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Review

Reciprocal regulation between nitric oxide and vascular endothelial growth factor in angiogenesis

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Physiologically, angiogenesis is tightly regulated, or otherwise it leads to pathological processes, such as tumors, inflammatory diseases, gynecological diseases and diabetic retinopathy. The vascular endothelial growth factor (VEGF) is a potent and critical inducer of angiogenesis. The *VEGF* gene expression is regulated by a variety of stimuli. Hypoxia is one of the most potent inducers of the *VEGF* expression. The hypoxia inducible factor 1 (HIF-1) plays as a key transcription factor in hypoxia-mediated *VEGF* gene upregulation. Nitric oxide (NO) as well as hypoxia is reported to upregulate the *VEGF* gene by enhancing HIF-1 activity. The Akt/protein kinase B (PKB) pathway may be involved in NO-mediated HIF-1 activation in limited cell lines. There are some reports of negative effects of NO on HIF-1 and VEGF activity. These conflicting data of NO effects may be attributed mainly to the amount of released NO. Indeed, NO can be a positive or negative modulator of the *VEGF* gene under the same conditions simply by changing its amounts. The VEGF-mediated angiogenesis requires NO production from activated endothelial NO synthase (eNOS). Activation of eNOS by VEGF involves several pathways including Akt/PKB, Ca^{2+}/calmodulin, and protein kinase C. The NO-mediated *VEGF* expression can be regulated by HIF-1 and heme oxygenase 1 (HO-1) activity, and the VEGF-mediated NO production by eNOS can be also modulated by HIF-1 and HO-1 activity, depending upon the amount of produced NO. These reciprocal relations between NO and VEGF may contribute to regulated angiogenesis in normal tissues.

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Abbreviations: HIF, hypoxia inducible factor; HRE, hypoxia response factor; NOS, nitric oxide synthase; cNOS, inducible NOS; eNOS, endothelial NOS; nNOS, neuronal NOS; PI3K, phosphatidyl inositol 3-kinase; pVHL, protein von Hippel-Lindau; SNP, sodium nitroprusside; VEC, vascular smooth muscle cells; VEGF, vascular endothelial growth factor; VSMC, vascular smooth muscle cells.

NITRIC OXIDE

Nitric oxide (NO) was discovered to be a potent vasodilator in 1979 (Gruetter *et al.*, 1979), and later identified as an endothelium-relaxing factor (ERDF) (Furchgott & Zawadzki, 1980). This simple molecule is a short-lived free radical, which has a number of physiological functions including smooth muscle relaxation, inhibition of platelet aggregation, and nonadrenergic-noncholinergic neurotransmission (Ignarro, 1996; Schmidt & Walter, 1994). NO is a water-soluble gas but a lipophilic molecule that can easily permeate biological membrane barriers. Physiological NO concentrations range from 5 nM to 4 μM. As it is highly reactive and unstable *in vivo* (3–5 s), newly synthesized NO can be biologically active within a limited area. Some effects of NO are linked to its intracellular second messenger nature, and other effects result from its paracrine actions, mediated by activation of the guanylate cyclase/3′,5′-cyclic guanosine monophosphate (GC/cGMP) pathway (Ignarro, 1992; Mayer, 1994). NO is synthesized through the enzymatic conversion of L-arginine and molecular oxygen to L-citrulline by nitric oxide synthases (NOS) (Knowles & Moncada, 1994). Three distinct isoforms of NOS have been identified with the different localization and regulation.

These are: neuronal NOS (nNOS; also known as NOS-1), inducible NOS (iNOS; also known as NOS-2), and endothelial NOS (eNOS; also known as NOS-3). The eNOS is the membrane-bound isoform first found in vascular endothelial cells (VEC), whereas the nNOS is the cytosolic isoform first found in neuronal tissues, and both isoforms are constitutively expressed and activated by intracellular calcium-dependent binding of calmodulin (CaM). In contrast, the iNOS can be inducible in a variety of cells including vascular cells, tumor cells and macrophages. The iNOS gene is activated under stimulation mostly by inflammatory signals, such as cytokines and endotoxin, and insensitive to intracellular calcium levels (Knowles & Moncada, 1994).

VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF)

Vessel formation occurs mainly through two sequential mechanisms (Carmeliet, 2000). *De novo* formation of blood vessels during embryonic development is called vasculogenesis. Mesoderm-derived stem cells (hemangioblasts) form aggregates (blood islands), and they develop into primitive hematopoietic and endothelial cells (angioblasts). Angioblasts differentiate and proliferate *in situ* to form a primitive network. On the other hand, the formation of new capillaries from preexisting vessels is called angiogenesis. The principle mechanism of vessel formation in adults is angiogenesis. Angiogenesis is a tightly regulated process, required for a number of physiological processes, such as wound healing, ovulation and menstruation as well as embryonic development. Excessive angiogenesis is seen in a wide range of diseases including tumors, inflammatory diseases, psoriasis, rheumatoid arthritis and diabetic retinopathy. Most of embryonic vessels and proliferating endothelial cells under angiogenesis express receptors for VEGF, suggesting that VEGF play a key role in vasculogenesis and angiogenesis. The family of VEGF includes VEGF-A, -B, -C, -D, -E, and placenta growth factor. VEGF (denoted as VEGF-A) was initially named vascular permeability factor VPF for its ability to induce vascular permeability. Later this VEC-specific mitogen was named VEGF for its ability to promote proliferation of VEC. The main receptors which seem to initiate signal transduction cascades in response to VEGF binding consist of three kinds of tyrosine kinases: VEGFR-1 (previously known as Flt-1), VEGFR-2 (previously known as KDR/Flk-1) and VEGFR-3 (previously known as Flt-3). Among them, VEGFR-2 may mediate the major action of cell growth and permeability of VEGF.

REGULATION OF *VEGF* EXPRESSION BY NO (Fig. 1)

A number of angiogenic stimuli have been found to induce *VEGF* expression, such as cytokines, hormones, phorbol esters, oncogenes, transitional metals, iron chelator and hypoxia (Klagsbrun & D'Amore, 1996). Hypoxia is a key inducer of VEGF *in vitro* and *in vivo*, whose mechanisms have been extensively investigated. Inducibility by hypoxia is

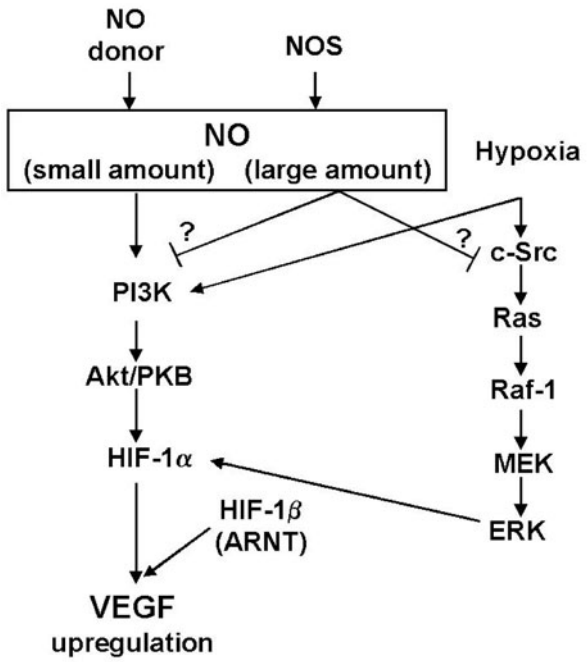

Figure 1. Mechanisms of *VEGF* upregulation by NO and hypoxia.

An optimal amount of NO may upregulate the *VEGF* gene expression probably through a PI3K-Akt pathway in limited cell lines while an excessive amount of NO inhibits the *VEGF* expression through an unidentified pathway.

conferred by the hypoxia response element (HRE), which is located within the 5′ promoter of the *VEGF* gene. Compared with HREs of erythropoietin and several glycolytic enzyme genes, these sequences reveal a high homology and similar protein-binding characteristic as hypoxia inducible factor 1 (HIF-1). HIF-1 is composed of two distinct subunits, both of which belong to the basic helix-loop-helix-per-arnt-sim protein family, HIF-1α and HIF-1β. HIF-1α was found as a novel protein, but HIF-1β was identical to aryl hydrocarbon receptor nuclear translocator (ARNT) (Wang & Semenza, 1995). Transcriptional activation of the VEGF gene is dependent upon HIF-1 binding activity and its protein level (Semenza *et al.*, 1997), although mRNA stabilization under hypoxia is also important for increase in VEGF expression (Ikeda *et al.*, 1995; Levy *et al.*, 1996).

Recently NO has been reported as a regulator of *VEGF* expression. Initially NO was reported to induce the *VEGF* gene in tumor cells (Chin *et al.*, 1997), followed by several contradictory reports. Tsurumi *et al.* (1997) demonstrated that sodium nitroprusside (SNP), a NO donor, downregulated the VEGF promoter activity and its synthesis in vascular smooth muscle cells (VSMC) by interfering binding of the AP-1 transcription factor. Other reports showed that NO inhibits hypoxic induction of the *VEGF* gene through attenuation of HIF-1 binding activity by abrogating accumulation of HIF-1α protein in VSMC and tumor cell lines (Huang *et al.*, 1999; Liu *et al.*, 1998; Sogawa *et al.*, 1998). However, SNP was commonly used as a NO donor in these experiments. SNP has a distinct effect from other NO donors. Even a small amount of SNP inhibited *VEGF* expression, and its inhibitory effect is not ascribed to NO production (Dulak *et al.*, 2000; Kimura *et al.*, 2002).

More recently, reports have been published that NO is an inducer of VEGF synthesis under normoxia. We have shown that NO donors except SNP induced *VEGF* expression by enhancing the HIF-1 binding activity and accumulation of HIF-1 protein in tumor cell lines, independent of a cGMP pathway (Kimura *et al.*, 2000). A detailed reporter gene analysis revealed that the HIF-1 binding site and its adjacent downstream sequence are the *cis*-elements for the transcriptional activation of VEGF by NO as well as hypoxia (Kimura *et al.*, 2001). Dulak *et al.* (2000) demonstrated that endogenous NO in eNOS-transduced VSMC

upregulated VEGF synthesis. Sandau *et al.* (2001) showed that endogenous NO released from overexpressed iNOS as well as NO donors provoked HIF-1 accumulation in tubular LLC-PK cells.

Why does NO show these conflicting effects on HIF-1 and *VEGF* expression? No answer is obtained so far, but we have to consider several experimental conditions. Effect of NO or NO donors on *VEGF* expression may depend on the absolute NO concentrations, pharmacological actions of NO donors, environmental oxygen tension and cells types. Several representative NO donors were tested for effects on the *VEGF* promoter activity and its mRNA level in tumor cells (Kimura *et al.*, 2002). The NO donors except SNP activated the VEGF transcription under normoxia, whereas these NO donors could act positively or negatively on the *VEGF* promoter activity under hypoxia dependent upon their concentrations. Under the low oxygen tension, NO donors at low concentrations have a synergetic effect on the *VEGF* gene activation, but NO donors at high concentrations inhibit the hypoxic induction. We also should be cautious about the cytotoxic effect of NO at a high concentration, as NO sensitivity in terms of cytotoxicity varies greatly among cell lines (Wink & Mitchell, 1998).

The mechanism of regulation of HIF-1 by hypoxia has been extensively studied. Although HIF-1β is continuously expressed, HIF-1α expression is enhanced under hypoxia by the protein stabilization rather than by an increase in its mRNA level. HIF-1α is ubiquitinated and subject to proteasomal degradation under non-hypoxic conditions (Huang *et al.*, 1998; Kallio *et al.*, 1999; Salceda & Caro, 1997). It is generally believed that the ubiquitination of HIF-1α is mediated by the von Hippel-Lindau protein (pVHL), which binds directly to the oxygen-dependent degradation domain (ODDD) (Cockman *et al.*, 2000; Maxwell *et al.*, 1999; Tanimoto *et al.*, 2000). Deletion of this region leads to stabilization of HIF-1α under normoxia. Interaction of HIF-1α and pVHL is regulated through hydroxylation of a proline residue by prolyl hydroxylase (PHD) (Bruick & McKnight, 2001; Epstein *et al.*, 2001). Since the activity of PHD depends on the concentrations of oxygen and iron (McNeill *et al.*, 2002), depletion of these molecules might limit the hydroxylation of a proline residue, thereby inhibiting interaction of HIF-1α and pVHL, and stabilizing HIF-1α protein. However, hypoxic activation of HIF-1 usually takes a long time, varying from a few hours to more than 12 h depending on cell lines (Kimura *et al.*, 2000). This finding might suggest that there is another mechanism other than the above simple explanation. Cobalt chloride and iron chelator induce HIF-1α expression by suppressing HIF-1α ubiquitination, and dissociate pVHL from HIF-1α (Maxwell *et al.*, 1999). In contrast, hypoxia does not cause pVHL dissociation from HIF-1α. The involvement of pVHL in the HIF-1α accumulation by NO remains unclear.

Several pathways are identified which generally regulate gene expression under hypoxia. They include phosphatidylinositol 3-kinase (PI3K)-Akt pathway, the ERK1 and ERK2 (also known as p42 and p44 mitogen-activated protein kinase (MAPK)) pathway, Ca^{2+}/CaM pathway, the 3′,5′-cyclic adenosine monophosphate (cAMP)-protein kinase A (PKA) pathway, and stress-activated protein kinase (SAPK, also known as p38 kinase) pathway.

A variety of growth factors and cytokines (e.g. insulin-like growth factor, epidermal growth factor, interleukin-1) induced HIF-1α expression by activation of PI3K under normoxia in certain cell types (Feldser *et al.*, 1999; Stiehl *et al.*, 2002; Zelzer *et al.*, 1998; Zhong *et al.*, 2000). Some reports describe an inhibitory effect of PI3K inhibitors LY294002 and wortmannin on hypoxia-induced HIF-1 activity (Blancher *et al.*, 2001; Zhong *et al.*, 2000; Zundel *et al.*, 2000). In contrast, the inhibition of PI3K had no effect on the induction of the HIF-1 protein and its transcriptional activity in several cell lines (Alvarez-Tejado *et al.*,

2002; Arsham *et al.*, 2002). Similar conflicting results were obtained with NO-induced HIF-1 activation. The kinase inhibitor genistein and blockers of PI3K attenuated NO-induced HIF-1 accumulation and DNA binding in LLC-PK cells (Sandau *et al.*, 2000), but high doses of LY294002 and wortmannin could only partially attenuate HIF-1 protein levels and its binding activity in glioblastoma cells (our unpublished data). These data suggest that the PI3K-Akt pathway may be involved in HIF-1 activation in limited cell lines, and specificity of the effects of PI3K inhibitors need to be confirmed in these experiments. Unlike NO, activation of MAPK may be associated with the enhancement of HIF-1α activity under hypoxia by direct phosphorylation, not by accumulation, of HIF-1α (Berra *et al.*, 2000; Richard *et al.*, 1999). In hypoxia, PI3K-Akt and ERK pathways can activate HIF-1α synergistically by phosphorylation of the transactivation domain and the ODDD, respectively, of HIF-1α in 3T3 and COS cells (Sodhi *et al.*, 2001). There is also an evidence that HIF-1α can be regulated at translational level by epidermal growth factor receptor 2 (HER2, also known as neu) through the PI3K-Akt pathway in breast cancer cells (Laughner *et al.*, 2001). In contrast, no pathway other than the possible involvement of PI3K/Akt is known for the NO-mediated HIF-1 activation, and how NO at a high concentration attenuates the HIF-1 activity under hypoxia is still to be investigated.

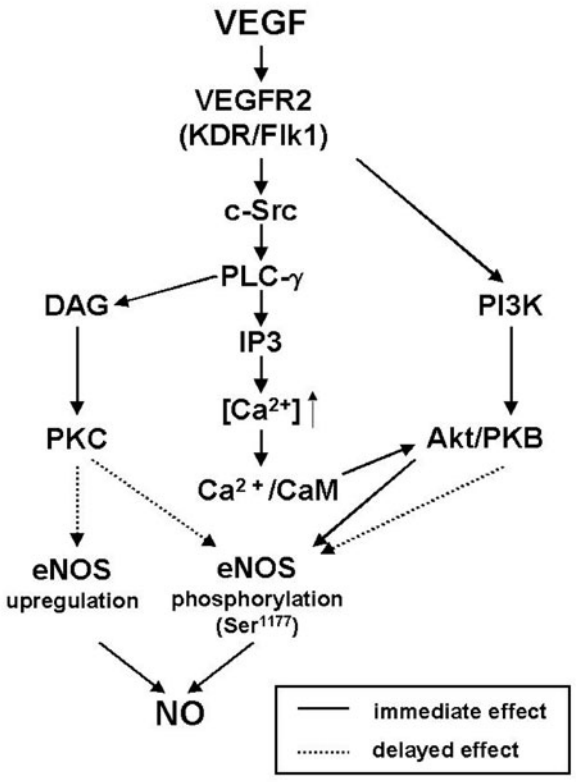

Figure 2. Signaling pathways for VEGF-mediated NO synthesis.

VEGF induces immediate NO synthesis through the CaM-Akt pathway. Delayed NO synthesis is mediated by the PI3K-Akt pathway or induced by upregulaton of the eNOS gene through PKC activation.

REGULATION OF NO PRODUCTION BY VEGF (Fig. 2)

The angiogenic and inflammatory effects of VEGF can be mediated by NO, which is produced by VEGF-activated eNOS in VEC (Murohara *et al.*, 1998; Papapetropoulos *et al.*, 1997; Parenti *et al.*, 1998). It has been reported that VEGFR-2 plays a major role in angiogenesis, and its autophosphorylation leads to eNOS activation (Feng *et al.*, 1999; He *et al.*, 1999; Kroll & Waltenberger, 1998; Thuringer *et al.*, 2002). A large number of research have been performed to elucidate the mechanism of VEGF-induced NO production. The eNOS is regulated by the level of intracellular calcium. Calcium binds to CaM, and this complex associates with eNOS to cause the enzyme activation. Intracellular calcium release by VEGF in VEC results from phospholipase C (PLC-γ) activation, which subsequently generates diacylglycerol (DAG) and activates inositol 1,4,5-triphosphate (IP_3). IP_3 induces the influx of calcium (Busse & Mulsch, 1990; Wu *et al.*, 1999; Xia *et al.*, 1996). The use of PLC inhibitors, CaM antagonists or intracellular calcium chelators attenuated Akt phosphorylation. In addition, the use of calcium ionophore induced Akt activation

and phosphorylated eNOS at Ser^{1177} (human). Furthermore, the blockade of Ca^{2+}/CaM-dependent Akt phosphorylation abrogated immediate NO production, whereas the inhibition of PI3K-dependent Akt phosphorylation was unrelated to immediate NO production (Gelinas *et al.*, 2002). These data suggest that immediate NO synthesis requires the Ca^{2+}/CaM-dependent Akt pathway. In contrast, delayed NO production from eNOS seems to be mediated by different pathways. It has been reported that VEGF inhibits apoptosis of VEC by activating antiapoptotic Akt/PKB *via* a PI3K-dependent pathway (Gerber *et al.*, 1998; Thakker *et al.*, 1999). The use of PI3K inhibitors prevented eNOS Ser^{1177} phosphorylation induced by VEGF and had an inhibitory effect on delayed NO production (Gelinas *et al.*, 2002). These results are in agreement with the positive effect of Akt on delayed calcium-independent NO production (Brouet *et al.*, 2001; Dimmeler *et al.*, 1999). PLC-γ promotes not only IP_3-dependent calcium release but calcium-dependent protein kinase C (PKC) activation through DAG synthesis. Treatment with PKC inhibitors abolished VEGF-induced eNOS upregulation (Shen *et al.*, 1999) and attenuated eNOS Ser^{1177} phosphorylation (Gelinas *et al.*, 2002), both of which lead to delayed NO production.

RECIPROCAL REGULATION BETWEEN NO PRODUCTION, NOS AND VEGF EXPRESSION (Fig. 3)

As described above, NO at an appropriate concentration induces VEGF synthesis through an HIF-1 mediated pathway, and VEGF enhances NO production by eNOS. These actions may lead to promotion of angiogenesis. However, angiogenesis in normal tissues should be strictly regulated to avoid vascular disaster, and there must be a reciprocal regulation between NO and VEGF.

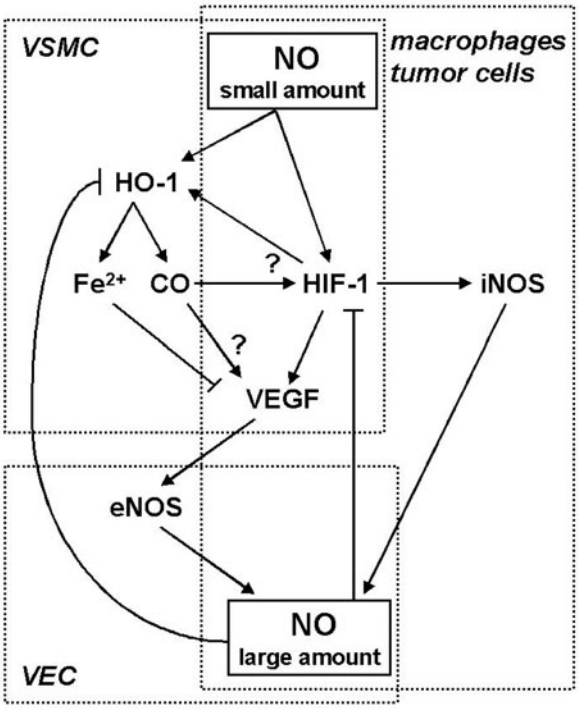

Figure 3. Reciprocal relationship between NO and VEGF.

NO is synthesized mainly by eNOS in VSMC and by iNOS in macrophages and tumor cells NO may modulate positively or negatively *VEGF* expression through HIF-1 and/or HO-1-mediated pathways.

Carbon monoxide (CO) has been reported as a modulator of *VEGF* expression. Unlike NO, CO is a stable gas, not a free radical, but both gases are endogenously produced. They have similar physiological functions, such as vasodilatation, inhibition of platelet aggregation and neurotransmission, and can act as second messenger molecules (Brann *et al.*, 1997; Carvajal *et al.*, 2000). Some reports described inhibitory effects of CO on hypoxia-induced HIF-1 and *VEGF* expression (Huang *et al.*, 1999; Liu *et al.*, 1998). In these experiments, the cells were cultured under hypoxia and treated with high concentration of CO. However, other groups demonstrated that CO at much lower concentrations enhanced *VEGF* expression under normoxia (Dulak *et al.*, 2002; Kramer *et al.*, 1997; Marti & Risau, 1998), and induced HIF-1α expression in kidneys of rats (Rosenberger *et al.*, 2002). Heme

oxygenase (HO) is responsible for generating CO and especially HO-1 is inducible after stimulation of cytokines, hypoxia and NO. Dulak *et al.* (2002) demonstrated that cytokine-induced VEGF synthesis in VSMC is dependent upon HO activity, and CO and ferrous ion (Fe^{2+}), both of which are HO-derived compounds, may serve as an inducer and an inhibitor, respectively, of the VEGF synthesis.

NO itself can act positively or negatively on HIF-1-mediated *VEGF* gene expression in various tissues. In the vascular wall, a small amount of NO induces activation of the VEGF synthesis in VSMC, and a positive feedback of VEGF leads to more NO production by eNOS in VEC. However, an excessive amount of NO acts negatively on the VEGF synthesis probably by limiting the HIF-1 activity. The iNOS is highly expressed in macrophages and tumor cells, and can generate several orders of magnitude more NO than the other constitutive NOS. This means that most of NO effect by these cells may be attributed to iNOS activity. NO as well as hypoxia can also regulate iNOS expression by modulating HIF-1 activity, because iNOS transcription can be regulated by HIF-1 (Semenza *et al.*, 1997). Due to unregulated angiogenesis, where the oxygen as well as nutrition demand of tumor cells exceeds their supply, tumor cells are always exposed to hypoxia. In the severely low oxygen tension, HIF-1 and thus iNOS is highly expressed. The negative feedback of a large amount of NO on the VEGF gene expression may be disregarded because of the strong induction of HIF-1 activity, often seen in tumors.

CONCLUSIONS

NO may have a pivotal role in normal vessel development, because it is a both upstream and downstream mediator of the VEGF-mediated angiogenesis in the vessel wall. The mechanism of the regulation of NO production by VEGF has been extensively studied and several signal transduction pathways seem to be involved in this regulation. In contrast, we are at the beginning of understanding the mechanism of the regulation of VEGF by NO, and we have not reached the consensus about the effect of NO on *VEGF* expression. However, at least it is certain that the amount of NO is critical for the effect of NO on HIF-1 and *VEGF* expression under normoxia and hypoxia. As NO is unstable, NO donors have been commonly used to elucidate the effect of NO *in vitro* and *in vivo*, but the selection of NO donors sometimes misleads the results because of their pharmacological effects unrelated to NO. Thus the confirmation of the results by endogenously produced NO is desirable. The use of NOS genes-transduced cells is preferred in *in vitro* studies (Dulak *et al.*, 2000; Sandau *et al.*, 2001). Recently gene targeting has made it possible to disrupt a specific gene in animals, and mice lacking NOS genes have become such powerful tools in defining the role of NO without the use of NO donors or NOS inhibitors.

In most of *in vitro* studies, 20% of oxygen (152 mmHg) has been used as "normoxia" and less than 5% of oxygen (38 mmHg) as "hypoxia". *In vivo* measurements of oxygen tension in air-breathing mice reveal that mean pO_2 is identical to 5% O_2 in normal tissues, and is less than 1.2% O_2 (1–9 mmHg) in most tumors (Adam *et al.*, 1999). This finding suggests that 20% of oxygen is not "normoxia" *in vivo* in the true meaning. In less oxygen tension, NO has a longer half-life and even a small amount of NO is sufficient for biologically active. We are prone to misunderstanding biological effects of NO unless we consider "hypoxic conditions of cells in normal tissues".

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