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GUIDELINES FOR BASIC SCIENCE

# Nitric oxide and cancer

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## Abstract

Nitric oxide (NO) is a lipophilic, highly diffusible and short-lived physiological messenger which regulates a variety of important physiological responses including vasodilation, respiration, cell migration, immune response and apoptosis. NO is synthesized by three differentially gene-encoded NO synthase (NOS) in mammals: neuronal NOS (nNOS or NOS-1), inducible NOS (iNOS or NOS-2) and endothelial NOS (eNOS or NOS-3). All isoforms of NOS catalyze the reaction of L-arginine, NADPH and oxygen to NO, L-citrulline and NADP. NO may exert its cellular action by cGMP-dependent as well as by cGMP-independent pathways including postranslational modifications in cysteine (S-nitrosylation or S-nitrosation) and tyrosine (nitration) residues, mixed disulfide formation (S-nitrosoglutathione or GSNO) or promoting further oxidation protein stages which have been related to altered protein function and gene transcription, genotoxic lesions, alteration of cell-cycle check points, apoptosis and DNA repair. NO sensitizes tumor cells to chemotherapeutic compounds. The expression of NOS-2 and NOS-3 has been found to be increased in a variety of human cancers. The multiple actions of NO in the tumor environment is related to heterogeneous cell responses with particular attention in the regulation of the stress response mediated by the hypoxia inducible factor-1 and p53 generally leading to growth arrest, apoptosis or adaptation.

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**Key words:** Nitric oxide; Cancer; Carcinogenesis

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## INTRODUCTION

### *Discovery of nitric oxide*

Nitric oxide (NO) is a lipophilic, highly diffusible and short-lived physiological messenger[1]. NO regulates a variety of important physiological responses including vasodilation, respiration, cell migration, immune response and apoptosis. Ignarro *et al*[2] and Moncada *et al*[3] identified simultaneously the endothelium-derived relaxing factor (EDRF) as NO. Hibbs *et al*[4] demonstrated that the reaction using L-arginine as substrate results in the formation of L-citrulline and the end products, $NO_2^-$/$NO_3^-$. The 1990s brought several landmarks to the field including the molecular characterization of the NO synthase (NOS) family of enzymes[5-8], the discovery of peroxyntrite ($ONOO^-$)[9], the importance of NO-mediated posttranslational protein modifications[10], the regulation of mitochondrial function by NO[11-14] and the chemistry of NO diffusion/reactivity[15].

### *Nitric oxide synthase isoenzymes*

NO is synthesized by three differentially gene-encoded NOS in mammals: neuronal NOS (nNOS or NOS-1), inducible NOS (iNOS or NOS-2) and endothelial NOS (eNOS or NOS-3). All three isoforms share similar structures and catalytic modes, yet the mechanisms that control their activity in time and space are quite diverse. The expression of NOS-2 is induced by inflammatory stimuli while NOS-1 and NOS-3 are more or less constitutively expressed[16]. The full active NOS form requires two NOS monomers associated with two $Ca^{2+}$-binding protein calmodulin (CaM). NOS contains relatively tightly-bound cofactors such as (6R)-5,6,7,8-tetrahydrobiopterin (BH4), FAD, FMN and iron protoporphyrin IX (haem) and catalyze the reaction of L-arginine, NADPH and oxygen to NO, L-citrulline and NADP[16]. The reaction is composed of two sequential steps involving the hydroxylation of guanidino nitrogen of L-arginine generating the intermediate $N^{\omega}$-hydroxy-L-arginine (NOHA) which is oxidized to NO and L-citrulline[17]. $HB_4$ acts as a redox cofactor in the second reduction step and prevents the uncoupling of NOS and generation of anion superoxide ($O_2^-$). All NOSs require similar amounts of L-arginine, BH4 and NADPH for activity. NOS isoforms are differentially regulated at transcriptional, translational and post-translational levels. However, the activity of NOS-1 and NOS-3 is highly dependent upon intracellular $Ca^{2+}$ concentration whereas NOS-2 forms an active complex with CaM. NOS-2 is already maximally activated by $Ca^{2+}$/CaM even at basal levels of intracellular $Ca^{2+}$[16]. Several inhibitory and activator phosphorylated sites in NOS-1 and NOS-3 tightly regulate their NO production[18].

The intracellular localization is relevant for the activity of NOS. It appears that there are compartments that allow full activation of NOS with free access to substrates and cofactors as well as the presence of activators[19]. In this sense, accumulating evidence indicates that NOSs are subject to specific targeting to subcellular compartments (plasma membrane, Golgi, cytosol, nucleus and mitochondria) and that this trafficking is crucial for NO production and specific posttranslational modifications of target proteins[20,21].

## NITRIC OXIDE CELL SIGNALING

The biological activity of NO is classified by cGMP-dependent and cGMP-independent pathways, both attributed to physiological and pathological conditions[22-24]. cGMP-dependent protein kinases, cyclic-nucleotide-gated ion channels and cGMP-regulated phosphodiesterases mediate several cellular effects. However, during the last decade, cGMP-independent reactions have gained considerable interest. A variety of effects are achieved through its interactions with targets *via* redox and additive chemistry that may promote covalent modifications of proteins as well as oxidation events that do not require attachment of the NO group. In fact, NO is the prototypic redox-signaling molecule more versatile than $O_2^-$ or $H_2O_2$ and clearly better identified with redox-related modifications of intracellular proteins[25].

### *Nitric oxide cGMP-independent pathways*

The most prominent and recognized NO reaction with thiols groups of cysteine residues is called S-nitrosylation or S-nitrosation which leads to the formation of more stable nitrosothiols[26]. However, other modifications such as disulfide, mixed disulfide formation with reduced glutathione (S-nitrosoglutathione or GSNO) or oxidation towards sulfenic acid are also important since they are reversible. Higher thiol oxidation states such as sulfinic or sulfonic acids are irreversible modifications with subsequent loss of functional control. Nitrosothiol formation can be the result of a direct reaction with NO or of an oxidative nitrosylation reaction involving the preformation of $ONOO^-$[27]. The pattern of nitrosylated proteins is specific, probably dependent of the presence of specific consensus motifs which influence the accessibility of the thiols groups to NO[28]. Different proteins such as NMDA and ryanodine receptors, ras, caspases, glyceraldehyde-3-phosphate dehydrogenase and DNA repair proteins are widely post-translationally modified by nitrosylation[29].

Oxidative and nitrosative stress is sensed and closely associated with transcriptional regulation of multiple target genes[30]. The net effect of NO on gene regulation is variable and ranges from activation to inhibition of transcription. S-nitrosation of specific cysteines in active zinc fingers sequences in SP-1, EGR-1 and glucocorticoid receptors, induces $Zn^{2+}$ release, concomitant conformational changes and reduced DNA-binding[31]. The impact of NO in other transcription factors such as NF-$\kappa$B may affect at different levels such as I$\kappa$B expression and stability, NF-$\kappa$B activation, nuclear translocation or cysteine residue modification involving alteration of DNA binding. The administration of NO donors reduces NF-$\kappa$B activation and downstream expression of anti-apoptotic gene products[32] which is relevant for NO-dependent sensitization chemotherapy-resistant tumor cells[33,34]. It now seems more certain that reducing conditions are required in the nucleus for NF-$\kappa$B DNA binding whereas oxidizing conditions in the cytoplasm promote NF-$\kappa$B activation[30]. AP-1 is a transcription factor that belongs to the basic leucine zipper (bZip) family in which a single cysteine residue is present that confers redox sensitivity[35]. NO, mostly by S-nitrosylation[36] and glutathiolation[37] of cysteine, inhibits c-Jun and c-Fos DNA binding in a reversible manner. p53 also binds to its specific DNA sites in a reducing environment and mutations of cysteine residues in the p53 core binding domain (loop-sheet-helix motif linked to a loop-helix motif) prevents DNA binding and p53-induced transcription[38]. HIF-1$\alpha$ has a single cysteine in the basic-helix-loop-helix of the carboxyl-terminal trans-activating domain which participates in protein–protein interactions that activate transcription[39]. Other transcription factors whose binding to DNA is facilitated under reducing conditions include c-Myb, USF, NFI, NF-Y, HLF, PEBP2, GABPa, TTF-1 and Pax-8[30].

The generation of $O_2^{\cdot-}$ and NO may lead the production of the harmful molecule $ONOO^-$[40]. $ONOO^-$ may result in S-nitrosylation and tyrosine nitration of proteins with a concomitant change in their function[41]. The generation of $ONOO^-$ may exert a negative feedback regulation on the NO production. In this sense, the reaction of $ONOO^-$ with Akt and BH4 altered NO production generated by NOS[42,43]. Proteins that can be nitrated on tyrosine residues include actin, histone proteins, protein kinase C, prostacyclin synthase, manganese superoxide dismutase, tyrosine hydroxylase, cytochrome P450B1, transcription factor STAT1 and p53[44]. Also, different proteins appeared to be nitrated in cultured human hepatocytes[45]. Alternatively, NO may indirectly induce gene transcription *via* activation/modulation of signaling pathways such as mitogen-activated protein kinases (MAPK), G-proteins, Ras pathway or phosphatidylinositol-3 kinase (PI3K) pathways[46].

## NITRIC OXIDE, CELL PROLIFERATION AND CANCER

Nitrogen oxide chemistry is critical in the nitrogen cycle, converting nitrate($NO_3^-$) and nitrite ($NO_2^-$) to ammonia ($NH_4^+$), an essential component of protein synthesis as well as in the vascular tone and cell signaling regulation. However, it has also been associated to the deleterious/cytotoxic effects in air pollution, antibacterial in the preservation of food as well as the generation of carcinogenic nitrosamines[47]. In this sense, NO may participate in the induction of genotoxic lesions as well as promoting angiogenesis, tumor cell growth and invasion[48].

### *Participation of nitric oxide in carcinogenesis*

The infectious and non-infectious generation of chronic injury and irritation initiates an inflammatory response[49]. A subsequent respiratory burst, an increased uptake of oxygen that leads to the release of free radicals from leukocytes, including activated macrophages, can damage surrounding cells. This process can drive carcinogenesis by altering targets and pathways that are crucial to normal tissue homeostasis. NO and NO-derived reactive nitrogen species induce oxidative and nitrosative stress which results in DNA damage (such as nitrosative deamination of nucleic acid bases, transition and/or transversion of nucleic acids, alkylation and DNA strand breakage) and inhibition of DNA repair enzymes (such as alkyltransferase and DNA ligase) through direct or indirect mechanisms[50]. However, the diversity of reactive species produced during chronic inflammation under different cellular microenvironments has impaired identification of a clear biomarker that identifies the involvement of a single reactive species in the carcinogenic process[51]. Chronic inflammation contributes to about one in four of all cancer cases worldwide[49]. The induction of mutations in cancer-related genes or post-translational modifications of proteins by nitration, nitrosation, phosphorylation, acetylation or polyADP-ribosylation are some key events that can increase the cancer risk. In particular, high levels of NO are genotoxic through formation of carcinogenic nitrosamines or by directly modifying DNA or DNA repair proteins. It was found that aerobic solutions of NO, $NO_2$ and $N_2O_3$ led to deamination of nucleic acids[52]. Unlike oxidation by $ONNO^-$ or reactive oxygen species (ROS) that preferentially results in transversions, nitrosative mixtures of $NO_2/N_2O_3$ mediate transitions[53]. However, NO may also influence the carcinogenesis process by alteration of cell-cycle checkpoints[54], apoptosis[55] and DNA repair[56]. NO donors sensitize tumor cells to chemotherapeutic compounds by nitrosilation of critical thiols in DNA repair enzymes in hepatoma cell line[57]. Other studies have demonstrated increased susceptibility to chemotherapy to cisplatin[58] and melphalan[59] by NO donors in different cell lines. These results implied substantial modification of key biological target(s) including DNA repair proteins and transcription factor known to be inhibited by NO.

### *Cell signaling of NO in carcinogenesis*

It is difficult to identify the specific role of NO in carcinogenesis because it is dependent on its concentration, interaction with other free radicals, metal ions and proteins, and the cell type and the genetic background that it targets. The expression of NOS-2 has been found to be increased in a variety of human cancers[60-62]. However, NOS-3 has also been suggested to modulate different cancer-related events (angiogenesis, apoptosis, cell cycle, invasion and metastasis)[63]. NO can both cause DNA damage and protect from cytotoxicity, can inhibit and stimulate cell proliferation and can be both pro- and anti-apoptotic[64-67]. Lancaster and Xie[68] suggest that the multiple actions of NO in the tumor environment are related to its chemical (post-translational-related modifications) and biological heterogeneity (cellular production, consumption and responses). However, one of the critical insights into this dichotomy may be the regulation by NO of the stress response mediated by the hypoxia inducible factor-1 (HIF-1)[69] and p53[70] generally leading to growth arrest, apoptosis or adaptation[71].

### *Nitric oxide and p53*

The biological outcomes of p53 activity include apoptosis, inhibition of cell cycle progression, senescence, differentiation and accelerated DNA repair. The types of stress that promote p53 activation include many conditions associated with cancer initiation and progression such as direct DNA damage, chromosomal aberrations, illegitimate activation of oncogenes, hypoxia, telomere shortening *etc*. p53 is found at very low steady state in non stress conditions by the mouse double minute (Mdm2, the human orthologue form is named Hdm2) protein[72]. Mdm2 displays an E3 ubiquitin ligase activity towards p53 for ubiquitin-dependent proteasomal degradation, although non-proteasomal mechanisms for p53 degradation may also play a significant role under certain

circumstances[73]. The induction of cell death or antitumoral properties of NO has been extensively related to nuclear p53 accumulation[74-77]. NO donors induce p53 accumulation and apoptosis through JNK-1/2, but not ERK1/2 or p38, activation in RAW 264.7 macrophages[78]. A peptide corresponding to the JNK binding site on p53 protein efficiently blocks its ubiquitination and consequently increases p53 half-life[79]. The nuclear accumulation of p53 is mainly regulated by posttranslational modifications by phosphorylation[80] or tyrosine nitration[81]. S-nitrosoglutathione and NO donors prevent poly-ubiquitinated-dependent p53 degradation by proteasome which it is antagonized by reducing agents[82]. Ubiquitin-activating enzyme (E1), a key component of ubiquitinization process, has a cysteine residue that is S-nitrosylated by NO donors[83].

The overexpression of NOS-2 reduced *in vitro* cell proliferation[84-86] and *in vivo* tumor progression in xenograft experimental models[84,86] using different carcinoma cell lines. Le *et al*[87] have recently shown that NOS-2 overexpression exerts antitumor activity *in vitro* and *in vivo* dose dependently, regardless of its up-regulation of protumor factors. Also, NOS-2-derived NO suppresses lymphomagenesis even in a $p53^{-/-}$ back-ground by promoting apoptosis and decreasing tumor cell proliferation[88]. NOS-2 overexpression has also been shown to induce radiosensitization through p53 accumulation in *in vitro* and *in vivo* xenograft models[89,90]. Furthermore, wild-type p53-induced transrepression of NOS-2 provides a protective mechanism against prolonged exposure to pathological conditions of NO[75,91]. The frequency of p53 mutations occurs in about 50% of all human tumors suggesting that can be an early event in the process of hepatocellular carcinogenesis[92,93]. The expression of NOS-2 has been associated with increased expression of p53 mutated isoforms in liver sections from patients with hemochromatosis and Wilson diseases[94], ulcerative colitis[95], colon cancer[96] and stomach, brain and breast cancers[60,62,97]. Ambs *et al*[84] have observed that NOS-2 overexpression in cells with mutated p53 accelerated tumor growth, increased vascular endothelial growth factor (VEGF) expression and neovascularization. These studies indicate that exposure of cells to high levels of NO and its derivatives during chronic inflammation in the absence of wild-type p53, and therefore the negative NOS-2 regulation, may increase the susceptibility to cancer. Therefore, loss of functional p53 may lead to a reduction of NO sensitivity and transfer to other stress survival response such as HIF-1 that may promote a selective tumoral growth advantage. However, recent study has shown that NOS-2 overexpression abrogated the growth of various human tumor cells with different p53 functional status (wild-type, mutated and gene loss)[87]. The contradictory results among studies showing the potential role of p53 in high NO production may be explained by the different overall genetic background of tumoral cell lines as well as the stromal cell-derived NO in xenograft models that may modulate the response of surrounding tumor cells.

### *Nitric oxide and HIF-1*

NO has also revealed an impact on the redox-sensitive target HIF-1. HIF-1 is predominantly active under hypoxic conditions because the HIF-1α subunit is rapidly degraded in normoxic conditions by proteasomal degradation[98]. Different genes involved in erythropoiesis and iron metabolism (erythropoietin or transferrin), glucose/energy metabolism (glucose transporters), cell pro liferation/viability decisions (transforming growth factor-β), vascular development/remodeling and/or vasomotor tone (VEGF or NOS-2) contain HRE (hypoxia responsive element)[99]. HIF-1α is overexpressed as a re sult of intratumoral hypoxia and/or genetic alterations affecting key oncogenes and tumor suppressor genes in human cancer[100]. Different signals other than hypoxia such as growth factors, reactive oxygen species, cytokines, NO and/or NO-derived species participate in hypoxic signaling[101]. NO, through cGMP-dependent pathways, regulates different modifications during drosophila development in oxygen deprivation conditions[102]. However, thiol groups in HIF-1 or the proteins that are involved in the regulation of HIF-1 are also potential targets for post-translational modifications by NO. GSNO or selected NO donors enhance S-nitrosylation of propyl hydroxylase which lead to HIF-1α accumulation[103,104] and HIF-1 DNA-binding activity in cell systems[105]. However, small NO concentrations induce a faster but transient HIF-1α accumulation than higher doses of the same donor[106]. NO-related HIF-1 activation mediates up-regulation of VEGF expression in normoxic human glioblastoma and hepatoma cells[107]. Different studies have also shown that phosphorylation mechanism by PI3K/Akt pathway is also involved in GSNO-induced HIF-1 accumulation[108].

As described above, HIF-1 is predominantly active under hypoxic conditions in which the generation of oxygen species, specifically $H_2O_2$, is supposed to attenuate HIF-1 activation. Similarly, the redox cycler DMNQ (2,3-dimethoxy-1,4-naphthoquinone) generating $O_2^-$ and/or $H_2O_2$ (derived from superoxide dismutase-triggered conversion of $O_2^-$ to $H_2O_2$) attenuated NO-derived reactive nitrogen species-elicited HIF-1α accumulation[108]. The attenuation by NO of hypoxia-evoked reporter gene activation has been extended to several genes such as insulin-like growth factor binding protein (IGFBP-1), endothelin-1 and VEGF[101]. In this condition, NO has been shown to prevent HIF-1 accumulation in Hep3B and PC-12 cells which it reduced by addition of a lipophilic glutathione analog or $ONOO^-$ scavenger[109]. If indeed the steady state of $O_2^-$ increases under hypoxia, it may be hypothesized that hypoxia in the presence of NO-derived reactive nitrogen species delivery promotes formation to the strong oxidant $ONOO^-$. $ONOO^-$ in turn may not only oxidize reduced glutathione but also damage mitochondria. The differential behavior of NO in normoxic and hypoxic conditions may also be related to its capacity to regulated mitochondrial oxidative phosphorylation which may limit ROS generation and HIF-1 accumulation in hypoxic conditions[110]. In addition, the

interference of NO signaling by mitochondrial $O_2^-$ generation can be rationalized by the diffusion-controlled radical interaction which may redirect signaling qualities of NO towards other species, i.e. $ONOO^-$ that may not share the ability to stabilize HIF-1α. Hypoxic intracellular environment is characterized by a complex network of radical pattern generation that in conjunction with variable amounts of defense-systems may reveal a variable HIF-response to NO.

## CONCLUSION

Different studies have shown that increased and continuous NO production plays a pivotal role in the regulation of carcinogenic process. The alteration of redox status and transcriptional pattern modifications induced by NO in tumoral cells increase cell death and exerts antineoplastic properties. In this sense, more studies should be done in order to identify the temporal, spatial and concentration-dependent intra- and extra-cellular NO generation that exerts its maximum antitumoral activity either as monotherapy or combined treatment with chemotherapy.

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