Nitric Oxide and Cancer: The Emerging Role of S-Nitrosylation

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Abstract: Nitric oxide ($NO^{\bullet}$) is a short-lived, endogenously produced gas that is highly diffusible across cell membranes and acts as a signaling molecule in the body. The redox state and chemistry of $NO^{\bullet}$ facilitate its interaction with various proteins thus regulating various intracellular and intercellular events. One of the key mechanisms by which $NO^{\bullet}$ regulates the function of various target proteins is through the coupling of a nitroso moiety from NO-derived metabolites to a reactive cysteine leading to the formation of a S-nitrosothiol (SNO), a process commonly known as S-nitrosylation. S-nitrosylation signaling events within the cell have led to the discovery of many other physiological functions of $NO^{\bullet}$ in many other types of cells including cancer cells. Only recently are the diverse roles of S-nitrosylation in cancer beginning to be understood. In the present review we discuss the recent evidence for the diverse roles of $NO^{\bullet}$/SNO-related mechanisms in cancer biology and therapy, including the participation of $NO^{\bullet}$ in the pathogenesis of cancer, its duality in protecting against or inducing cancer cell death and the contribution of $NO^{\bullet}$ to metastatic processes. In addition, $NO^{\bullet}$ can be therapeutically used in the reversal of tumor cell resistance to cytotoxic drugs and as a sensitizing agent to chemo- and radiotherapy. Finally, recent studies providing evidence for NO-related mechanisms of epigenetic gene expression regulation will also be discussed. Undoubtedly, new exciting results will contribute to this rapidly expanding area of cancer research.

Keywords: Angiogenesis, apoptosis, cancer, epigenetics, nitric oxide, S-nitrosylation, therapy.

INTRODUCTION

Nitric oxide ($NO^{\bullet}$) is a simple, diatomic molecule that was regarded as an atmospheric pollutant until it was found in 1987 and the following years that it is synthesized *in vivo* and acts as a signaling molecule in the body [1]. $NO^{\bullet}$ is responsible for a range of physiological processes such as vasodilation, inhibition of platelet aggregation, neurotransmission and anti-microbial activity [2-5]. $NO^{\bullet}$ is synthesized by the metabolism of L-arginine to L-citrulline through a complex reaction catalyzed by the enzyme nitric oxide synthase (NOS), of which there are several isoforms [6, 7]. NOS1 (nNOS) and NOS3 (eNOS) were initially cloned in neural cells and endothelial cells, respectively, are dependent on Ca^{+2}-calmodulin and are constitutively expressed. The NOS2 (iNOS) enzyme was initially identified in macrophages and its binding to calmodulin is strong even at low concentration of intracellular calcium, so it is independent of calcium and can consistently produce high levels of $NO^{\bullet}$ for prolonged periods [8, 9]. It has been shown that many cell types (hepatocytes, vascular smooth muscle cells, fibroblasts and epithelial cells) express one or more of these isoforms [7, 10]. $NO^{\bullet}$ excess or deficiency is currently believed to participate in numerous pathophysiological conditions, such as arthritis, atherosclerosis, cancer, diabetes, numerous degenerative neuronal diseases, stroke and myocardial infarction [10]. Regarding cancer, various studies have demonstrated roles for $NO^{\bullet}$ in the pathogenesis of cancer, its duality in protecting against or inducing cancer cell death and the contribution of $NO^{\bullet}$ to metastatic processes. The complex chemistry involving $NO^{\bullet}$ has complicated the investigation of its biological roles, with discussions from fundamental chemistry and biochemistry as they relate to normal physiology and disease processes.

The redox state and chemistry of $NO^{\bullet}$ facilitate its interaction with a variety of proteins thus regulating various intracellular and intracellular events. Because $NO^{\bullet}$ is a relatively weak oxidant, a large body of evidence attributes the NO-mediated effect to its other reactive metabolites [11]. Many of $NO^{\bullet}$ actions are mediated *via* stimulation of the hemoprotein soluble guanylyl cyclase (sGC). It is through the specific interaction of $NO^{\bullet}$ with the sGC heme that sGC is activated resulting in the production of the second messenger cyclic GMP, thus regulating vascular tone and neurotransmission [12]. However, a large number of studies in recent years have provided ample evidence that $NO^{\bullet}$ can trigger or modulate cell signaling by modifying other proteins. Hence, one key mechanism by which $NO^{\bullet}$ regulates the function of various target proteins is through the coupling of a nitroso moiety to a reactive thiol group in specific cysteine residues leading to the formation of a S-nitrosothiol (SNO), a process commonly known as S-nitrosylation [13-15]. As long as NO itself is a poor nitrosating agent, some mechanisms have been described for SNO formation within the biological environment [16]. Thus, dinitrogen trioxide (N_2O_3) and peroxynitrite ($ONOO^-$) which are formed from O_2 and $NO^{\bullet}$ are the likely S-nitrosating species in biological

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systems. S-nitrosylation may also occur *via* the intermediacy of a ferric heme nitrosyl species [16, 17]. Importantly, the nitroso function can be transferred from one thiol to another in a transnitrosylation reaction that occurs *via* nucleophilic attack on the nitrogen atom of the SNO [16, 17].

A large number of studies have provided evidence that S-nitrosylation of proteins may play a regulatory role similar to phosphorylation. In fact, although multiple cysteine residues in a protein are available for S-nitrosylation, few are specifically modified and are responsible for the modification of protein function [18-20]. Specific motifs within proteins [21, 22] and the interaction or subcellular co-localization with NOS isoforms [23-26] are among the factors contributing to this selective S-nitrosylation. In addition, proteins that directly introduce $NO^{\bullet}$ groups *via* trans-nitrosylation reactions into target proteins, and that may be considered "protein nitrosylases", have been described [27-30]. On the other hand, the physiological relevance of denitrosylases, including the nitrosoglutathione reductase (GSNOR) and the thioredoxin/thioredoxin reductase (Trx/TR) enzymatic systems are now well established [31-33]. S-nitrosylation plays a crucial role in the control of mammalian gene transcription with numerous transcription factors regulated by this modification [34]. A broad spectrum of human diseases is associated with a deregulation of protein S-nitrosylation and, given the importance of $NO^{\bullet}$ in cancer, this important aspect must also be taken into account when investigating tumor biology. A growing body of evidence suggests that many of the NO-mediated effects observed in tumors are conveyed through S-nitrosylation of proteins. Thus, S-nitrosylation of key proteins in cancer biology (Table **1**) may explain the role of $NO^{\bullet}$ in the pathogenesis of cancer, its duality in protecting against or inducing cancer cell death and the contribution of $NO^{\bullet}$ to metastatic processes.

NITRIC OXIDE AND THE PATHOGENESIS OF CANCER

Since the early research on $NO^{\bullet}$, a clear association of NOS with several tumors in humans became evident [35-38] and further studies have provided evidence that NOS isoforms are widely expressed and often up-regulated in multiple tumor tissues [39]. The effects of $NO^{\bullet}$ in tumors depend on the concentration and duration of exposure to $NO^{\bullet}$, location and activity of NOS isoforms, cell type and microenvironment and its sensitivity to $NO^{\bullet}$ [10, 40]. All three NOS isoforms have been associated with the promotion or inhibition of cancer. Tumor cells often express iNOS and in some cases eNOS or nNOS (i.e. brain, lung or breast) depending on tumor type and stage [41-43]. Tumor-associated stromal fibroblasts and immune cells commonly express iNOS, whereas tumor vascular endothelial cells predominantly express eNOS [39].

There is a clinical evidence that $NO^{\bullet}$ derived from tumor cells can both promote and inhibit tumor progression. Low levels of NO would facilitate tumor progression and high $NO^{\bullet}$ levels would exert inhibitory effects. This is dependent on the type and level of NOS expression and the local concentration of $NO^{\bullet}$ within the tumor cells. The activation of sGC and the mitogen-activated protein kinase (MAPK) pathway has been shown to enhance migration and invasion of colon cancer and breast cancer cells [44, 45]. In breast carcinomas, nuclear epithelial growth factor receptor (EGFR) positively correlates with iNOS, and interaction of STAT3 and nuclear EGFR has been shown to be involved in transcriptional activation of iNOS in highly proliferative cells [46]. Besides, several studies have shown that the upregulation of tumor VEGF expression, which promotes angiogenesis and tumor progression, is mediated by iNOS induction in tumor cells [47, 48]. As will be discussed later, the S-nitrosylation of transcriptional regulators of VEGF expression may explain the promotion of angiogenesis in tumor cells. However, conflicting observations have been reported regarding NO-mediated tumor angiogenesis, invasion and growth in human cancers. Some studies showed the relationship between angiogenic activity or high VEGF expression and iNOS expression in human brain [49, 50], head and neck [51, 52], lung [42, 53], breast [54, 55], and colon [56] cancers. However, histological examination of several human cancers has revealed that iNOS expression does not show any correlation with tumor progression. In some cases, iNOS expression in tumor cells was inversely correlated with tumor stage, grade and progression, and positively correlated with apoptosis and patient survival [42].

Inflammation is now regarded as one of the hallmarks of cancer. Although many cancers arise *de novo* without an identifiable predisposing disease, it is now believed that chronic inflammation is a strong supportive factor in tumor development and associated with a higher cancer risk [39, 57]. The genetic ablation of iNOS in mice has been shown to abolish the spontaneous development of lymphomas associated to *Cryptosporidium parvum*–induced inflammation [58]. Also, in a model of chronic inflammatory bowel disease, infection with *Helicobacter hepaticus* led to infiltration of macrophages and neutrophils into the colon, up-regulation of iNOS expression at the site of infection, increased $NO^{\bullet}$ production, severe inflammation, hyperplasia, dysplasia, and colon cancer. Notably, concurrent administration of an iNOS inhibitor in this model prevented $NO^{\bullet}$ production, abrogated the epithelial pathology and inhibited the onset of cancer [59]. Activated Src-kinase, a non-receptor tyrosine kinase which play important roles in the regulation of both inflammation and cancer, co-localizes with and phosphorylates iNOS on Tyr^{1055} and thus stabilizes active iNOS under inflammatory conditions and in cancer cells [60].

There is an increasing interest in the role of $NO^{\bullet}$ in regulating tumor-stroma communication and inflammation. It has long recognized that some tumors are densely infiltrated by cells of both the innate and

Table 1. Some S-Nitrosylated Proteins and their Relevance in Cancer Biology

Protein name	S-nitrosylation consequences [Ref.]	Cancer-process involved
AGT[a]	Promotion of proteasomal degradation of AGT, higher incidence of liver tumors [75]	Tumor initiation
Bcl-2	Stabilization of Bcl-2 through inhibition of ubiquitination and proteasomal degradation, inhibition of apoptosis [90, 91]	Resistance to chemotherapy
Caspases	Inhibition of caspase activity, inhibition of apoptosis [87]	Resistance to chemotherapy
Caveolin-1	Prevention of caveolin-1 proteasomal degradation, promotion of anoikis resistance and metastasis [133]	Metastasis
β-catenin	Disassembly of adherens junctions in endothelial cells and increased vascular permeability to tumor cells [136] Degradation of nuclear β-catenin and tumor cell proliferation [163]	Metastasis?, tumor progression
Fas receptor	Redistribution of Fas receptor to lipid rafts and formation of the death-induced signaling complex, promotion of apoptosis [84]	Sensitization to chemotherapy
GAPDH	Nuclear translocation of GAPDH and promotion of apoptosis, transnitrosylation of other nuclear proteins [82, 83]	Sensitization to chemotherapy
HDAC2	Dissociation of HDAC2 from CREB-regulated promoters [200], inhibition of deacetylase activity [201]	Epigenetic deregulation?
HIF-1α	Stabilization of accumulation and activity, promotion of angiogenesis [107, 108]	Angiogenesis, tumor progression
Mdm2	Disruption of Mdm2-p53 binding [73]	Tumor progression?
MGMT	Inhibition of DNA repair activity, sensitization to nitrosourea treatment [183]	Sensitization to chemotherapy
NF-kappaB	Inhibition of NF-kappa B binding to DNA and downregulation of anti-apoptotic signaling, promotion of apoptosis [78-80]. Downregulation of the NF-kappaB/Snail/RKIP circuitry, reversal of tumor cell invasion and resistance to chemotherapy [130]	Sensitization to chemotherapy
PTEN	Inhibition of PTEN activity and increased stability of HIF-1α [110]	Angiogenesis, tumor progression
Ras	Activation of wild-type Ras proteins in Ras-mutated tumors [74]	Tumor initiation and progression
c-Src	Disruption of E-cadherin junctions and enhancement of cell invasion [20]	Metastasis

[a]AGT: O^6-alkylguanine-DNA alkyltransferase; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; HDAC2: histone deacetylase 2; HIF-1α: hypoxia-inducible factor 1α; MGMT: O^6-methylguanine-DNA methyltransferase; PTEN: Phosphatase and tensin homolog.

adaptive arms of the immune system and thereby mirror inflammatory conditions arising in non-neoplastic tissues. Tumor-associated macrophages are frequently observed to be immunosuppressive and functionally polarized to promote tumor growth and metastasis. Recently, the induction of macrophage-dependent iNOS expression within the tumor microenvironment has been shown to be critical for the success of immunotherapy with IL-2/alpha-CD40 in a murine model of lung metastases [61]. Furthermore, the authors also reported that treatment of tumor-bearing mice with the $NO^{\bullet}$ donor JS-K significantly reduced metastases by downregulating matrix metalloproteinases (MMPs), which play important roles in matrix remodeling and the metastatic process [61]. Therefore, iNOS expression *via* macrophages may be decisive for the control of metastatic spread and context-dependent application of $NO^{\bullet}$ donors may prevent metastatic disease.

A variety of *in vitro* and *in vivo* models show that iNOS/$NO^{\bullet}$ signaling can also induce COX-2, which itself is also a promising link between inflammation and cancer [62]. In fact, targeting iNOS and COX-2 in inflammatory cells has proven to exert cancer prevention activities in several rodent models of inflammation-associated carcinogenesis [63]. The constitutively activated MAPK pathway in melanoma has been shown to stimulate activation of NF-kappaB, which, in turn, drive iNOS expression and support melanoma tumorigenesis [64]. Moreover, published data on INOS expression in primary cutaneous melanomas reveal that the expression of this protein can exist at very early stages of malignancy [65]. Accordingly, studies on the prognostic value of iNOS expression strongly suggest that iNOS expression is an independent prognostic marker for stage III melanomas [66, 67]. The expression of iNOS has also been related with poorer prognosis in gastric [68] and liver cancer

[69], although its prognostic significance in other human cancers remains to be elucidated [70, 71].

Differences in tumor type, local $NO^{\bullet}$ levels and cellular responsiveness to $NO^{\bullet}$ may dictate the overall tumor response to $NO^{\bullet}$. In this regard, it has been shown that the response of tumor cells to iNOS induction is dependent of their p53 status [47]. The induction of iNOS increased VEGF expression, angiogenesis and tumor growth in p53-mutant tumor cells but decreased the growth of p53 wild type tumors. The nuclear p53 accumulation has been described in NO-stressed cells [72]. In unstressed cells, p53 and its negative regulator Mdm2 shuttle between the nucleus and the cytoplasm with 26S-proteasomal degradation of p53. In NO-stressed cells, p53 is trapped in the nucleus, is phosphorylated, ubiquitinated and transcriptionally active. Interestingly, $NO^{\bullet}$ may indirectly control p53 by means of S-nitrosylation of a critical cysteine residue in Mdm2 that disrupts Mdm2-p53 binding [73].

Effects of eNOS on tumorigenesis have been largely attributed to its activity in endothelial cells. However, a recent study has shown that the continual need for PI3K-AKT signaling during initiation and maintenance of oncogenic Ras-driven tumor growth is due, at least in part, to activation of tumor-expressed eNOS, which in turn leads to S-nitrosylation and activation of the other wildtype Ras family members [74]. These results suggest a key role for tumor-expressed eNOS in the tumorigenic process, at least in oncogenic Ras-driven cancers. However, the reason why a cancer cell that already has one Ras family member oncogenetically activated would rely on activation of the other Ras family members remains a mystery.

A recent study has underscored the importance of protein S-nitrosylation in the pathogenesis of liver cancer. Previously generated mice harboring a targeted deletion of GSNOR, $GSNOR^{-/-}$ mice that accumulate S-nitrosothiols and have increased levels of protein S-nitrosylation [32], were found to have a 10-fold higher incidence of spontaneous hepatocellular carcinoma (HCC) than controls [75]. The livers of $GSNOR^{-/-}$ mice exhibited substantial S-nitrosylation and proteasomal degradation of the key DNA repair enzyme O^6-alkylguanine-DNA alkyltransferase (AGT). Significantly, predisposition to HCC, S-nitrosylation and depletion of AGT was abolished in mice deficient in both GSNOR and iNOS. Moreover, Liu and coworkers found that GSNOR abundance and activity was significantly decreased in 50% of patients with HCC [75]. These data raise new perspectives for therapeutic applications, including the suitability of iNOS as a target molecule in liver cancer.

ROLE OF NITRIC OXIDE AND S-NITROSYLATION IN TUMOR APOPTOSIS

The dual activity of $NO^{\bullet}$ in protecting against or inducing cancer cell death may also explain the multifaceted role of $NO^{\bullet}$ in the pathogenesis and progression of cancer. Overall, it appears that high $NO^{\bullet}$ levels from extracellular sources induce apoptosis and cell death by several mechanisms including direct membrane damage, inhibition of ribonucleotide reductase and inhibition of cellular ATP generation by mitochondrial electron transport enzymes, aconitase and mitochondrial glyceraldehyde-3-phosphate dehydrogenase GAPDH [76]. However, accumulating evidence points to protein S-nitrosylation involvement in cell death signaling [77]. $NO^{\bullet}$ can promote cell death *via* S-nitrosylation of key target proteins which participate in the apoptotic signaling cascade (Fig. **1**). One of the mechanisms of NO-induced apoptosis is the S-nitrosylation of NF-kappaB which causes the downstream inhibition of several antiapoptotic proteins [78-80]. Other studies have revealed further mechanisms of NO-mediated apoptosis and cytotoxicity. In this regard, the cytosolic glycolytic enzyme GAPDH has been associated with transcriptional regulation of apoptosis by $NO^{\bullet}$. A signaling pathway has been reported in which $NO^{\bullet}$ generation elicits S-nitrosylation of GAPDH, which augments its binding to the E3 ubiquitin ligase Siah1. Subsequently, the nuclear localization signal of Siah1 mediates nuclear translocation of GAPDH and apoptosis [81]. Nuclear S-nitrosylated GAPDH is acetylated by the acetyltransferase p300/CBP and, in turn, stimulates these proteins to acetylate and activate downstream targets, such as p53. This cascade seems to mediate the NO-triggered GAPDH cell death pathway [82]. Additionally, a recent study has shown that S-nitrosylated GAPDH physiologically transnitrosylates other nuclear proteins, including the deacetylating enzyme sirtuin-1, histone deacetylase-2 and DNA-activated protein kinase [83]. On the other hand, since S-nitrosylation of caspase-3 on their catalytic-site cysteine by endogenous $NO^{\bullet}$ production inhibits apoptosis, other NO-related mechanism regulating cell death is the active denitrosylation of cytosolic S-nitrosylated caspase-3 by thioredoxin-1 [33]. Recently revealed mechanisms of NO-mediated apoptosis involves Fas receptor [33, 84]. This receptor, also known as CD95, is a death receptor that mediates Fas ligand-induced apoptosis through sequential activation of caspases. Many cancer cells express Fas receptor but do not undergo Fas-mediated apoptosis. Interestingly, a recent study demonstrated the sensitization of cancer cells to Fas ligand-induced apoptosis after $NO^{\bullet}$ induced S-nitrosylation of cysteine residues in the cytoplasmic part of Fas receptor. The NO-induced modification of the receptor promoted its redistribution to lipid rafts and formation of the death-inducing signal complex [84]. In addition, upon stimulation of Fas, thioredoxin-2 mediates denitrosylation and activation of mitochondria-associated caspase-3, promoting apoptosis [33].

NO-induced modifications of proteins involved in the regulatory circuitry of cell death can also generate anti-apoptotic effects. Some caspases are subjected to inhibitory S-nitrosylation at their active site Cys, and

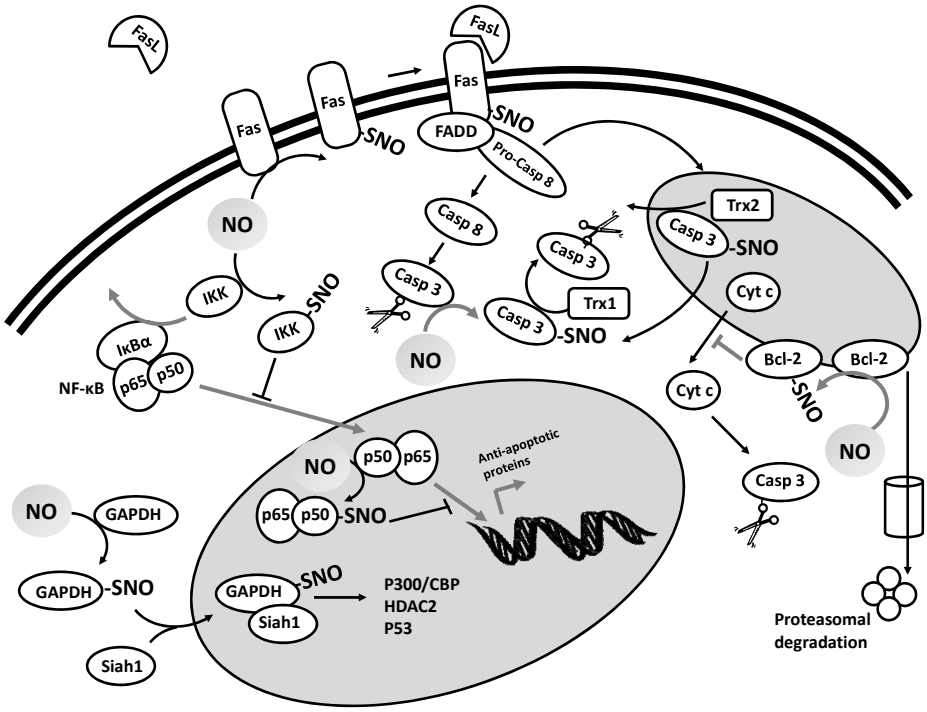

Fig. (1). Apoptotic and anti-apoptotic effects of NO in tumor cells. NO can promote or inhibit tumor cell death *via* S-nitrosylation (-SNO) of key target proteins which participate in the apoptotic signaling cascade. Apoptotic and anti-apoptotic actions are indicated as black and grey arrows respectively. This dual activity of NO in protecting against or inducing cancer cell death contribute to the multifaceted role of NO in the pathogenesis and progression of cancer. Bcl-2: B-cell CLL/lymphoma 2; FADD: Fas-Associated protein with Death Domain; GAPDH: Glyceraldehyde 3-Phosphate Dehydrogenase; HDAC2: Histone Deacetylase 2; IκBα: IκBα Kinase; IKK: IκBα Kinase; Siah1: E3 Ubiquitin-protein Ligase. See text for further details.

stimulation of death receptors of the tumor necrosis factor superfamily results in caspase denitrosylation [85, 86]. Particularly, mitochondrial executioner caspase-3 is constitutively S-nitrosylated at the active site Cys, and engagement of the Fas receptor promotes its release into the cytoplasm and subsequent denitrosylation [87]. A recent study has related the lower apoptosis observed in epithelial ovarian cancer with the coexpression of myeloperoxidase (MPO) and iNOS in tumor cells, but not in normal ovarian epithelium. MPO is an enzyme that utilizes $NO^{\bullet}$, produced by iNOS, as a one-electron substrate generating the nitrosating species NO+, that S-nitrosylates caspase-3 and induce apoptosis resistance in epithelial ovarian cancer cells [88]. The antiapoptotic protein Bcl-2 was discovered to be overexpressed in human B-cell lymphomas as it is located near chromosomal translocation break points frequently found in those cancers [89]. Interestingly, $NO^{\bullet}$ generation has been found to regulate lung cancer cells sensitivity to cisplatin-induced-apoptosis through S-nitrosylation of Bcl-2, which inhibits its ubiquitination and subsequent proteasomal degradation [90, 91]. This finding has been suggested as a mechanism to explain the previously observed correlation between increased NOS activity and chemotherapeutic resistance in lung carcinomas [91]. Also in this regard, a recent study has shown that Cr(VI)-transformed lung cells evaded apoptosis by a mechanism involving S-nitrosylation and stabilization of Bcl-2 protein, elucidating a novel mechanism that potentiates malignant transformation of nontumorigenic lung cells in response to a human carcinogen [92].

NO-MEDIATED MECHANISMS REGULATING TUMOR ANGIOGENESIS

A major event in tumor growth and expansion is an alteration in the balance of pro-angiogenic and anti-angiogenic molecules that leads to tumor neovascularization. This “angiogenic switch” is essential in tumor progression so that nutrients can reach tumor cells and to enable them to eliminate metabolic waste. The new vessels not only help to meet the growing metabolic demands of the tumor but also favor tumor dissemination and metastasis. Angiogenesis is stimulated by several protein growth factors and steroids. Among these, the vascular endothelial growth factor (VEGF) family plays a major role. VEGF-A is essential for vasculogenesis and angiogenesis during embryonic development and similarly serves as a major angiogenic factor in tumors [93]. Based on its key role in angiogenesis, VEGF-A has attracted most attention to date as a molecular target for inhibiting tumor angiogenesis [94]. VEGF-A stimulates proliferation and motility of endothelial cells by binding to VEGF receptor-2 (VEGFR2) and to a lesser extent VEGFR1.

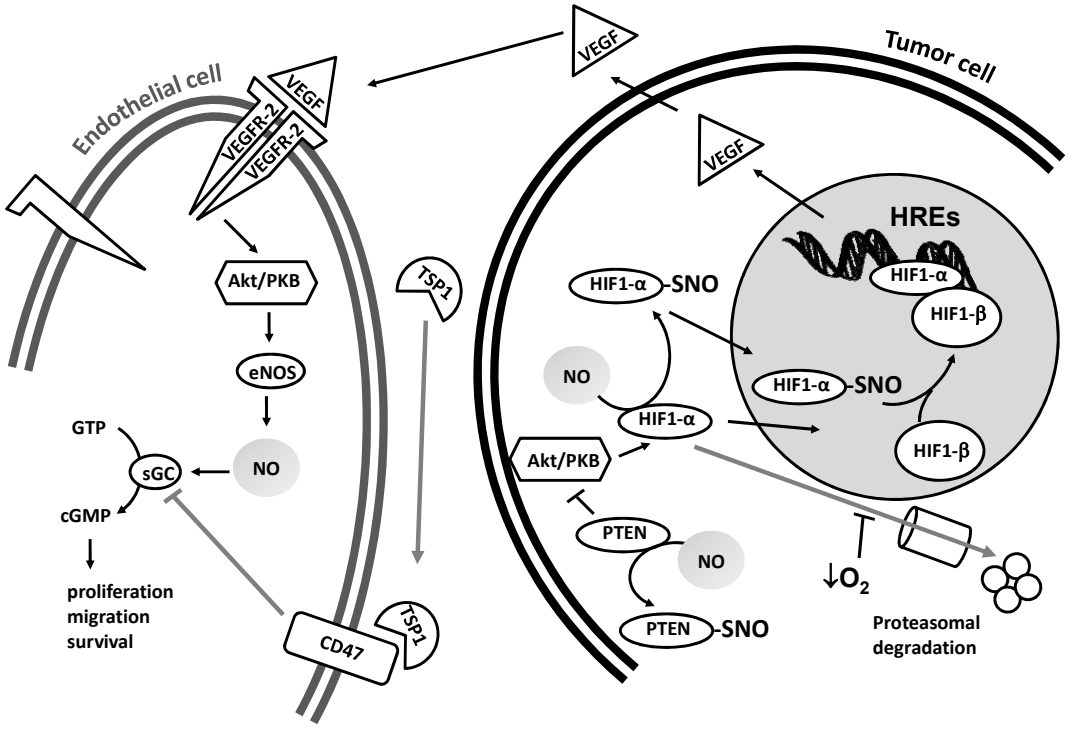

Fig. (2). NO-mediated mechanisms regulating tumor angiogenesis. NO mediates the pro-angiogenic activities of VEGF and a number of additional angiogenic factors that support the neovascularization of tumors. Some of these activities are regulated *via* S-nitrosylation (-SNO) of target proteins which modulate oxygen sensing in tumor cells. Angiogenic and antiangiogenic events are indicated as black and grey arrows respectively. HIF1-α: Hypoxia Inducible Factor α; HREs: Hypoxia Response Elements; PTEN: Phosphatase and Tensin Homolog; TSP1: Thrombospondin-1; VEGF: Vascular Endothelial Growth Factor; VEGFR2: Vascular Endothelial Growth Factor Receptor 2. See text for further details.

$NO^{\bullet}$ mediates pro-angiogenic activities of VEGFA and a number of additional angiogenic factors that support the neovascularization of tumors (Fig. **2**). VEGF-A binding to VEGFR2 on endothelial cells activates its Tyr kinase activity which in turn activates the serine/threonine protein kinase Akt/PKB, which phosphorylates eNOS at Ser1177 and induces $NO^{\bullet}$ synthesis [95]. $NO^{\bullet}$ produced by eNOS binds to the prosthetic haem on sGC to stimulate cyclic GMP synthesis, activating downstream targets that increase endothelial cell proliferation, migration, survival and permeability [96]. On the other hand, thrombospondin-1 (TSP1), an endogenous angiogenesis inhibitor whose expression is frequently lost during cancer progression [97], is a potent antagonist of $NO^{\bullet}$ signaling in vascular endothelial cells [98]. TSP1 signaling through CD47 redundantly inhibits $NO^{\bullet}$ signaling at the level of sGC and cGMP-dependent protein kinase [97].

A major mechanism mediating adaptive responses of tumor cells to reduced O_2 availability (hypoxia) is the regulation of transcription by hypoxia-inducible factor 1 (HIF-1). The interplay between $NO^{\bullet}$ and HIF-1 is extremely complex, and readers are referred to excellent recent reviews on this subject [99,100]. HIF-1 is a proangiogenic transcription factor that interacts with specific promoter sequences, called hypoxia response elements (HREs), in the regulatory region of target DNA to stimulate the transcription of multiple angiogenic factors, including VEGF, and also genes involved in cell proliferation and survival [101,102]. HIF-1 is a heterodimeric transcription factor composed of two subunits, HIF-1α and HIF-1β. The HIF-1α subunit is rapidly degraded under normal O_2 conditions (normoxia), while the β subunit is constitutively expressed. Under hypoxia, HIF-1α is stabilized and dimerizes with HIF-1β to form an active transcription factor. Oxygen dependent hydroxylation at proline residues 402 and 564 in the oxygen-dependent degradation domain (ODD) within HIF-1α affects both the stability of the protein and its function as a transcription factor. The hydroxylated HIF-1α is then ubiquitinated and targeted for proteasomal degradation. Under hypoxic conditions, HIF-1α remains unhydroxylated, accumulates and, after translocation to the nucleus, heterodimerizes with HIF-1β to bind HREs [101,102]. This tightly regulated activity of HIF-1 in both hypoxic and normoxic conditions in normal tissues is deregulated by the activation of oncogenic pathways and the loss of tumor suppressor function (e.g. PTEN and p53) in many cancers, which causes HIF-1 to accumulate regardless of oxygen tension [103-105]. Initial studies using various, chemically distinct $NO^{\bullet}$ donors in different cell types provided evidence that $NO^{\bullet}$ also provoked HIF-1α stabilization, HIF-1 DNA binding and HIF-1 transactivation under normoxia [106-108]. In one of these studies, the fact that GSNO, a

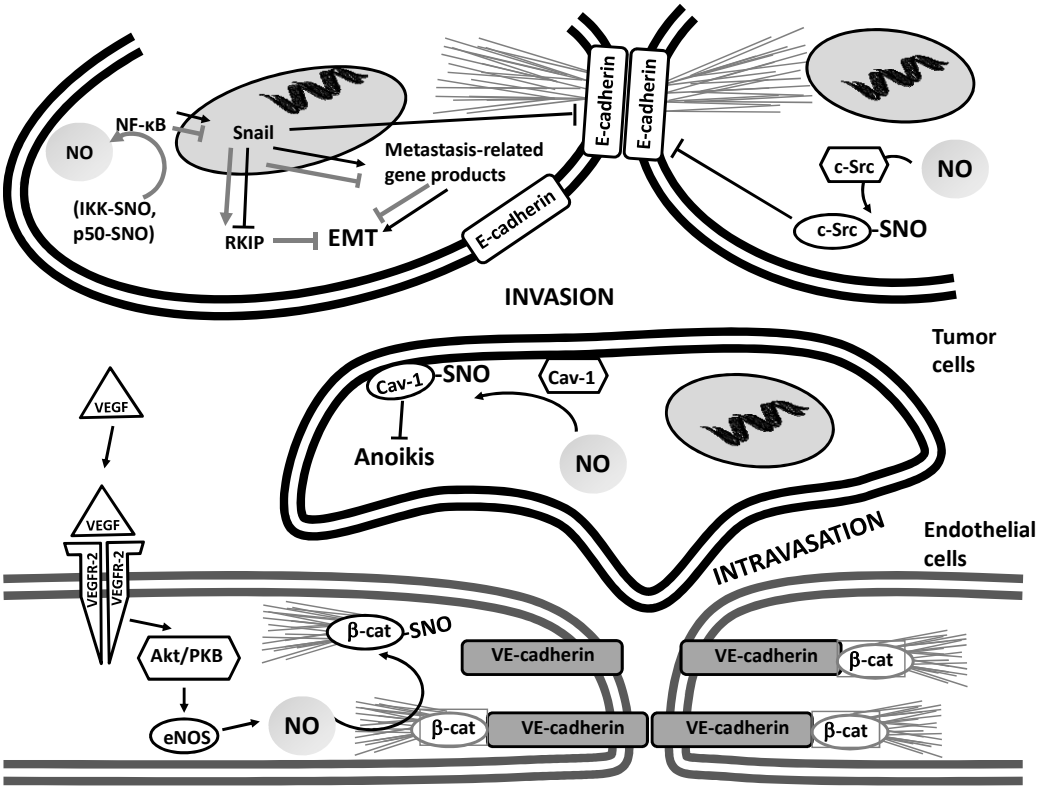

Fig. (3). Participation of NO in the metastatic process. NO is an important factor for the failure or the accomplishment of some steps of the invasion-metastatic cascade. High NO levels may reverse the epithelial-mesenchymal transition (EMT) regulatory program by downregulating the NF-κB/Snail/RKIP circuitry. However, NO may mediate the disruption of E-cadherin and VE-cadherin junctions in tumor and endothelial cells, respectively. NO-mediated mechanims may also suppress the detachment-induced apoptosis (anoikis) of tumor cells. Some of these NO effects are exerted *via* S-nitrosylation (-SNO) of target proteins. Pro-metastatic and anti-metastatic events are indicated as black and grey arrows respectively. Cav-1: Caveolin-1; β-Cat: β-Catenin; IKK: IκBα Kinase; RKIP: Raf Kinase Inhibitory Protein; VEGF: Vascular Endothelial Growth Factor; VEGFR2: Vascular Endothelial Growth Factor Receptor 2. See text for further details.

nitrosonium donor, provoked HIF-1α accumulation and that the GSNO effect could be reversed by dithiothreitol as well as acivicin, a proposed inhibitor of GSNO breakdown, made the authors to suggest that S-nitrosylation reactions stabilize HIF-1α accumulation and activity [108]. Subsequently, strong evidence was provided that $NO^{\bullet}$ mediated stabilization of HIF-1α is largely mediated by S-nitrosylation of the Cys533 in the ODD domain, which leads to the inhibition of HIF-1α degradation pathway [109]. This study was primarily conducted in murine tumors that were exposed to ionizing radiation and the authors demonstrated that selective disruption of this S-nitrosylation, by replacing Cys533 residue in the murine HIF-1α by a serine, significantly attenuated both radiation-induced and macrophage-induced activation of HIF-1α in tumors. In another study, the S-nitrosylation and subsequent inhibition of PTEN activity was suggested to be responsible for the increased stability and activity of HIF-1α in primary pulmonary vascular endothelial cells [110]. Therefore, $NO^{\bullet}$ may regulate HIF-1-dependent gene expression through several redundant pathways.

The implications of $NO^{\bullet}$ and eNOS in tumor growth by stimulating angiogenesis through VEGF signaling have motivated the investigation of polymorphisms of eNOS gene and their association with cancer risk, particularly in breast cancer. The 894 G>T (E298D) polymorphism of eNOS results in a 298 Glu>Asp substitution that alters susceptibility to cleavage [111] and leads to reduced $NO^{\bullet}$ levels [112]. Meta-analyses studies have demonstrated that 298 Glu>Asp substitution in eNOS confers a higher breast cancer risk [113, 114]. A 786T>C variant in the promoter region of eNOS gene has also been related to breast cancer risk [114]. However, E298D polymorphism has not found to be associated with prostate cancer risk [115] and none of the two aforementioned eNOS polymorphisms have been associated with survival in patients with colorectal cancer [116].

ROLE OF NITRIC OXIDE IN THE METASTATIC PROCESS

The multistep process of invasion and metastasis has been schematized as a sequence of discrete steps, often termed as the invasion-metastasis cascade [117]. This succession of molecular and cellular changes begins with local invasion of primary tumor cells, then they enter into the circulation

(intravasation), are transported through the circulation until their arrest in microvessels, invade the parenchyma of distant tissues (extravasation), and form micrometastatic nodules, some of which eventually grow into macroscopic metastases, the last process being termed "colonization". Much like to the participation of $NO^{\bullet}$ in other aspects of cancer biology, its role in the metastatic process is only partially understood. However, some studies reveal that $NO^{\bullet}$ is an important factor for the failure or the accomplishment of some steps of the invasion-metastasis cascade (Fig. **3**). Elevated $NO^{\bullet}$ and $NO^{\bullet}$ synthase levels have been associated with invasion and metastasis in many human cancers including melanoma [118], breast [37, 119, 120], head and neck [121], lung [122] and colon cancer [123]. Clinical data indicate that NOS activity is positively associated with human breast cancer progression [120] and in murine mammary tumors the sequential activation of NOS, GC and MAPK pathways is an essential step in invasion and metastasis [44].

Epithelial-mesenchymal transition (EMT) is a developmental regulatory program that has become prominently implicated as a means by which transformed epithelial cells can acquire the abilities to invade, to resist apoptosis, and to disseminate [124, 125]. The process was originally identified during specific stages of embryogenesis, in which a set of pleiotropically acting transcription factors, such as Snail, Slug, Twist and Zeb1/2, orchestrate the epithelial cell migration and colonization of various embryonic territories to form different organs. These transcriptional regulators are expressed in malignant tumors types, program cellular invasion in carcinoma experimental models of carcinoma formation and elicit metastasis when ectopically overexpressed [124]. Several of these transcription factors directly repress E-cadherin gene expression, thereby depriving neoplastic epithelial cells of this key suppressor of motility and invasiveness. The administration of $NO^{\bullet}$ donors has been reported to attenuate TGF-beta1-induced EMT in alveolar epithelial cells [126] and to suppress TGF-beta1-induced EMT and apoptosis in mouse hepatocytes [127]. Furthermore, a recent study has shown that high levels of $NO^{\bullet}$ derived from the $NO^{\bullet}$ donor DETANONOate inhibits EMT and reverses both the mesenchymal phenotype and the invasive properties of human prostate metastatic cells [128]. This NO-mediated EMT inhibition was shown to be related to the inhibition of both Snail expression and DNA binding activity that paralleled the upregulation of the metastasis suppressor Raf-1 kinase inhibitor protein (RKIP) and E-cadherin. Since Snail is transcriptionally regulated by NF-kappaB and in turn, Snail repress RKIP and E-cadherin transcription [129], these findings reveal a mechanism of NO-mediated reversal of cell invasion and resistance to apoptosis in cancer cells. Accordingly, the downregulation by $NO^{\bullet}$ of the NF-kappaB/Snail/RKIP circuitry has been also shown to sensitize tumor cells to apoptosis by both chemotherapeutic drugs and cytotoxic immuno-therapeutic ligands [130]. The abovementioned studies suggest that high $NO^{\bullet}$ levels may reverse EMT and invasive capabilities of malignant cells. However, others studies indicate positive effects of $NO^{\bullet}$ in this step of the metastasis cascade. For example, the disruption of E-cadherin junctions and enhancement of cell invasion by β-estradiol stimulation of breast cancer cells has been reported to be dependent on $NO^{\bullet}$ production and mediated by the S-nitrosylation of c-Src tyrosine kinase [20].

$NO^{\bullet}$ has also been implicated in the suppression of detachment-induced apoptosis, or anoikis, a process that is a principal mechanism of inhibition of tumor cell metastasis [131]. Caveolin-1 is a structural protein component of the plasma membrane microdomains termed caveolae that acts as a negative regulator of anoikis and its elevated expression is closely associated with increased metastasis and poor survival [132]. Interestingly, a study has reported that the NO-mediated down-regulation of anoikis in lung cancer cells involves S-nitrosylation of caveolin-1, which prevents its proteasomal degradation [133]. In addition NO has been shown to inhibit MMPs [61], while other studies have reported the positive correlation between iNOS activity and MMPs expression [134, 135]. Therefore, NO-mediated regulation of caveolin-1 and MMPs could be important mechanisms of anoikis resistance and metastasis in tumors.

Metastatic cells that survive the harsh circulatory stream and reach distant tissues still need to extravasate to begin colonization. The $NO^{\bullet}$ fluxes in the metastatic microenvironment may play a critical role determining metastatic cell dynamics and, consequently, the success or failure of extravasation. The eNOS derived $NO^{\bullet}$ participates in both VEGF-induced endothelial cell permeability and lymphangiogenesis, two processes that contribute to metastasis formation. In an elegant study, Gratton and coworkers have identified β-catenin as a target of S-nitrosylation in response to VEGF stimulation of endothelial cells [136]. The authors demonstrate how the specific S-nitrosylation of β-catenin on cys619 promotes its dissociation from VE-cadherin and contributes to VEGF-stimulated disassembly of adherens junctions and increased vascular permeability. Other study has shown that genetic deletion of eNOS, as well as blockade of $NO^{\bullet}$ synthesis attenuates peritumor lymphatic hyperplasia and decreases tumor cell dissemination to the lymph nodes in both fibrosarcoma and melanoma animal experimental models [137]. These findings raise the appealing possibility that the blockade of $NO^{\bullet}$ production in tumors could simultaneously block tumor-associated angiogenesis, decrease tumor vascular permeability and reduce lymphatic metastasis.

The effects of $NO^{\bullet}$ in the acquisition of invasive capabilities of cancer cells seem to depend on the concentration and duration of exposure to $NO^{\bullet}$, tumor type and secondary organ microenvironment. *In vivo* and *in vitro* experimental evidence indicates that during

the interactions between endothelial cells and intravascular tumor cells, $NO^{\bullet}$ plays a key role as a cytototoxic natural defensive effector. This has been demonstrated in the case of liver, which is a paradigmatic example of a common site for metastasis development. Both anatomical/hemodynamical and microenvironmental factors collaborate trapping and destroying circulating cancer cells, but also make this organ one of the most common sites for cancer [138]. A natural defense mechanism against metastatic cells after their arrest in the liver microvasculature is the endothelial $NO^{\bullet}$ release, which leads to sinusoidal cancer cell killing and reduced hepatic metastasis formation [139]. Accordingly, eNOS-deficient mice developed liver tumors more frequently in response to carcinogens compared with control animals, while eNOS overexpression in the tumor microenvironment attenuated both the number and size of tumor implants in a surgical model of pancreatic cancer [140].

Data derived from studies summarized above indicate that different NO-mediated mechanisms are implicated in most steps of the metastatic cascade. Thus, high $NO^{\bullet}$ levels suppress metastasis inhibiting EMT *via* downregulation of NF-kappaB/Snail/RKIP circuitry and exerting cytotoxic effects against tumor cells in colonization sites. However, the S-nitrosylation of c-Src, caveolin-1 and β-catenin mediate disruption of E-cadherin junctions, anoikis resistance and increased vascular permeability, respectively, thus contributing to the metastatic process. However, further studies are needed to clarify the exact role played by $NO^{\bullet}$ in metastatic dissemination. The metastatic cell cross-talk with stromal and endothelial cells along all the steps of metastatic cells spread is determinant of metastasis success or failure. The precise role of $NO^{\bullet}$ in these molecular interactions is beginning to be dissected, and undoubtedly could help in the design of new therapeutic approaches in the metastatic setting.

USE OF NITRIC OXIDE IN ANTI-CANCER THERAPY

Early observations indicated that $NO^{\bullet}$ is the predominant species responsible for the cytotoxic action of activated macrophages or interferon-activated endothelial cells against malignant cells [141, 142]. Cancer therapy with $NO^{\bullet}$ has been considered mainly as a means of sensitizing tumor cells to conventional treatments, such as radiotherapy and chemotherapy, as will be discussed later. However, a number of investigations have demonstrated that $NO^{\bullet}$, on its own, can exhibit potent anticancer properties, generally without significant toxicity. Several methods have been employed to generate therapeutic levels of $NO^{\bullet}$ in cancer cells, including administration of $NO^{\bullet}$ donor drugs, iNOS induction and iNOS gene therapy (Table **2**).

Many nitrogen-containing compounds will release $NO^{\bullet}$ by a variety of mechanisms. In the case of organic nitrates, such as glyceryl trinitrate, there is a rapid $NO^{\bullet}$ release after bioactivation by mitochondrial aldehyde dehydrogenase 2 [143]. In the case of diazeniumdiolates, also known as NONOates, such as the NO-donors DEA/NO and PAPA/NO, the release of $NO^{\bullet}$ is due to spontaneous decomposition [144, 145]. This latter class of compounds consists of a diolate group [N(O-)N=O] bound to a nucleophile adduct (a primary or secondary amine or polyamine) *via* a nitrogen atom, and their rate of decomposition is dependent on their structure, with half-lives varying from seconds to hours [145]. The *in vitro* anti-proliferative effects of NONOates have been reported in human melanoma [146], leukemia [147, 148], and breast cancer cells [149]. S-nitrosothiols comprise another class of $NO^{\bullet}$ donors that covers a vast array of different compounds, such as S-nitroso-N-acetylpenicillamine (SNAP) or S-nitrosoglutathione (GSNO), which contain a single chemical bond between a thiol (sulphydryl) group (R-SH) and the $NO^{\bullet}$ moiety [145]. The $NO^{\bullet}$ donor SNAP has been reported to induce apoptosis in human ovarian cancer, glioblastoma and glioma cells [150-152]. Besides, S-nitrosylated human serum albumin induced apoptosis and inhibited the growth of murine tumor cells both *in vitro* and *in vivo* [153, 154].

A novel approach to the design of NO-releasing compounds are the hybrid $NO^{\bullet}$ donor drugs, a range of established drugs that have been structurally modified to incorporate NO-releasing moieties. Of such agents, NO-NSAIDs (nitric oxide nonsteroideal anti-inflammatory drugs) were intended to modify the gastrointestinal toxicity associated with NSAIDs and later were also demonstrated to involve attenuated NDAIDs cardiotoxicity [145]. NO-NSAIDs were designed for use in arthritis and other pain treatments, but their chemopreventive properties against a variety of cancers have been demonstrated in preclinical models including cell culture systems [155-157] and animal tumor models [158, 159]. Interestingly, recent investigations indicate that some of the anti-cancer effects of NO-NSAIDs and other hybrid NO-donor drugs may be conveyed through S-nitrosylation. Two different NO-NSAIDs (NO-aspirin and NO-naproxen) having a different NSAID, spacer and NO-releasing moiety have been reported to inhibit human colon cancer cell growth, and this inhibition was shown to depend on S-nitrosylation of NF- kappaB p65 protein and subsequent suppression of anti-apoptotic signaling through this transcription factor [160]. JS-K, a hybrid NO-donor prodrug that has been designed to be selectively bioactivated by glutathione S-transferase enzymes, has shown promising *in vitro* and *in vivo* results as an anti-cancer drug [161]. The glutathione S-transferase enzymes are responsible for cleaving the compound to expose the diolate group and release $NO^{\bullet}$, but despite the ubiquitous nature of these enzymes, JS-K slows the growth of cancer cells without harming healthy cells [162]. Besides, JS-K has been reported to inhibit the proliferation of Jurkat T leukemia cells by S-nitrosylation and subsequent degradation of nuclear β-catenin [163]. Although the anti-cancer properties of NO-donors drugs strongly suggest their

Table 2. NO-Therapy Against Human Tumor Cells

Source of NO	Human tumor cells (NO-monotherapy, radiosensitization or chemosensitization)	References
Diazeniumdiolates (NONOates)		
PAPA/NO	Melanoma cells	[146]
PAPA/NO, DETA/NO	Leukemia cells	[147,148]
DETA/NONOate	Breast cancer cells	[149]
DETA/NO	Radiosensitization of breast cancer cells	[175]
DETA/NONOate	Radiosensitization of colorectal cancer cells (*in vivo* and *in vitro*)	[176]
DETA/NONOate	Chemosensitization of colorectal cancer cells to cisplatin (*in vitro* and *in vivo*)	[186]
S-nitrosothiols		
SNAP	Glioblastoma cells	[151]
SNAP	Radiosensitization of glioma cells	[152]
SNAP	Chemosensitization of ovarian cancer cells to cisplatin (*in vivo* and *in vitro*)	[150]
GSNO	Radiosensitization of neuroblastoma cells	[159]
Hybrid NO-donor drugs		
NO-NSAIDs	Pancreatic, colorectal, prostate, lung, and tongue cancer cells	[155]
NO-NSAIDs	Prostate and bladder cancer cells	[157]
NO-NSAIDs	Colorectal cancer cells (*in vitro* and *in vivo*)	[156, 160]
NO-NSAIDs	Radiosensitization of prostate cancer cells	[178]
NO-NSAIDs	Chemosensitization of ovarian cancer cells to cisplatin (*in vitro* and *in vivo*)	[184]
NO-NSAIDs	Chemosensitization of colorectal cancer cells to oxaliplatin or 5-fluorouracil	[159]
JS-K	Leukemia cells (*in vitro* and *in vivo*)	[161, 163]
JS-K	Breast cancer cells	[162]
Gene therapy or iNOS induction		
CMV-iNOS	Thyroid cancer cells (*in vivo*)	[164]
Ad.iNOS	Pancreatic, colorectal, gastric, prostate, bladder, breast, ovarian, renal and fibrosarcoma cancer cells (*in vitro* and *in vivo*)	[168]
CMV/iNOS, hOC/iNOS, PSMA/iNOS	Prostate cancer cells (*in vitro* and *in vivo*)	[169, 170]
Ad.iNOS	Radiosensitization of colorectal cancer cells (*in vitro* and *in vivo*)	[167, 181]
pE9.iNOS	Radiosensitization of colorectal cancer cells (*in vitro* and *in vivo*)	[166, 182]
Cytokine cocktail	Radiosensitization of fibrosarcoma and breast cancer cells	[180]

use in anti-cancer monotherapy, their potential efficacy in human cancer remains to be demonstrated.

Another approach for the targeted generation of $NO^{\bullet}$ in malignant tissue is gene therapy using iNOS, the high-output isoform of NOS. The first demonstration of this strategy came from studies where the injection of naked iNOS plasmids directly into murine thyroid experimental tumors halved their growth rate [164]. Antitumoral effects in murine sarcomas were also observed with direct liposomal delivery of a construct incorporating the pE9 promoter driving iNOS, that establishes a positive feedback loop leading to enhanced promoter activation by $NO^{\bullet}$ [165]. This promoter, which was derived from the cyclin dependent kinase inhibitor, p21(WAF1), is preferentially activated in tumors undergoing radiotherapy and provides as well efficacy in radiosensitization approaches [166]. Other efficient method for gene delivery is adenoviral infection, which has been used to achieve increased iNOS expression and $NO^{\bullet}$ generation in human tumors xenografted in mice [167, 168]. Gene delivery in iNOS gene therapy has also been accomplished by fusing the iNOS gene to promoters exhibiting tumor-specific properties. For instance, human osteocalcin (hOC) expression in prostate tumors is almost exclusively restricted to androgen-independent cell lines where current treatment strategies are ineffective. The transfection of the hOC promoter in tandem with the iNOS gene increased iNOS expression and $NO^{\bullet}$ generation in androgen-independent human prostate

cancer cells, an effect that was not observed in normal and androgen-dependent cells [169]. Similarly, prostate specific membrane antigen (PSMA) is up-regulated after androgen deprivation, indicating potential value for gene therapy in hormone refractory prostate cancer. Accordingly, the same authors demonstrated that the intra-tumoral injection of hOC-iNOS or PSMA-iNOS constructs caused tumor growth delays in human prostate cancer xenografts [170].

Hypoxia is not only a hallmark of solid tumors, but a fundamental problem for cancer radiotherapy. Dioxygen directly reacts with a radical DNA, formed in response to ionizing radiation, to yield stable adducts, a reaction known as the "oxygen effect" [171]. Since this reaction is of critical importance for the stabilization of DNA lesions that would otherwise be fully reversible, hypoxic niches escaping radiotherapy constitute nodes for tumor recurrence after treatment completion. One of the first described effects of $NO^{\bullet}$ in mammalian cells is that it can radiosensitize hypoxic cells as efficiently as oxygen [172]. Compared to oxygen, $NO^{\bullet}$ has a similar potential to bind to other free radicals and was suggested to behave as an intrinsic radiosensitizer that could mimic the "oxygen effect" in a hypoxic tumor environment [173]. Several NO-donor drugs, including NONOates, NO-NSAIDs and S-nitrosothiols, have been investigated for their capability to radiosensitize *in vitro* human glioma [152], neuroblastoma [174], breast [173, 175], colorectal [176], lung [177], and prostate cancer cells [178]. In other studies, tumor cells were radiosensitized through treatment with a cytokine cocktail for the induction of cellular iNOS [179, 180]. Gene therapy with iNOS has also been used as a method of radiosensitization of tumor cells. Adenoviral gene transfer of iNOS (AdiNOS) into human colorectal cancer cell lines significantly enhanced the effects of radiation [167]. Moreover, the AdiNOS treatment in the analogous xenografted tumors combined with radiation led to a significant tumor growth delay compared with radiation alone [167, 181]. Additionally, one of these studies showed that $NO^{\bullet}$ and ionizing radiation synergistically activated p53 by augmenting its phosphorylation at Ser15 [181]. As described earlier an appropriate approach to radiosensitization of tumor cells is the delivery of the iNOS gene under the control of radiation-inducible promoters. A series of studies have demonstrated that the combination of intratumoral injection of the radiation-inducible pE9/iNOS construct and radiation in human colon cancer xenografts significantly delayed tumor growth compared to the gene therapy treatment alone [166, 182].

Shortly after the demonstration of the $NO^{\bullet}$ ability to radiosensitize mammalian cells [172], a series of experiments indicated that DEA/NO treatment sensitized rat hepatoma cells to the nitrosourea BCNU [183]. The $NO^{\bullet}$ chemosensitization effect was attributed to inhibition by S-nitrosylation of the DNA repair enzyme O^6-methylguanine-DNA-methyltrans-ferase. Later studies have tested the delivery of $NO^{\bullet}$ *in vivo* in combination with platinum compounds, which are one of the most widely used classes of drugs in cancer therapy. The combination of NO-donor drugs and cisplatin has been investigated in a number of studies. Bratasz *et al.* [184] evaluated the combination of cisplatin with NCX-4040, a NO-NSAID consisting of an aspirin backbone attached to a NO-donor moiety. Their results showed that NCX-4040, in combination with cisplatin, was more effective than cisplatin alone in inhibiting the growth of cisplatin-resistant ovarian cancer xenografts in mice [184]. The low response to cisplatin therapy has been linked to high intracellular glutathione levels and to the overexpression of glutathione-S-transferase enzymes which catalyze coupling of glutathione to multiple reactive substrates [185]. Significantly, Bratasz and coworkers found that NCX-4040 depleted thiol levels in the tumor xenografts, and they proposed the formation of intracellular nitrosothiols, such as GSNO, as one of the possible mechanisms of thiol depletion [184]. NCX-4040 in combination with 5-fluorouracil or oxaliplatin has also been reported to enhance the inhibition of tumor growth *in vitro* and *in vivo* and to induce apoptosis in human colon cancer cells [159]. The sensitization by DETA/NONOate has been observed in nude mice bearing human cisplatin-resistant colon tumor xenografts and treated with the combination of DETA/NONOate and cisplatin. Besides, xenografts revealed significant upregulation of apoptosis-inducing factor (AIF) and increased apoptosis by DETA/NONOate and cisplatin combination treatment [186]. However, other authors claim that blocking NOS activity and $NO^{\bullet}$ production cause cisplatin sensitivity in human cancer cells. For example, the chemical inhibition of iNOS activity in mice bearing human melanoma xenografts was reported to downregulate the antiapoptotic protein Bcl-2, which was associated with increased susceptibility to cisplatin-mediated tumor death [187]. Moreover, the combination therapy of targeted iNOS inhibition and cisplatin was demonstrated in the same study to be more effective than either treatment alone [187]. Additionally, depletion of endogenous $NO^{\bullet}$ in melanoma cells by $NO^{\bullet}$ scavengers enhanced cisplatin-induced apoptosis and cell cycle arrest in a p53-dependent manner [188]. Cisplatin sensitization induced by high levels of a $NO^{\bullet}$ donor in ovarian cancer cells directly involves p53 upregulation [150]. However, cisplatin sensitization induced by inhibition of all three forms of NOS in cisplatin-resist ovarian cancer cells did not change p53 levels in ovarian cancer cells. Thus, the role of p53 in cisplatin sensitization appears to be different in melanoma and ovarian cancer cells. Data from this study suggest that endogenous eNOS/nNOS activity in ovarian cancer cells, especially cisplatin-resistant cells, produces low-level anti-apoptotic $NO^{\bullet}$, thus contributing to resistance against cisplatin. However, the role of iNOS in ovarian cancer cells appears to be different, contributing to, rather than suppressing, cisplatin-induced apoptosis [150].

The evidences provided by the aforementioned studies indicate that $NO^{\bullet}$ possess the ability to sensitize tumors to radiation or chemotherapy at clinically

relevant doses. Additional studies are needed to evaluate the true therapeutic gain for some of these treatments, before they can be considered for their extension to clinical trials. Nevertheless several clinical trials, mainly phase II trials, regarding $NO^{\bullet}$ and cancer have been, or are being, conducted. $NO^{\bullet}$ as chemosensitizer in cancer chemotherapy has progressed to clinical research in one phase II trial exploring the clinical benefit of adding the NO-donor nitroglycerin to chemotherapy in non-small cell lung cancer (NSCLC) patients with inoperable disease [189]. Patients were randomly assigned to a vinorelbine/cisplatin combination therapy, with transdermally applied nitroglycerin or with placebo patch. Following tumor size, a significant increase in response rate was observed in the group receiving the combination therapy with $NO^{\bullet}$. Notably, both the progression-free survival and overall survival was significantly extended in the $NO^{\bullet}$ group. Based on these clinical results, other two randomized phase II trials are now being conducted to assess the benefits of adding nitroglycerin to paclitaxel-carboplatin treatment in patients with previously untreated stage III or stage IV NSCLC (ClinicalTrials.gov identifier NCT00616031) or to concurrent chemoradiotherapy in stage III NSCLC (NCT00886405). Also, a randomized phase II trial (NCT01171170) has been designed to assess the effects of adding nitroglycerin patches to the standard first line treatment (carboplatin-paclitaxel-bevacizumab) of metastatic non-squamous NSCLC.

NEW HORIZONS: NITRIC OXIDE AS AN EPIGENETIC REGULATOR

Epigenetic changes are defined as those heritable changes in genome function that occur without a change in DNA sequence. Therefore, epigenetic inheritance is defined as cellular information, other than the DNA sequence itself, that is heritable during cell division [190]. It is now recognized that epigenetic modifications of DNA and histone proteins influence the overall structure of chromatin, regulating the access of transcription factors to gene promoters and driving transcription. Consequently, epigenetics has been recently redefined as "the structural adaptations of chromosomal regions so as to register, signal or perpetuate altered activity states" [191].

The deep scientific understanding of basic cellular processes mediated by $NO^{\bullet}$ is providing new direct evidences that $NO^{\bullet}$ is an epigenetic molecule. $NO^{\bullet}$ may diffuse from the cytoplasm to the nucleus, but since $NO^{\bullet}$ is a short-lived molecule this diffusion may be insufficient to modulate chromatin structure. However, there is evidence to suggest synthesis of $NO^{\bullet}$ within the nucleus due to the presence of tetrahydrobiopterin (a cofactor for NOS) biosynthetic enzymes [192] and at least the eNOS and iNOS isoforms [193, 194]. Indirect epigenetics effects of $NO^{\bullet}$ include its impact on gene expression through S-nitrosylation and modulation of transcription factors (reviewed in [34]), such as NF-kappaB, p53, β-catenin and HIF-1α [73, 79, 109, 136].

A more direct $NO^{\bullet}$ in epigenetics has emerged, as $NO^{\bullet}$ has been recently found to affect the functional activity of histone-modifying enzymes [195]. Among chromatin modifications, histone acetylation plays a pivotal role in the epigenetic regulation of transcription and other functions in cells. Histone acetyltransferases (HATs) and histone deacetylases (HDACs), catalyze the acetylation and deacetylation, respectively, at lysine residues [196, 197]. The interplay between HATs and HDACs alters the net balance of histone acetylation levels, thereby remodeling chromatin structure. Overall, HATs promote a more open, relaxed transcriptionally active chromatin, whereas HDACs often function as a component of the transcriptional repressor complex to silence gene expression and induce chromatin compaction [198, 199]. Not surprisingly, epigenetic alterations caused by aberrant activity of these enzymes are linked to the establishment and maintenance of the cancer phenotype and, importantly, are potentially reversible, since they do not involve genetic mutations in the underlying DNA sequence [198, 199]. Several studies have reported that $NO^{\bullet}$ regulates the function of histone modifying enzymes. Recently, the histone modifying enzyme HDAC2 has been identified as a key nuclear target of $NO^{\bullet}$ in neurons and muscle cells [200, 201]. S-nitrosylation at Cys262 and Cys274 residues of HDAC2 in developing neurons does not affect deacetylase activity but induces the dissociation of HDCAC2 from CREB-regulated promoters activating genes that are associated with neural development [200]. The $NO^{\bullet}$ dependent inhibition of HDAC2 functions has also been reported in muscle cells [201]. In a mouse model of muscular dystrophy, the therapeutic response to $NO^{\bullet}$ donors was associated to S-nitrosylation of HDCA2 on yet unidentified cysteine residues that caused a decrease in deacetylase activity [201]. The discrepancy in the inhibition of HDAC2 enzymatic activity by S-nitrosylation may reflect a difference of cysteine residues targeted by $NO^{\bullet}$ in neurons and muscle cells. Thus, S-nitrosylation of specific cysteine residues (Cys262 and Cys274) within HDAC2 protein may modulate its interaction with CREB-regulated promoters in neurons, whereas S-nitrosylation of yet unidentified cysteine residues of HDAC2 affects its deacetylase activity in muscle cells. In other study, Illi and coworkers showed that the treatment of human endothelial cells with $NO^{\bullet}$ donors induced nuclear localization of HDAC4 and HDAC5 [202]. Interestingly, the same group has recently shown that the NO-induced nuclear retention of HDACs 4 and 7, is associated with global histone deacetylation, chromatin remodeling, and mesodermic differentiation in mouse embryonic stem cells [203]. GAPDH is other important S-nitrosylated protein that may possibly play a key role in NO-mediated epigenetic control of gene expression. As described before, once S-nitrosylated, GAPDH interacts with Siah1, whose nuclear localization signal mediates nuclear translocation of GAPDH. This S-nitrosylated GAPDH-Siah1 complex transnitrosylates other nuclear proteins, including the histone deacetylating enzymes sirtuin-1, and HDAC2

[81, 83]. NO-dependent nuclear GAPDH also acts as an enhancer of p300/CBP autoacetylation and thereby its catalytic activity [82]. Of note, a recent report describes that the histone hyperacetylation observed in oral cancer is associated to the NO-mediated nuclear translocation of GAPDH and the subsequent increase of HAT activity of p300/CP [204]. These studies suggest that $NO^{\bullet}$ generation and cytosolic GAPDH, conveys a signal to the nucleus, and then may modulate epigenetic regulation of gene expression through histone modifications.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Since the early studies the multifaceted role of nitric oxide in cancer biology became evident. A series of recent seminal studies have begun to disentangle the complex web of interrelationships between this simple molecule and the critical molecular pathways involved in the initiation and progression of tumors. There is sufficient clinical and experimental evidence showing that $NO^{\bullet}$ can both promote and inhibit tumor progression, depending on concentration and duration of exposure to $NO^{\bullet}$, location and activity of NOS isoforms, cell microenvironment and sensitivity to $NO^{\bullet}$. The interplay of these critical factors ultimately define the $NO^{\bullet}$ targets in the tumoral environment. S-nitrosylation has emerged as a key molecular mechanism involved in $NO^{\bullet}$ signaling, and evidence is accumulating that this posttranslational modification has an important role in the NO-mediated regulation of tumorigenesis. However, few mechanisms regarding $NO^{\bullet}$/SNO and cancer have been clearly established, and more strong genetic evidence is required. The complexity of the signaling $NO^{\bullet}$ network confounds the simple translation of preclinical data from bench to bedside. This may explain why, despite huge amount of research spanning more than two decades, anticancer NO-based therapies have not made it to the clinic. Nonetheless, the identification of NO-modified proteins in tumors will have obvious implications in the recognition of potential targets for anti-tumor therapy. In this regard, an increasing progress of new analytical methods and strategies over the past several years will allow the investigation of the "S-nitrosoproteome" in tumors on a global scale [205-207]. Besides, the available evidence, suggest that the combination of NO-related therapies with the currently available targeted therapies, including antiangiogenic agents, must be tested in future clinical cancer trials. Undoubtedly, forthcoming studies will expand not only our current knowledge of the role of $NO^{\bullet}$ in cancer, but will also contribute to the tuning of therapeutic weapons against this intricate disease.

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