Nitric Oxide Signaling in Biology

Article *in* Messenger · March 2013

DOI: 10.1166/msr.2013.1018

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Nitric Oxide Signaling in Biology

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Over the last two decades, the role of NO in cellular processes and signaling has been extensively investigated. Under physiological and pathophysiological conditions, NO is produced primarily by specific NO synthase enzymes and also through the process of nitrite reduction. NO is an important biological signaling molecule in the vasculature and tissues. NO contributes to hypoxic signaling, vasodilation, regulation of intracellular oxygen availability, and cytoprotection. In cells, NO interacts with various target proteins and organelles such as mitochondria and endoplasmic reticulum. Its interaction with intracellular proteins also results in the formation of second messenger molecules such as cyclic guanosine monophosphate. Post translational modification of proteins by NO may be involved in regulating cellular functions and the pathogenesis of human diseases. In NO-mediated intercellular communication, various cellular molecules are involved in its storage and transport. Production of NO by effector cells and the process of its conversion to form reactive nitrogen species modulates the balance of its *in vivo* actions in cytoprotection and cytotoxicity. Therefore, understanding the cellular signaling processes and biological effects of NO in health and disease can facilitate the development of improved therapeutic approaches to prevent or ameliorate disease. This mini review summarizes the processes of NO formation and NO-mediated cellular signaling and their role in physiology and disease.

Keywords: NO, Messenger, Signaling, Nitric Oxide Synthase, Nitrite, Peroxynitrite, Nitrosothiol, Guanylate Cyclase.

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INTRODUCTION

The discovery of the role of nitric oxide (NO) as a cellular signaling molecule has greatly influenced our understanding of normal physiology and disease. Endogenously generated NO has many diverse functions. It is synthesized constitutively or on demand by both enzymatic and non-enzymatic processes (Chen et al., 2010a; Zweier et al., 1995). NO signaling forms an integrated signaling web that regulates many physiological and pathophysiological processes (Erusalimsky and Moncada, 2007; Gotoh and Mori, 2006; Zweier and Talukder, 2006). The chemical interaction between NO and biological targets is an important factor in its role as signaling molecule (Erusalimsky and Moncada, 2007; Gotoh and Mori, 2006; Suryo Rahmanto et al., 2012). In humans, in order to improve our understanding and treatment of disease, it is important to understand the biological effects of NO and its cellular signaling functions. This review focuses on the processes of NO generation and the biochemical actions of NO through soluble guanylyl cyclase (sGC), post translational modification of protein thiols (PrSH), iron (Fe) and reduced glutathione (GSH) mobilization, mitochondria, endoplasmic reticulum (ER), and their possible relationship to cellular homeostasis and pathophysiology.

Nitric Oxide or Nitrogen Monoxide (NO)

NO is a gaseous, inorganic, uncharged, small diatomic molecule (Toledo and Augusto, 2012). It is a relatively stable free radical containing an unpaired electron (Toledo and Augusto, 2012). In mammalian cells, NO is a signaling/messenger molecule generated both enzymatically and non-enzymatically (Chen et al., 2010a; Zweier et al., 1995). NO exerts diverse physiological and pathological actions and is essential to the homeostasis of cells and organisms (Zweier and Talukder, 2006). It is well known that NO has a physiological role as a short-lived messenger. NO has two major functions in cells, regulation and cytotoxicity. The regulated homeostatic production versus persistent high level production of NO differentiates between the messenger and the cytotoxic properties of NO. While low levels of NO exert a number of regulatory and cytoprotective effects, higher levels are potentially deleterious. Moreover, many of the harmful effects of NO are not directly due to NO itself, but are mediated by its oxidative reaction products.

Physiological Sources of NO

In humans, NO is produced by both enzymatic and non-enzymatic pathways under various physiological and pathophysiological conditions. In healthy human adults, the calculated rate of NO synthesis is reported to be approximately 0.6 μmol kg^{-1} h^{-1} (Siervo et al., 2011). In addition, the rate of NO synthesis is increased by

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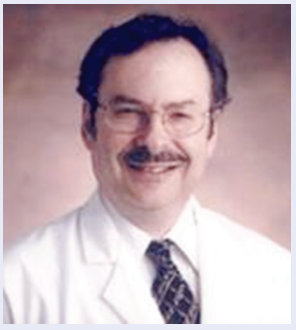
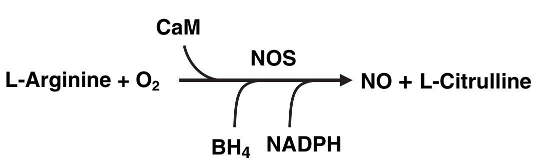


Jay L. Zweier received his baccalaureate degrees in Physics and Mathematics at Brandeis University in 1976. After Ph.D. training in Biophysics at the Albert Einstein College of Medicine, he pursued medical training at the University of Maryland School of Medicine and received his MD in 1980. Subsequently he completed his residency in internal medicine followed by his cardiology fellowship at Johns Hopkins in 1986. In 1987, he joined the faculty of The Johns Hopkins University School of Medicine. At Hopkins, he established the molecular and cellular biophysics laboratories and the institutional Electron Paramagnetic Resonance Center. He served as co-director of The Johns Hopkins Specialized Center of Research (SCOR) in Ischemic Heart Disease and the Ischemic Heart Disease Heart Research Program. In 1998 he was promoted to the rank of Professor and in 2000, he was appointed as Chief of Cardiology Research. In addition to serving as Professor of Medicine in the Cardiology and Pulmonary Divisions, Dr. Zweier also held appointments as Professor of Radiology and Environmental Health Sciences in the Schools of Medicine and Public Health, respectively. He was elected as a fellow in the American College of Cardiology in 1995 and the American Society of Clinical Investigation in 1994. In July of 2002, Dr. Zweier joined The Ohio State University College of Medicine as Director of the Davis Heart & Lung Research Institute and as the John H. and Mildred C. Lumley Chair in Medicine. Dr. Zweier has published over 400 peer reviewed published manuscripts in the fields of cardiovascular research, free radical biology, nitric oxide biology and chemistry, and magnetic resonance. He has served on the editorial boards of numerous journals including the Journal of Clinical Investigation, Circulation, Circulation Research, British Journal of Pharmacology, Antioxidants and Redox Signaling, Current Topics in Biophysics, Methods in Enzymology and the American Journal of Physiology. He has been the recipient of many endowed lectureships and awards in the US and abroad. These awards include the Virchow Prize for medical research, the WW Smith award for innovative cardiology research, the Upjohn Award for outstanding investigation from the International Society for Heart Research, the Louis N. Katz Basic Research Award from the American Heart Association, the Clinician Scientist and Established Investigator awards from the American Heart Association, the DuPont-Merck award for research excellence, and the Alice Hamilton Award for Biological Sciences.

several orders of magnitude under infectious and inflammatory conditions (Siervo et al., 2011). A widely accepted range for the physiological concentration of NO is 1–100 nM (Toledo and Augusto, 2012). NO is produced by many cell types such as endothelial cells, neurons, myocytes, smooth muscle cells, and activated immune cells. In cells, NO synthase enzymes are typically the major source of NO synthesis (Chen et al., 2010a; Forstermann and Sessa, 2012; Zhou and Zhu, 2009). In addition, nitrate (NO_3^-) and nitrite (NO_2^-) can also be reduced to NO through enzymatic and non-enzymatic reactions (Gladwin and Kim-Shapiro, 2008; Jansson et al., 2008; Li et al., 2009). In humans, NO_2^- infusions at near physiological concentrations cause vasodilation, and dietary supplementation with NO_3^-, that is reduced to NO_2^-, causes a reduction in blood pressure (Jansson et al., 2008).

Nitric Oxide Synthases (NOSs)

In cells, NO is formed from the amino acid precursor L-arginine by the enzyme nitric oxide synthase (NOS), in the presence of 5,6,7,8-tetrahydrobiopterin (BH_4), reduced nicotinamide-adenine-dinucleotide phosphate (NADPH), molecular oxygen (O_2), and calcium (Ca^{2+})/calmodulin (CaM), Scheme 1 (Chen et al., 2010a; Forstermann and Sessa, 2012). Three distinct isoforms of NOS derived from separate genes are well characterized: neuronal NOS (nNOS, NOS1), inducible NOS (iNOS, NOS2), and endothelial NOS (eNOS, NOS3). nNOS and eNOS are constitutively expressed; therefore, they are also called cNOS. The expression of iNOS is regulated by transcriptional activation in response to inflammation and cytokine production (Wu, 1995). iNOS is a high-flux enzyme highly regulated by substrate availability. The activity of all NOS isoforms is regulated by CaM binding. In cNOS isoforms, CaM binding is brought about by an increase in intracellular Ca^{2+} (half-maximal activity between 0.2 and 0.4 μM) (Forstermann and Sessa, 2012). In iNOS, the amino acid structure for CaM binding is different; hence, it binds CaM at extremely low intracellular Ca^{2+} concentrations (below 40 nM) (Cho et al., 1992; Hemmens and Mayer, 1998). Moreover, once expressed, iNOS is constantly active and not regulated by intracellular Ca^{2+} concentrations (Forstermann and Sessa, 2012). CaM binding facilitates the flow of electrons from NADPH in the reductase domain to the heme in the oxygenase domain.



Scheme 1. Conversion of L-arginine to NO and L-citrulline is catalyzed by nitric oxide synthase in the presence of oxygen, calmodulin, 5,6,7,8-tetrahydrobiopterin, and NADPH.

NO AND MOLECULAR SIGNALING

Endogenously generated NO is generally thought to be freely diffusible in cells. NO is an unusual messenger molecule with numerous molecular targets. Hence, the chemical interaction between NO and biological targets is an important factor in its role as a signaling agent. A fundamental understanding of the biochemistry of NO is essential to understand its role in cellular signaling and biological/physiological utilization.

NO and sGC in Vascular Smooth Muscle Cell Relaxation and Vasodilation

The NO produced by constitutively expressed NOS (cNOS) is involved in signal transduction. Under normal physiological conditions, NO produced by eNOS in the endothelium can rapidly diffuse across the cell membrane to smooth muscle cells where it activates soluble guanylate cyclase (sGC) and induces various cellular signaling pathways involved in vascular smooth muscle relaxation (vasodilation).

Present in cardiomyocytes and smooth muscle cells, sGC is a heterodimeric protein and an authentic heme-based sensor for NO. sGC selectively and efficiently binds NO while having no affinity for O_2 (Derbyshire and Marletta, 2009). In the active site, sGC has a five-coordinate ferrous (Fe^{2+}) heme containing a proximal histidine ligand (His-105) (see Fig. 1) (Zhao et al., 1998). The signaling roles of NO are mediated by its high affinity binding to ferrous iron in the heme group of sGC (see Fig. 1). sGC selectively and efficiently binds NO even in the presence of O_2 (μM) (Derbyshire and Marletta, 2009). NO rapidly reacts with the pentacoordinated heme iron of sGC with a calculated rate constant for the reaction of 7×10^8 M^{-1} s^{-1} (Winger et al., 2007). NO binding causes the dissociation of His-105 resulting in the formation of the pentacoordinated nitrosyl heme sGC (NO-sGC) (see Fig. 1) (Martin et al., 2012). The dissociation constant K_D for NO-sGC to form NO is 27 s^{-1} (Martin et al., 2012). Binding of NO to sGC leads to strong activation of its enzyme activity by several hundred-fold (Derbyshire and Marletta, 2009). sGC is key for catalysis of the formation of the second messenger cyclic guanosine monophosphate (cGMP) from guanosine 5'-triphosphate (GTP) (see Fig. 1) (Derbyshire and Marletta, 2009). cGMP in turn regulates protein kinases (cGK), phosphodiesterases (PDE), and ion-gated channels. cGMP signaling plays an important role in many physiological and pathophysiological processes such as vasodilation, neurotransmission and platelet aggregation (Derbyshire and Marletta, 2009).

More recent studies have shown that NO binding to sGC is complex (Derbyshire and Marletta, 2009; Martin et al., 2012) and that sGC has a second NO binding site (see Fig. 1). In the presence of excess NO, a second molecule of NO binds to sGC to form transient dinitrosyl complex

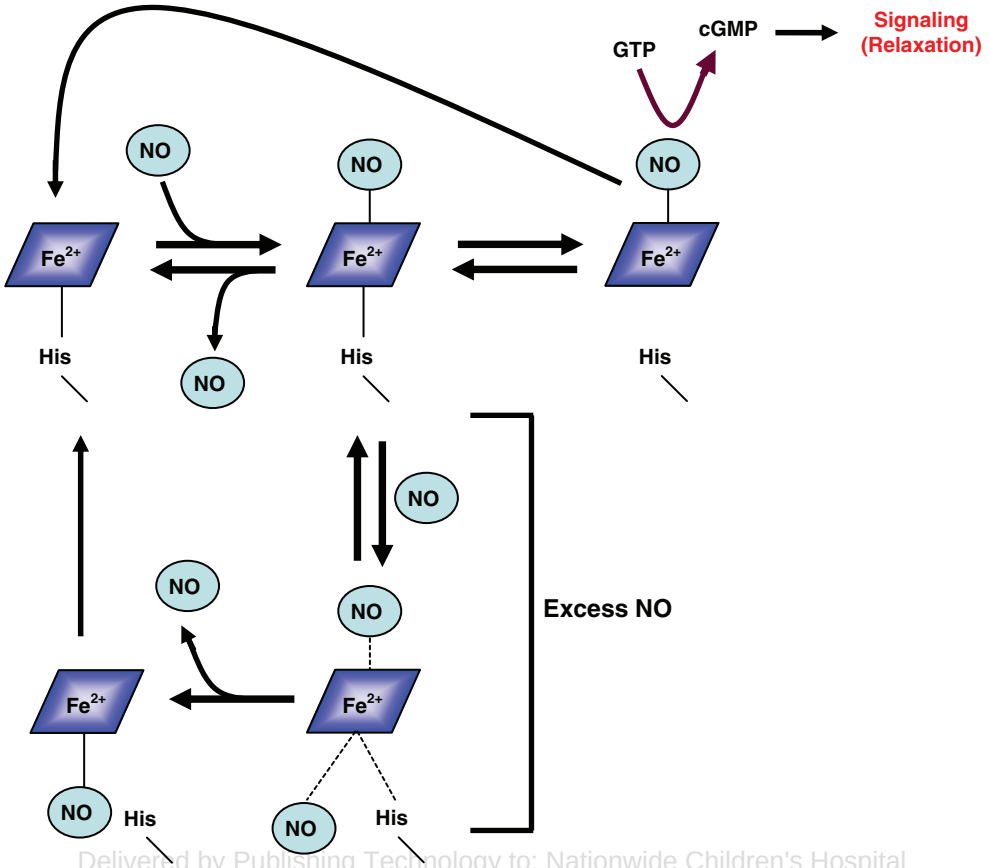
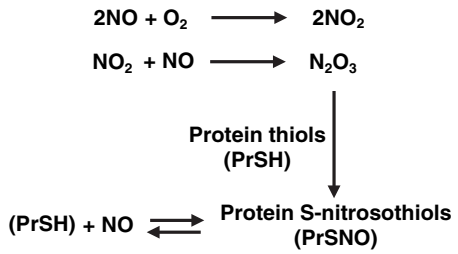


Figure 1. Model of activation of soluble guanylyl cyclase (sGC) by NO. Activation of sGC leads to increased synthesis of cyclic guanosine monophosphate (cGMP) from guanosine 5'-triphosphate (GTP). Production of second messenger cGMP activates various signaling pathways including smooth muscle cell relaxation. NO binds to the heme of sGC at a diffusion-controlled rate to form a six-coordinate complex. Excess NO–sGC reacts with stoichiometric amount of NO to form the six-coordinate complex and further conversion to a five-coordinate complex exhibits NO concentration dependence.

$(NO)_2$-sGC, which is a hexacoordinated complex as shown in Figure 1 (Derbyshire and Marletta, 2009; Martin et al., 2012). Subsequently, $(NO)_2$-sGC converts to a pentacoordinated complex with NO bound in the proximal heme pocket as shown in Figure 1 (Derbyshire and Marletta, 2009; Martin et al., 2012). Other molecular mechanisms for the NO activation of sGC have also been reported (see the following section) (Derbyshire and Marletta, 2009) and the molecular mechanisms involved in the NO activation of sGC remain under investigation. Importantly, both NOS and sGC are critical for cellular homeostasis, and dysfunction in this NO signaling pathway has been linked to several diseases (Bredt, 1999). Hence, understanding the molecular mechanisms involved in the NO/sGC/cGMP pathway is very important for the rational design of therapeutic agents for the treatment of disease.

NO and Post Translational Modifications of Protein Thiols (PrSH)

Post translational modification of proteins can modulate the function of proteins involved in signaling cascades, redox systems, ion channels, receptors, DNA repair, O_2 transport (heme proteins), transcription factors, and apoptosis (Broillet, 1999; Handy and Loscalzo, 2006; Stamler et al., 1992). Many biological effects of NO are mediated through cGMP-independent pathways via post translational modification of proteins by S-nitrosylation of target proteins. NO-mediated nitrosylation of thiol-containing proteins is increasingly recognized as an important post translational modification in cell signaling and pathology. In cells with a high concentration of NO, one of the most important and feasible post translational modifications of proteins is S-nitrosylation of protein thiols (PrSH) by NO or its higher oxides (Broillet, 1999; Handy and Loscalzo, 2006; Stamler et al., 1992). This is a reversible process that may modulate enzyme activity. It is generally believed that higher oxides of nitrogen such as N_2O_3 mediate S-nitrosylation of protein thiols (Fukuto et al., 2012; Handy and Loscalzo, 2006). To form N_2O_3, NO reacts with O_2 to form the nitrogen dioxide (NO_2) intermediate, which further reacts with another NO as shown in Scheme 2 (Fukuto et al., 2012; Handy and Loscalzo, 2006). Formation of N_2O_3 also depends on NO



Scheme 2. NO-mediated nitrosylation of thiol-containing proteins.

concentration. Thus, N_2O_3 reacts with PrSH to form protein S-nitrosothiols (PrSNO), and this is reversible (see Scheme 2) (Handy and Loscalzo, 2006).

PrSNO are regarded as NO storage/transport compounds. In cells with a low concentration of NO, PrSNO formation occurs after intermediate oxidation of PrSH by NO_2 or other oxidants to form a susceptible protein thiyl radical ($PrS^{\bullet}$), that can further react with another NO (Heo and Campbell, 2004; Jourd'heuil et al., 2003). In another study, mitochondria have been shown to be crucial for the formation of PrSNO (Handy and Loscalzo, 2006). It has also been suggested that there is an important role for peroxynitrite in the S-nitrosylation of PrSH (van der Vliet et al., 1998). Moreover, other molecular pathways/mechanisms have also been proposed for NO-mediated S-nitrosylation of PrSH (Handy and Loscalzo, 2006). However, many questions remain regarding *in vivo* formation of PrSNO.

The post translational modification of PrSH by NO is implicated in protein regulation and signaling in intact cells (Handy and Loscalzo, 2006; van der Vliet et al., 1998). Moreover, experimental evidence indicates that only certain proteins are S-nitrosylated. However, the biochemical basis for this selectivity is not clear. eNOS and iNOS are susceptible to NO-induced thiol modifications (Erwin et al., 2006; Rosenfeld et al., 2010). S-nitrosylation of NOS enzymes regulates the NO production suggesting a feedback mechanism to control its activity (Erwin et al., 2006; Rosenfeld et al., 2010). In endothelial cells PrSNO levels are increased under various conditions (Handy and Loscalzo, 2006). NO-mediated inhibition of apoptosis or programmed cell death has been reported to occur due to the S-nitrosylation of caspases and inhibition of their subsequent signaling cascade (Kim and Tannenbaum, 2004; Rossig et al., 1999). NO targets the mitochondrial proteins complex I and complex IV involved in respiration to form PrSNO (Clementi et al., 1998; Erusalimsky and Moncada, 2007). The NO-mediated accumulation of PrSNO in mitochondria increases the production of reactive oxygen species (ROS) (Erusalimsky and Moncada, 2007). It has been reported that in the presence of excess NO, S-nitrosylation of NO-sGC results in increased activity in the conversion of GTP to cGMP (see Fig. 1) (Derbyshire and Marletta, 2009). There are other cellular functions also regulated by PrSNO (Abrams et al., 2012; Handy and Loscalzo, 2006). It has also been shown that S-nitrosylation plays important roles in neurodegenarative disorders such as Alzheimer's disease and Parkinson's disease (Cho et al., 2009; Chung et al., 2004; Uehara et al., 2006). Hence, NO messenger mediated cellular signaling via PrSNO plays a major role in various cellular functions and disease pathophysiology.

In cells, PrSNO are less stable and decompose to yield NO and other products due to various factors such as excess thiols, transition metals, antioxidants, and light (Handy and Loscalzo, 2006). This limited stability makes it challenging to identify and quantify PrSNO *in vivo* (Samouilov and Zweier, 1998). A thorough understanding of the chemistry of PrSNO formation and degradation is important to evaluate its relevance or likelihood as an intermediate in signaling pathways. There is much experimental evidence for the *in vivo* formation of PrSNO (Abrams et al., 2012; Handy and Loscalzo, 2006); however, many questions remain and this is currently an active area of investigation.

NO Storage and Transport and Role of Iron and GSH

In recent years, the importance of the chemical biology of NO has been increasingly appreciated. It has been suggested that NO plays an important role in cellular iron homeostasis and/or metabolism (Suryo Rahmanto et al., 2012). It has been demonstrated that NO has high affinity for iron and forms complexes with a variety of important iron-containing proteins such as ribonucleotide reductase (Lepoivre et al., 1991), heme-containing proteins such as sGC (Ignarro, 1991), [Fe-S]-containing proteins such as ferro-chelatase (Kim et al., 1995), and the iron storage protein ferritin (Lee et al., 1994). Studies have also investigated the effect of NO on cellular iron mobilization from cells (Suryo Rahmanto et al., 2012). From these investigations, a model has been proposed for NO-mediated iron mobilization from cells in the presence of metabolizable D-glucose (see Fig. 2) (Suryo Rahmanto et al., 2012). Glucose is transported into cells and subsequently converted into glucose 6-phosphate (G-6-P) by glucose 6-phophatase (G6P). G-6-P is further metabolized into ATP and NADPH by the tricarboxylic acid cycle (TCA) and pentose phosphate pathway (PPP), respectively. NADPH is utilized for the synthesis of reduced glutathione (GSH, antioxidant), which is important for the NO-mediated iron release from cells (see Fig. 2).

NO can be transported into cells either by protein-disulfide isomerase (PDI) (Zai et al., 1999) or diffusion through the cell membrane. Inside the cells, NO reacts with protein-bound iron or iron en route to ferritin. The high affinity of NO for iron results in a dinitrosyl-dithiol-iron complex (DNIC) (see Scheme 3) (Suryo Rahmanto et al., 2012). In cells, GSH is often present at millimolar

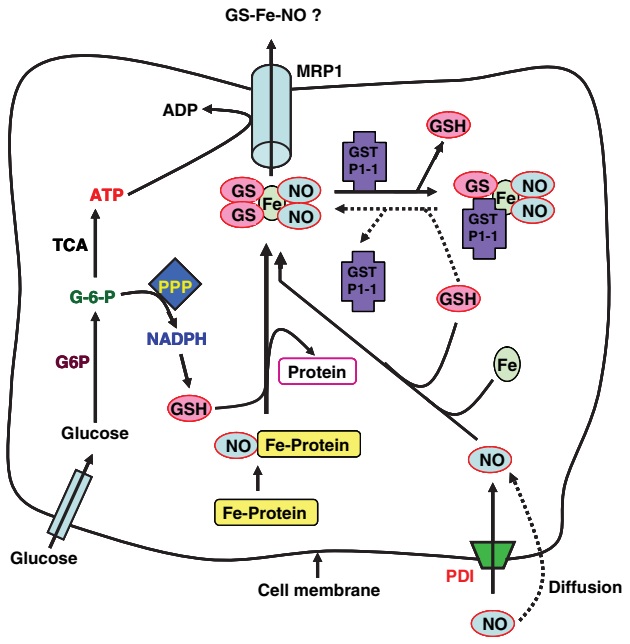
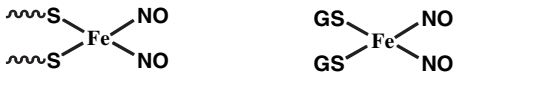


Figure 2. A proposed model of NO-mediated iron and GSH mobilization from cells. NO-mediated iron mobilization from cells is dependent on the transport of glucose (D-glucose) into cells and its metabolism. Inside the cells glucose is converted to glucose 6-phosphate (G-6-P) by glucose 6-phophatase (G6P), which is further converted to ATP and NADPH by the tricarboxylic acid cycle (TCA) and pentose phosphate pathway (PPP), respectively. NO can be transported into cells either by protein-disulfide isomerase (PDI) or diffusion through cell membrane. GSH: reduced glutathione, GST P1-1: Glutathione transferase P1-1, MRP1: multidrug resistance protein 1, GS-Fe-NO: glutathione conjugate of iron and NO.

concentration (1–10 mM) (Schafer and Buettner, 2001). Hence, at physiological conditions the relevant DNIC is dinitrosyl-diglutathionyl-iron complex (DNDGIC), where GSH acts as a ligand (see Scheme 3) (Suryo Rahmanto et al., 2012). The formation and release of DNICs were observed in endothelial cells in which eNOS was induced (Mulsch et al., 1993). It has also been suggested that DNICs serve as a NO reservoir for PrSNO formation in cells (Bosworth et al., 2009). However, the molecular mechanisms involved in the formation of DNIC/DNDGIC have not been fully elucidated.

It has been shown that multidrug resistance protein (MRP) 1 plays a role in the transport of GSH conjugates (see Fig. 2) (Ballatori et al., 2005). MRP1 is ubiquitously expressed (Flens et al., 1996) and requires ATP hydrolysis (Chang, 2010; Hou et al., 2002) to efflux various



Scheme 3. NO formation of iron containing complexes with thiol containing proteins and GSH.

complexes of antimony and arsenic (Ballatori et al., 2005; Leslie et al., 2004), and anticancer drugs (Ballatori et al., 2005; Chang, 2010) often as conjugates of GSH. It has been shown that there is an increase in NO-mediated iron and GSH efflux in human breast cancer cells (Suryo Rahmanto et al., 2012). In addition, NO-mediated iron and GSH efflux was prevented by the MRP1 inhibitors and GSH-depleting agent, respectively (Suryo Rahmanto et al., 2012; Watts et al., 2006). An electron paramagnetic resonance (EPR) spectroscopic study demonstrated that MRP1 inhibitors increased the DNIC signal in cells after NO treatment, indicating inhibition of release of the DNIC from the cell via MRP1 (Watts et al., 2006). Moreover, iron release from cells was demonstrated in murine embryonic fibroblasts transfected with iNOS or with addition of two exogenous NO generators (Lok et al., 2012). It is important to note that MRP1 is responsible only for NO-mediated iron mobilization and does not play a role in iron release in the absence of NO. Hence, NO mediates release of both iron and GSH from cells via MRP1 (see Fig. 2). In human HaCaT keratinocytes, it has been demonstrated that UVA irradiation increases GSH efflux and results in apoptosis (He et al., 2003). It is also well known that iron efflux from cells using chelators results in antitumor/anticancer activity and neuroprotection (Li et al., 2011; Richardson et al., 1995).

Glutathione transferases (GSTs) catalyze the nucleophilic attack by GSH on compounds that contain an electrophilic carbon, nitrogen, or sulfur atom. It has been suggested that GST P1-1 that binds DNDGIC could act as an intermediate form of NO for regulating various biological processes (Cesareo et al., 2005). In cells, MRP1 forms an integrated detoxification mechanism along with GST, which eliminates toxic endogenous and exogenous agents as GSH conjugates (see Fig. 2). MRP1 transports iron and GSH in the form of DNIC/DNDGIC (Suryo Rahmanto et al., 2012). The enzyme GST P1-1 has high affinity for DNDGICs ($K_d = 10^{-9}$ to 10^{-10} M) (Cesareo et al., 2005). DNDGIC binds with human GST P1-1 and displaces one thiol (GS) ligand and it is reversible (Cesareo et al., 2005). It has been shown that the release of iron from cells is decreased in NO treated GST-transfected human cancer cells (Lok et al., 2012). In addition, similar results were observed in cells that were transfected with iNOS or NO provided extracellularly (Lok et al., 2012). Thus, GST P1-1 binds DNICs and prevents their release from cells via MRP1. As a result, GSTs may act as a protective mechanism against high levels of DNICs or as a NO store to regulate the release of DNICs via MRP1.

It has been reported that DNICs are the most abundant NO-derived adduct in cells, greatly exceeding the production of S-nitrosothiols (Hickok et al., 2011). In cells, NOS, GSTs, MRP1 and GSH play a major role in NO metabolism (see Fig. 2). Elucidating their communication and interactions is important in formulating an integrated

model of NO metabolism and its link to the mobilization of iron and GSH. This is important for understanding NO and its effector functions under various physiological and pathophysiological conditions.

NO AND ORGANELLE SIGNALING

In cells, NO interacts with various organelles such as mitochondria, endoplasmic reticulum, and the nucleus (Erusalimsky and Moncada, 2007; Gotoh and Mori, 2006; Kroncke et al., 1997). In this review, we limit our focus to the interactions of NO with mitochondria and endoplasmic reticulum.

NO and Mitochondrial Signaling

Mitochondria are critical intracellular organelle targets of NO (Erusalimsky and Moncada, 2007). It is well recognized that mitochondria play an important role in the generation of energy (Saraste, 1999), ROS (Turrens, 2003; Velayutham et al., 2011), calcium homeostasis (Berridge et al., 2000), and the orchestration of apoptosis (Green and Reed, 1998). NO closely resembles O_2 and therefore can bind to O_2-binding sites. In the literature, it has been shown that NO at physiological concentrations modulates mitochondrial respiration through the reversible inhibition of the enzyme cytochrome c oxidase (CcO, complex IV) in the electron transport chain (ETC), in competition with O_2 (see Fig. 3) (Brown and Cooper, 1994; Cleeter et al., 1994; Erusalimsky and Moncada, 2007; Schweizer and Richter, 1994). It has been reported that the affinity K_D of NO for the O_2-binding site of CcO is 0.2 nM (Mason et al., 2006), confirming that concentrations of NO that have been detected in tissues (10–450 nM) (Malinski et al., 1993; Shibuki and Okada, 1991) would be sufficient to compete with intracellular O_2. The potential biological importance of the endogenously generated NO interaction with CcO and modulation of O_2 consumption has been demonstrated in various cells, isolated tissues, and whole animals (Erusalimsky and Moncada, 2007). It has been proposed that NO inhibition could be a physiological control mechanism and may contribute to disease pathophysiology (Mason et al., 2006).

In mammalian cells, mitochondria, endoplasmic reticulum, and peroxisomes are the major sources of ROS under physiological and pathophysiological conditions (Antonenkov et al., 2010; Kakihana et al., 2012; St-Pierre et al., 2002). Under normal physiological conditions ~1–2% of the O_2 consumed by the mitochondria is converted into ROS (Chance et al., 1979; O'Rourke et al., 2005). Electron leakage from the ETC in mitochondria occurs with partial reduction of O_2 with generation of ROS (see Fig. 3) (Giorgio et al., 2007). In the ETC, complex I and complex III are thought to be the major sites of $O_2^{\bullet-}$ production as shown in Figure 3 (Brand, 2010; Murphy,

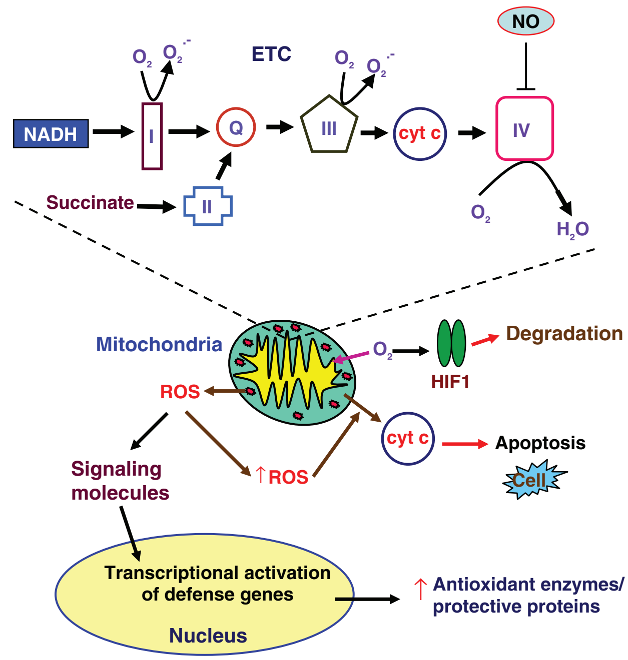


Figure 3. NO and mitochondrial signaling. In the mitochondrial electron transport chain molecular oxygen is completely or partially reduced. Partial (one electron) reduction of oxygen produces superoxide radical ($O_2^{\bullet-}$). NO reversibly binds and inhibits cytochrome *c* oxidase (CcO/complex IV), which modulates mitochondrial respiration and production of ROS. The production of ROS activates various cytoprotective and cytotoxic signaling pathways. ROS: reactive oxygen species, HIF1: hypoxia inducible factor 1.

2009). Very recently, it has also been shown that mitochondrial protein cyt *c* can also be involved in the generation of ROS (Velayutham et al., 2011). In isolated rat heart mitochondria, treatment with NO enhances the generation of $O_2^{\bullet-}$ (Poderoso et al., 1996). Incubation of RAW 246.7 cells and HUVECs at 3% O_2 resulted in a NO-dependent increase in $O_2^{\bullet-}$ levels (Palacios-Callender et al., 2004). Cu, Zn-superoxide dismutase (SOD1) in the mitochondrial intermembrane space dismutates $O_2^{\bullet-}$ to hydrogen peroxide (H_2O_2) (Fukai and Ushio-Fukai, 2011). ROS ($O_2^{\bullet-}$ and H_2O_2) in turn may act as a second messenger (Velayutham et al., 2007). The increased production of ROS from mitochondria thus initiates downstream signaling events (Erusalimsky and Moncada, 2007). It has been proposed that under physiological conditions the interaction between NO and CcO increases the mitochondrial ROS, which would act as a second messenger to maintain high constitutive levels of cytoprotective (antioxidant or phase II) enzymes (see Fig. 3) (Erusalimsky and Moncada, 2007; Velayutham et al., 2007).

Hypoxia inducible factor (HIF) 1, a heterodimeric (α and β) transcription factor, plays an important role in the response of tissues to low partial pressures of O_2 (Semenza, 2000). The protein stability of HIF1-α is regulated in an O_2-dependent manner by a family of prolyl hydroxylases (Bruick and McKnight, 2001; Epstein et al.,

2001; Pugh and Ratcliffe, 2003; Wenger, 2002). At high O_2 concentrations, HIF1 undergoes degradation. A study has shown that competition between NO and O_2 at the level of CcO is responsible for the inhibition of HIF1-α stabilization in the presence of NO (Hagen et al., 2003). Under hypoxia or low intracellular concentration of O_2, NO binds with CcO and increases the availability of O_2 in the presence of NO. The increased availability of O_2 is redistributed to non-respiratory O_2-dependent targets such as prolyl hydroxylases, which results in the degradation of HIF1 (see Fig. 3). This signaling leads to a situation in which the cell may fail to register hypoxia. In addition, NO may also be involved in the attenuation of adaptive responses to low level O_2 and/or hypoxia.

It has been well recognized that mitochondria play a critical role in apoptotic and necrotic cell death (Finkel, 2012). Apoptosis is associated with an increase in the generation of ROS from the mitochondria (Zamzami et al., 1995). Release of cyt *c* from the inner mitochondrial membrane into the cytosol is an early step in apoptosis (Kluck et al., 1997; Yang et al., 1997). It has been demonstrated that NO can induce apoptotic as well as necrotic cell death (Kroncke et al., 1997). Hence, under various pathophysiological conditions the increased production of NO interacts with CcO and increases the production of ROS in mitochondria. The increased production of ROS in mitochondria can induce the release of cyt *c* from the inner membrane (see Fig. 3). Subsequently, cells undergo death via apoptosis.

In cells, mitochondrial ROS are increasingly viewed as important signaling molecules (Finkel, 2012). The NO-induced release of mitochondrial ROS is interpreted as a signal that a stress has been encountered. The amount and duration of ROS release by mitochondria are potential determining factors in the ultimate biological outcome. The increased production of ROS may lead to peroxidation of lipids, destruction of Fe-S clusters, inhibition of manganese superoxide dismutase (SOD2) via nitration (MacMillan-Crow et al., 1996), and calcium efflux from the mitochondria (Richter et al., 1994). All these processes together cause irreversible damage to the mitochondria. Consequently, in susceptible cells NO can induce ROS-mediated apoptotic as well as necrotic cell death (Kroncke et al., 1997).

NO and Endoplasmic Reticulum (ER) Signaling

The endoplasmic reticulum is another important intracellular organelle targeted by NO. Important post translational modifications involving glycosylation, formation of disulfide bonds, oxidative folding, and assembly of newly synthesized proteins occur in the ER (Csala et al., 2012; Hagiwara and Nagata, 2012; Ramming and Appenzeller-Herzog, 2012). In the ER, proteins must be properly modified and folded into a proper conformation. Importantly, the unfolded or misfolded proteins can not be delivered to the Golgi apparatus (Hagiwara and Nagata, 2012). Hence, the accumulation of these abnormal proteins in ER perturbs ER function and cell survival. ER also functions as a cellular Ca^{2+} store (Csala et al., 2012; Palty et al., 2012; Talukder et al., 2008). It plays an important role in Ca^{2+} homeostasis by pumping Ca^{2+} into the lumen via sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) and by releasing Ca^{2+} from the lumen by the ryanodine receptor (RyR) (see Fig. 4) (George et al., 2011; Talukder et al., 2008). A high concentration of Ca^{2+} in ER is also necessary for ER functions such as folding and disulfide bond formation of newly synthesized proteins because functions of various ER chaperones, such as protein disulfide isomerase, calnexin, and calreticulin depend on a high concentration of Ca^{2+} (Csala et al., 2012). Hence, disruption of Ca^{2+} homeostasis in the ER disturbs ER function (Talukder et al., 2008).

It has been reported that excess NO disturbs ER Ca^{2+} homeostasis in pancreatic β-cells (Oyadomari et al., 2002). It was reported that NO, through ROS-mediated $ONOO^-$, inhibits SERCA activity by tyrosine nitration or thiol nitrosylation within the channel-like domain (see Fig. 4) (Gotoh and Mori, 2006; Viner et al., 1999). NO also increases the activity of RyR by S-nitrosylation (see Fig. 4) (Xu et al., 1998). On the other hand, the overexpression of calreticulin increases the Ca^{2+} content of ER and protects

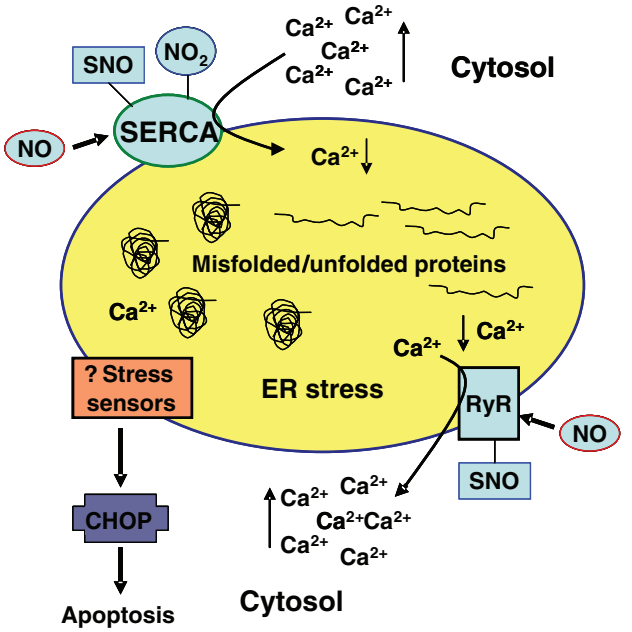


Figure 4. NO and endoplasmic reticulum (ER) stress-induced apoptosis pathway. SERCA, sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase; RyR, ryanodine receptor; CHOP, C/EBP homologous protein. NO depletes ER Ca^{2+}, increases unfolded and misfolded proteins, causes ER stress, activates transcription of CHOP transcription factor and leads to apoptosis. Molecular mechanisms involved in the CHOP-induced apoptosis are not fully elucidated.

against NO-mediated apoptosis in β-cells (Oyadomari et al., 2001). In sea urchin eggs, NO mobilizes intracellular Ca^{2+} stores from the ER, via the cyclic adenosine diphosphate ribose (cADPR) signaling pathway (Willmott et al., 1996). Here, NO increases the production of cGMP from GTP by sGC (Derbyshire and Marletta, 2009; Willmott et al., 1996). The increased production of cGMP enhances the synthesis of second messenger, cADPR, from NAD^+ by ADP-ribosyl cyclase (CD38) (Galione et al., 1993; Lee, 2011; Lee, 2012). In mammalian cells and urchin eggs, cADPR activates the ryanodine-sensitive Ca^{2+} release channels, ryanodine receptors (RyRs), in the intracellular Ca^{2+} stores (Galione et al., 1991; Meszaros et al., 1993; Takasawa et al., 1993). In rat pancreatic exocrine cells, NO-induced cGMP increases the production of inositol 1,4,5-triphosphate (IP_3) (Moustafa et al., 2011). The increased production of IP_3 activates the Ca^{2+} release by IP_3 receptors (IP_3R) in ER (Berridge, 1993; Patel et al., 1999; Rooney et al., 1996; Shawl et al., 2009; Streb et al., 1983; Thatcher, 2010). Thus, NO or NO-derived $ONOO^-$ or NO/cGMP-induced second messengers such as cADPR and IP_3 deplete ER Ca^{2+}, increase unfolded and misfolded proteins, cause ER stress, and lead to apoptosis. When ER function is disturbed, ER stress response sensors are activated (Gotoh and Mori, 2006). Further, when ER functions are severely impaired, apoptotic pathways are activated to protect the organs and whole body by eliminating the damaged cells.

Under ER stress, the transcription of the gene for C/EBP homologous protein (CHOP) is activated (Wang et al., 1996). It has been reported that in various cells, NO-induced apoptosis is mediated by an ER stress pathway involving CHOP (see Fig. 4) (Gotoh et al., 2002; Oyadomari et al., 2002). CHOP itself is a transcription factor and the transcription of the *chop* gene is regulated by ER stress sensor signaling pathways (Ron and Habener, 1992). Islet cells from *chop* knockout mice are resistant to NO-induced apoptosis (Oyadomari et al., 2001). However, the molecular mechanisms involved in CHOP-mediated apoptosis are not fully understood. Many unanswered questions remain regarding the mechanism of ER stress-induced apoptosis (Gotoh and Mori, 2006).

Excess production of NO by induction of NOS2 occurs in inflammatory cells such as macrophages (MacMicking et al., 1997). It has been shown that the ER stress pathway including CHOP expression is activated in macrophages (Zhou et al., 2005). Activated macrophages play a major role in the pathogenesis of human cardiovascular diseases such as atherosclerosis (Charo and Taub, 2011; Hotamisligil, 2010; Ignarro, 2000; MacMicking et al., 1997). Therefore, NO-mediated activation of the ER stress pathway is thought to play an important role in the pathophysiology of human disease. Recently, it has been suggested that ER stress and mitochondrial pathways are involved in excess cholesterol-triggered smooth muscle cell death (Kedi et al., 2009). In addition, endothelial ER stress is activated *in vivo* at atherosusceptible arterial sites (Civelek et al., 2009). Hence, NO-mediated activation of ER stress pathway may be involved in the dysfunction of endothelial and other vascular cells.

NO IN INTERCELLULAR SIGNALING/COMMUNICATIONS

As described above, it is clear that NO is produced in various cell types such as neurons, endothelium, myocytes, smooth muscle cells, and immune cells. Cellular communication can be mediated by the exchange of small molecules, such as NO. Importantly, the functional communication, integrated cellular signaling, and cross-talk among the various cell types are critical for the development, protection, and environmental adaptation of various organs in multicellular organisms. In the immediate vicinity of a cultured cell monolayer, it has been calculated that a steady-state concentration of NO was about 4–5 μM (Laurent et al., 1996). However, the molecular mechanisms involved in the secretion of NO by the effector cell are not fully understood. The secreted NO then diffuses to a target cell located nearby and contacts its plasma membrane. NO can easily diffuse through the hydrophobic and fluid-phase membranes (Vanderkooi et al., 1994). The diffusion distance of NO secreted by a single cell is estimated to be 150–300 μm in 4–15 s and is further effective when cells are located in clusters (Kroncke et al., 1997). After diffusion into the target cell, NO can interact with target molecules and activate second messenger and intracellular signaling pathways. The diagnostic and therapeutic potential of NO-based communication is emerging.

Endothelium and Cardiac Myocytes/Smooth Muscle Cells

NO is a key regulator of vascular tone. Vascular NO is derived primarily from eNOS in the endothelial cells under normal physiological conditions (Chen et al., 2010a; Zweier and Talukder, 2006). Physiological factors such as shear stress and blood flow can activate eNOS in endothelial cells (see Fig. 5) (Suryo Rahmanto et al., 2012). iNOS is also induced in endothelial cells by cytokine stimulation (Gotoh and Mori, 2006; Teng et al., 2002; Wu, 1995). It has been demonstrated that NO produced in vascular endothelial cells plays an important physiological role in the regulation of tissue mitochondrial respiration in cardiomyocytes and skeletal muscle, (Loke et al., 1999; Shen et al., 1995). It has also been demonstrated that endothelium-derived NO regulates postischemic myocardial oxygenation and oxygen consumption by modulation of mitochondrial electron transport (Zhao et al., 2005). Hence, endothelium-derived NO plays an important role in mitochondrial oxygen consumption (see Fig .3) in smooth

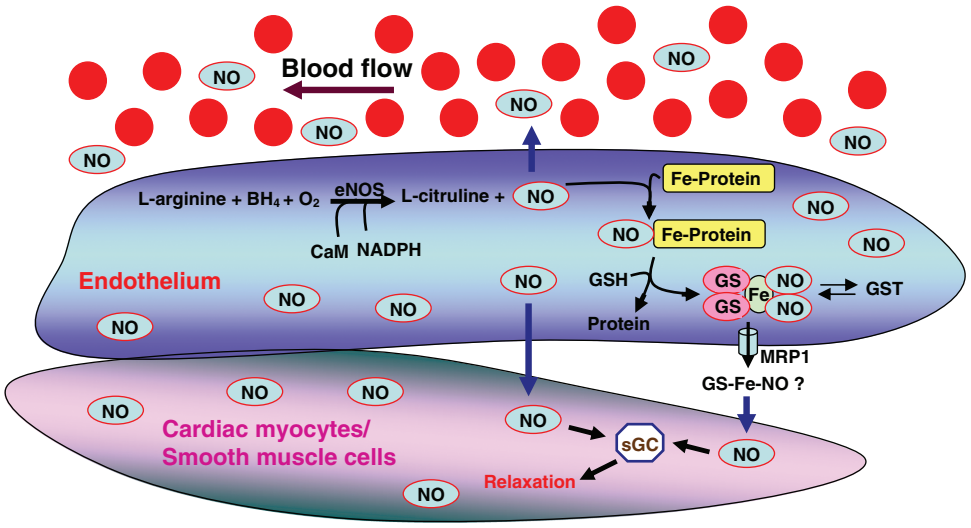


Figure 5. Intercellular NO signal transduction pathways. NO secreted by a donor endothelial cell (blue) is taken up by an acceptor smooth muscle or cardiac myocyte cell (pink). NO synthesized by endothelial nitric oxide synthase (eNOS) in endothelium diffuses across cell membranes to target cells such as cardiac myocytes/smooth muscle cells. In target cells NO activates soluble guanylate cyclase (sGC), which converts guanosine 5'-triphosphate (GTP) to cyclic guanosine monophosphate (cGMP) (see Fig. 1). Production of second messenger cGMP activates various signaling pathways, including the smooth muscle cell relaxation pathway. GST: Glutathione transferase, MRP1: multidrug resistance protein 1, GS-Fe-NO: glutathione conjugate of iron and NO.

muscle cells under physiological and pathophysiological conditions.

Under normal physiological conditions, NO produced by eNOS in the endothelium can rapidly diffuse across a cell membrane and contribute to the regulation of vasomotor tone and blood pressure by activating sGC in vascular smooth muscle, resulting in vasodilation (see Fig. 5). The unique chemistry of NO provides this molecule with the capacity to diffuse rapidly into nearby cells and activates sGC and stimulates cGMP formation (Derbyshire and Marletta, 2009; Ignarro, 1991). NO is known to mediate its effects by binding to the ferrous heme moiety of its effector, sGC, with the subsequent activation of cGMP dependent signaling (see Fig. 1). cGMP acts as a second messenger in different cell types and triggers different cellular signaling pathways. The interaction between NO synthesized in one cell and cytosolic sGC-bound heme located in adjacent target cells to generate the NO-sGC represents a novel and widespread signal transduction mechanism that links extracellular NO to the biosynthesis of cGMP in target cells. This NO messenger-mediated intercellular communication allow the initiation of increased blood flow, inhibition of platelet-induced thrombosis and other cellular functions (Ignarro, 1991). Hence, the intercellular signaling/communication between endothelium and myocytes is important for the protection of organs such as the heart from various pathological conditions such as ischemia and reperfusion.

It is important to note that NO can escape from the cytosol of the producer cell without reacting with the many intracellular targets and without causing damage to the producer cell itself. Therefore, to protect producer cells from the inadvertent reactions, either harmless NO transport molecules or special intracellular transport routes for NO must exist. It has been suggested that GST P1-1 that binds DNICs could act as an intermediate form of NO for regulating vascular tone and effect on blood pressure (Suryo Rahmanto et al., 2012). The formation and release of DNICs were observed in aortic endothelial cells in which eNOS was induced (Mulsch et al., 1993). It has been suggested that DNICs are involved in the transmembraneous transport of iron and NO. (Mulsch et al., 1993). Studies have shown that DNICs activate purified sGC (Mulsch et al., 1991; Severina et al., 2003). In addition, incubating rabbit pre-contracted femoral artery segments with DNICs has been shown to increase relaxation (Mulsch et al., 1991). Similar results were also observed in rat aortic rings (Alencar et al., 2003). In rat abdominal aortic rings, a long-lasting vasorelaxing effect was observed with increasing concentrations of DNICs (Vanin et al., 2007). In experimental animals, treatment with DNICs induced the prolonged/long lasting hypotensive effect (Vanin, 2009). In humans, GSTP1 polymorphisms significantly decreased the risk of developing preeclampsia, a condition marked by high blood pressure during pregnancy (Canto et al., 2008). From these studies, it is clear that DNICs may play a major role in NO-mediated intercellular communication as shown in Figure 5.

Macrophages and Atherosclerosis

Atherosclerosis is the primary cause of stroke, coronary artery disease, and peripheral vascular disease (Charo and Taub, 2011; Raman et al., 2008). Atherosclerosis

is a condition in which an arterial blood vessel wall thickens as a result of the accumulation of low density lipoprotein (LDL) molecules in the sub-endothelial layer (Charo and Taub, 2011). The accumulation of LDL causes chronic inflammation with accumulation of macrophages, which results in the formation of atherosclerotic lesions or plaques (Charo and Taub, 2011; Hotamisligil, 2010). Endothelium-dependent vascular relaxation is impaired in atherosclerosis (Esaki et al., 1997; Wilcox et al., 1997).

In human aorta, the expression of eNOS in endothelium is decreased in advanced atherosclerotic lesions (Wilcox et al., 1997). iNOS is primarily expressed in activated mononuclear leukocytes and macrophages, such as those found in atherosclerotic lesions (Esaki et al., 1997; Wilcox et al., 1997). However, the expression of nNOS and iNOS are reported to be increased in endothelium, macrophages, intimal cells, and smooth muscle cells (Esaki et al., 1997; Gotoh and Mori, 2006; Wilcox et al., 1997). In mice, deficiency in iNOS results in reduced atherosclerosis lesions (Detmers et al., 2000). Once iNOS is expressed, it produces large toxic bursts of NO for long periods of time (hours to days) (MacMicking et al., 1997; Rosenfeld et al., 2010). In addition, iNOS protein content in fully activated cells may be higher than the constitutive NOS content (MacMicking et al., 1997).

It has been reported that the ER stress pathway including CHOP expression (see Fig. 4) is activated in macrophages, which invade the vascular wall at all stages of atherosclerosis (Hotamisligil, 2010; Zhou et al., 2005). Hence, the ER stress pathway activated by increased production of NO and secondary RNS can be involved in the pathogenesis of atherosclerosis and its complications (Charo and Taub, 2011; Gotoh and Mori, 2006; Hotamisligil, 2010; Zhou et al., 2005). Furthermore, other atherosclerotic lesion cell types also show signs of ER stress (Civelek et al., 2009; Kedi et al., 2009).

Thus, in atherogenesis the expression and activation of NOS along with concurrent inflammation and oxidative stress leads to cytotoxicity. The increased production of NO and secondary RNS contribute to the overall process of atherogenesis by increasing cell death and necrosis, which are deleterious to the function of the arterial blood vessel wall (Charo and Taub, 2011; Kedi et al., 2009; Zhou et al., 2005). Increased production of NO acts as a messenger between different cell types involved in atherogenesis. Moreover, NO-mediated intercellular communication plays a major role in the pathophysiology of atherosclerosis.

Macrophages and Cancer Cells

iNOS can be induced with lipopolysaccharides (LPS) and cytokines in murine and human macrophages (Suryo Rahmanto et al.,2012). The activated macrophages produce very high levels of NO (MacMicking et al., 1997). Various mammalian cancer cells are killed by NO produced by activated macrophages (MacMicking et al., 1997). Very recently it has been shown that co-cultivation with activated macrophages caused cell killing in cancer cells (Kim et al., 2012). It has also been shown that NO plays a role in the inhibition of mitochondrial respiration and DNA replication in target cells by activated macrophages (Hibbs et al., 1984). The increased production of NO by activated macrophages has been determined to mediate cancer cell death through various molecular mechanisms (Suryo Rahmanto et al., 2012). The dual action of NO mobilizing iron and GSH could play an important role in macrophage-mediated cytotoxicity against tumor cells (Handy and Loscalzo, 2006; Hibbs et al., 1984). It has been demonstrated that DNICs are the most abundant intracellular NO-derived adduct formed in activated macrophages (Hickok et al., 2011). The proximity of the cancer cell to activated macrophages is a critical determinant of the cytotoxic effect induced by NO. However, the cooperative roles of DNIC, GST, and MRP1 in the cytotoxic effector mechanism of activated macrophages against tumor cells remain to be investigated.

BENEFICIAL AND DELETERIOUS EFFECTS OF NO

Studies have shown that NO plays an important role in synaptic signaling events (Forstermann and Sessa, 2012). NO has been implicated in modulating physiological functions such as learning, memory, and neurogenesis (Zhou and Zhu, 2009). Under physiological conditions, NO is involved in the regulation of blood pressure, gut peristalsis, vasodilation, and penile erection. (Forstermann and Sessa, 2012). NO also regulates gene transcription, mRNA translation, and produces post-translational modifications of proteins. (Forstermann and Sessa, 2012). The increased production of NO in induced macrophages is essential for the control of parasitic microorganisms, intracellular bacteria, and killing of cancer cells and tumor tissues. (Forstermann and Sessa, 2012; MacMicking et al., 1997; Nathan and Hibbs, 1991; Suryo Rahmanto et al., 2012; Wei et al., 1995). NO interacts with the DNA of target cells and causes strand breaks and fragmentation. (Fehsel et al., 1993; Wink et al., 1991). It has been demonstrated that cytokine-induced non-immune cells, such as hepatocytes kill malaria sporozoites (Green et al., 1990). Moreover, cytokine-activated endothelial cells can lyse tumor cells (Li et al., 1991).

The release of endothelial NO towards the vascular lumen is involved in vasoprotection by inhibiting platelet aggregation and adhesion to the vascular wall (Forstermann and Sessa, 2012). NO prevents the platelet-derived growth factors that stimulate smooth muscle proliferation. Endothelium-derived NO prevents leukocyte adhesion to the vascular endothelium and its migration in to the vascular wall and protects against early phases of

atherogenesis (Li and Forstermann, 2000). NO also controls mitogenesis, proliferation of vascular smooth muscle cells, and expression of genes involved in atherogenesis. (Forstermann and Sessa, 2012). Endothelium-derived NO is involved in the stimulation of angiogenesis and activation of endothelial progenitor cells (Forstermann and Sessa, 2012). Various studies have suggested that NO interaction with mitochondria may act as a signaling mechanism for the regulation of cytoprotection and adaptive responses to hypoxia and external stress (Erusalimsky and Moncada, 2007).

The increased production of NO by iNOS and eNOS plays an important role in IPC and cardioprotection (Bolli et al., 2007; Granfeldt et al., 2009; Talukder et al., 2010). In IPC, there are two distinct windows of cardioprotection. The first window of cardioprotective effects of IPC, known as "early preconditioning," wanes 2–3 h after the onset of the IPC insult (Granfeldt et al., 2009). The second window of cardioprotection occurs 24 h following the onset of the IPC insult and lasts for 48–72 h. This phenomenon is known as "delayed/late preconditioning" (Bolli et al., 2007; Hausenloy and Yellon, 2010; Kuzuya et al., 1993). A major difference between the two phases of cardioprotective mechanisms of IPC is that early preconditioning results in the modification or turnover/translocation of existing myocardial proteins, whereas late preconditioning is exerted by newly synthesized cardioprotective proteins (Bolli et al., 2007). In the late preconditioning, endothelial NO production and iNOS are increased and upregulated, respectively. (Hausenloy and Yellon, 2010; West et al., 2008). The increased endothelial NO production activates various transcription factors and triggers multiple signaling pathways (Bolli, 2001). These lead to the increased *de novo* synthesis of various proteins, including iNOS. (Bolli, 2001; Hausenloy and Yellon, 2010). Mechanistic studies of delayed preconditioning have demonstrated that NO plays a central role in mediating cardioprotection (Bolli, 2001; West et al., 2008). It also protects the heart from ischemic and reperfusion injury (West et al., 2008). Hence, under various conditions NO has regulatory and protective functions in system biology by direct action or by inducing defense mechanisms (Erusalimsky and Moncada, 2007; Forstermann and Sessa, 2012; Gotoh and Mori, 2006). However, uncontrolled levels of NO or secondary RNS exhibit cytotoxic effects (Erusalimsky and Moncada, 2007; Forstermann and Sessa, 2012; Gotoh and Mori, 2006).

NO has several cytotoxic effects such as reactions with proteins and nucleic acids, and causes apoptosis and cell death (Dimmeler and Zeiher, 1997; Kroncke et al., 1997). High levels of NO produce energy depletion due to inhibition of mitochondrial respiration and glycolysis (Brown, 2010; Erusalimsky and Moncada,2007). NO secreted by activated macrophages or microglia kills neurons (Boje and Arora, 1992; Chao et al., 1992). The overproduction of NO is associated with pathological conditions such as diabetes mellitus, neurodegenerative disorders, cerebral infarction, septic shock, and atheroscelerosis (Ignarro, 2000; Kroncke et al., 1997). Increased production of iNOS and nNOS is found in early and advanced atherosclerotic lesions associated with macrophages, endothelial cells and intimal cells (Wilcox et al., 1997). It is believed that large amounts of NO produced in atherosclerotic lesions are related to cell death. The production of NO by iNOS is increased in various types of inflammatory diseases and mediates various symptoms of inflammation (Rafiee et al., 2003; Wong and Billiar, 1995). High level NO production is also a major cause of the vasodilation and hypotension seen in septic shock. (Lange et al., 2009; Wong and Billiar, 1995). NO-induced islet cell death is involved in the pathogenesis of Type 1 diabetes (Oyadomari et al., 2002). It has been suggested that iNOS-derived NO is involved in non-specific allograft rejection. (Langrehr et al., 1991).

Many of the toxic/harmful effects of NO are not only due to NO itself; but, are mediated by its oxidative reaction products. An important pathway of inactivation/oxidation of NO is its reaction with $O_2^{\cdot -}$ to form the RNS $ONOO^-$ (Goldstein and Czapski, 1995). The superoxide radical ($O_2^{\cdot -}$) has a very short half-life *in vivo* (<50 ms) (Kim et al., 2012) and rapidly reacts with NO to produce peroxynitrite (Goldstein and Czapski, 1995). The calculated rate constant for the reaction is 4.3×10^9 $M^{-1}s^{-1}$ (Goldstein and Czapski, 1995). Peroxynitrite is not a radical; however, it is a strong oxidant, highly reactive, and very unstable with a half-life of less than a second *in vivo*. $ONOO^-$ can cause oxidative damage, nitration, and S-nitrosylation of biomolecules such as DNA, lipids, and proteins (Lee et al., 2003; Mikkelsen and Wardman, 2003; Ridnour et al., 2004). The increased production of $ONOO^-$ contributes to a number of brain pathologies. (Brown and Neher, 2010). It has been suggested that $ONOO^-$ contributes to excitotoxicity via activation of poly ADP-ribose polymerase (PARP) and/or the mitochondrial permeability transition. (Forstermann and Sessa, 2012). In the postischemic heart $ONOO^-$ formation has been shown to occur and lead to myocardial injury (Wang and Zweier, 1996). $ONOO^-$ can irreversibly oxidize BH_4 to BH_2 and various other metabolites and results in the decreased bioavailability of BH_4 (Chen et al., 2010b; Dumitrescu et al., 2007; Nishijima et al., 2011; Sun et al., 2010; Vasquez-Vivar, 2009). A decreased bioavailability of BH_4 results in uncoupled NOS (Dedkova and Blatter, 2009; Settergren et al., 2009; Xia et al., 1998). The uncoupled NOS produces increased level of $O_2^{\cdot -}$ and little or no NO. (Cardounel et al., 2005; Wever et al., 1997; Xia et al., 1998). $ONOO^-$ reacts with protein tyrosines to form protein nitro-tyrosine which has been found to be increased in a number of human pathologies (Greenacre and Ischiropoulos, 2001).

Therefore, under pathological conditions the increased production of NO and its metabolites play a major role in oxidative damage to cellular components, cytotoxicity, and cell and organ death. Although NO has many

regulatory functions under physiological conditions and exhibits direct cytoprotective effects, at high concentrations it is capable of causing cytodamaging effects. Knowledge pertaining to the basis for the cytotoxic effects of NO and how these can be prevented is steadily increasing.

SUMMARY AND CONCLUSIONS

In humans, NO is generated from various sources primarily by NOS enzymes that convert L-arginine to NO but also by nitrite reductase and non-enzymatic pathways under pathophysiological conditions. There are many important and diverse signaling functions associated with NO. Under physiological conditions, constitutively-produced NO acts as an endogenous regulator of intracellular O_2 availability in mammalian cells. Important insights have been gained regarding the regulation of the cellular effects of NO by S-nitroso-modified protein thiols. In mitochondria, NO binds with mitochondrial CcO and affects O_2 consumption, increases the formation of $O_2^{\cdot-}$, and can trigger apoptosis. In ER, NO disturbs the Ca^{2+} homeostasis and induces apoptosis. Under pathological conditions such as non-specific immune response, NO acts as a cytotoxic effector molecule. In susceptible cells, various effects of NO and its conversion to RNS may lead to cellular apoptosis or necrosis depending on the cell type and local NO and ROS concentrations. Therefore, NO on one hand acts as a physiological intracellular and intercellular messenger and on the other can exert cytotoxity. NO plays an important role as a signaling molecule throughout an organism. Understanding the biological trafficking mechanisms involved in the metabolism of NO is vital for elucidating its many roles in cellular signaling and cytotoxicity. While much work has been done, many critical questions remain and further work is needed to better understand the precise mechanisms by which NO and its products serve as messengers and effectors contributing to human health and disease.

Conflict of Interest

There are no conflicts of interest.

LIST OF ABBREVIATIONS

Ca^{2+}; calmodulin, CaM; caveolin-1, Cav-1; C/EBP homologous protein, CHOP; cyclic adenosine diphosphate ribose, cADPR; cyclic guanosine monophosphate, cGMP; cytochrome *c*, cyt *c*; cytochrome *c* oxidase, CcO/complex IV; cytochrome *c* reductase, complex III; dimethyl-L-arginine, ADMA; dinitrosyl-diglutathionyl-iron complex, DNDGIC; dinitrosyl-dithiol-iron complex, DNIC; electron paramagnetic resonance, EPR; electron transport chain, ETC; endoplasmic reticulum, ER; endothelial nitric oxide synthase, eNOS/NOS3; glucose 6-phophatase, G6P; glucose 6-phosphate (G-6-P); glutathione transferases, GSTs; guanosine 5'-triphosphate, GTP; heat shock protein 90, Hsp90; hydrogen peroxide, H_2O_2; hypoxia inducible factor 1, HIF1; inducible nitric oxide synthase, iNOS/NOS2; inositol 1,4,5-triphosphate, IP_3; IP_3 receptors, IP_3R; iron, Fe; ischemic preconditioning IPC; mitochondrial nitric oxide synthase, mtNOS; monomethylarginine, L-NMMA; multidrug resistance protein 1, MRP1; neuronal nitric oxide synthase, nNOS/NOS1; nitrate, NO_3^-; nitric oxide, NO; nitric oxide synthase, NOS; nitrite, NO_2^-; oxygen, O_2; pentose phosphate pathway, PPP; phosphodiesterases, PDE; poly ADP-ribose polymerase, PARP; protein S-nitrosothiols, PrSNO; protein thiols, PrSH; reactive oxygen species, ROS; reduced glutathione, GSH; ryanodine receptor, RyR; sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase, SERCA; soluble guanylyl cyclase sGC; superoxide radical, $O_2^{\cdot-}$; tetrahydrobiopterin, BH_4; tricarboxylic acid cycle, TCA.

Acknowledgments: Authors thank Craig Hemann, James J. Boslett, and Dr. M.A. Hassan Talukder for critical reading of this manuscript. This work was supported by National Institute of Health Grants HL63744, HL65608, and HL38324.

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Received: 21 May 2012. Accepted: 13 September 2012.