules can also directly affect target proteins through the formation of *S*-nitrosothiols at critical thiol residues in protein active sites, including those of nuclear protein transcription factors (for review, see 60). Most studies indicate that activation of guanylyl cyclase by NO did not modulate VEGF synthesis (17, 25, 29, 30, 49). In our work as well, 1*H*-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one (ODQ) and methylene blue, inhibitors of guanylyl cyclase, did not influence cytokine- or gene transfer-mediated induction of VEGF synthesis in VSMC (17, 25). In line with this observation, treatment of cells with an analogue of cGMP did not enhance VEGF production (17). Similar results excluding the role of cGMP were demonstrated in keratinocytes (29, 30), tumor cells (49), and kidney epithelial cells (73). On the other hand, in glioblastoma tumor cells, enhancement of VEGF synthesis by NO donors was mediated by cGMP (14). Thus, the effect of cGMP on VEGF production might be cell type-dependent.

### *HIF-1 transcription factor mediates the NO effect on VEGF synthesis*

NO influences VEGF transcription by mechanisms both in common with and distinct from those exerted by hypoxia. Kimura *et al.* (49, 50) recently demonstrated that NO mediates stabilization of the HIF-1α subunit in normoxic conditions in human hepatoma and glioblastoma cells, raising HIF-1 binding capacity and increasing the transcriptional rate of the VEGF promotor. Their studies indicate that the NO-responsive *cis* elements are the HIF-1 binding site (5′TACGTG; −975 bp to −968 bp) and an adjacent hypoxia ancillary sequence (5′CAGGT) located immediately downstream (−962 bp to −956 bp) within the HRE. Additionally they demonstrated that VEGF gene regulation by NO, as well as by hypoxia, was potentiated by the AP-1 element located next to the HRE (−937 bp to −932 bp) (50).

In LLK3 proximal tubular kidney cells (73), bovine pulmonary aortic endothelial cells, or rat VSMC (68), NO donors induced HIF-1α accumulation in normoxic conditions. Similarly, overexpression of NOS induced HIF-1α accumulation in LLK3 cells, suggesting a role for NO as an intracellular activator for HIF-1 (73). In contrast, NO attenuated upregulation of HIF-1α evoked by $CoCl_2$, whereas the deferoxamine-elicited HIF-1α signal remained unaltered (74).

Activation of HIF-1 by NO is independent of cGMP. Instead, NO rather stabilizes the normoxic expression of HIF-1α by *S*-nitrosylation of the VHL E3 ligase component (Fig. 2), in this way blocking its ubiquitination activity (67, 68). Interestingly, chondrocytes treated with IL-1β (iNOS inducer) or SNAP had lower collagen prolyl hydroxylase activity, and L-NMA ($N^G$-monomethyl-L-arginine, NOS inhibitor) was partially able to reverse the effects of IL-1β (13). Based on new discoveries revealing the crucial role of proline hydroxylation in HIF-1α degradation, it might be speculated that NO can also affect the HIF-1α-specific prolyl hydroxylase and in this way inhibit its activity, enhancing HIF stability and activation of VEGF promoter in normoxic conditions (Fig. 2).

NO can also influence p53 interaction with HIF-1 as p53 has been found to inhibit HIF-1-stimulated transcription by

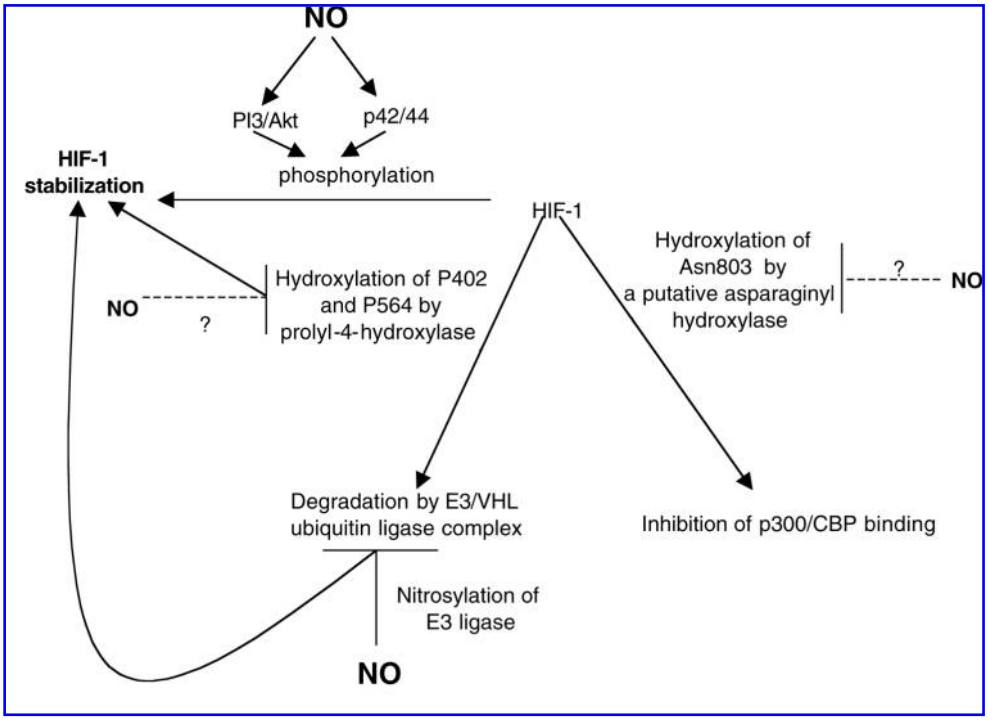


**FIG. 2. Possible mechanisms of activation of HIF-1 by NO.** HIF-1α is degraded in normoxic conditions due to hydroxylation of specific proline residues, performed by newly discovered prolyl-4-hydroxylase enzymes. We suggest that, similarly to hypoxia, NO can enhance VEGF synthesis by blocking HIF-1α degradation through inhibition of proline hydroxylation. It has been shown recently that NO also augments HIF-1α stability through nitrosylation of E3 ubiquitin ligase (67). Stabilization of HIF-1α results in increased synthesis of VEGF.

blocking the HIF/p300 transactivating effect (11) and promoting HIF-1α ubiquitination and degradation (12, 69). Loss of p53 activity resulted in increased HIF-1α expression and enhanced tumor vascularization (69). It can be speculated that a mutation in p53 somehow facilitated the NO-mediated HIF-1 activation and induction of VEGF expression in tumor cells (3).

NO can influence gene expression by direct control of iron regulatory proteins (IRP) (48, 71). IRP-1 and IRP-2 are proteins that posttranscriptionally regulate cellular iron storage and uptake by ferritin and transferrin, respectively. In conditions of limited iron supply, IRP binding to iron regulatory elements (IRE) present in the 5′ UTR region of ferritin mRNA and the 3′ UTR of transferrin receptor mRNA blocks ferritin translation and stabilizes transferrin receptor mRNA. The opposite scenario develops when iron in the labile pool is plentiful. NO caused a significant decrease in IRP-2 binding to IRE and induced IRP-2 degradation, and in this way decreased the transferrin mRNA level and increased ferritin synthesis (48). NO can also facilitate iron removal from ferritin, a process recently demonstrated to be dependent on glutathione (84). NO is thereby capable of depleting intracellular iron storage, imitating the effect of iron chelators. As depletion of iron by chelators is known to activate VEGF expression through HIF-1 stabilization (62), one can speculate that the putative influence of NO on iron metabolism contributes to the observed VEGF increase.

In addition to the involvement of HIF-1α, VEGF gene regulation by NO is potentiated by the AP-1 responsive element of the gene promoter (49) (Fig. 3). We also observed that SNAP treatment augmented AP-1 binding in VSMC (17). However, another report showed the inhibitory influence of SNAP on AP-1 activity in VSMC (81). Indeed, numerous studies showed that the effect of NO on this transcription factor appears to be cell type-specific and NO concentration-dependent (60); this might account for the discrepant results. Further studies are necessary to elucidate the role of AP-1 in NO-mediated VEGF induction.

## *The role of phosphatidylinositol 3-kinase (PI3)/Akt and p42/44 kinases in NO-mediated induction of VEGF synthesis*

Several mechanisms upstream of activation of transcription factors by NO have been proposed. It has been shown that NO and TNF-α signaling in LLC-PK$_1$ cells required phosphorylation events, especially activation of the PI3/Akt, whereas activation of HIF-1 by deferoxamine did not involve Akt kinase (12, 72, 74).

The role of p42/44 kinase in VEGF regulation has been extensively studied by Pouyssegur *et al.* (for review, see 9). Analyzing the activation of VEGF promoter coupled to a luciferase reporter gene, they found that sequences between −88 and −66 (upstream of the transcription initiation site) were absolutely necessary for basal and p42/44 kinase-stimulated promoter activity. In this region a chain of the following sequences is present: Sp1 (5′GGGCGG), AP-2 (5′GCCGGG), and Sp1 (5′GGGCGG). All three of those

binding sites were required for normal VEGF induction (70). Additionally, p42/44 was found to phosphorylate HIF-1α protein directly to enhance HIF-1 transcriptional activity. The kinase p42/44 can be activated by NO (6, 47), and our data point to the contribution of this kinase in NO-mediated VEGF synthesis in VSMC. PD98059, a specific inhibitor of this kinase, diminished VEGF synthesis in cells stimulated with cytokines or in VSMC overexpressing the eNOS gene (25), so possibly the effect of NO on HIF-1-mediated VEGF synthesis can be exerted through this signaling pathway (Fig. 3).

## HEME OXYGENASE-1 (HO-1)— A MODULATOR OF NO-INDUCED VEGF SYNTHESIS

HO-1 is a stress-inducible enzyme, generating carbon monoxide (CO), iron, and biliverdin from its substrate heme (for review, see 59). HO-1 expression is induced or enhanced in response to numerous stimuli, including NO and reactive oxygen species (ROS) (for review, see 26, 59). Interestingly, we demonstrated that NO-mediated up-regulation of VEGF expression in VSMC is dependent on the induction of HO-1 (24). Inhibition of HO activity was associated with reduction of VEGF synthesis, whereas cells over expressing HO-1 produced more VEGF than did cells transfected with control plasmids (23, 24, 44). In line with our observations, it has been reported recently that VEGF expression in placenta of pregnant rats was greatly enhanced after transfection with HO-1 adenoviral vectors (53).

As HO-1 and iNOS are induced in hypoxic conditions (26, 65), we aimed to elucidate the role of those metabolic pathways in hypoxia-induced VEGF synthesis. Unlike in normoxic conditions, induction of iNOS expression seems not to play a role in hypoxia-mediated induction of VEGF production. In contrast, inhibition of HO activity abrogated hypoxia-induced VEGF generation (24). These results indicate that HO products are involved in enhancement of VEGF synthesis in normoxia as well as in hypoxia, whereas the hypoxic pathway does not necessarily involve NO.

We investigated the possible role of all HO-1-derived compounds in modulating VEGF synthesis. Neither biliverdin nor bilirubin influenced basal VEGF production. Iron ions (both $Fe^{2+}$ and $Fe^{3+}$), the other product of HO-1 activity, attenuated VEGF generation. As the stimulatory effect of HO-1 induction or overexpression has been observed, it can be suggested that CO enhances VEGF synthesis more potently than iron inhibits it. In accordance with such a hypothesis, cells kept for 24 h in an atmosphere containing 1% CO generated significantly more VEGF than VSMC did in normoxic conditions (23, 24).

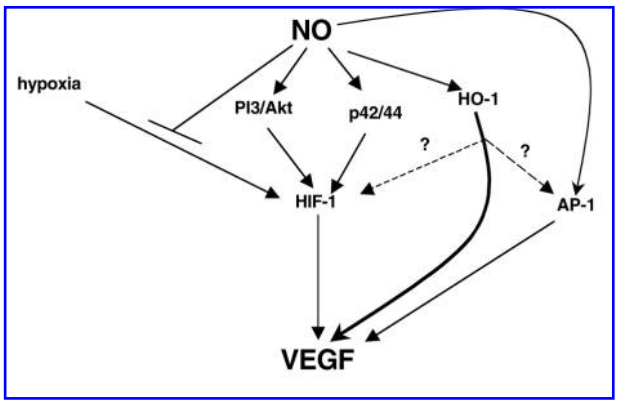


**FIG. 3. The effect of NO on VEGF synthesis.** In normoxic conditions, NO can activate VEGF expression influencing HIF-1 and AP-1 transcription factors. The intermediate pathway involves protein kinases p42/44 and PI3/Akt, as well as HO-1. In hypoxic conditions, NO may inhibit hypoxia-induced stabilization of HIF-1, but the effect appears to be cell type-dependent (see text for discussion).

## CONTROVERSIES ABOUT THE REGULATION OF VEGF SYNTHESIS BY NO

Besides numerous studies demonstrating the stimulatory effect of NO on VEGF synthesis, there are, however, other articles showing the opposite results. Here we discuss those data, suggesting possible explanations for the observed discrepancies.

First, the effect of NO on VEGF synthesis could be cell type-dependent, although this is not always the case, as VEGF synthesis in rat VSMC was inhibited (81) or enhanced (21, 43) by NO-releasing substances. Tsurumi *et al.* (81) have shown that VSMC treated with SNP or SNAP expressed less VEGF than control cells did. In our work, however, VSMC treated with different NO donors generated more VEGF, with the exception of cells stimulated with SNP, which was inhibitory. As SNP also releases iron and cytotoxic cyanides (for references, see 21, 51), the inhibitory effect of this substance is not surprising and should not be regarded as an indication of the attenuating action of NO on VEGF expression.

Another explanation for the discrepant data may rest on the different effects of various NO donors used in the experiments. Indeed, the inhibitory effect of NO has been reported when SNP was used as the source of NO (34, 51, 81, 82). We have tested several compounds and found that SNAP, SIN-1, DETA, and GSNO enhanced VEGF synthesis, whereas only SNP was inhibitory (Fig. 1). Similarly, the stimulatory effect of several NO donors, but not SNP, was observed when HIF-1 activation was investigated (51). *In vivo* the NO-dependent effect of SNP may be predominant, as SNP augmented VEGF synthesis in rat skeletal muscles (8).

Alternatively, the effect of NO can differ in normoxic and in hypoxic conditions, as we suggested recently (18). Several reports demonstrated the inhibitory effect of NO on HIF-1 activity (33, 34) and VEGF synthesis in hypoxia (1, 34, 36, 58). Again in some experiments performed under hypoxia, SNP was used as the source of NO (36, 58, 78). Inhibition of VEGF expression by NO in hypoxia may be attributed to the higher concentration of NO released from NO donors under hypoxia than under normoxia, as exposure to excessive amounts of NO could be toxic (37). The effect of NO on hypoxia-induced VEGF can be also cell type-dependent: GSNO at low concentrations (50 μ*M*) attenuated activation of

HRE in VSMC (58), whereas at much higher concentrations (500 μ*M*) GSNO enhanced VEGF promoter activity in A-172 glioblastoma cells (51). Generally, however, very high concentrations of NO donors appear to inhibit VEGF expression, although exceptions can occur, as in the case of NOR4 (51). The same may apply to the observed inhibitory effect on VEGF or HIF-1 of extremely high concentrations of CO (5, 10, or 80%) applied to cells growing in hypoxia (36, 58, 78).

Furthermore, Yin *et al.* (87) reported that iNOS overexpression in hypoxic C6 glioma cells inhibits HIF-1 activity and VEGF transcription. Pretreatment of C6 cells with *N*-acetyl-L-cysteine nullified the inhibitory effect of iNOS, suggesting the involvement of ROS in hypoxia/NO-mediated HIF-1 inhibition (87). In other studies as well, ROS were claimed to be responsible for NO-mediated inhibition of HIF-1 accumulation in hypoxia (2).

On the other hand, it may be suggested that contradictory data on the effect of NO in VEGF synthesis are due to the action of the different NOS isoforms (76). However, our studies demonstrated that VEGF expression can be enhanced both by low doses of NO (such as those generated by eNOS) and by higher amounts of NO derived from iNOS (21, 43).

The effect of targeted disruption of iNOS and eNOS can be different *in vivo.* Indeed, opponents of the stimulatory role of NO in VEGF synthesis are supported by the results of some *in vivo* experiments. Recently, Matsunaga *et al.* (61) reported enhancement of VEGF synthesis after systemic (oral) L-NAME treatment in ischemic hearts of experimental dogs. Zhao *et al.* (90) demonstrated that L-NAME enhanced VEGF synthesis in rat myocardium, whereas Kang *et al.* (46) showed similar activity in kidney. Those experiments suggest that NO inhibits VEGF synthesis *in vivo,* but we propose that blocking of NO generation *in vivo* may not necessarily attenuate production of VEGF, if a compensatory stimulatory pathway is induced concomitantly. Thus, the final output of NO inhibition on the VEGF expression *in vivo* may be a sum of direct and indirect effects influenced by decreased NO synthesis. In fact, inhibition of NO generation *in vivo* raises blood pressure due to the augmentation of angiotensin II (Ang II) synthesis (for review, see 42). Ang II has been shown to induce redox-sensitive transcription of VEGF in vascular wall cells (85). The effect of Ang II might be mediated by enhanced production of hydrogen peroxide, which was demonstrated to increase VEGF expression and protein synthesis (54).

The same mechanism may apply to eNOS knockout mice, which had higher VEGF levels under nonstimulated conditions than wild-type ones (16). Mice with the targeted eNOS gene are hypertensive and have increased renin–angiotensin system activity (77).

Another compensatory mechanism, increasing VEGF synthesis while NO generation is blocked, can operate when iNOS expression is inhibited by oxidized low-density lipoprotein (oxLDL) (19, 22). The mechanism behind such an effect is under investigation. As HO-1 expression is induced by oxLDL, Ang II, and hydrogen peroxide (59), it might be supposed that HO-1-derived CO stimulates VEGF synthesis.

## CONCLUSIONS

A growing body of evidence indicates that NO is both an upstream and a downstream mediator of VEGF-dependent angiogenesis (Fig. 4). It is well demonstrated that VEGF enhances NO generation by binding to its receptor(s) on endothelial cells. It is possible that such released NO can reach the underlying smooth muscle cells, or keratinocytes (in wounds), or macrophages and tumor cells (in tumors), and in this way may stimulate VEGF synthesis. Hence, the positive feedback may be involved in the synthesis and activity of VEGF (Fig. 4). Such a relationship may be implicated in the potent enhancement of angiogenesis in tumors.

In recent years, plenty of evidence has accumulated demonstrating that NO can enhance VEGF synthesis in different cell types. It has been shown that NO influenced the activity of

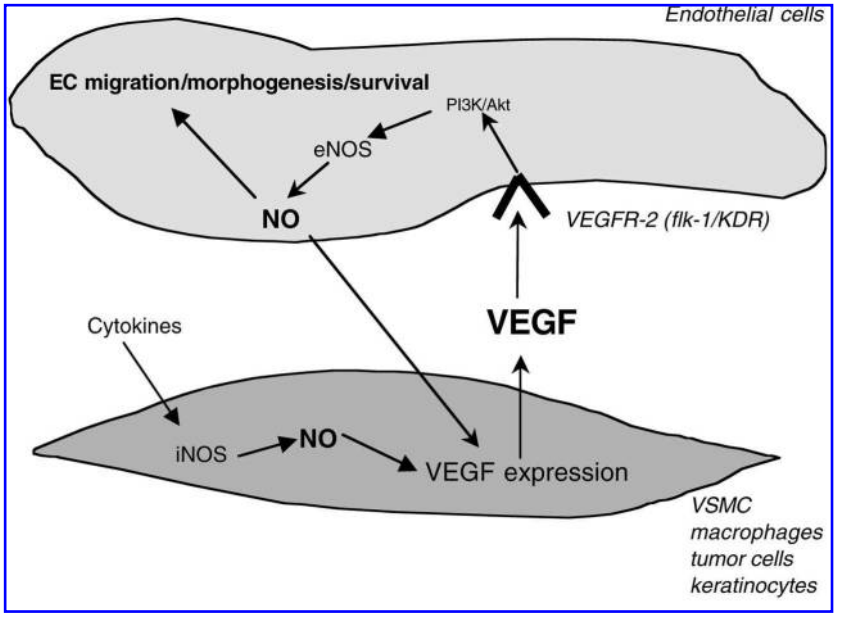


**FIG. 4. Possible reciprocal relationship between VEGF and NO.** NO is an upstream and downstream modulator of VEGF-mediated angiogenesis. NO induces VEGF synthesis in numerous cell types. The angiogenic effect of VEGF exerted on endothelial cells (EC) is dependent on NO production by activated eNOS. It is postulated that endothelium-derived NO can augment angiogenesis by stimulating VEGF production in adjacent cells.

HIF-1 and AP-1 transcription factors, resulting in increased VEGF transcription. Activation of p42/44 kinase and Akt kinase appears to mediate those effects. However, cGMP, the classical signaling molecule of the NO pathway, is not involved in those processes, but S-nitrosylation of VHL E3 ligase appears to be a prerequisite for stabilization of HIF-1α.

Our recent data indicate that HO-1 may be a modulator of NO-induced VEGF synthesis and actually may provide both stimulatory (CO) and inhibitory (iron) signals. Thus, HO-1 activity may represent an intricate, elaborate modulatory pathway governing both the stimulatory and inhibitory arms of stress-induced VEGF synthesis. The existence of both positive and negative feedback loops is known in many signaling networks (10). Further studies are needed to clarify the molecular feedback mechanisms governing NO–HO–VEGF relationships.

Numerous *in vitro* and *in vivo* data support the stimulatory effect of NO on VEGF synthesis. We have discussed the conflicting reports that showed the inhibitory effect. The NO-independent inhibitory effects of the compounds used can explain some of the controversies. Additionally, the NO effect has often been tested under the conditions of decreased oxygen tension, but the influence of NO in hypoxia probably differs from that in normoxia (18).

Augmentation of VEGF synthesis is being tested as a way to accelerate blood vessel formation in ischemic tissues (88). Genetic augmentation of NO-generating enzymes may offer a possible therapeutic strategy for treatment of impaired angiogenesis in cardiovascular diseases or for enhancement of wound healing. Further studies using more targeted delivery of well defined molecules releasing NO, or applying regulated overexpression or disruption of NOS genes, should be helpful in elucidating the role of NO in VEGF production, and may lead to therapeutic applications.

## ACKNOWLEDGMENTS

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## ABBREVIATIONS

Ang II, angiotensin II; AP-1 and AP-2, activator protein-1 and -2, respectively; carboxy-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt; cGMP, 3′5′-cyclic guanosine monophosphate; CO, carbon monoxide; eNOS, endothelial nitric oxide synthase; GSNO, *S*-nitrosoglutathione; HIF-1, hypoxia-inducible factor; HO-1, heme oxygenase-1; HRE, hypoxia-responsive element; IL-1β, interleukin-1β; iNOS, inducible nitric oxide synthase; IRE, iron regulatory elements; IRP, iron regulatory proteins; L-NAME, $N^{\omega}$-nitro-L-arginine methyl ester; NO, nitric oxide; NOS, nitric oxide synthase; $O_2^-$, superoxide anion; oxLDL, oxidized low-density lipoprotein; PGE, prostaglandin E; $PGI_2$, prostacyclin; PI3, phosphatidylinositol 3-kinase; ROS, reactive oxygen species; SIN-1, *S*-morpholinosydnonimine; SNAP, *S*-nitroso-*N*-acetylpenicillamine; SNP, sodium nitroprusside; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α; UTR, untranslated region; VEGF, vascular endothelial growth factor; VHL, von Hippel–Lindau; VSMC, vascular smooth muscle cells.

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