REVIEW ARTICLE

Molecular regulation of tumour angiogenesis by nitric oxide

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ABSTRACT. As tumors grow, their original vasculature can be insufficient to supply the growing tissue mass, and consequently local hypoxia develops. Thus neovascularisation is a key feature determining growth and metastasis of malignant tumors. This is, at least in part, mediated by humoral factors known to stimulate angiogenesis, such as vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2). Among the multiple angiogenic modulators released by tumor and stromal cells, a key role is played by nitric oxide (NO). Beside its capacity to regulate permeability and blood flow, NO has been reported to exert angiogenic properties in various tumor models. The focus of this review will be the proangiogenic role of NO in the tumor microenvironment and its multiple mechanism of action on vascular endothelium. Particular attention will be devoted to the role of NO in regulating metalloproteinase activity on cultured microvascular endothelium and in the *in vivo* rabbit cornea assay. Finally, the potential clinical outcomes and expectations related to this topic will be discussed.

Keywords: angiogenesis, endothelial cell, metalloproteinases, nitric oxide, tumor

FEATURES OF TUMOR VASCULARISATION

Angiogenesis is the main process mediating the expansion of the blood vessel network during development, tissue regeneration and in pathological conditions such as cancer.

The growth of new blood vessels is regulated by a sequential cascade of cellular events which involves:

– the directional sprouting of outgrowing endothelial cells to form a solid cord

– their attractive and repulsive positioning with subsequent network formation and establishment of flow

– the maturation of the resulting vasculature with recruitment of periendothelial mural cells and acquisition of the quiescent vascular phenotype. This series of events is associated with distinct endothelial phenotypes and corresponding molecular signatures designed as tip cells (invading lamellipodia- and filopodia-rich cells), stalk cells (following remodelling and lumen-forming cells) and phalanx cells (quiescent endothelial cells).

In tumors, the expanded vasculature provides nutrients required for tumor growth, but the newly formed and remodelled blood vessels have multiple abnormalities that distinguish them from normal vessels, and limit their efficiency. Tumor blood vessels are highly irregular, tortuous, have arterio-venous shunts, blind ends, lack smooth muscle and innervation, and have incomplete endothelial linings and basement membranes. All components of the vessel wall, including endothelial cells, pericytes and basement membrane are indeed abnormal. Structural defects results in impaired endothelial barrier function, vessel leakiness, poor blood flow, hypoperfusion and increased interstitial pressure, thus limiting the diffusion and efficacy of anticancer drugs.

Typical tumor angiogenic factors such as vascular endothelial growth factor (VEGF), and fibroblast growth factors (FGFs) are able to drive the early stages of angiogenesis and induce the abnormal vessel phenotype. The process is nonetheless controlled by the balance of multiple and complex angiogenic factors and inhibitors. Among them, nitric oxide (NO) has been reported to contribute to tumor biology and vascularization with multiple cellular and molecular effects and sometimes with divergent properties. Examining the role of NO in angiogenesis and tumor cell development can help the design of novel drugs potentially aimed at producing vessel normalization thus improving drug delivery and anticancer treatments.

NO AND CANCER

After the initial discovery of NO, several actions in both physiology and pathological conditions have been attributed to this gaseous mediator. Most of them are divergent, depending on the concentration, the duration of its release, the cell type and the presence of scavengers or

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other reactive molecules in the microenvironment, which may impair or otherwise amplify the effects of NO.
In the last three decades, the role of NO in tumor cell biology and tumor angiogenesis has been firmly established. However, many articles have been published suggesting contradicting results (see [1-4] as recent reviews).
A solid tumor consists of cancer cells and host-derived cells, including tumor-infiltrating leucocytes and cells of the tumor vasculature, especially endothelial cells. One or more of these cellular constituents may be responsible for the production of NO in the tumor microenvironment [5] *(figure 1)*. Functional roles for tumor-derived NO in cancer progression and spreading represent a complex combination of NO-mediated effects on tumor cell proliferation and invasiveness and the functions of immune/stromal cells infiltrating tumors. It has been proposed that NO also promotes tumor growth by regulating tumor blood flow and maintaining the vasodilated tone of tumor microvasculature. In addition to angiogenesis stimulation, NO can promote metastasis by increasing vascular permeability, and up-regulating matrix metalloproteases (MMPs). Recently, it has been reported that NO released by metastatic tumor cells may impair the immune system, which facilitates their escape from immunosurveillance and metastasis of tumor cells [2, 6]. Moreover, an association between NO production, resistance to chemotherapeutic drugs and angiogenesis has been demonstrated [7].
In this intricate scenario, the present review will be particularly focused on the proangiogenic role of NO in tumor angiogenesis, providing experimental data in support of it.

NO AND NO SYNTHASES (NOS) INVOLVED IN TUMOR ANGIOGENESIS

NO is a short-lived, gaseous, free radical that is produced by the activity of specific NOS isoforms starting from the precursor L-arginine [8]. NO is a highly diffusive, hydrophobic molecule and it is therefore a key signalling molecule in inflammation-driven diseases including cancer [9].
For a comprehensive description of NO biosynthesis and NOS isoform regulation and expression we refer to pertinent literature [10]. In this review, particular attention will be paid to the endothelial constitutive (eNOS) and inducible (iNOS) isoforms. The first is mainly present on vascular endothelium and produces nanomolar amounts of NO in a calcium-dependent manner. The inducible isoform is overexpressed in most of the solid tumors

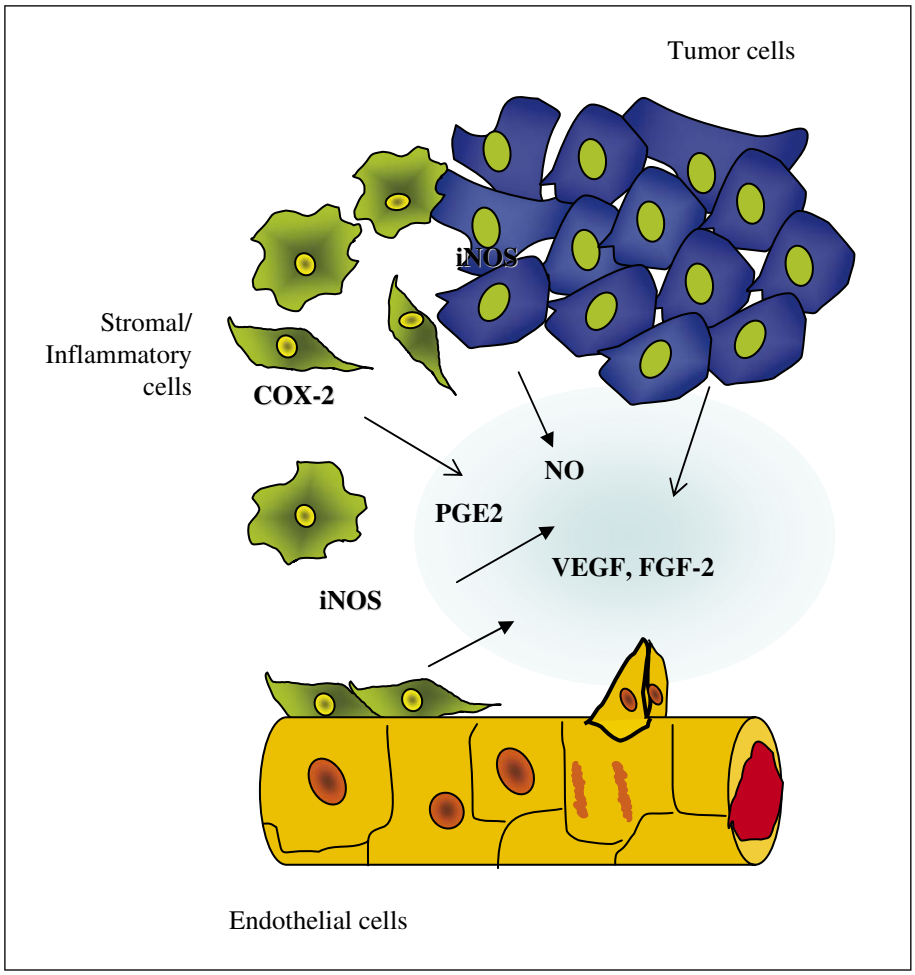

Figure 1
Interplay between angiogenic factors, prostanoids and nitric oxide produced and released by stromal/inflammatory and tumor cells in promoting angiogenesis. The tumor microenvironment is particularly rich in these factors (as VEGF, FGF-2, NO, PGE_2), and many of these mediators cooperate and synergize in stimulating endothelial cells toward an angiogenic phenotype.

analyzed so far, and produces micromolar concentrations of NO in a calcium-independent manner. The distinction, however, is not so clear since there is evidence that iNOS can be induced in the endothelium by for example inflammatory cytokines, while many tumor cells express eNOS [11]. This finding highlights the problem of lack of efficacy by NOS inhibitors designed to be specific for the various synthases and proposed as antitumor strategies.

NO CONTRIBUTION TO VASCULAR BIOLOGY

NO contributes to cardiovascular regulation by multiple mechanisms, such as vascular tone (vasodilation), vascular remodelling (inhibition of smooth muscle cell proliferation), and cell-cell interactions in blood vessels (inhibition of platelet adhesion and aggregation; inhibition of monocyte adhesion) [12]. NO is involved in the regulation of basal systemic, coronary, and pulmonary vascular tone through the production of cyclic guanosine 3′,5′-monophosphate (cGMP) in smooth muscle cells, inhibition of the vasoconstrictor peptide endothelin-1, and inhibition of norepinephrine release from sympathetic nerve terminals [12].

eNOS-dependent NO production has been also shown to contribute significantly to the endothelium-protective effect of vasodilating peptides (as substance P and bradykinin) [13-15], drugs such as angiotensin-converting enzyme inhibitors [16] and growth/vasopermeabilizing factors such as VEGF [17].

MULTIPLE ROLES OF NO IN TUMOR ANGIOGENESIS

Early experimental studies have shown that induction of iNOS in tumor cells promotes angiogenesis (by upregulating VEGF expression), which increases microvascular density and tumor progression [18-22]. Inhibition of NOS, genetically or with pharmacological agents, has been shown to reduce VEGF levels and inhibit tumour angiogenesis [23-26].

The strongest data supporting a fundamental role for NO in tumor angiogenesis come, however, from the histological examination of tumor specimens, revealing a significant relationship between high angiogeneic activity (*i.e*. microvessel density or VEGF expression) and iNOS expression in human brain, head and neck, lung, breast, stomach, colon tumors, etc. (see [27-36] among others). Together these findings definitely indicate that cancer-derived NO mediates tumor angiogenesis, invasion and growth.

The contribution of NO to tumor angiogenesis is multifaceted. NO has been shown to mediate angiogenesis by direct and indirect mechanisms. Beside its direct, stimulating effects on endothelial cells, NO has been demonstrated to be a mediator of angiogenic factor activity and to control transcriptionally angiogenic stimuli expression in endothelial, tumor and stromal cells. Conversely, it has been reported that antiangiogenic molecules and drugs lead to NOS inhibition and that NO downregulates angiogenesis-inhibitor expression. The permissive action of exogenous or endogenously-produced NO on angiogenesis will be particularly examined.

Firstly, NO exposure increases DNA synthesis, cell proliferation and migration of endothelial cells through the soluble guanylate cyclase-cGMP pathway, as well as through *S*-nitrosylation or nitration of specific target proteins [13, 37-40]. Recently, a role for NO in the mobilization of stem and progenitor cells has also been described [41].

Secondly, NO has been shown to mediate the function of many angiogenic factors. VEGF, sphingosine-1-phosphate, angiopoietins, oestrogen, shear stress and metabolic stress activate eNOS through phospholipase-C/Ca^{2+}-calmodulin binding and phosphoinositide 3-kinase (PI3K)-Akt-induced and adenylate-cyclase-protein-kinase-A-induced phosphorylation [42-49]. VEGF can also activate eNOS by the recruitment of heat shock protein 90 [41, 50, 51] and upregulation of eNOS mRNA and protein [52, 53]. Further, VEGF increases angiogenesis in both $iNOS^{+/+}$ and $iNOS^{-/-}$ mice, but not in $eNOS^{-/-}$ mice, supporting a predominant role for eNOS in VEGF-induced angiogenesis and vascular permeability [41].

Additionally, NO or reactive nitroderivative species interfere with the synthesis and activation of the pro-metastatic and pro-angiogenic family of matrix metalloproteinases (MMP), enzymes involved in the degradation of the basal membrane of blood vessels [38, 54, 55]. The role of NO in the balance between MMPs and their tissue inhibitors (TIMPs) has been studied *in vitro* in microvascular endothelial cells and *in vivo* in the avascular rabbit cornea model. Data reported in *figure 2* indicate that endogenously-produced NO is able to promote MMP-2 activity in endothelium stimulated by VEGF, by promoting MMP-2 gene up-regulation and down-regulating TIMP-1 and 2 expression *(figure 2A-C)*. These VEGF-promoted effects are eNOS- and cGMP-dependent, since they can be blocked by preincubation with selective inhibitors of NOS (L-NMMA) or soluble guanylate cyclase (ODQ), and reproduced by a stable analogue of the intracellular mediator of NO (8-Br-cGMP) or exogenous NO (the NO donor S-nitroso-N-acetyl-l,l-penicillamine, SNAP). The relevance of the NO pathway and the MMP/TIMP balance in VEGF-induced neovascularization *(figure 3)* was substantiated in the rabbit cornea, where the neovascular growth induced by VEGF was impaired by pre-treating the animals with a NOS inhibitor (L-NAME) in the drinking water, or enriching the corneal microenvironment with TIMP-2 microinjection *(figure 2D)*.

According to the altered balance between MMPs and TIMPs by exogenous and endogenous NO in the microvascular endothelium, the regulation of both endothelial MMP-13 and TIMP-4 by direct amino acid nitration has been recently reported [56, 57].

These findings, taken together, reinforce the concept of abnormal vessel morphology and function in tumors, accompanied by increased basement membrane degradation.

In cell culture models, eNOS is a central mediator of several other endothelium growth stimulators, such as prostaglandin E_2 (PGE_2; 58). Also, PGE_2 activates the phosphatidylinositol 3-kinase (PI3K)/Akt pathway and promotes endothelial cell sprouting (the first step in neoangiogenesis) through the NO/cGMP pathway [58].

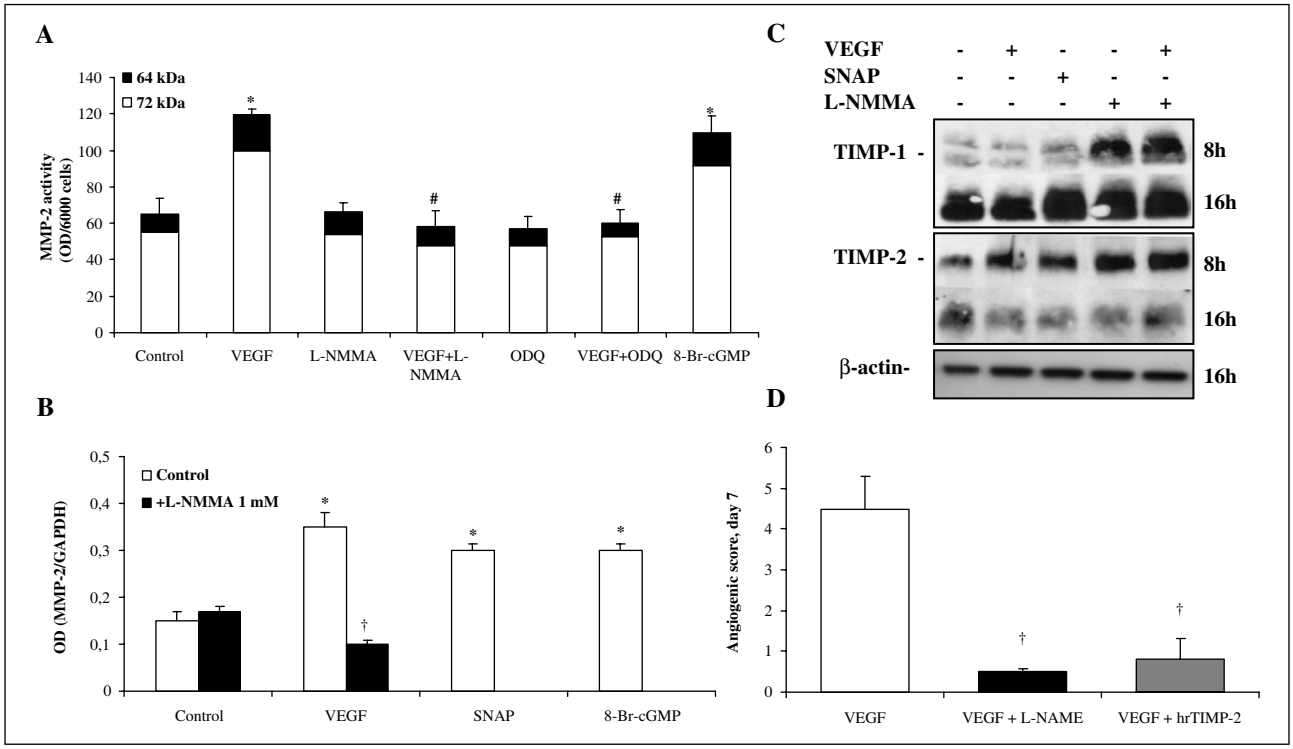

Figure 2

Role of the MMP/TIMP balance in the angiogeneic activity elicited by VEGF through the NOS/cGMP pathway.

A) Effect of the NO-cGMP pathway inhibition on MMP-2 release and activity in media of coronary venular endothelial cells (CVEC, [74]) treated with VEGF. Serum-starved CVEC were pre-incubated for 1 h with a NOS inhibitor (L-NMMA; 1 mM) or soluble guanylate gyclase inhibitor (ODQ; 10 μM) and then stimulated for 24 h with VEGF (20 ng/mL) or 8-Br-cGMP (100 μM). MMP-2 activity was measured in the supernatant by gelatine zymography [75]. Open bars represent the 72 kDa form, solid bars the 64 kDa form of MMP-2. Results are expressed as optical density (OD) of the zymograms. The OD of each band was normalized for the corresponding cell number. n = 3. * $p < 0.01$ versus control condition and # $p < 0.01$ *versus* VEGF alone.

B) Role of NO in MMP-2 mRNA expression. Serum-starved CVEC were stimulated for 24 h with VEGF (20 ng/mL), with or without the NOS inhibitor L-NMMA (1 mM), with the NO donor drug SNAP (100 μM) or 8-Br-cGMP (100 μM). MMP-2 mRNA expression was measured by RT-PCR [76]. Amplification products were quantified by densitometry of the gels. Data are expressed as means ± SEM of MMP-2/GAPDH optical density (OD); n = 3. * $p < 0.05$ *versus* control condition, † $p < 0.05$ *versus* VEGF alone.

C) Western blotting for TIMP-1 and TIMP-2 expression in CVEC pretreated for 1 h with L-NMMA (1mM) and then stimulated for 8 and 16 h with VEGF (20 ng/mL) or SNAP (100 μM). Results were normalised with beta actin. One representative gel out of three is shown.

D) Effect of NOS inhibition and hrTIMP-2 on VEGF-induced angiogenesis *in vivo*. Animals were treated either with 0.5 g/L L-NAME in the drinking water for 1 week before and for 10 days after the implant of VEGF (100 ng/pellet), or the corneal stroma was enriched by microinjection of hrTIMP-2 (25 ng/cornea) one day before VEGF-implant [13]. The vehicle as well as Elvax empty pellets did not affect the neovascular growth. Data are reported as angiogenic score at day seven. n = 4. † $p < 0.05$ *versus* VEGF alone.

Thirdly, NO is an important modulator of the expression of endogenous angiogenic factors. An NO donor induces VEGF expression [19] and microvascular endothelial cell proliferation through the upregulation of FGF-2 [13] *(figure 3)*.

NO activates the transcription factor hypoxia-inducible factor 1 α (HIF1 α), which, in turn, upregulates VEGF, thereby promoting angiogenesis [59, 60]. NO induces HIF1 α synthesis through MAPK and PI3K under normoxic conditions [61]. It also impairs normoxic degradation of HIF1α by inhibiting the action of prolyl hydroxylases [62]. A recent study found expression of iNOS and/or eNOS in all cases of HIF1α-positive oral squamous-cell carcinomas, which suggests that there is an NO-induced HIF1α accumulation and subsequent tumour-promoting effects in cancer [63].

At low levels, endogenous or exogenous NO can also serve as an intracellular second messenger for the induction of expression of the IL-8 gene in tumor cells, which represents an indirect angiogenesis factor [64, 65]. Moreover, in tumor and stromal cells NO can regulate the expression of inflammatory molecules as nuclear factor-KB (NF-kB) and cycloxygenase-2 [66].

The commonly held concept regarding cooperation among inflammatory mediators is indeed generally accepted both in inflammatory-based diseases and in cancer. Overexpression, elevated secretion, or abnormal activation of proinflammatory mediators, such as cytokines, chemokines, prostaglandins, and nitric oxide, and an intricate network of intracellular signalling molecules including upstream kinases and transcription factors facilitate tumor promotion and progression [67]. In the case of colorectal cancer for example, the interaction of NO with cyclooxygenase (COX)-2 seems to mediate a cooperative effect, culminating in the increased production of VEGF [68].

CONCLUSION AND PERSPECTIVES

Many points on the role of NO in vessel biology and in particular in tumor angiogenesis remain to be elucidated. First of all, the controversial proposal that vasodilator nitric oxide donors be used as therapeutic adjuvant to increase tumor blood flow and oxygenation in order to

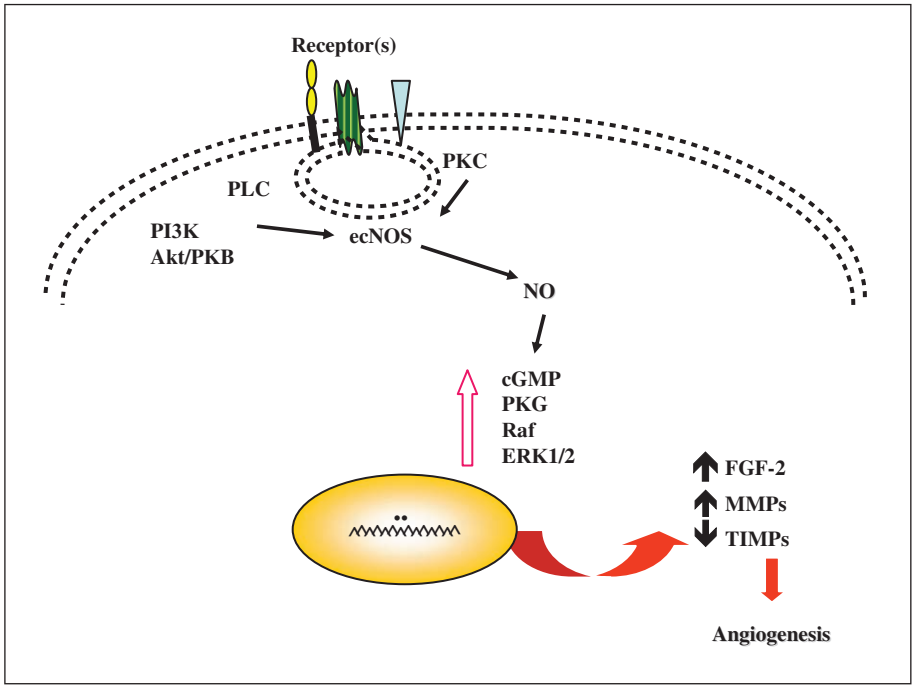

Figure 3
Schematic representation of the molecular cascades involved in NO-mediated angiogenesis. The invaginations of the microvascular endothelial cell membrane where receptors (tyrosine kinase or G-coupled receptors for angiogenic factors or vasoactive peptides) and key signalling enzymes are concentrated are the caveolae. The activation of NO-dependent MAPK pathway is then responsible for increased FGF-2 and MMPs transcription, with concomitant TIMP-1 and 2 downregulation. These events are ultimately responsible for endothelial cell migration, increased basement membrane degradation and proliferation, thus leading to neovascular growth [13-15, 44, 46].

increase the response to radiotherapy or p53-dependent anticancer drugs [69]. However, NO combination therapy for solid tumors does not have approval at this time.
Among the open questions we want to stress *i)* the unsuccessful effort to develop selective and safe strategies to inhibit NOS isoforms present at tumor level, *ii)* the paucity of clinical trials involving NO, and *iii)* the genetic variations present in genes encoding for NOS isoforms or NO pathway-related proteins in relation to tumor risk.
A phase I clinical trial designed to use the NOS inhibitor N-nitro-L-arginine (L-NNA) in different solid tumors documented a correlation between the L-NNA plasma area under the curve and the reduction in tumor blood volume [70].
Genetic comparison studies on healthy people and cancer patients have shown that gene polymorphisms in NOS are associated with the development of multiple cancers [71-73]. Although the functional effect of NOS SNPs has yet to be determined in large studies, these data support the hypothesis that abnormal NOS genes might drive tumorigenesis in humans.

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