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Forum Review

Constitutive and Inducible Nitric Oxide Synthase: Role in Angiogenesis

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ABSTRACT

Since the initial report of nitric oxide (NO) activity, enormous progress has been made over the last two decades in the field of NO research. Whereas most physiological responses triggered by moderate concentrations of NO are mediated by soluble guanylate cyclase activation and the subsequent production of cyclic GMP as the major signaling messenger, recent studies have provided evidence of alternative signaling pathways triggered by high concentrations of NO. These signals operate in part through redox-sensitive regulation of transcription factors, gene expression, transcription, cellular activation, proliferation, and cell death. Numerous results converge to indicate a role for NO in physiological and pathological angiogenesis. Experimental data indicate that NO synthase, depending on the isoforms, the timing, and the degree of activation, may display contradictory effects, expressed during both physiological and pathological angiogenesis. The dual personality of NO will be reviewed in the context of the angiogenesis process. *Antioxid. Redox Signal.* 4: 817–823.

INTRODUCTION

In mammals, the synthesis of nitric oxide (NO) is catalyzed by nitric oxide synthase (NOS), which exists in three distinct isoforms (32). Under physiological conditions, the concentration of NO fluctuates at rather low levels controlled by constitutively expressed endothelial (eNOS) and neuronal (nNOS) isotypes. Under pathological conditions (*e.g.*, during inflammation), temporarily high levels of NO are produced in the body after induction of the expression of the inducible type of NOS (iNOS). iNOS can be induced by endotoxin and by inflammatory cytokines such as interleukin 1 or tumor necrosis factor-α in macrophages and many other cell types (26, 32) and requires a delay of 6–8 h before the onset of NO production. However, once induced, this enzyme is active for hours to days and produces NO in 1,000-fold larger quantities than the constitutive isotypes.

At low concentrations, NO stimulates guanylate cyclase activity and induces the formation of cyclic GMP (cGMP), which mediates most of the physiological functions of NO, such as the control of vascular homeostasis, hormonal control, the male sexual function, platelet aggregation, and neurotransmission (6, 50, 51). Temporarily high levels of NO produced by iNOS are responsible for the antibacterial, antiparasital, antiviral, and tumoricidal effects of NO (6, 26, 41). In conditions in which high NO concentrations are produced, recent studies have provided evidence of alternative signaling pathways beside the classical NO–cGMP signaling. When persistent high amounts of NO are produced, NO can react with oxygen, generating nitrogen oxide intermediates (RNOI) or with concomitantly produced superoxide anions, generating toxic compounds such as peroxynitrite and hydroxyl radicals. These signals play a key role in the redox-sensitive regulation of gene expression, transcription, cellular activation, proliferation, and cell death.

This review will focus on the role of the NO in angiogenesis. Depending on its concentration, the duration, and the context of its synthesis, NO can act as a universal intercellular messenger that affects important signaling pathways and, on a more long-term scale, modulates gene expression or can

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cause nitrosative stress by intracellular redox regulation. The two molecular mechanisms by which NO affects cell signaling will be considered in angiogenesis.

CONSTITUTIVE NOS AND ANGIOGENESIS

Angiogenesis indicates the mechanism of blood vessel formation in the adult. The steps involved for new vessel growth are biologically complex and comprise rapid modifications occurring in minutes, including modification of cell–cell interaction, changes in permeability, and vasodilation, and structural changes that require longer time (from hours to days), including degradation and remodeling of extracellular matrix proteins surrounding preexisting vessels, migration and proliferation of endothelial cells, apoptosis, and/or survival of vascular cells. NO derived from the endothelium has been shown to be involved in postdevelopmental vascular remodeling and angiogenesis, as well as in the formation of limb vasculature during embryogenesis (38). Works from our laboratory and others have demonstrated that the constitutive NOS activity is involved in all the steps of the angiogenesis process.

A very early event in capillaries undergoing angiogenesis is vasodilation, a process involving constitutive NO production. Experimental evidence of a molecular/biochemical link between vasodilation and angiogenesis comes from the observation that many vasodilating peptides possess angiogenic properties (29, 36, 53). We demonstrated that NO can act as a modulator of angiogenesis *per se,* mediating endothelial cell growth and migration *in vitro* and *in vivo.* Mediators of different chemical natures that activate the NOS pathway, like substance P and bradykinin, as well as NO donors, promoted endothelial cell proliferation and migration *in vivo* and *in vitro,* whereas inhibitors of NOS suppressed these responses (52, 53). NO inhibits vascular smooth muscle cell proliferation (6, 12) and migration (34) and stimulates endothelial cell mobilization and reorganization (33, 53), phenotypes necessary for vascular remodeling. Genetic evidence in support of endothelium-derived NO as a major regulator of vascular architecture stems from the inability of NOS knockout mice to remodel their carotid arteries in response to a decrease in blood flow (38).

The secreted endothelial-specific growth factor, vascular endothelial growth factor (VEGF), is known to initiate and participate in the complete angiogenesis process *in vivo* (10). Vascular permeability, an early step of the angiogenesis process, is increased in response to VEGF and is mediated by a redistribution of adhesion molecules on the endothelial cell surface. VEGF effects on permeability and vascular tone are coupled with eNOS activation (21, 47), and we demonstrated that NO production significantly contributed to the angiogenesis activity of VEGF. Postcapillary endothelial cell mobilization and growth and *in vivo* angiogenesis induced by VEGF were blocked by the NOS inhibitor N^G-methyl-L-arginine (L-NMMA) and by the guanylate cyclase inhibitor LY 83583 (28, 36).

The NOS pathway contributes to the angiogenic switch of endothelial cells at various steps of the cascade triggered by VEGF. The activation of the mitogen-activated protein kinases induced by VEGF is dependent on the activation of eNOS (36). The cGMP-dependent protein kinase (PKG) mediating VEGF-induced raf-1 and extracellular signal-regulated kinase-1/2 (ERK-1/2) activation (14, 36) lies downstream from the activation of constitutive NOS.

The NO–cGMP signaling pathway not only mediates the growth-promoting activity of VEGF, but can also affect the transcriptional events involved in endothelial cell differentiation and survival. The serine/threonine kinase Akt is an important regulator of various cellular processes, including metabolism and cell survival (5). Recently, Morales-Ruiz and collegues demonstrated that the ability of VEGF to stimulate cell migration and actin rearrangement in microvascular endothelial cells is mediated by the Akt/eNOS pathway (27). The relevance of the VEGF/Akt/eNOS pathway is further supported by the observations that inhibition of NOS blocks VEGF-induced NO production, endothelial cell migration, formation of endothelial tube-like structures *in vitro,* and angiogenesis *in vivo* (22, 36, 53, 54). Once assembled in new vessels, endothelial cells become quiescent and survive for years. The importance of endothelial survival is demonstrated by findings that vascular regression in the embryo occurs as a result of conditions affecting cell survival (3). In this contest, the contribution of NO in the VEGF-mediated prosurvival/proangiogenic program of capillary endothelium plays an essential role. Gene manipulation demonstrated that VEGF-induced angiogenesis is markedly attenuated in eNOS knockout mice (31). Despite the high number of components of the intracellular pathway mediating the prosurvival/proangiogenic program of VEGF via NO, the molecular mechanisms responsible for endothelial survival and angiogenesis still need to be elucidated. We demonstrated that NO contributes to the angiogenic process by inducing endogenous fibroblast growth factor-2 (FGF-2) in capillary endothelium. Even though FGF-2-induced angiogenesis occurs independently of NO production, NO exert its prosurvival effect through the up-regulation of FGF-2 production by endothelial cells, which in turn stimulates proliferation and urokinase-type plasminogen activator expression (54). Consistently, NO mediates endothelial cell–matrix interaction via the expression of $\alpha v\beta 3$ integrin (23). Thus, during the angiogenic switch, the production of NO controlled by the constitutive NOS appears to regulate an autocrine loop in microvascular endothelium, which involves minute/tonic NO production, cGMP elevation, and endogenous FGF-2 expression ultimately leading to modulation of cell survival (Fig. 1).

INDUCIBLE NOS AND ANGIOGENESIS

Increased NO levels have been found in numerous inflammatory diseases, as well as in human tumors (4, 11, 19, 20, 45). Most of the cellular components of the tumor mass (the tumor cells themselves, the immune cell infiltrate, and the stromal cells) have been shown to generate NO *in vitro.* Although a body of evidence indicates a role for NO in tumor growth, the end point of NO up-regulation is matter of dispute because increased NO levels have been equally documented to favor and to impair tumor progression.

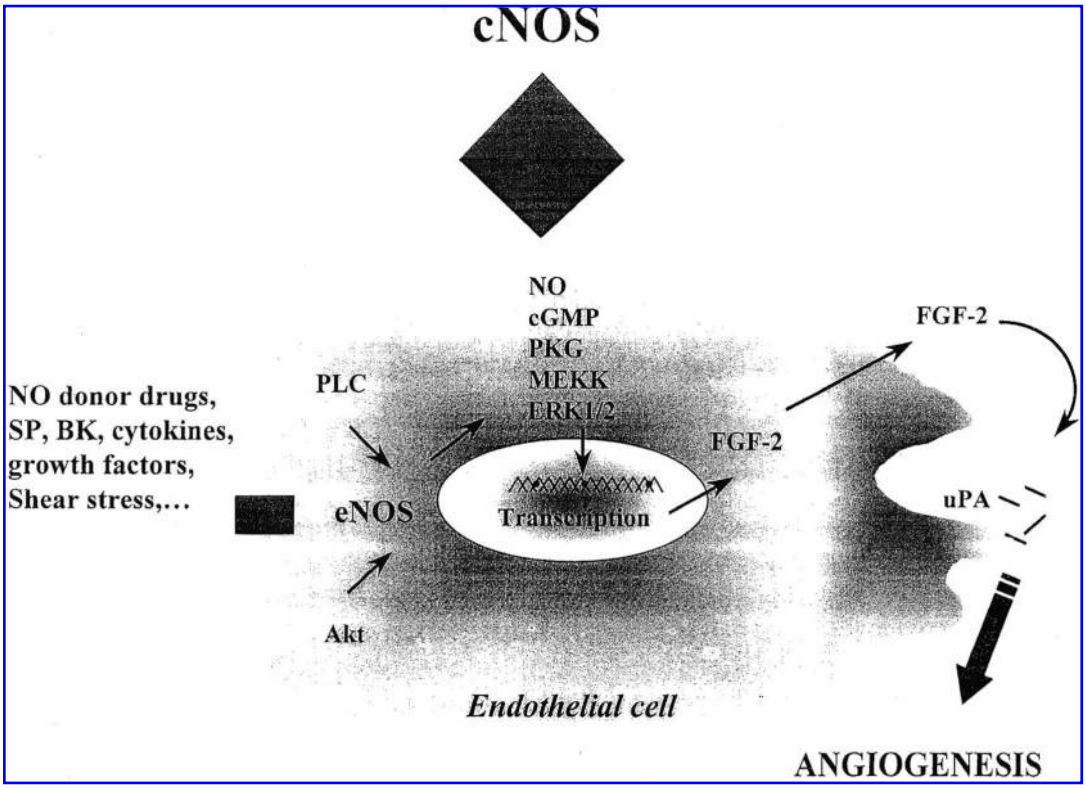

FIG. 1. Schematic representation of the described signaling pathway activated by constitutive eNOS in angiogenesis. Following the stimulation of specific receptors for angiogenic factors or vasoactive substances on endothelial cell surface, eNOS is activated and NO produced. NO can stimulate soluble guanylate cyclase (sGC) in an autocrine manner in endothelial cells or diffuse outside. Following the stimulation of sGC, cGMP is formed and activates kinase cascades including the PKG and the mitogen-activated protein kinase MEKK and ERK1/2, leading the membrane-linked signal to the nuclear level. The transcription of specific genes, as well as the growth factor FGF-2, leads to the cellular events associated with angiogenesis as proliferation, migration and matrix degradation or cell survival. BK, bradykinin; PLC, phospholipase C; SP, substance P; uPA, urokinase-type plasminogen activator.

In experimental models, NO facilitates tumor growth and vascularization. Transfection of the iNOS into a colon adenocarcinoma line gave a cell line that promoted tumors that grew more rapidly and were more vascularized than wild-type cells (16). The enhancement of tumor growth and angiogenic potential *in vivo* resulting from the transfection of VEGF into a human breast carcinoma cell line was selectively linked to the activation of the NOS pathway (54). Other observations in agreement with NO being a specific signal for tumor vascularization show that blocking iNOS activity retarded the growth of xenografted tumors (17, 35). Conversely, an excessive production of NO sustaining tumor growth was correlated with *in vitro* invasive ability of tumor cells and *in vivo* induced tumor angiogenesis (9, 15). Furthermore, administration of the selective NOS inhibitors markedly reduced the *in vivo* growth of the iNOS-transduced human colonic adenocarcinoma and the iNOS-expressing EMT6 mammary tumor (46) and blocked the angiogenic activity of iNOS-expressing murine endothelioma and of the VEGF transfectants (29, 54). At the clinical level, we found increased iNOS activity in human head and neck cancer specimens, which was associated with elevated cGMP levels and correlated with tumor vascularization. *In vivo* experiments demonstrated that transplant of tumor samples and cell line from squamous cell carcinoma produced angiogenesis in the rabbit cornea, which was made to regress by treatment with NOS inhibitors (11).

Several reports have also indicated that an increased production of NO can result in a potential antitumor strategy. Cell lines engineered to produce very high levels of NO have been shown to cause lysis of bystander tumor cells once injected in proximity of a tumor mass (49). It was shown that NO and its intermediates released by macrophages reduced tumor cell survival and induced tumor cell death (48, 49). It has also been shown that iNOS-overexpressing murine melanoma cells have poor growth and survival *in vitro* and *in vivo,* losing their ability for metastasis *in vivo.* The antitumor function of NO has been attributed to RNOI. NO can react with molecular oxygen, transition metals, and superoxide, resulting in intermediates that are responsible for cell injury. In the presence of superoxide anion, NO is converted into peroxynitrite, a highly potent toxic molecule (1) (Fig. 2).

Overall the dual function of NO in tumor progression is not contradictory. Several interpretations can be proposed: (a) the source of NO generation (produced by eNOS or iNOS) (6); (b) the extent of total NO-RNOI produced by the different

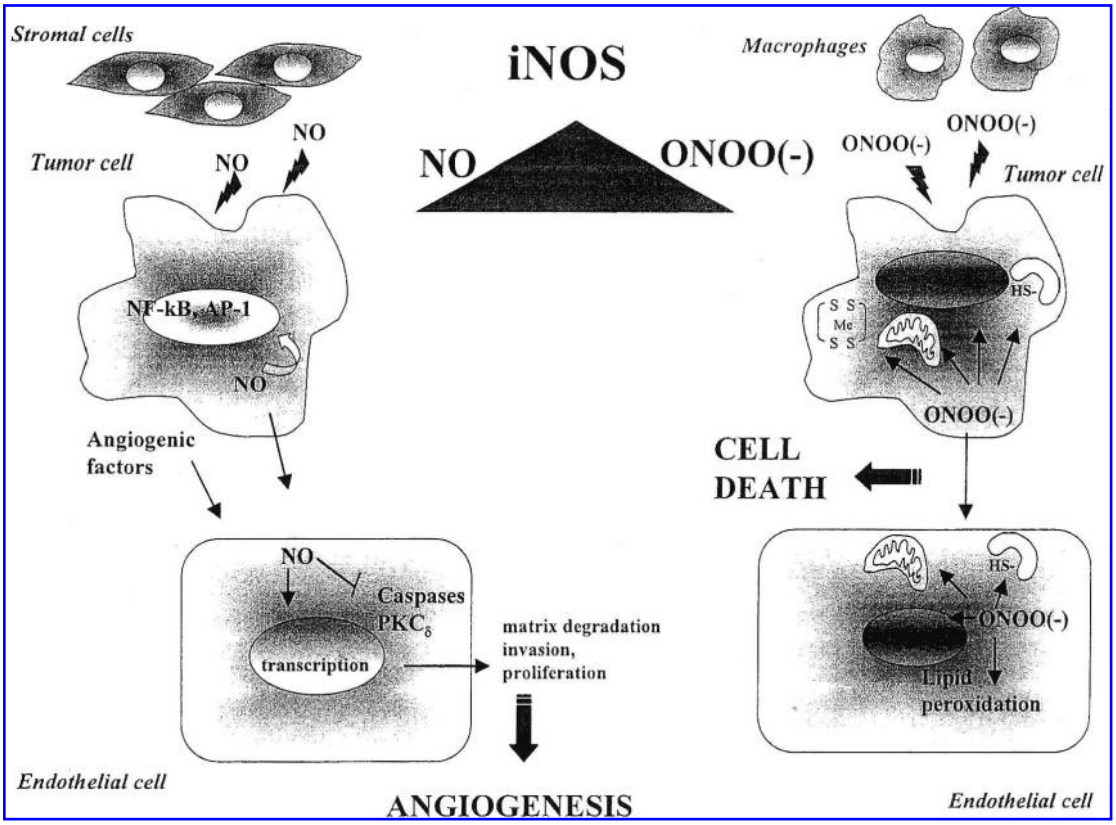

FIG. 2. Modulation of signaling cascade by iNOS activation. High levels of NO produced by the iNOS activity in tumor cells or in stromal and endothelial cells can affect different intracellular signaling pathways. NO can act on gene transcription via *S*-nitrosylation of redox-sensitive transcription factors like AP-1 and NF-κB, leading to the transcription of angiogenic factor by tumor cells. Moreover, iNOS activity can promote migration and proliferation of endothelial cells by inhibiting PKCδ activity or the endothelial cell survival by blocking the caspase signaling pathway. Conversely, high levels of NO-RNOI can induce nitrosative stress and death in endothelial and tumor cells. The molecular targets are heme moieties and proteins with metal–sulfur clusters or thiol groups, whereas the organelle targets are the nucleus and the mitochondria. ONOO(−) peroxynitrite.

cells types inside the tumor; (c) the different ratio and/or interaction between the cellular components of the tumor (stromal cells or inflammatory cells) (42); (d) the microenvironmental conditions (hypoxia or normoxia) (2); (e) the intrinsic biological difference of the tumor (*i.e.,* mutant p53 versus wild type) (25); and finally (f) the extent of cell necrosis potentially responsible for total RNOI release (37). For example, reactive oxygen species, as well as NO, have been reported to increase protein kinase C (PKC) activity (18). Recently, Shizukuda *et al.* demonstrated that VEGF-induced endothelial cell migration and proliferation depend on the reduction of PKCδ, and that this effect requires the activation of iNOS (44). Alternatively, O'Donnell and Freeman have shown that a high level of NO can potentiate inflammatory injury to vascular cells, enhancing lipid oxidation reactions, and consequently promoting endothelial cell death (34) (Fig. 2).

The role of NO and RNOI has been intensively studied in relation to apoptotic cell death. Although NO is established as a potent inducer of apoptosis, in certain cell types contradictory effects have been reported, with NO displaying antiapoptotic effects in lymphocytes, B cells, hepatocytes, and endothelial cells (8). Cells vary greatly in their sensitivity to NO, and the response of a cell can be modulated by the simultaneous release of oxygen radicals. Among the several mechanisms proposed for the antiapoptotic effects of NO, a direct inhibition of caspases by reversible *S*-nitrosylation, and therefore an interaction between NO and RNOI and the executioners of apoptotic signal transduction, has been consistently reported (13, 24). In this context, iNOS expression may exert a protectve effect and may be one of the rationales for the clinical use of NO donor drugs or inducers in cerebral ischemia and stroke, to improve vasodilation through neovessel growth, tissue reperfusion, and reoxygenation (39).

An additional mechanism for the role of NO in tumor biology is gene regulation. NO can alter transcription factors that are sensitive to changes in the cellular oxidation-reduction (redox) status. Although the molecular mechanisms by which NO affects gene expression are still poorly understood, two well defined transcription factors, nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) were shown to be regulated by the intracellular redox state (43). Single conserved cysteine residues within the DNA-binding domains of c-Fos and c-Jun, the major components of AP-1, seem to be responsible for redox regulation of AP-1 activity. Our experimental data

indicate that in a cell line from squamous cell carcinoma, iNOS activation enhances the binding activity of AP-1 and promotes the gene expression of VEGF. *In vivo,* the iNOS activation appears to be responsible for the acquisition of the angiogenic phenotype of squamous cell carcinoma (Donnini, unpublished observations).

CONCLUSIONS

Angiogenesis involves the proliferation of endothelial cells under the control of local peptide growth factor and physical forces. In microvascular endothelium, NO at moderate concentrations significantly contributes to integrate both chemical and physical influences and is involved in the growth-promoting effect of vasodilating peptides and VEGF. The angiogenic responses triggered by modulation of constitutive NOS in endothelial cells are mediated by the activation of soluble guanylate cyclase with the subsequent production of cGMP as the principal intracellular messenger.

Recent studies have provided evidence of alternative signaling pathways triggered by high concentrations of NO that might lead to apoptosis by altering transcriptional events.

In summary, NO, depending on the nature, the timing, and the degree of the enzyme activity releasing it, may display opposite effects on endothelial cells resulting in physiological and/or pathological angiogenesis. Thus, at the clinical level, the manipulation of NO levels by the use of pharmacological strategies, selected to minimize the negative effects and maximize the beneficial effects, can be exploited for a variety of angiogenesis-dependent diseases.

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ABBREVIATIONS

AP-1, activator protein-1; cGMP, cyclic GMP; eNOS, endothelial nitric oxide synthase; ERK, extracellular signal-regulated kinase; FGF-2, fibroblast growth factor-2; iNOS, inducible type of nitric oxide synthase; NF-κB, nuclear factor-κB; ; NO, nitric oxide; NOS, nitric oxide synthase; PKC, protein kinase C; PKG, cyclic GMP-dependent protein kinase; RNOI, nitrogen oxide intermediates; VEGF, vascular endothelial growth factor.

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