Biomolecules & Therapeutics, **18(4)**, 351-362 (2010)
www.biomolther.org

ISSN: 1976-9148(print)/2005-4483(online)
DOI: 10.4062/biomolther.2010.18.4.351

Invited Review

Therapeutic Application of Nitric Oxide in Human Diseases

Seung NAMKOONG[1], and Young-Myeong KIM[2,*]

[1]*Medical & Bio-Material Research Center and Department of Physical Therapy, College of Health and Welfare, Kangwon National University, Samcheok 245-907,*
[2]*Vascular System Research Center and Department of Molecular and Cellular Biochemistry, School of Medicine, Kangwon National University, Chuncheon 200-701, Republic of Korea*

(Received September 10, 2010; Revised September 13, 2010; Accepted October 5, 2010)

Abstract – Nitric oxide (NO), synthesized from L-arginine by three isoforms of NO synthase (NOS), is a gaseous signaling molecule with an astonishingly wide range of biological and pathophysiological activities, including vasorelaxation, angiogenesis, anti-inflammation, and anti-apoptosis in mammalian cells. Recent studies have shown that NO donors and inhaled NO convert to biologically active NO under biological conditions and act as a signaling molecule in pathophysiological conditions. This review will discuss the roles of NO and its potential therapeutic implication in various human diseases, such as tumor, vascular regeneration, hypertension, wound healing, and ischemia-reperfusion injury.

Keywords: Nitric oxide, Therapy, Nitric oxide donors, Nitric oxide synthase, Tumor, Ischemia-reperfusion injury

INTRODUCTION

NO, first characterized as a major endothelial-derived relaxing factor (Furchgott and Zawadzki, 1980), is a gaseous molecule with a wide range of physiological and pathological activities (Ignarro *et al.*, 1987), including smooth muscle relaxation, inhibition of platelet aggregation, and neurotransmission. NO, synthesized from L-arginine by the reaction of three NOS isozymes, such as eNOS expressed in endothelial cells, iNOS induced in immune cells including macrophages, and nNOS expressed in neuronal cells, has received intense media coverage due to its role as a biological messenger and was named 'molecule of the year' in 1992 by the journal *Science*. Moreover, this molecule has been investigated for it's critical role in vascular physiology, immune response to bacterial infections, and neurotransmission. In 1998, Drs. Furchgott, Ingnarro and Murad shared the Noble Prize in Physiology and Medicine for 'the first discovery that a gas can act as a signal molecule', emphasizing that NO produced by mammalian cells is an important signaling mediator in various biological systems such as immunology, physiology, and neuroscience.

Various biological activities of NO are highly associated with chemical reactivity for intracellular target molecules, such as transition metal, free thiol (sulfhydryl) group, tyrosine residues, superoxide anion, and molecular oxygen. The earliest described intracellular receptors for NO are heme and non-heme iron-containing proteins, including hemoglobin, soluble guanylyl cyclase (sGC), and aconitase. In particular, binding of NO to the heme group of sGC promotes its catalytic activity, increasing the conversion of GTP to cGMP, which in turn activates protein kinase G (Murad, 1986). The NO-cGMP pathway plays an important role in NO-mediated physiological events, such as vasodilation and penile erection. Despite identification of sGC as the first receptor against NO for eliciting cellular function, it has become clearly demonstrated that NO can exert most of its cellular influence in a cGMP-independent manner. NO interacts with sulfhydryl groups of proteins and non-protein biomolecules to generate *S*-nitrosothiol, designated *S*-nitrosylation. *S*-nitrosylation exhibits a wide rage of cellular effects of NO on pathophysiological processes in the cardiovascular system and vascular disorders (Lima *et al.*, 2010). In addition, *S*-nitrosylation on catalytic thiols of caspase family proteases prevents cells from apoptotic cell death (Kim *et al.*, 1997b; Li *et al.*, 1997).

*Corresponding author
Tel: +82-33-250-8831 Fax: +82-33-244-3286
E-mail: ymkim@kangwon.ac.kr

Reaction of NO with superoxide anion generates the strong oxidant peroxynitrite, leading to cytotoxicity or apoptotic cell death. However, NO reaction with molecular oxygen decreases its biological effect in pathophysiological conditions as a result of reduced bioavailability via the production of the stable and inert oxidation products, nitrite and nitrate. Taken together, NO plays versatile functions in various pathophysiological conditions via reactions with different intracellular receptors and target molecules. Here, we review the biological roles and pathophysiological mechanisms of action of NO and discuss the therapeutic potential of NO in various human diseases.

NO AND NITRIC OXIDE SYNTHESES

NO is a small, odorless, colorless, endogenous, and free radical molecule, which highly diffuses in water and through cellular membranes. However, its high reactivity limits its short half-life in biological systems. Interestingly, NO elicits different reactions in cells in a concentration- dependent manner. At low concentrations, it is a potent biological messenger in a variety of tissues, with a wide range of physiological functions such as vasodilation, inhibition of platelet aggregation, regulation of neurotransmission and natural defense of the immune system. Conversely, high concentrations of NO are involved in the immune system during cytotoxicity of tumor cells, infection of microorganisms, and inflammation (Nathan, 1997).

NO is endogenously produced by a group of homodimeric NOS enzymes through enzymatic oxidation of the guanidine group of L-arginine in the presence of oxygen (Ignarro *et al.*, 1987; Nathan, 1992). It is hydroxylated to generate L-hydroxyarginine, which is further oxidized, yielding L-citrulline and NO (Marletta *et al.*, 1998) (Fig. 1). All three NOS isozymes possess reductase and highly conserved oxygenase polypeptide domains within each monomer; thus, this enzyme catalyzes two sequential NADPH- and O_2-dependent mono-oxygenase reactions to produce NO from L-arginine. These isoforms are denoted by descriptive terms, based on the requirement of intracellular calcium oscillation for full activity as well as gene expression (Table I). Two NOS isoforms, eNOS and nNOS, are constitutively expressed and operate their catalytic activities in a calcium-dependent manner, consequently producing NO at low concentrations (nM - pM) (Forstermann *et al.*, 1995; Gath *et al.*, 1996). These constitutive NOS isoforms require a transient increase in intracellular calcium levels and several cofactors for their enzymatic activity, which promotes the release of NO over the course of several minutes. Specifically, these NOS isoforms increase their enzymatic activity at a specific level of intracellular calcium, but inactivate at a low level of calcium. These constitutive isoforms are key modulators in physiological processes such as memory, long-term potentiation, and depression in the nervous system and regulate blood pressure (vasorelaxation) in the vascular system.

The comparatively small quantities of NO produced by

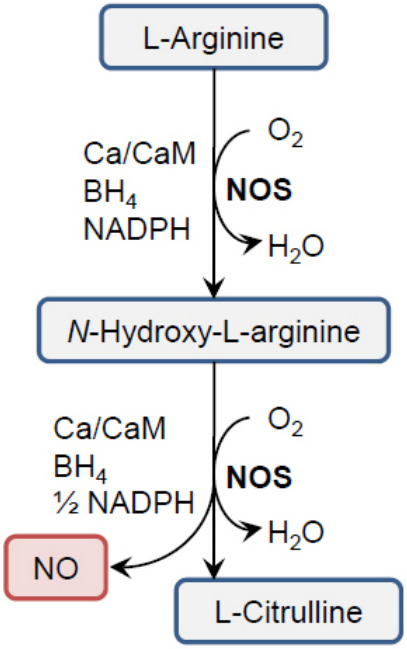

Fig. 1. Reaction pathway of NO production from L-arginine by NOS.

Table I. Isoforms of nitric oxide synthases

Name	Expression type	M.W (kDa)	Regulated by	Location	Main function
Neuronal NOS (nNOS, NOS1)	Constitute	160	Ca^{2+}/calmodulin	Brain, other neuronal tissues/cells	Neurotransmission
Inducible NOS (iNOS, NOS2)	Inducible	125	Cytokine, endotoxin	Macrophages, neutrophiles, hepatocytes, chondrocytes	Cytotoxicity against tumor cells and bacteria
Endothelial NOS (eNOS, NOS3)	Constitute	135	Ca^{2+}/calmodulin	Endothelial cells, cardiac myocytes	Vasodilation

constitutive NOS isoforms is important for cellular signaling events such as blood pressure regulation and neurotransmission. The other isoform iNOS is induced in response to cytokines and/or bacterial products such as interferon-γ, interleukins, TNF-α, and lipopolysaccharides (LPS) in various cell types including macrophages, hepatocytes, dendritic cells, fibroblasts, chondrocytes, osteoclasts, astrocytes, and epithelial cells, which results in the production of large amounts of NO for several days (Moncada *et al.*, 1991; Jun *et al.*, 1994). The catalytic activity of iNOS is independent of intracellular calcium level, but is required for cofactors. Although the low level of NO produced by constitutive NOS isoforms has been known to play an important physiological role in neuronal and vascular systems, the larger amounts of NO generated by iNOS functions both as a regulator and effecter during infection and inflammation. One effecter function includes direct cytotoxicity toward tumor cells, microorganisms, and host cells. The cytotoxicity capacity of NO has been associated with *S*-nitrosylation, nitrosyl-iron complex formation, gene expression, and DNA mutation, which have been confirmed in numerous subcellular systems using diverse cell targets, such as cytosol, mitochondria, and nuclei (Kroncke *et al.*, 1997). In addition, there are some other spliced variants or isoforms of NOS, such as mitochondrial NOS (mtNOS) and NOS in skeletal muscle. mtNOS is an α-isoform of nNOS that is localized at the inner mitochondria membrane in rat liver mitochondria (Tatoyan and Giulivi, 1998; Elfering *et al.*, 2002), and NOS in skeletal muscle is a μ-isoform of nNOS which is localized beneath the sarcolemma of fast twitch muscle fibers (Silvagno *et al.*, 1996). The NOS isoforms are phosphorylated by various kinases such as cyclic AMP-dependents protein kinase, cyclic GMP-dependents protein kinase, protein kinase C, or Ca^{2+}/calmodulin-dependent protein kinase (Nathan and Xie, 1994; Sase and Michel, 1997; Fleming *et al.*, 2001). Phosphorylation of NOS by these kinases regulates an increase or decrease in its catalytic activity. For example, the phosphatidylinositol-3-OH-kinase (PI3K)/Akt pathway has been shown to directly activate eNOS by phosphorylation at serine-1177, which is induced by various cytokines and mechanical forces such as shear stress (Dimmeler *et al.*, 1999). Furthermore, eNOS is co- and post-translational modified by myristoylation, palmitoylation or acylation, which targets it to the cytoplasmic membrane or other intracellular organelles from the cytosol (Sase and Michel, 1997). Therefore, the enzymatic activity of NOS can be regulated by various post-translational modifications.

PATHOPHYSIOLOGICAL ACTION MECHANISMS OF NO

NO is a small, hydrophobic molecule that can easily pass through membranes and possesses autocrine and/or paracrine functions. In addition, NO is a diffusible, multifunctional, transcellular messenger that has been implicated in numerous physiological and pathological conditions. The biological activity of NO is dependent on complex formation with iron, thiol groups of cysteine, and tyrosine residues in proteins or enzymes. In many cells, NO reacts with the cofactor heme-iron in sGC, which results in conformational changes that triggers its enzymatic activity to produce cGMP from GTP. The cGMP engages various downstream targets including protein kinases, phosphodiesterases, and ion channels, resulting in modulation of cell functions such as smooth muscle relaxation, platelet aggregation, and synaptic plasticity. These cellular events are associated with the activation of cGMP-dependent kinase and involves various physiological functions, such as regulation of vascular tone and neurotransmission. However, some studies have reported that regulation of cell function by NO can be associated with a diverse cGMP-independent pathway (Schmidt *et al.*, 1993; Chung *et al.*, 2001). Low concentrations of NO by nNOS and eNOS are mainly involved in the cGMP-dependent pathway, but high concentrations of NO by iNOS are implicated in physiological and biological functions through either cGMP-dependent or independent pathways (Kim *et al.*, 1997b; Chung *et al.*, 2001). Indeed, NO can inhibit apoptosis via inhibition of caspase activity through the activation of cGMP-dependent protein kinase and redox-based *S*-nitrosylation of cysteine residues in the catalytic site of caspase (Kim *et al.*, 1997a; Li *et al.*, 1997; Dash *et al.*, 2003). We have previously reported that high doses of NO may inhibit apoptosis through both cGMP-dependent and independent mechanisms (Kim *et al.*, 1997b; Kim *et al.*, 1999). cGMP can also activate myosin light chain phosphatase (Surks, 2007), Ca^{2+} channels (Spedding *et al.*, 1986), PI3K-dependent Akt (Ha *et al.*, 2003), and ATP-sensitive K^+ channels (Archer *et al.*, 1994), as well as regulate smooth muscle cell growth (Garg and Hassid, 1989) and gene expression (Kroncke, 2003).

NO can modify proteins through direct chemical reactions, without the requirement of enzymatic activity. NO directly interacts with a sulfhydryl group and iron of various intracellular molecules and proteins, leading to conformation changes. This modification is a well-known cGMP-independent pathway of NO and requires larger amounts of NO than mechanisms involving activation of sGC. The

interaction of NO with a sulfhydryl group results in the production of a RS-NO form via the direct attachment of NO to the thiol group of cysteine residues in various proteins including albumin, hemoglobin, Ras, and caspase family proteins. In addition, many ion channels, such as ryanodine receptors, NMDA receptor, and L-type Ca^{2+} channels, which are *S*-nitrosylated are nearly as broad as mechanisms controlled by cGMP (Ahern *et al.*, 2002). *S*-nitrosylation is emerging as an important post-translational modification of ion channels, which provides a route by which NO can regulate electrical activity without stimulating production of cGMP. Biological functions of S-nitrosylation are associated with the regulation of apoptosis via caspases, gene expression by NF-κB, HIF-1α or IRE, and blood flow by hemoglobin (Foster *et al.*, 2009). In addition, formation of a nitrosyl-iron complex is an important cellular event for inhibiting the catalytic activity of several enzymes, such as aconitase, ferrochelatase, and cytochrome *c* oxidase (Kim *et al.*, 1995b; Taylor and Moncada, 2010), resulting in an increase in cytotoxicity and mitochondrial dysfunction. These observations indicate that NO-mediated cGMP production is associated with its beneficial role in physiological and pathological conditions and that cGMP-independent cellular action is linked to consequences of either cytotoxicity or cytoprotection in a pathophysiological condition.

NO THERAPY FOR HUMAN DISEASES

NO is an autocrine and paracrine signaling molecule whose half-life and diffusion gradient are limited by scavenging reactions with hemoglobin, myoglobin, small iron-sulfur complexes, molecular oxygen, and radicals. Interestingly, nitrite, a primary one-electron oxidation product of NO by reaction with macular oxygen, can act as a selective NO donor because of reduction back to NO by several mechanisms, including, but not limited to, deoxyhemoglobin, deoxymyoglobin, xanthine oxidoreductase, acidic disproportionation, and mitochondrial complex IV (Huang *et al.*, 2005; Dezfulian *et al.*, 2007). Therefore, NO donors, nitrite, and inhaled NO can be considered as endocrine molecules that are transported in the blood, accumulate in tissue, and have the potential to be converted back to NO under physiological and pathologic conditions, resulting in an increase in cGMP production, protein modification by *S*-nitrosylation, and cellular signaling events (Cosby *et al.*, 2003; Kumar *et al.*, 2008). Based on these reasons, inhaled NO and administrated NO donors and nitrite are used as therapeutic drugs for several human diseases.

Effect of NO on tumor therapy

The first report that NO can suppress or arrest tumor growth was initially suggested by several studies which demonstrated that immune-activated murine macrophages synthesize nitrite and nitrate leading to cytotoxicity against tumor cells and bacteria (Stuehr and Marletta, 1985; Hibbs *et al.*, 1987; Hibbs *et al.*, 1988). Macrophages infiltration into tumors synthesize NO, which attributes to the formation of iron-nitrosyl complex, as determined by EPR spectroscopy, which is responsible for cytostatic and cytotoxic effects on tumor cells, including inhibition of mitochondrial respiration and DNA replication (Granger *et al.*, 1980; Yim *et al.*, 1993). This cytotoxic effect during *in vivo* tumor progression was inhibited by continuous infusions of the NOS inhibitor N-monomethyl-L-arginine into tumor-bearing mice. However, a study by Moncada demonstrated that two human colorectal adenocarcinoma primary cell lines, SW-480 and SW620, express Ca^{2+}- independent NOS and produce NO (Radomski *et al.*, 1991). Tumor-derived NO inversely correlates with their metastatic potential. Additionally, when injected with tumor cells, tumor growth and metastasis was greater in iNOS-deficient mice than in wild-type mice (Wei *et al.*, 2003), indicating that the physiological expression of iNOS in host cells directly inhibits tumor growth and metastasis. These evidences support the initial hypothesis for the cytotoxic and cytostatic effects of NO production on tumor progression.

The anti-tumor mechanism of NO is directly associated with the stimulation of tumor cell apoptosis by the tumor suppressor p53 up-regulation and caspase activation (Forrester *et al.*, 1996; Messmer and Brune, 1996) as well as inhibition of tumorigenesis and tumor growth by cell cycle arrest and necrotic cell death (Shimaoka *et al.*, 1995; Lala and Chakraborty, 2001; Trikha *et al.*, 2001; Le *et al.*, 2005). Exogenous and endogenous NO production has been shown to increase p53 protein accumulation, and overexpression of p53 in a variety of human tumor cell lines results in down-regulation of iNOS expression through inhibition of its promoter activity (Forrester *et al.*, 1996). This observation reveals that there is a negative feedback loop mechanism between iNOS expression and p53 accumulation in which p53 safeguards against DNA damage through p53-mediated suppression of iNOS gene overexpression, reducing the potential for NO-induced DNA damage and mutation. In addition, NO initiates apoptosis or necrotic cell death by alterations in the expression of pro- and anti-apoptotic Bcl-2 family members, cytochrome c relocation, activation of caspase family proteases, mitochondrial dysfunction, chromatin condensation, and

DNA fragmentation (Brune *et al.*, 1998). These evidences indicate that NO generated in the intratumoral microenvironment may prove to be a useful tumor therapy by promoting direct cellular toxicity (apoptotic and necrotic cell death) and cytostatic effects (cell cycle arrest).

Conversely, NO may be acting as part of a signaling cascade for tumor neovascularization *in vivo*, leading to the promotion of tumor metastasis, whereas *in vitro* cytotoxic properties contribute to the apparent slowing of tumor growth. Therefore, NO function in tumors still remains controversial with evidence suggesting its contribution to both pro- and anti-tumor effects. This contradiction has been suggested to be dependent on the concentration of NO, with high concentrations responsible for anti-tumor activity through the promotion of cytotoxicity and lower NO concentrations which lead to the promotion of tumor growth and metastasis by tumor angiogenesis (Chinje and Stratford, 1997). Although the concentrations of NO at which this switch occurs are still not clear, the opposite effects of NO on tumor cells can depend on cell type or the concentration of certain cellular components, such as iron or thiols (Kim *et al.*, 2000).

The mechanism by which NO acts as a molecule possessing pro-tumor activity can be associated with the inhibition of tumor cell apoptosis by inhibition of the seven members of the caspase family via *S*-nitrosylation (Kim *et al.*, 1997a; Li *et al.*, 1997), inhibition of the Apaf-1/caspase-9 apoptosome (Zech *et al.*, 2003), accumulation and nitration of p53 (Forrester *et al.*, 1996; Chazotte-Aubert *et al.*, 2000) and the induction of heme oxygenase-1 and heat-shock protein 70 (Kim *et al.*, 1995a; Kim *et al.*, 1995b). In addition, several studies have shown promotion of tumorigenesis by NO in an *in vivo* model. For examples, iNOS-deficient mice decreased adenoma development (Ahn and Ohshima, 2001), B16-F1 melanoma growth (Konopka *et al.*, 2001) and mouse lung tumorigenesis (Kisley *et al.*, 2002), probably by negative regulation of vascular endothelial growth factor (VEGF) expression. Furthermore, NO can directly interact with DNA molecules, resulting in G:C →A:T transitions and DNA strand breakage by causing *N*-nitrosylation of deoxynucleotides and yielding deaminated DNA bases (Kroncke *et al.*, 1997). In addition, NO reacts with intracellular superoxide anion to produce the strong oxidant peroxynitrite, which induces DNA strand breakage via N-nitrosamine formation and subsequent alkylation reaction. Indeed, NO has been shown to induce oxidative DNA damage in immune-activated macrophages (deRojas-Walker *et al.*, 1995) and to inhibit enzymes involved in DNA repair (Kroncke *et al.*, 1997). These results indicate that NO can increase tumor growth via inhibition of caspase activity as well as initiate tumor promotion via promotion of nucleotide base mutations.

The dual role of NO in tumors is still a dilemma, but some investigations have shown that administration of several types of NO donors reveals the beneficial effects of NO in tumor therapy. Treatment with the non-specific NOS inhibitor, N^G-monomethyl-L-arginine, exhibits increases in survival rate in MDA-MB-231 cells under hypoxic conditions (Matthews *et al.*, 2001), whereas NOS inhibitors selectively reduce tumor blood flow in murine adenocarcinoma and melanoma *in vivo* (Andrade *et al.*, 1992), suggesting that NO can play a dual role in tumor toxicity and tumor angiogenesis. The iNOS inducer (N-(4-Hydroxyphenyl) retinamide) and NO pro-drug (diethylamine NONOate; DEA/NO, which decomposes spontaneously in solution at physiological pH and temperature, to generate up to 2 molar equivalents of NO) have been shown to exhibit apoptosis and anti-invasive effects against breast cancer cells and their bone metastases in an animal model (Simeone *et al.*, 2006). In addition, JS-K, a pro-drug designed to release NO following reaction with glutathione S-transferases, induces apoptosis in human myeloma cells, which was associated with caspase protease activation, PARP cleavage, increased Fas/CD95 expression, anti-apoptotic Mcl-1 cleavage, and Bcl-2 phosphorylation, as well as the release or relocation of cytochrome c, apoptosis-inducing factor, and endonuclease G release via DNA damage and JNK activation (Kiziltepe *et al.*, 2007). These studies suggest that exploitation of NO concentration-dependent biological effects on tumor growth, angiogenesis, and apoptosis offers a new opportunity to improve the efficacy of anti-tumor therapy.

Effect of NO on therapeutic angiogenesis

Angiogenesis is the formation of new blood vessels from pre-existing vessels providing new routes for blood circulation and is strongly regulated in many physiological and pathological conditions (Folkman and Shing, 1992). Angiogenesis is a fundamental step in a variety of physiological (wound healing and embryonic development) and pathological (tumor progression and metastasis, rheumatoid arthritis, psoriasis, ischemic disease, cardiac infarction) conditions. Chronic organ and tissue ischemia due to defective blood circulation is a feature of several human diseases such as myocardial infarction, cerebral ischemic injury, and limb ischemia, for which minimal therapeutic strategies exist. Angiogenesis is an important process for supplying metabolic needs such as nutrients, growth factors, and molecular oxygen to sites of tissue repair or regeneration. Improvement of the angiogenic process can

repair ischemia-associated tissue damage, and thus has served as a significant tool for therapeutic angiogenesis. Therefore, angiogenic inducers including the proangiogenic factor VEGF have been used as therapeutic drugs for ischemic disease, wound healing, and tissue regeneration (Folkman and Shing, 1992).

NO has been shown to increase neovascularization *in vivo* (Namkoong *et al.*, 2008) and is a component of the pathways underlying VEGF-activated endothelial cell proliferation. However, the mechanism of action by which NO acts as an upstream or a downstream mediator of VEGF has not been clearly elucidated. VEGF promotes phosphorylation-dependent eNOS activation and the release of NO via activation of the phosphatidylinositol 3-kinase (PI3K)/Akt cascade (Papapetropoulos *et al.*, 1997). The NOS inhibitor, N^G-nitro-L-arginine methyl ester, blocks VEGF-induced angiogenesis in rabbit cornea (Ziche *et al.*, 1997). In eNOS-deficient mice, the angiogenic response to hind-limb ischemia was impaired and could not be reversed by administration of VEGF (Murohara *et al.*, 1998). On the other hand, there is a growing body of evidence which shows that NO up-regulates VEGF (Dulak *et al.*, 2000; Zhang *et al.*, 2003). NO has been shown to both increase and decrease VEGF production *via* modulation of HIF-1α expression. Indeed, some authors have reported that NO mediates the suppression of hypoxia-induced production of VEGF in smooth muscle cells by decreasing HIF-1α DNA binding activity (Sogawa *et al.*, 1998; Huang *et al.*, 1999), whereas others have shown enhanced HIF-1 binding activity in response to NO in human glioblastoma and rat smooth muscle cells (Dulak *et al.*, 2000; Kimura *et al.*, 2000). This opposing mechanism may be due to different concentrations of NO treated in cells. Although VEGF promotes angiogenesis by eNOS-dependent NO production *in vitro* and *in vivo* (Ziche *et al.*, 1997; Namkoong *et al.*, 2008), the capillary growth stimulated by bFGF does not require NO production, and its angiogenic activity was inhibited by treatment with exogenous NO donors (Ray-Chaudhury *et al.*, 1996; Ziche *et al.*, 1997). These observations indicate that VEGF and bFGF activate distinct angiogenesis signaling pathways.

Many researches for development and identification of new angiogenic inducers have recently gained a growing interest for the treatment of ischemic diseases and wound healing. NO acts both as a positive regulator of HIF-1α activation and as an upstream or a downstream mediator of VEGF, which places the small radical NO at a central position in the angiogenic process (Papapetropoulos *et al.*, 1997; Brune and Zhou, 2007), suggesting that NO therapy would be very beneficial for therapeutic angiogenesis. The low concentrations of NO produced by eNOS activation play a critical role in modulating angiogenesis activity, because genetic deletion of this enzyme diminishes ischemia-induced angiogenesis and pericyte recruitment (Fukumura *et al.*, 2001; Yu *et al.*, 2005). Consistent with these observations, transfection with the eNOS gene has been shown to promote wound healing and cardioprotective effects including augmentation of angiogenic responses (Lee *et al.*, 1999; Yu *et al.*, 2005). Various agents that promote eNOS-dependent NO production induce therapeutic angiogenesis (Chung *et al.*, 2008; Schgoer *et al.*, 2009; Chung *et al.*, 2010), leading to improved function and tissue integrity in mouse hindlimb ischemia, penile dysfunction, and wound healing models. A recent study demonstrates that treatment with sodium nitrite as an *in vivo* NO-generating compound promotes vascular angiogenesis and restores blood flow in a murine model system of hind-limb ischemia (Kumar *et al.*, 2008). We also demonstrated that treatment with the NO donor *S*-Nitroso-N-penicillamine (SNAP) increases angiogenesis in eNOS-deficient mice and blood supply in a mouse model (Namkoong *et al.*, 2008). Taken together, these observations indicate that elevation of NO production would be beneficial for therapeutic angiogenesis, yet there are currently no effective means to selectively deliver NO to target organs (ischemic tissues and wound sites) to promote angiogenesis.

Effect of NO on hypertension

The initial study by Furchgott and Zawadzki (1980) reported that endothelial cells were responsible for vasorelaxation by producing endothelium-derived relaxing factor, which was later identified as NO (Ignarro *et al.*, 1987). Numerous studies have confirmed the vasodilatory effects of NO in several animal models and humans (Cosby *et al.*, 2003; Hunter *et al.*, 2004; Tsuchiya *et al.*, 2005). Knocking out the gene encoding eNOS in mice results in significant hypertension, which is unaffected by treatment with NOS inhibitors, and endothelium-intact aortic rings removed *ex vivo* from these animals display no relaxation to acetylcholine (Huang *et al.*, 1995), indicating that non-endothelial isoforms of NOS may be involved in maintaining blood pressure. These results indicate that elevation of NO production in the vasculature can be an important therapeutic strategy for hypertension. Indeed, exogenous NO delivery is currently the first line of treatments for post-operative pulmonary hypertension or primary pulmonary hypertension of the newborn (PPHN) being managed on the intensive care unit, as it reduces pulmonary arterial pressure quickly (Beghetti *et al.*, 1995; Steudel *et al.*, 1999). PPHN

is a condition that is associated with a high pulmonary vascular resistance and extremely low systemic oxygenation. Delivery of sodium nitrite by aerosol in sheep models of PPHN elicited a rapid and sustained reduction (approximately 65%) in hypoxia-induced pulmonary hypertension, with a magnitude approaching that of the effects of 20 ppm NO gas inhalation (Hunter *et al.*, 2004). This reduction was directly associated with the immediate appearance of NO in expiratory gas. Notably, from a therapeutic standpoint, short-term delivery of nitrite dissolved in saline through nebulization sustained pulmonary vasodilation with no clinically significant increase in blood methemoglobin levels. These data support the concept that nitrite is a vasodilator acting through conversion to NO and evinces a new, simple and inexpensive potential therapy for neonatal pulmonary hypertension.

It was found that NO produced by endothelial cells diffuses into smooth muscle cells and interacts with heme as a cofactor of sGS, which is a heterodimeric enzyme with subsequent formation of cGMP. Cyclic GMP activates protein kinase G, which causes phosphorylation of myosin light chain phosphatase, and therefore inactivation of myosin light-chain kinase ultimately leading to the dephosphorylation of the myosin light chain, resulting in smooth muscle relaxation, reduction of pulmonary arterial pressure, and increased oxygenation (Surks, 2007). NO absorbed from the lungs into the systemic circulation is quickly deactivated by direct interaction with hemoglobin, minimizing its effect on the systemic circulation. Inhaled NO is a selective pulmonary vasodilator that has the ability to produce vasodilation in the pulmonary vascular bed without affecting systemic circulation (Thebaud *et al.*, 1999; Lowson, 2004). This property of inhaled NO has made it a useful therapy in the management of both adult and pediatric patients with a variety of conditions associated with pulmonary hypertension, with or without hypoxia. Although these studies indicate that NO can significantly reduce blood pressure in models of hypertension, more detailed studies are warranted to determine if NO therapy can lower blood pressure to the same extent as the current standard of care. To overcome this problem, various types of NO donors have been developed over the past 20 years based on understanding the chemical biology and physiology of NO (Thatcher, 2005). NONOates (diazeniumdiolate NO donors; DEA/NO, PAPA/NO and NOC-5) have been chemically synthesized and extensively studied for preclinical use. In addition, new NO donors are developed for generating NO in a specific organ or tissue. For example, V-PYRRO/NO is a stable NO donor fashioned from a pyrrolidine diazeniumdiolate ion with attached pro-drug groups predicted to be metabolized by cytochrome p-450 and hepatic epoxidase. Intravenous administration of V-PYRRO/NO selectively replenishes NO in the liver sinusoids in a mouse model of portal hypertension, leading to sinusoidal dilation and therefore, decreased hepatic vascular resistance (Edwards *et al.*, 2008). This liver-selective drug did not systemically produce NO and subsequently did not affect systemic vascular hemodynamics.

The cytosolic heme-containing sGC is one of the key enzymes in the NO-mediated signaling pathway as well as NO-dependent vascular physiology. The vasodilatory properties of NO have been exploited for over a century in cardiovascular disease, but NO donor drugs and inhaled NO are associated with significant shortcomings, such as resistance to NO in some disease states, the development of tolerance during long-term treatment, non-specific delivery to target sites, and non-specific effects such as post-translational modification of proteins. Thus, development of pharmacological agents capable of directly stimulating the NO receptor, sGC, is highly desirable. The benzylindazole compound YC-1 was the first generation of sGC stimulators to be identified; based on its chemical structure, the second generation of sGC stimulators with improved potency and specificity against sGC has been developed, including CFM-1571, BAY 41-2272, BAY 41-8543, and BAY 63-2521. A sGC stimulator, BAY 63-2521 (Riociguat), is currently in clinical development as an oral therapy for patients with pulmonary hypertension (Stasch and Hobbs, 2009). A recent study has reported that phase 2 trial of BAY 63-2521 exhibits significant promotion of pulmonary hypertension and pulmonary arterial hypertension (Ghofrani *et al.*, 2010). These results indicate that NO donors or sGS stimulators, which elevate the intracellular levels of cGMP, can be potentially useful therapeutic agents for patients with hypotension.

Effect of NO on ischemia and reperfusion (I/R) injury

Endogenous or exogenous NO has proven to be therapeutically effective in various models of I/R injury. An animal model of single organ IR injury is generally developed by occlusion of the vascular supply of an organ of interest for a period of time, then releasing the occlusion to permit reperfusion. NO at low concentrations protects cardiomyocytes from I/R injury *via* sGC activation and subsequent cGMP formation (Kanno *et al.*, 2000; Jones *et al.*, 2004). NO provides cytoprotective effects in different organs, including liver (Duranski *et al.*, 2005), heart (Webb *et al.*, 2004; Duranski *et al.*, 2005), kidney (Tripatara *et al.*, 2007), and brain (Jung *et al.*, 2006). Furthermore, several studies have also shown protective effects of NO in vari-

ous I/R conditions, including stroke (Jung *et al.*, 2006), renal ischemia (Tripatara *et al.*, 2007), and chronic limb ischemia (Kumar *et al.*, 2008). These effects are associated with a significant increase in blood levels of S-nitrosothiols, which are endogenous transporters of NO used in activator of cGMP-dependent and -independent signaling pathways. All together, the promising animal data discussed here indicate that NO possesses the characteristics of a useful adjunctive therapy for stroke and other conditions of I/R injury.

To date, human clinical trials of NO therapy for organ I/R injury have been conducted with inhaled NO. Although inhaled NO improved oxygenation and decreased pulmonary artery pressure without systemic circulatory effects (Date *et al.*, 1996), beneficial effects of inhaled NO therapy have been recently reported for liver and lung I/R injuries (Lang *et al.*, 2007; Yerebakan *et al.*, 2009), demonstrating pharmacological efficacy of NO-based therapy in the setting of I/R injury. However, conventional NO donors have shown pharmacologically significant limitations, including indiscriminate NO release, vehicle toxicity, and triggers of NO generation not practical in an I/R setting model, such as light irradiation (Scatena *et al.*, 2005; Miller and Megson, 2007). Several types of NO-releasing agents are available. Sodium nitroprusside was first described as a vasodilator in 1929 and is still used to manage hypertensive crisis, as well as try to treat I/R injury in the rat liver (Kuroki *et al.*, 2004); however, this compound can exert cytotoxicity because it generates NO in an one step reaction (half life time, 2-26 min) and simultaneously releases the cytotoxic chemical cyanide (CN^-). Exogenous N-bound diazeniumdiolated NO donors, such as 2-(N,N-diethylamino)- diazenolate 2-oxide (DEA/NO) and 2,2'-(hydroxynitrosohydrazono)-bis-ethanimine (DETA/NO), which spontaneously release a bolus of NO into body fluid may be harmful if NO generates the strong oxidant $ONOO^-$ by reacting with large concentrations of superoxide anion produced during reperfusion (Schulz *et al.*, 2004). In contrast, S-nitrosothiols (SNAP and S-nitrosoalbumin), which are useful NO donors for treating I/R injury (Bell *et al.*, 2003), are the endogenous transporters of NO used in NO-signaling pathways that release NO through direct trans-nitrosation with thiol groups of proteins and non-protein or via decomposition initiated by enzymatic metal centers. Recently, dendritic glutathione-conjugated SNAP (G4-SNAP) has been developed and exhibits NO release characteristics similar to free small molecule nitrosothiols, like SNAP, in solution. However, these dendritic NO release vehicles were more effective at inhibiting platelet aggregation than their small molecule NO donor counterparts. This compound is a strong potential of NO-based therapy for I/R injury in the presence of appropriate concentrations of GSH (Johnson *et al.*, 2010).

Elevation of NO by NOS gene transfer has shown some beneficial effects, such as wound healing, erectile dysfunction, vascular surgery, and transplantation (Schwentker and Billiar, 2002). Genetic overexpression of eNOS in mice attenuated myocardial infarction after myocardial I/R but fails to significantly protect against post-ischemic myocardial contractile dysfunction in mice (Jones *et al.*, 2004), indicating that regulation of endogenous NO production attributes protection of organ injury after I/R. Furthermore, cardiomyoctye-specific eNOS overexpression displayed decreased infarct size and preservation of cardiac function compared with systemic transgenic overexpression of eNOS in an *in vivo* murine model of myocardial I/R injury (Elrod *et al.*, 2006). These results provide evidence that a site-specific increase in NO production by eNOS gene therapy may be more advantageous in limiting myocardial I/R injury. In addition, NO production by direct intramyocardial injection of adenoviral vector carrying iNOS gene (Ad5/iNOS) in mice increased local iNOS protein expression and activity and markedly reduced infarct size, without perturbation of systemic hemodynamics (Li *et al.*, 2003). These results indicate that endogenous NO production by delivery of NOS gene or NO donors can mediate the cytoprotective effects that mitigate ischemia/reperfusion injury.

CONCLUSIONS

In summary, NO, generated by constitutive expression or inducible expression of NOS in cells, plays an important role in physiological and pathological conditions, such as the regulation of vascular tone, neurotransmission, and pathogenesis of various diseases. NO elicits opposing mechanisms of action in vascular inflammation, neovascularization, cell survival, tumor growth, and I/R injury in a concentration-dependent manner. Thus, maintenance of appropriate levels of NO is critically important for human health. These evidences indicate that the regulation of endogenous NO levels offers the beneficial opportunity to improve the efficacy of therapeutic applications in human diseases. Several ongoing trials using techniques with NO-generating compounds and NOS gene delivery will provide important insights into the potential benefits of NO therapies, new information about strategic designs for NO donor development, and additional clinical trials aimed at evaluating the therapeutic effects of NO.

ACKNOWLEDGMENTS

This work was supported by a grant from the Korean Ministry of Education, Science, and Technology (Regional Core Research Program/Medical & Bio-Materials Research Center). We thank Mrs. Elaine Por for helpful comments and critical reading of this manuscript.

REFERENCES

Ahern, G. P., Klyachko, V. A. and Jackson, M. B. (2002). cGMP and S-nitrosylation: two routes for modulation of neuronal excitability by NO. *Trends Neurosci.* **25**, 510-517.

Ahn, B. and Ohshima, H. (2001). Suppression of intestinal polyposis in Apc (Min/+) mice by inhibiting nitric oxide production. *Cancer Res.* **61**, 8357-8360.

Andrade, S. P., Hart, I. R. and Piper, P. J. (1992). Inhibitors of nitric oxide synthase selectively reduce flow in tumor-associated neovasculature. *Br. J. Pharmacol.* **107**, 1092-1095.

Archer, S. L., Huang, J. M., Hampl, V., Nelson, D. P., Shultz, P. J. and Weir, E. K. (1994). Nitric oxide and cGMP cause vasorelaxation by activation of a charybdotoxin-sensitive K channel by cGMP-dependent protein kinase. *Proc. Natl. Acad. Sci. USA.* **91**, 7583-7587.

Beghetti, M., Habre, W., Friedli, B. and Berner, M. (1995). Continuous low dose inhaled nitric oxide for treatment of severe pulmonary hypertension after cardiac surgery in paediatric patients. *Br. Heart J.* **73**, 65-68.

Bell, R. M., Maddock, H. L. and Yellon, D. M. (2003). The cardio-protective and mitochondrial depolarising properties of exogenous nitric oxide in mouse heart. *Cardiovasc. Res.* **57**, 405-415.

Brune, B., von Knethen, A. and Sandau, K. B. (1998). Nitric oxide and its role in apoptosis. *Eur. J. Pharmacol.* **351**, 261-272.

Brune, B. and Zhou, J. (2007). Nitric oxide and superoxide: interference with hypoxic signaling. *Cardiovasc. Res.* **75**, 275-282.

Chazotte-Aubert, L., Hainaut, P. and Ohshima, H. (2000). Nitric oxide nitrates tyrosine residues of tumor-suppressor p53 protein in MCF-7 cells. *Biochem. Biophys. Res. Commun.* **267**, 609-613.

Chinje, E. C. and Stratford, I. J. (1997). Role of nitric oxide in growth of solid tumours: a balancing act. *Essays Biochem.* **32**, 61-72.

Chung, B. H., Kim, J. D., Kim, C. K., Kim, J. H., Won, M. H., Lee, H. S., Dong, M. S., Ha, K. S., Kwon, Y. G. and Kim, Y. M. (2008). Icariin stimulates angiogenesis by activating the MEK/ERK- and PI3K/Akt/eNOS-dependent signal pathways in human endothelial cells. *Biochem. Biophys. Res. Commun.* **376**, 404-408.

Chung, B. H., Lee, J. J., Kim, J. D., Jeoung, D., Lee, H., Choe, J., Ha, K. S., Kwon, Y. G. and Kim, Y. M. (2010). Angiogenic activity of sesamin through the activation of multiple signal pathways. *Biochem. Biophys. Res. Commun.* **391**, 254-260.

Chung, H. T., Pae, H. O., Choi, B. M., Billiar, T. R. and Kim, Y. M. (2001). Nitric oxide as a bioregulator of apoptosis. *Biochem. Biophys. Res. Commun.* **282**, 1075-1079.

Cosby, K., Partovi, K. S., Crawford, J. H., Patel, R. P., Reiter, C. D., Martyr, S., Yang, B. K., Waclawiw, M. A., Zalos, G., Xu, X., Huang, K. T., Shields, H., Kim-Shapiro, D. B., Schechter, A. N., Cannon, R. O. 3rd and Gladwin, M. T. (2003). Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation. *Nat. Med.* **9**, 1498-1505.

Dash, P. R., Cartwright, J. E., Baker, P. N., Johnstone, A. P. and Whitley, G. S. (2003). Nitric oxide protects human extravillous trophoblast cells from apoptosis by a cyclic GMP-dependent mechanism and independently of caspase 3 nitrosylation. *Exp. Cell. Res.* **287**, 314-324.

Date, H., Triantafillou, A. N., Trulock, E. P., Pohl, M. S., Cooper, J. D. and Patterson, G. A. (1996). Inhaled nitric oxide reduces human lung allograft dysfunction. *J. Thorac. Cardiovasc. Surg.* **111**, 913-919.

deRojas-Walker, T., Tamir, S., Ji, H., Wishnok, J. S. and Tannenbaum, S. R. (1995). Nitric oxide induces oxidative damage in addition to deamination in macrophage DNA. *Chem. Res. Toxicol.* **8**, 473-477.

Dezfulian, C., Raat, N., Shiva, S. and Gladwin, M. T. (2007). Role of the anion nitrite in ischemia-reperfusion cytoprotection and therapeutics. *Cardiovasc. Res.* **75**, 327-338.

Dimmeler, S., Fleming, I., Fisslthaler, B., Hermann, C., Busse, R. and Zeiher, A. M. (1999). Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature* **399**, 601-605.

Dulak, J., Jozkowicz, A., Dembinska-Kiec, A., Guevara, I., Zdzienicka, A., Zmudzinska-Grochot, D., Florek, I., Wojtowicz, A., Szuba, A. and Cooke, J. P. (2000). Nitric oxide induces the synthesis of vascular endothelial growth factor by rat vascular smooth muscle cells. *Arterioscler. Thromb. Vasc. Biol.* **20**, 659-666.

Duranski, M. R., Greer, J. J., Dejam, A., Jaganmohan, S., Hogg, N., Langston, W., Patel, R. P., Yet, S. F., Wang, X., Kevil, C. G., Gladwin, M. T. and Lefer, D. J. (2005). Cytoprotective effects of nitrite during *in vivo* ischemia-reperfusion of the heart and liver. *J. Clin. Invest.* **115**, 1232-1240.

Edwards, C., Feng, H. Q., Reynolds, C., Mao, L. and Rockey, D. C. (2008). Effect of the nitric oxide donor V-PYRRO/NO on portal pressure and sinusoidal dynamics in normal and cirrhotic mice. *Am. J. Physiol. Gastrointest. Liver Physiol.* **294**, G1311-1317.

Elfering, S. L., Sarkela, T. M. and Giulivi, C. (2002). Biochemistry of mitochondrial nitric-oxide synthase. *J. Biol. Chem.* **277**, 38079-38086.

Elrod, J. W., Greer, J. J., Bryan, N. S., Langston, W., Szot, J. F., Gebregzlabher, H., Janssens, S., Feelisch, M. and Lefer, D. J. (2006). Cardiomyocyte-specific overexpression of NO synthase-3 protects against myocardial ischemia-reperfusion injury. *Arterioscler. Thromb. Vasc. Biol.* **26**, 1517-1523.

Fleming, I., Fisslthaler, B., Dimmeler, S., Kemp, B. E. and Busse, R. (2001). Phosphorylation of Thr(495) regulates Ca(2+)/calmodulin-dependent endothelial nitric oxide synthase activity. *Circ. Res.* **88**, E68-75.

Folkman, J. and Shing, Y. (1992). Angiogenesis. *J. Biol. Chem.* **267**, 10931-10934.

Forrester, K., Ambs, S., Lupold, S. E., Kapust, R. B., Spillare, E. A., Weinberg, W. C., Felley-Bosco, E., Wang, X. W., Geller, D. A., Tzeng, E., Billiar, T. R. and Harris, C. C. (1996). Nitric oxide-induced p53 accumulation and regulation of inducible

nitric oxide synthase expression by wild-type p53. *Proc. Natl. Acad. Sci. USA.* **93**, 2442-2447.

Forstermann, U., Kleinert, H., Gath, I., Schwarz, P., Closs, E. I. and Dun, N. J. (1995). Expression and expressional control of nitric oxide synthases in various cell types. *Adv. Pharmacol.* **34**, 171-186.

Foster, M. W., Hess, D. T. and Stamler, J. S. (2009). Protein S-nitrosylation in health and disease: a current perspective. *Trends Mol. Med.* **15**, 391-404.

Fukumura, D., Gohongi, T., Kadambi, A., Izumi, Y., Ang, J., Yun, C. O., Buerk, D. G., Huang, P. L. and Jain, R. K. (2001). Predominant role of endothelial nitric oxide synthase in vascular endothelial growth factor-induced angiogenesis and vascular permeability. *Proc. Natl. Acad. Sci. USA.* **98**, 2604-2609.

Furchgott, R. F. and Zawadzki, J. V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* **288**, 373-376.

Garg, U. C. and Hassid, A. (1989). Nitric oxide-generating vasodilators and 8-bromo-cyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells. *J. Clin. Invest.* **83**, 1774-1777.

Gath, I., Closs, E. I., Godtel-Armbrust, U., Schmitt, S., Nakane, M., Wessler, I. and Forstermann, U. (1996). Inducible NO synthase II and neuronal NO synthase I are constitutively expressed in different structures of guinea pig skeletal muscle: implications for contractile function. *FASEB J.* **10**, 1614-1620.

Ghofrani, H. A., Hoeper, M. M., Halank, M., Meyer, F. J., Staehler, G., Behr, J., Ewert, R., Weimann, G. and Grimminger, F. (2010). Riociguat for chronic thromboembolic pulmonary hypertension and pulmonary arterial hypertension: a phase II study. *Eur. Respir. J.* **36**, 792-799.

Granger, D. L., Taintor, R. R., Cook, J. L. and Hibbs, J. B. Jr. (1980). Injury of neoplastic cells by murine macrophages leads to inhibition of mitochondrial respiration. *J. Clin. Invest.* **65**, 357-370.

Ha, K. S., Kim, K. M., Kwon, Y. G., Bai, S. K., Nam, W. D., Yoo, Y. M., Kim, P. K., Chung, H. T., Billiar, T. R. and Kim, Y. M. (2003). Nitric oxide prevents 6-hydroxydopamine-induced apoptosis in PC12 cells through cGMP-dependent PI3 kinase/Akt activation. *FASEB J.* **17**, 1036-1047.

Hibbs, J. B. Jr., Taintor, R. R. and Vavrin, Z. (1987). Macrophage cytotoxicity: role for L-arginine deiminase and imino nitrogen oxidation to nitrite. *Science* **235**, 473-476.

Hibbs, J. B. Jr., Taintor, R. R., Vavrin, Z. and Rachlin, E. M. (1988). Nitric oxide: a cytotoxic activated macrophage effector molecule. *Biochem. Biophys. Res. Commun.* **157**, 87-94.

Huang, L. E., Willmore, W. G., Gu, J., Goldberg, M. A. and Bunn, H. F. (1999). Inhibition of hypoxia-inducible factor 1 activation by carbon monoxide and nitric oxide. Implications for oxygen sensing and signaling. *J. Biol. Chem.* **274**, 9038-9044.

Huang, P. L., Huang, Z., Mashimo, H., Bloch, K. D., Moskowitz, M. A., Bevan, J. A. and Fishman, M. C. (1995). Hypertension in mice lacking the gene for endothelial nitric oxide synthase. *Nature* **377**, 239-242.

Huang, Z., Shiva, S., Kim-Shapiro, D. B., Patel, R. P., Ringwood, L. A., Irby, C. E., Huang, K. T., Ho, C., Hogg, N., Schechter, A. N. and Gladwin, M. T. (2005). Enzymatic function of hemoglobin as a nitrite reductase that produces NO under allosteric control. *J. Clin. Invest.* **115**, 2099-2107.

Hunter, C. J., Dejam, A., Blood, A. B., Shields, H., Kim-Shapiro, D. B., Machado, R. F., Tarekegn, S., Mulla, N., Hopper, A. O., Schechter, A. N., Power, G. G. and Gladwin, M. T. (2004). Inhaled nebulized nitrite is a hypoxia-sensitive NO-dependent selective pulmonary vasodilator. *Nat. Med.* **10**, 1122-1127.

Ignarro, L. J., Buga, G. M., Wood, K. S., Byrns, R. E. and Chaudhuri, G. (1987). Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc. Natl. Acad. Sci. USA.* **84**, 9265-9269.

Johnson, T. A., Stasko, N. A., Matthews, J. L., Cascio, W. E., Holmuhamedov, E. L., Johnson, C. B. and Schoenfisch, M. H. (2010). Reduced ischemia/reperfusion injury via glutathione-initiated nitric oxide-releasing dendrimers. *Nitric Oxide* **22**, 30-36.

Jones, S. P., Greer, J. J., Kakkar, A. K., Ware, P. D., Turnage, R. H., Hicks, M., van Haperen, R., de Crom, R., Kawashima, S., Yokoyama, M. and Lefer, D. J. (2004). Endothelial nitric oxide synthase overexpression attenuates myocardial reperfusion injury. *Am. J. Physiol. Heart. Circ. Physiol.* **286**, H276-282.

Jun, C. D., Choi, B. M., Hoon, R., Um, J. Y., Kwak, H. J., Lee, B. S., Paik, S. G., Kim, H. M. and Chung, H. T. (1994). Synergistic cooperation between phorbol ester and IFN-γ for induction of nitric oxide synthesis in murine peritoneal macrophages. *J. Immunol.* **153**, 3684-3690.

Jung, K. H., Chu, K., Ko, S. Y., Lee, S. T., Sinn, D. I., Park, D. K., Kim, J. M., Song, E. C., Kim, M. and Roh, J. K. (2006). Early intravenous infusion of sodium nitrite protects brain against *in vivo* ischemia-reperfusion injury. *Stroke* **37**, 2744-2750.

Kanno, S., Lee, P. C., Zhang, Y., Ho, C., Griffith, B. P., Shears, L. L. 2nd and Billiar, T. R. (2000). Attenuation of myocardial ischemia/reperfusion injury by superinduction of inducible nitric oxide synthase. *Circulation* **101**, 2742-2748.

Kim, Y. M., Bergonia, H. and Lancaster, J. R. Jr. (1995a). Nitrogen oxide-induced autoprotection in isolated rat hepatocytes. *FEBS Lett.* **374**, 228-232.

Kim, Y. M., Bergonia, H. A., Muller, C., Pitt, B. R., Watkins, W. D. and Lancaster, J. R. Jr. (1995b). Loss and degradation of enzyme-bound heme induced by cellular nitric oxide synthesis. *J. Biol. Chem.* **270**, 5710-5713.

Kim, Y. M., Chung, H. T., Kim, S. S., Han, J. A., Yoo, Y. M., Kim, K. M., Lee, G. H., Yun, H. Y., Green, A., Li, J., Simmons, R. L. and Billiar, T. R. (1999). Nitric oxide protects PC12 cells from serum deprivation-induced apoptosis by cGMP-dependent inhibition of caspase signaling. *J. Neurosci.* **19**, 6740-6747.

Kim, Y. M., Chung, H. T., Simmons, R. L. and Billiar, T. R. (2000). Cellular non-heme iron content is a determinant of nitric oxide-mediated apoptosis, necrosis, and caspase inhibition. *J. Biol. Chem.* **275**, 10954-10961.

Kim, Y. M., de Vera, M. E., Watkins, S. C. and Billiar, T. R. (1997a). Nitric oxide protects cultured rat hepatocytes from tumor necrosis factor-alpha-induced apoptosis by inducing heat shock protein 70 expression. *J. Biol. Chem.* **272**, 1402-1411.

Kim, Y. M., Talanian, R. V. and Billiar, T. R. (1997b). Nitric oxide inhibits apoptosis by preventing increases in caspase-3-like activity via two distinct mechanisms. *J. Biol. Chem.* **272**,

31138-31148.

Kimura, H., Weisz, A., Kurashima, Y., Hashimoto, K., Ogura, T., D'Acquisto, F., Addeo, R., Makuuchi, M. and Esumi, H. (2000). Hypoxia response element of the human vascular endothelial growth factor gene mediates transcriptional regulation by nitric oxide: control of hypoxia-inducible factor-1 activity by nitric oxide. *Blood* **95**, 189-197.

Kisley, L. R., Barrett, B. S., Bauer, A. K., Dwyer-Nield, L. D., Barthel, B., Meyer, A. M., Thompson, D. C. and Malkinson, A. M. (2002). Genetic ablation of inducible nitric oxide synthase decreases mouse lung tumorigenesis. *Cancer Res.* **62**, 6850-6856.

Kiziltepe, T., Hideshima, T., Ishitsuka, K., Ocio, E. M., Raje, N., Catley, L., Li, C. Q., Trudel, L. J., Yasui, H., Vallet, S., Kutok, J. L., Chauhan, D., Mitsiades, C. S., Saavedra, J. E., Wogan, G. N., Keefer, L. K., Shami, P. J. and Anderson, K. C. (2007). JS-K, a GST-activated nitric oxide generator, induces DNA double-strand breaks, activates DNA damage response pathways, and induces apoptosis *in vitro* and *in vivo* in human multiple myeloma cells. *Blood* **110**, 709-718.

Konopka, T. E., Barker, J. E., Bamford, T. L., Guida, E., Anderson, R. L. and Stewart, A. G. (2001). Nitric oxide synthase II gene disruption: implications for tumor growth and vascular endothelial growth factor production. *Cancer Res.* **61**, 3182-3187.

Kroncke, K. D. (2003). Nitrosative stress and transcription. *Biol. Chem.* **384**, 1365-1377.

Kroncke, K. D., Fehsel, K. and Kolb-Bachofen, V. (1997). Nitric oxide: cytotoxicity versus cytoprotection--how, why, when, and where? *Nitric Oxide.* **1**, 107-120.

Kumar, D., Branch, B. G., Pattillo, C. B., Hood, J., Thoma, S., Simpson, S., Illum, S., Arora, N., Chidlow, J. H., Jr., Langston, W., Teng, X., Lefer, D. J., Patel, R. P. and Kevil, C. G. (2008). Chronic sodium nitrite therapy augments ischemia-induced angiogenesis and arteriogenesis. *Proc. Natl. Acad. Sci. USA.* **105**, 7540-7545.

Kuroki, I., Miyazaki, T., Mizukami, I., Matsumoto, N. and Matsumoto, I. (2004). Effect of sodium nitroprusside on ischemia-reperfusion injuries of the rat liver. *Hepatogastroenterology* **51**, 1404-1407.

Lala, P. K. and Chakraborty, C. (2001). Role of nitric oxide in carcinogenesis and tumour progression. *Lancet Oncol.* **2**, 149-156.

Lang, J. D. Jr., Teng, X., Chumley, P., Crawford, J. H., Isbell, T. S., Chacko, B. K., Liu, Y., Jhala, N., Crowe, D. R., Smith, A. B., Cross, R. C., Frenette, L., Kelley, E. E., Wilhite, D. W., Hall, C. R., Page, G. P., Fallon, M. B., Bynon, J. S., Eckhoff, D. E. and Patel, R. P. (2007). Inhaled NO accelerates restoration of liver function in adults following orthotopic liver transplantation. *J. Clin. Invest.* **117**, 2583-2591.

Le, X., Wei, D., Huang, S., Lancaster, J. R. Jr. and Xie, K. (2005). Nitric oxide synthase II suppresses the growth and metastasis of human cancer regardless of its up-regulation of protumor factors. *Proc. Natl. Acad. Sci. USA.* **102**, 8758-8763.

Lee, P. C., Salyapongse, A. N., Bragdon, G. A., Shears, L. L. 2nd, Watkins, S. C., Edington, H. D. and Billiar, T. R. (1999). Impaired wound healing and angiogenesis in eNOS-deficient mice. *Am. J. Physiol.* **277**, H1600-1608.

Li, J., Billiar, T. R., Talanian, R. V. and Kim, Y. M. (1997). Nitric oxide reversibly inhibits seven members of the caspase family via *S*-nitrosylation. *Biochem. Biophys. Res. Commun.* **240**, 419-424.

Li, Q., Guo, Y., Xuan, Y. T., Lowenstein, C. J., Stevenson, S. C., Prabhu, S. D., Wu, W. J., Zhu, Y. and Bolli, R. (2003). Gene therapy with inducible nitric oxide synthase protects against myocardial infarction via a cyclooxygenase-2-dependent mechanism. *Circ. Res.* **92**, 741-748.

Lima, B., Forrester, M. T., Hess, D. T. and Stamler, J. S. (2010). S-nitrosylation in cardiovascular signaling. *Circ. Res.* **106**, 633-646.

Lowson, S. M. (2004). Alternatives to nitric oxide. *Br. Med. Bull.* **70**, 119-131.

Marletta, M. A., Hurshman, A. R. and Rusche, K. M. (1998). Catalysis by nitric oxide synthase. *Curr. Opin. Chem. Biol.* **2**, 656-663.

Matthews, N. E., Adams, M. A., Maxwell, L. R., Gofton, T. E. and Graham, C. H. (2001). Nitric oxide-mediated regulation of chemosensitivity in cancer cells. *J. Natl. Cancer Inst.* **93**, 1879-1885.

Messmer, U. K. and Brune, B. (1996). Nitric oxide-induced apoptosis: p53-dependent and p53-independent signalling pathways. *Biochem. J.* **319**, 299-305.

Miller, M. R. and Megson, I. L. (2007). Recent developments in nitric oxide donor drugs. *Br. J. Pharmacol.* **151**, 305-321.

Moncada, S., Palmer, R. M. and Higgs, E. A. (1991). Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.* **43**, 109-142.

Murad, F. (1986). Cyclic guanosine monophosphate as a mediator of vasodilation. *J. Clin. Invest.* **78**, 1-5.

Murohara, T., Asahara, T., Silver, M., Bauters, C., Masuda, H., Kalka, C., Kearney, M., Chen, D., Symes, J. F., Fishman, M. C., Huang, P. L. and Isner, J. M. (1998). Nitric oxide synthase modulates angiogenesis in response to tissue ischemia. *J. Clin. Invest.* **101**, 2567-2578.

Namkoong, S., Chung, B. H., Ha, K. S., Lee, H., Kwon, Y. G. and Kim, Y. M. (2008). Microscopic technique for the detection of nitric oxide-dependent angiogenesis in an animal model. *Methods Enzymol.* **441**, 393-402.

Nathan, C. (1992). Nitric oxide as a secretory product of mammalian cells. *FASEB J.* **6**, 3051-3064.

Nathan, C. (1997). Inducible nitric oxide synthase: what difference does it make? *J. Clin. Invest.* **100**, 2417-2423.

Nathan, C. and Xie, Q. W. (1994). Regulation of biosynthesis of nitric oxide. *J. Biol. Chem.* **269**, 13725-13728.

Papapetropoulos, A., Garcia-Cardena, G., Madri, J. A. and Sessa, W. C. (1997). Nitric oxide production contributes to the angiogenic properties of vascular endothelial growth factor in human endothelial cells. *J. Clin. Invest.* **100**, 3131- 3139.

Radomski, M. W., Jenkins, D. C., Holmes, L. and Moncada, S. (1991). Human colorectal adenocarcinoma cells: differential nitric oxide synthesis determines their ability to aggregate platelets. *Cancer Res.* **51**, 6073-6078.

RayChaudhury, A., Frischer, H. and Malik, A. B. (1996). Inhibition of endothelial cell proliferation and bFGF-induced phenotypic modulation by nitric oxide. *J. Cell. Biochem.* **63**, 125-134.

Sase, K. and Michel, T. (1997). Expression and regulation of endothelial nitric oxide synthase. *Trends in Cardiovasc. Med.* **7**, 28-37.

Scatena, R., Bottoni, P., Martorana, G. E. and Giardina, B. (2005). Nitric oxide donor drugs: an update on pathophysiology and therapeutic potential. *Expert Opin. Investig. Drugs* **14**, 835-846.

Schgoer, W., Theurl, M., Jeschke, J., Beer, A. G., Albrecht, K., Gander, R., Rong, S., Vasiljevic, D., Egger, M., Wolf, A. M., Frauscher, S., Koller, B., Tancevski, I., Patsch, J. R., Schratzberger, P., Piza-Katzer, H., Ritsch, A., Bahlmann, F. H., Fischer-Colbrie, R., Wolf, D. and Kirchmair, R. (2009). Gene therapy with the angiogenic cytokine secretoneurin induces therapeutic angiogenesis by a nitric oxide-dependent mechanism. *Circ. Res.* **105**, 994-1002.

Schmidt, H. H., Lohmann, S. M. and Walter, U. (1993). The nitric oxide and cGMP signal transduction system: regulation and mechanism of action. *Biochim. Biophys. Acta.* **1178**, 153-175.

Schulz, R., Kelm, M. and Heusch, G. (2004). Nitric oxide in myocardial ischemia/reperfusion injury. *Cardiovasc. Res.* **61**, 402-413.

Schwentker, A. and Billiar, T. R. (2002). Inducible nitric oxide synthase: from cloning to therapeutic applications. *World J. Surg.* **26**, 772-778.

Shimaoka, M., Iida, T., Ohara, A., Taenaka, N., Mashimo, T., Honda, T. and Yoshiya, I. (1995). NOC, a nitric-oxide-releasing compound, induces dose dependent apoptosis in macrophages. *Biochem. Biophys. Res. Commun.* **209**, 519-526.

Silvagno, F., Xia, H. and Bredt, D. S. (1996). Neuronal nitric- oxide synthase-mu, an alternatively spliced isoform expressed in differentiated skeletal muscle. *J. Biol. Chem.* **271**, 11204-11208.

Simeone, A. M., Colella, S., Krahe, R., Johnson, M. M., Mora, E. and Tari, A. M. (2006). N-(4-Hydroxyphenyl)retinamide and nitric oxide pro-drugs exhibit apoptotic and anti-invasive effects against bone metastatic breast cancer cells. *Carcinogenesis* **27**, 568-577.

Sogawa, K., Numayama-Tsuruta, K., Ema, M., Abe, M., Abe, H. and Fujii-Kuriyama, Y. (1998). Inhibition of hypoxia-inducible factor 1 activity by nitric oxide donors in hypoxia. *Proc. Natl. Acad. Sci. USA.* **95**, 7368-7373.

Spedding, M., Schini, V., Schoeffter, P. and Miller, R. C. (1986). Calcium channel activation does not increase release of endothelial-derived relaxant factors (EDRF) in rat aorta although tonic release of EDRF may modulate calcium channel activity in smooth muscle. *J. Cardiovasc. Pharmacol.* **8**, 1130-1137.

Stasch, J. P. and Hobbs, A. J. (2009). NO-independent, haem-dependent soluble guanylate cyclase stimulators. *Handb. Exp. Pharmacol.* 277-308.

Steudel, W., Hurford, W. E. and Zapol, W. M. (1999). Inhaled nitric oxide: basic biology and clinical applications. *Anesthesiology* **91**, 1090-1121.

Stuehr, D. J. and Marletta, M. A. (1985). Mammalian nitrate biosynthesis: mouse macrophages produce nitrite and nitrate in response to Escherichia coli lipopolysaccharide. *Proc. Natl. Acad. Sci. USA.* **82**, 7738-7742.

Surks, H. K. (2007). cGMP-dependent protein kinase I and smooth muscle relaxation: a tale of two isoforms. *Circ. Res.* **101**, 1078-1080.

Tatoyan, A. and Giulivi, C. (1998). Purification and characterization of a nitric-oxide synthase from rat liver mitochondria. *J. Biol. Chem.* **273**, 11044-11048.

Taylor, C. T. and Moncada, S. (2010). Nitric oxide, cytochrome C oxidase, and the cellular response to hypoxia. *Arterioscler. Thromb. Vasc. Biol.* **30**, 643-647.

Thatcher, G. R. (2005). An introduction to NO-related therapeutic agents. *Curr. Top. Med. Chem.* **5**, 597-601.

Thebaud, B., Arnal, J. F., Mercier, J. C. and Dinh-Xuan, A. T. (1999). Inhaled and exhaled nitric oxide. *Cell Mol. Life Sci.* **55**, 1103-1112.

Trikha, P., Sharma, N. and Athar, M. (2001). Nitroglycerin: a NO donor inhibits TPA-mediated tumor promotion in murine skin. *Carcinogenesis* **22**, 1207-1211.

Tripatara, P., Patel, N. S., Webb, A., Rathod, K., Lecomte, F. M., Mazzon, E., Cuzzocrea, S., Yaqoob, M. M., Ahluwalia, A. and Thiemermann, C. (2007). Nitrite-derived nitric oxide protects the rat kidney against ischemia/reperfusion injury *in vivo*: role for xanthine oxidoreductase. *J. Am. Soc. Nephrol.* **18**, 570-580.

Tsuchiya, K., Kanematsu, Y., Yoshizumi, M., Ohnishi, H., Kirima, K., Izawa, Y., Shikishima, M., Ishida, T., Kondo, S., Kagami, S., Takiguchi, Y. and Tamaki, T. (2005). Nitrite is an alternative source of NO *in vivo*. *Am. J. Physiol. Heart. Circ. Physiol.* **288**, H2163-2170.

Webb, A., Bond, R., McLean, P., Uppal, R., Benjamin, N. and Ahluwalia, A. (2004). Reduction of nitrite to nitric oxide during ischemia protects against myocardial ischemia-reperfusion damage. *Proc. Natl. Acad. Sci. USA* **101**, 13683-13688.

Wei, D., Richardson, E. L., Zhu, K., Wang, L., Le, X., He, Y., Huang, S. and Xie, K. (2003). Direct demonstration of negative regulation of tumor growth and metastasis by host-inducible nitric oxide synthase. *Cancer Res.* **63**, 3855-3859.

Yerebakan, C., Ugurlucan, M., Bayraktar, S., Bethea, B. T. and Conte, J. V. (2009). Effects of inhaled nitric oxide following lung transplantation. *J. Card. Surg.* **24**, 269-274.

Yim, C. Y., Bastian, N. R., Smith, J. C., Hibbs, J. B. Jr. and Samlowski, W. E. (1993). Macrophage nitric oxide synthesis delays progression of ultraviolet light-induced murine skin cancers. *Cancer Res.* **53**, 5507-5511.

Yu, J., deMuinck, E. D., Zhuang, Z., Drinane, M., Kauser, K., Rubanyi, G. M., Qian, H. S., Murata, T., Escalante, B. and Sessa, W. C. (2005). Endothelial nitric oxide synthase is critical for ischemic remodeling, mural cell recruitment, and blood flow reserve. *Proc. Natl. Acad. Sci. USA.* **102**, 10999-11004.

Zech, B., Kohl, R., von Knethen, A. and Brune, B. (2003). Nitric oxide donors inhibit formation of the Apaf-1/caspase-9 apoptosome and activation of caspases. *Biochem. J.* **371**, 1055-1064.

Zhang, R., Wang, L., Zhang, L., Chen, J., Zhu, Z., Zhang, Z. and Chopp, M. (2003). Nitric oxide enhances angiogenesis via the synthesis of vascular endothelial growth factor and cGMP after stroke in the rat. *Circ. Res.* **92**, 308-313.

Ziche, M., Morbidelli, L., Choudhuri, R., Zhang, H. T., Donnini, S., Granger, H. J. and Bicknell, R. (1997). Nitric oxide synthase lies downstream from vascular endothelial growth factor-induced but not basic fibroblast growth factor-induced angiogenesis. *J. Clin. Invest.* **99**, 2625-2634.