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Nitric oxide and carcinogenesis

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Abstract

Chronic inflammation induced by biological, physical and chemical factors is associated with the risk of cancer for many different tissues, including the lungs, liver and the oral cavity. Nitric oxide (NO) and several cytokines, particularly proinflammatory cytokines, are produced during the chronic inflammatory process. NO is one of the most important cytotoxic proinflammatory compounds. It is catalyzed by nitric oxide synthase (NOS) and has been associated with carcinogenesis. Previous studies have revealed the expression patterns of NOS in cancer. NO is able to suppress cellular immunity and stimulate cell proliferation. This small molecule also plays a major role in tumor angiogenesis, as well as tumor cell migration, invasiveness and metastasis. The purpose of this narrative review is to describe the role of the tumor-promoting effect of NO, and conversely its potential applications in cancer therapy.

Keywords: *nitric oxide, nitric oxide synthase, oxidative stress, carcinogenesis, angiogenesis, NO inhibitor*

1. Introduction

Inflammation activates various inflammatory cells, resulting in the induction and activation of several oxidant-generating enzymes, including nicotinamine dinucleotide phosphate oxidase (NADPH oxidase) (Witko-Sarsat, Khoa, Jungers, Drueke, & Descamps-Latscha, 1998), inducible nitric oxide synthase (iNOS) (Mazzio, Becker, & Soliman, 2003; Secco et al., 2003) and myeloperoxidase (Milla et al., 2004). These groups of enzymes produce high concentrations of diverse free radicals, such as superoxide anion and nitric oxide (NO). These radicals can react with each other to generate additional reactive oxygen and nitrogen substances that are more potent, such as peroxynitrite (Okada, 2002). They can damage DNA, RNA, lipids, and proteins by nitration, oxidation (Finkel, 2003), chlorination, and brominating reactions. This damage can lead to increased mutations and altered functions of enzymes and proteins (e.g., activation of oncogene products and/or inhibition of tumor-suppressor proteins) involved in the multistage carcinogenesis process (Ohshima, Tatemichi, & Sawa, 2003).

NO is a diatomic radical which plays a role in many regulatory functions *in viv*o. It is the product of the conversion of L-arginine to L-citrulline. This conversion from the L-arginine substrate is catalyzed by nitric oxide synthase (NOS). Oxygen, NADPH, and other cofactors, including tetrahydrobiopterin, flavin nonucleotide, flavin adenine dinucleotide, and heme, are essential in this reaction (Bentz, Simmons, Haines, & Radosevich, 2000). In addition to physiological functions, NO is linked to many inflammatory and neurodegenerative diseases and cancers. Although NO has been extensively studied, its roles in the pathogenesis of these diseases remain controversial. Previous work has noted the opposing effects of NO-dependence upon the experimental models, relative concentrations of NO, and surrounding environment where NO is produced.

A review on this topic has not been published previously in the Thai literature. The aim of this narrative review is to describe the biological effect of NO and its roles in promoting carcinogenesis and to explain its potential application in cancer therapy.

2. Search strategy and selection criteria

The search terms ‘nitric oxide synthase’, ‘nitric oxide’ or ‘nitric oxide and cancer’ were used as initial selection criteria. Publications indexed by PubMed/Medline during the period between 1990 and January 2012 were analyzed for relevant information. Certain pertinent articles were selected and included in this review. A language restriction was not applied.

3. Nitric oxide (NO)

NO has received much attention from researchers since its identification in 1978 (Freeman,

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Dyer, Juhos, St John, & Anbar, 1978). It can be classified into 3 isoforms which are encoded by distinct genes. Type I NOS, or neuronal NOS (nNOS/NOS-1), is a constitutive isoform. It is a Ca^{2+}- and calmodulin-dependent molecule, which can be found in high concentrations in the brain, peripheral nerves, skeletal muscle and pulmonary epithelium (Bentz, et al., 2000; Kendall, Marshall, & Bartold, 2001).

Type II NOS, or inducible NOS (iNOS/NOS-2), is an inducible, Ca^{2+}-independent isoform mainly in macrophages, but it can also be found in other cell types, including hepatocytes, vascular smooth muscle cells, fibroblasts, and epithelial cells, and in inflammatory areas (Forstermann, Nakane, Tracey, & Pollock, 1993).

Type III, or endothelial NOS (eNOS/NOS-3), is a constitutive isoform, which is Ca^{2+}- and calmodulin-dependent. It is found in the hippocampus, epithelium, platelets, cardiac myocytes and endothelial tissue (Forstermann, et al., 1993).

Both NOS-1 and NOS-3 are grouped together as constitutive NOS (cNOS). Their activities are regulated by intracellular calcium concentrations via calmodulin. In contrast, NOS-2 is not found in resting cells. Certain compounds, such as endotoxin and proinflammatory cytokines (e.g., interleukin-1 [IL-1], Interferon alpha [IFN α], and tumor necrosis factor-alpha [TNF-α]), induce iNOS expression. NOS-2 is bound to calmodulin even under basal intracellular calcium concentrations; therefore, the function of NOS-2 is not affected by intracellular calcium concentrations. Additionally, NOS-2 can produce much higher levels of NO than NOS-1 and NOS-3 are able to produce. These higher NO levels are linked to the bacterial cytotoxic characteristics of NOS-2-expressing cells (Forstermann, Boissel, & Kleinert, 1998; Schwartz et al., 1997).

3.1 Biology of nitric oxide

The wide range of roles for NO can be classified into three categories: i) an intracellular signal (Park et al., 1996), ii) a transcellular messenger (Ignarro, 1990), or iii) a cytotoxic species (Albina & Reichner, 1998). Direct effects of NO are mediated by the NO molecule itself, while its indirect effects are controlled by reactive nitrogen species (RNS) produced by the interaction of NO with oxygen (O_2) or superoxide radicals (O_2^{2-}).

Previously, NO was thought to be a fragile free radical. Currently, NO is considered a unique messenger with diverse physiological functions, including regulation of vascular smooth muscle tone, platelet activity, cytostatic actions of inflammatory cells, as well as non-adrenergic and non-cholinergic neurotransmission. Unlike other biomolecules, NO does not have a specific receptor. It diffuses from synthesis sites and interacts with different intracellular molecular sites. The characterized target site is guanylate cyclase. This enzyme stimulates enzymatic conversion of guanosine triphosphate (GTP) to cyclic guanosis monophostphate (cGMP). NO also mediates the guanylate cyclase-independent activity, which may be due to the inhibition of DNA synthesis by NO. This activity occurs via the inactivation of ribonucleotide reductase and deamination of DNA. The formation of peroxynitrite anion ($OONO^-$) from the reaction with super oxide anion (O_2^-) is cytotoxic. Host cells, which synthesize and release NO, possess some inherent protection against the toxic effects of this molecule (Wang, Spitzer, & Chamulitrat, 1999).

3.2 NOS expression in cancer

In vivo studies have shown that NO is formed by many cell types, including tumor cells and infiltrating immune cells (Thomsen & Miles, 1998). The presence of tumoral NOS expression raises the question about its roles during tumorigenesis.

Radomski et al. (Radomski, Jenkins, Holmes, & Moncada, 1991) first reported NOS-expression in human tumor cell lines. They studied colorectal adenocarcinoma cell lines from a primary tumor (SW-480) and a lymph node metastasis (SW-620) from the same patient. Both cell lines expressed the Ca^{2+}-independent NOS activity. Additional studies demonstrated the induction of iNOS in various human cell lines in response to cytokine stimulation. This response was observed in adenocarcinoma cell lines (Siegert, Rosenberg, Schmitt, Denkert, & Hauptmann, 2002), megakaryocytic cell lines (Battinelli & Loscalzo, 2000), melanoma cell lines (Joshi, Strandhoy, & White, 1996), and neuroblastoma cell lines (Fujisawa et al., 1994). Zellinger et al was the first to report on the expression and induction of iNOS in human breast cell lines (Zeillinger et al., 1996). Based on immunohistochemical analysis, Sappayatosok et al, 2009, reported the expression of iNOS in oral squamous cell carcinoma in Thai patients

(Sappayatosok et al., 2009). The effects of NO have been also reported in many cancer types, such as colon cancer (Roy et al., 2007), lung cancer (Edwards et al., 1996), melanoma (Tu et al., 2006), hepatocellular carcinoma (Sun et al., 2005), prostate cancer and urinary bladder cancer (Wolf, Haeckel, & Roessner, 2000)

3.3 Nitric oxide and carcinogenesis

During early stages of tumorIgenesis tumorigenesis, mutations caused by NO can induce several types of DNA damage (Jaiswal, LaRusso, Burgart, & Gores, 2000; Sakano, Oikawa, Hiraku, & Kawanishi, 2002; Wink et al., 1991), including transitions or transversions of bases or the inactivation of DNA repair proteins (Juedes & Wogan, 1996).

NO can mediate genotoxicity via many different mechanisms and is therefore considered a tumor initiation agent. NO can also influence other stages of carcinogenesis. The effects of NO are broad and often self-contradictory, involving cytostatic processes, cellular transformation, formation of neoplastic lesions, and regulation of various aspects of tumor biology.

Although humans have multiple antioxidant defenses and repair systems, derivatives of oxygen radicals and nitrogen oxide (e.g., peroxynitrite and NO_2) can significantly damage DNA, (Ahmad, Rasheed, & Ahsan, 2009). It is also thought that such damage leads to age-correlated cancer development. Additionally, NO can counteract the role of the tumor suppressor oncoprotein p53 (Popowich et al., 2010).

Excess NO results in oxidative stress and DNA damage, as well as in the disruption of energy metabolism, calcium homeostasis and mitochondrial function. All of these events can lead to cell death by apoptosis or necrosis, depending on the environment and severity of the damage (Gupta, 2003; Kanduc et al., 2002). Long-term elevation of cytosolic calcium activates a wide range of cell damage pathways. Small decreases in levels of adenosine tri-phosphate (ATP) can also lead to apoptosis. Mild oxidative stress can cause apoptosis, whereas severe oxidative stress contributes to extensive cellular damage and necrotic cell death (Slater, Nobel, & Orrenius, 1995). Alternatively, if the damage is insufficient to cause necrosis, the upregulation of *p*53 halts cell division, providing the opportunity for repair of damaged DNA (Colucci, el-Gehani, Flint, & Mothersill, 1997). Once cell damage has gone beyond the threshold level, the cell will undergo apoptosis, but the factors determining whether a cell undergoes death rather than repair remains unclear. Cell death via apoptosis can occur without inducing the expression of a new gene or protein synthesis; however, transcriptional changes can also initiate apoptosis. When apoptosis begins, self-destruction occurs gradually, preparing the cell remnants for removal by phagocytosis (Hale et al., 1996). ATP is essential to the apoptotic pathway, and if ATP is lacking, the mechanism of death will switch to necrosis.

Cancer growth is complicated. Several additional molecules also play an important role in cancer growth and metastasis. The immune system is involved in cancer growth through both stimulatory and inhibitory pathways. Similar to NO, the arginine metabolism of intratumoral macrophages is an example of the dual abilities of the immune system in inhibiting or stimulating tumor growth. Arginine metabolism in the tumor bed produces citrulline and NO, which favor tumor rejection, whereas production of ornithine and urea may promote tumor growth (Coffey, Phare, & Peters-Golden, 2000; Kim & Ponka, 2000).

It is important to note that the discrepancies described in the results above may be due to the genetic variability of cells, which may play a role in determining NO sensitivity or resistance. Some studies have proposed that the genetic make-up of tumor cells and the concentrations of NO in the tumor microenvironment are the main determinants of the role played by NO (Nadaud & Soubrier, 1996). During clonal evolution of tumors in the presence of high NO concentrations, NO-sensitive cells may be removed, and NO-resistant cells may emerge because of the mutations mediated by NO. In addition to NO resistance, the *p53*-mutation leads to the ability of cells to use NO for stimulating tumor progression.

Accumulation of p53 protein in many cells is stimulated by NO and, at least in part, by the inhibition of protein proteosomal reduction (Glockzin, von Knethen, Scheffner, & Brune, 1999). Many genes transcriptionally activated by *p*53 can initiate cell-cycle arrest (e.g., *P21* and cyclin G) and apoptosis (e.g. *BAX* and *FAS*). High concentrations of NO can result in *p*53-dependent cell-cycle arrest (cytostasis), as well as apoptosis (Tebbi, Guittet, Cottet, Vesin, & Lepoivre, 2011). A recent study demonstrated that inflammation can modulate miRNA expression *in vivo,* which is an event also

associated with cancer. Additionally, the expression alteration for specific miRNAs under an inflammatory microenvironment can be influenced by *p*53 and NO (Mathe et al., 2011)

Clinical and experimental studies have revealed the promoting role of NO in both tumor progression and metastasis. Expression of NOS has been associated with oral squamous cell carcinoma, angiogenesis and lymph node metastasis (Sappayatosok, et al., 2009).

Details pertaining to angiogenesis are beyond the scope of this review. Primary mediators of tumoral angiogenesis include cytokines and vascular endothelial growth factor (VEGF) (Vermeulen, van Golen, & Dirix, 2010). VEGF is produced by various cell types under hypoxic conditions, but its production in some tumors is independent of oxygenation status. It binds to specific receptors on vascular endothelium, which in turn stimulates two processes essential to angiogenesis. First, increased vascular permeability leads to the formation of a provisional fibrin matrix, which is a scaffold for endothelial cell migration. Second, the cytokine stimulates endothelial cell proliferation and migration into the provisional matrix. The process is modulated by many co-factors, including TNF-α (Alleva, Burger, & Elgert, 1994), transforming growth factor-beta (TGF-β_ (Alleva, et al., 1994), and basic fibroblast growth factor (bFGF) (Yang, Yan, Abraham, & Terjung, 2001), and the angiogenic activity of these molecules may also be regulated by NO. VEGF stimulates NO synthesis, resulting in vascular hyperpermeability (Boucherat et al., 2010; Leidi, Mariotti, & Maier, 2010). Because tumors require nutrients for growth, the angiogenic potential of the tumor and the vascular permeability, coupled with the effects of NO, contribute to rapid tumor growth (Bing et al., 2001; Lin, Chen, Ye, & Zhu, 2003).

Recently, studies have reported that the angiogenic activity of human monocytes also requires NOS and that NO plays a significant role in angiogenesis for normal wound healing (Tan, Qian, Rosado, Flood, & Cooper, 2006; Wink et al., 2011). NO functions as a vascular permeability factor. In addition to VEGF, bradykinin aids in increasing the vascular permeability of solid tumors and also activates eNOS (Korkmaz et al., 2006). A study conducted in Thai oral squamous cell carcinoma patients revealed a relationship between the expression of eNOS, iNOS and VEGF and tumor angiogenesis (Sappayatosok, et al., 2009). An additional study revealed the relationship between significant iNOS expression and the invasiveness of breast cancers in humans (Thomsen et al., 1995).

As previously stated, NO promotes tumor progression and angiogenesis by inducing angiogenesis (this is still circular logic. "Water makes something wet because it is water." A similarly circular statement that really doesn't tell anything) (Weidner, Semple, Welch, & Folkman, 1991). However, the detailed mechanism underlying NO regulation of angiogenesis remains unknown. Additionally, NO can promote tumor growth by non-vascular events. For example, NO induces mucin secretion of colonic adenocarcinoma cells (Gottke & Chadee, 1996).

NOS is also involved in the degradation of articular cartilage, and NO stimulates metalloproteinase enzymes in chondrocytes and cartilage in humans (Ishii et al., 2003), cattle and rabbits. Significant loss of integrity of the extracellular matrix and basement membranes induces angiogenesis, invasion and metastasis of tumors (Marcet-Palacios et al., 2003). Some studies, however, have also demonstrated that NOS may retard matrix metalloproteinases (Eagleton et al., 2002).

3.4 NO and cancer therapy

A therapeutic role for NO may be applicable for cancers due to its effect on tumor angiogenesis and its direct tumor cell toxicity. Angiogenesis is the process of new blood vessel development from pre-existing vessels, while vasculogenesis is the process of *de novo* blood vessel formation from an embryonic precursor. It occurs under different pathological conditions. For example, solid tumors require angiogenesis for their growth beyond a diameter of 2-3 mm (Folkman, 1972). NO donors can stimulate proliferation and migration of endothelial cells *in vitro*. Angiogenesis in the rabbit cornea, which is induced by vasoactive molecules (e.g., substance P and prostaglandin E), is blocked by the inhibition of NOS (Ziche et al., 1994) Similarly, NOS i*nhibitors* reduce angiogenesis in acetic acid-induced gastric ulcers in rats (Konturek, Brzozowski, Majka, Pytko-Polonczyk, & Stachura, 1993) and in human squamous cell carcinoma xenografts in the rabbit cornea (Gallo et al., 1998). Increased vascularity and growth of a colon cancer cell line in nude mice following iNOS transduction suggests an angiogenesis-promoting role of tumor-derived NO (Jenkins et al., 1995). Previous work

has shown that *iNOS* gene transfection corrects the delayed wound closure in *iNOS* knockout mice (Yamasaki et al., 1998). Additionally, *eNOS* knockout mice have impaired angiogenesis in ischemic hind limbs (Murohara et al., 1998) and have impaired wound healing (Konturek, et al., 1993). NO is the final mediator of angiogenesis induced by VEGF (Ziche et al., 1997). Functional eNOS is absolutely necessary for endothelial cell migration induced by VEGF. In an analysis of endothelial cells, NO mediated spontaneous micromotion (also known as "podokinesis") even in stationary cells, and VEGF transformed this scalar motion to vectorial motion (Noiri et al., 1998).

p53 mutations in oral and para-oral squamous cell carcinomas may be associated with iNOS upregulation, which promotes angiogenesis (Gallo et al., 2002). This hypothesis is supported by observed upregulation of iNOS in cancer-prone *p53* knockout mice (Ambs et al., 1998). iNOS induction and transduction increases both tumor growth and vascularity *in vivo* (Edwards, et al., 1996). When growth factor reduced Matrigel was implanted in mice subcutaneously, highly metastatic mammary tumor cell lines expressed increased eNOS expression and angiogenic potential (Jadeski, Hum, Chakraborty, & Lala, 2000). This model is well adaptable to human tumor cell xenografts in nude mice and seems to be more appropriate than other models for testing antiangiogenic drugs. For example, in the *in vitro* endothelial cell culture model, reconstitution of all cellular constituents of the tumor microenvironment is difficult. The chick chorioallantoic membrane assay cannot distinguish between angiogenesis and vasculogenesis, and the rat or rabbit cornea assay with human cells cannot exclude xenograft-induced angiogenesis (Jadeski, et al., 2000).

Certain cancers can be treated with selected NO-blocking drugs, either alone or in combination with other treatment modalities. In mice, NO-blocking agents have been shown to decrease tumor growth and metastasis. The omega 3 polyunsaturated fatty acids show inhibitory effect of on NO production and could contribute to their cancer chemopreventive influence especially on colon cancer (Hellmuth, Paulukat, Ninic, Pfeilschifter, & Muhl, 2004; Ohata, Fukuda, Takahashi, Sugimura, & Wakabayashi, 1997)The initial phase of neoplastic transformation for mouse fibroblasts is inhibited by arginine analogues. In contrast, NO-blocking agents may decrease pulmonary metastasis of Lewis lung carcinoma and B16 melanoma cells; therefore, inducible activation NO synthase may be an effective therapy for cancer metastasis (Xie & Fidler, 1998). iNOS inhibition has also shown beneficial therapeutic effects for brain tumors (Swaroops, Kelly, Holmes, Shinoda, & Whittle, 2001). Recent evidence demonstrated that transfection of RIF-1 tumor cells *in vitro* with cytomegalovirus (CMV)/iNOS significantly enhanced cisplatin cytotoxicity and that *in vivo* transfer of CMV/iNOS by direct injection into established RIF-1 tumors caused a significant delay in tumor growth (Adams et al., 2009). The combination of human osteocalcin and iNOS gene therapy has been found to be a beneficial option for treating hormone-refractory prostatecancer (McCarthy, Coulter, Worthington, Robson, & Hirst, 2007)

The therapeutic role of NO-blocking drugs on cancer growth and metastasis has been investigated in a variety of experimental tumor models. iNOS is expressed in endothelial cells within the tumor of rodent adenocarcinomas. Treatment with NG-nitro-L-arginine methyl ester (L-NAME; a NO inhibitor) can reduce the production of NO and delay tumor growth (de Wilt et al., 2000). Although NO exerts cytostatic effects induced by lipopolysaccharide and interferon in EMT-6 murine mammary tumor cells *in vitro*, it also accelerates the growth and metastasis of tumor cells *in vivo* (Edwards, et al., 1996).

Using a human colonic adenocarcinoma cell line, Jenkins et al. demonstrated that tumor growth and vascularity in nude mice is stimulated by iNOS transduction(Jenkins, et al., 1995), but these effects ceased when treated with the selective iNOS inhibitor 1400W (Thomsen et al., 1997). Spontaneously developing tumors are comprised of both eNOS-positive and eNOS-negative tumor cells in a variable ratio, whereas metastatic cells are primarily eNOS-positive.

Lastly, migration of a C3H/HeJ mammary tumor cell line, C3L5, is reduced in the presence of L-NAME in a concentration-dependent manner and is restored in the presence of excess L-arginine (NOS substrate). This confirms the migration-promoting role of endogenous NO (Jadeski, Chakraborty, & Lala, 2003). Parallel differences in the tumor cell lines can be observed in growth rates at primary sites of transplantation, invasive behavior *in vitro*, and angiogenic abilities *in vivo*. Based on this type of experiment, several studies have shown that NO-

mediated tumor progression is a result of the promotion of tumor invasiveness, differential regulation of matrix metalloproteinases (Orucevic et al., 1999) and the promotion of tumor cell migratory abilities (Jadeski, et al., 2000). L-NAME treatment decreases the angiogenic ability of C3L5 cells expressing high levels of eNOS but does not decrease this activity in C10 cells expressing lower levels of eNOS (Jadeski, et al., 2000). NO precursors, such as nitroglycerin, help improve the treatment outcome in patients with solid cancers (Yasuda, 2008). Chemopreventive nitric oxide-donating aspirin (NO-ASA) can also inhibit tumor growth in the BxPC-3 human pancreatic cancer cell model (Zhou, Huang, Sun, & Rigas, 2009).

The clinical applications of NO-blocking drugs as a single chemotherapeutic agent or as a part of a combined treatment requires further investigation; specifically, data representing long-term results, survival rates, cost-effectiveness, drug interaction and side effects in humans are needed.

4. Conclusion

NO plays many roles in carcinogenesis. It promotes cancer development by initiating tumor growth through DNA damage and by promoting tumor invasiveness, angiogenesis and metastasis. Studies have demonstrated the expression of NO and NOS in many cancers. The association of both NO and NOS with angiogenesis and the grading and staging of many tumors is well recognized. Treatment with NO-blocking drugs has been shown to reduce tumor growth and vascularity, characteristics necessary for tumor invasiveness and metastasis. Although there is therapeutic potential, certain aspects of NO, including its dual roles in the process of carcinogenesis and the therapeutic applications of NO-blocking drugs, merit future studies.

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