

Protein cysteine *S*-guanylation and electrophilic signal transduction by endogenous nitro-nucleotides

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Abstract Nitric oxide (NO), a gaseous free radical that is synthesized in organisms by nitric oxide synthases, participates in a critical fashion in the regulation of diverse physiological functions such as vascular and neuronal signal transduction, host defense, and cell death regulation. Two major pathways of NO signaling involve production of the second messenger guanosine 3',5'-cyclic monophosphate (cGMP) and posttranslational modification (PTM) of redox-sensitive cysteine thiols of proteins. We recently clarified the physiological formation of 8-nitro-guanosine 3',5'-cyclic monophosphate (8-nitro-cGMP) as the first demonstration, since the discovery of cGMP more than 40 years ago, of a new second messenger derived from cGMP in mammals. 8-Nitro-cGMP is electrophilic and reacts efficiently with sulfhydryls of proteins to produce a novel PTM via cGMP adduction, a process that we named protein *S*-guanylation. 8-Nitro-cGMP may regulate electrophilic signaling on the basis of its electrophilicity through induction of *S*-guanylation of redox sensor proteins. Examples include *S*-guanylation of the redox sensor protein Kelch-like ECH-associated protein 1 (Keap1), which leads to activation of NF-E2-related factor 2 (Nrf2)-dependent expression of antioxidant and cytoprotective genes. This *S*-guanylation-mediated activation of an antioxidant adaptive response may play an important role in cytoprotection during bacterial infections and oxidative stress. Identification of new redox-sensitive proteins as targets for *S*-guanylation may help development of novel therapeutics for oxidative stress- and inflammation-related

disorders and vascular diseases as well as understanding of cellular protection against oxidative stress.

Keywords Nitric oxide · Reactive oxygen species · Oxidative stress · Posttranslational modification · Redox signal · ROS signal · Electrophilic signal · Adaptive response

Introduction

Nitric oxide (NO) is one of the smallest and perhaps oldest known bioregulatory molecules. It modulates a number of different cellular functions in prokaryotes, plants, and animals. In stark contrast to the apparent simplicity of this diatomic molecule, its biological chemistry is surprisingly complex, which makes it one of the most versatile signaling molecules known. In the past 20 years, NO has been established as a gaseous free radical with critical and unforeseen roles in quite varied biological functions and organisms (Martinez-Ruiz and Lamas 2009), including regulation of vascular and neuronal signal transduction, host defense, and cell death regulation (Bredt et al. 1990; Manick 2007; Murad 1986; Patel et al. 2000). In mammals, NO is synthesized by three different NO synthases (NOSs): neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). These NOSs catalyze the oxidation of L-arginine to form NO and L-citrulline (Griffith and Stuehr 1995). Signal transduction of NO can be classified into cGMP-dependent (canonical NO/cGMP pathway) and cGMP-independent [noncanonical: NO-induced posttranslational modifications (PTMs)] (Madhusoodanan and Murad 2007; Martinez-Ruiz and Lamas 2009). The canonical NO/cGMP signaling paradigm involves NO-dependent activation of soluble guanylate cyclase, which results in formation

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of cGMP, which itself activates protein kinases, cyclic nucleotide-gated ion channels, and phosphodiesterases (Feelisch 2007; Madhusoodanan and Murad 2007).

NO readily reacts with oxygen radicals and metals to produce chemically reactive compounds named collectively reactive nitrogen oxide species (RNOS). RNOS such as peroxynitrite (ONOO^-) and nitrogen dioxide (NO_2) can produce several types of PTMs (Bartesaghi et al. 2007; Eiserich et al. 1998; Radi 2004; Schopfer et al. 2003) including nitration, oxidation, and nitrosylation of amino acid residues in proteins (Akaike 2000; Alvarez and Radi 2003; Yamakura et al. 2005). Protein tyrosine nitration has been associated with several pathological conditions such as neurodegenerative diseases (Beckman et al. 1993), inflammatory airway diseases (Sugiura et al. 2004), and aging (Hong et al., 2007), and hence, regarded as biomarker for biological RNOS formation. Recently, accumulated evidence has suggested that PTMs of redox-sensitive protein thiols contribute to the regulation of NO signaling via cGMP-independent mechanisms (Martinez-Ruiz and Lamas 2009). A well-characterized example of a PTM of redox-sensitive protein thiols by NO is *S*-nitrosylation, i.e., the conjugation of an NO moiety to a reactive cysteine (Cys) thiol (Cys thiolate) to form *S*-nitrosothiol (Akaike 2000; Hess et al. 2005; Stamler et al. 2001).

PTMs mediated by electrophilic metabolites of NO also play an important role in the NO signaling network. Recent studies suggested that biomolecules such as fatty acids and nucleotides react with NO and RNOS to form electrophilic metabolites and that these electrophiles also modify protein thiols to regulate NO signaling. Several groups reported the biological formation of nitrated fatty acids (Baker et al. 2005; Freeman et al. 2008), which has been found to cause thiol modification via *S*-nitroalkylation (Batthyany et al. 2006). However, RNOS are also known to induce nitration of nucleic acids (Fig. 1). During the past several years, nitrated guanine derivatives such as 8-nitroguanine and

8-nitroguanosine were identified in diverse cultured cells and in tissues from humans with lung diseases and different organisms with viral pneumonia, cancer, and other inflammatory conditions (Akaike et al. 2003; Ohshima et al. 2006; Sawa and Ohshima 2006; Sawa et al. 2006; Tazawa et al. 2007; Terasaki et al. 2006; Yasuhara et al. 2005; Yoshitake et al. 2004, 2008). The redox activity of 8-nitroguanosine in particular suggests that guanine nitration may have potent biological effects (Sawa et al. 2003). We recently discovered that another nitrated cyclic nucleotide, 8-nitroguanosine 3',5'-cyclic monophosphate (8-nitro-cGMP), is produced in cells expressing iNOS (Sawa et al. 2007). 8-Nitro-cGMP has an extremely potent signaling function in biological systems because of its dual nature in signal transduction, i.e., the canonical NO/cGMP pathway and noncanonical electrophilic signaling; among the nitrated guanine derivatives studied, it possessed the strongest redox-active property (Sawa et al. 2007). Because of its potent electrophilic behavior, 8-nitro-cGMP effectively reacts with sulfhydryl groups of particular Cys residues to form a novel PTM via formation of a protein-S-cGMP adduct, named *S*-guanylation (Fig. 2).

In this review article, we will discuss NO-mediated signal transduction and its physiological role in the context of protein Cys thiol modification by the novel nitrated nucleotide derivative 8-nitro-cGMP.

Cys thiol, a prominent target for electrophilic signal transduction

Cys is the most nucleophilic amino acid and readily reacts with oxidants (e.g., reactive oxygen species, ROS) and electrophiles of endogenous and exogenous origins. Not all Cys thiols are important as redox sensors, inasmuch as most protein thiols do not react with oxidants under conditions and at concentrations found in cells. However,

Fig. 1 Chemical structures of 8-nitroguanine-related compounds including various 8-nitroguanine nucleotides

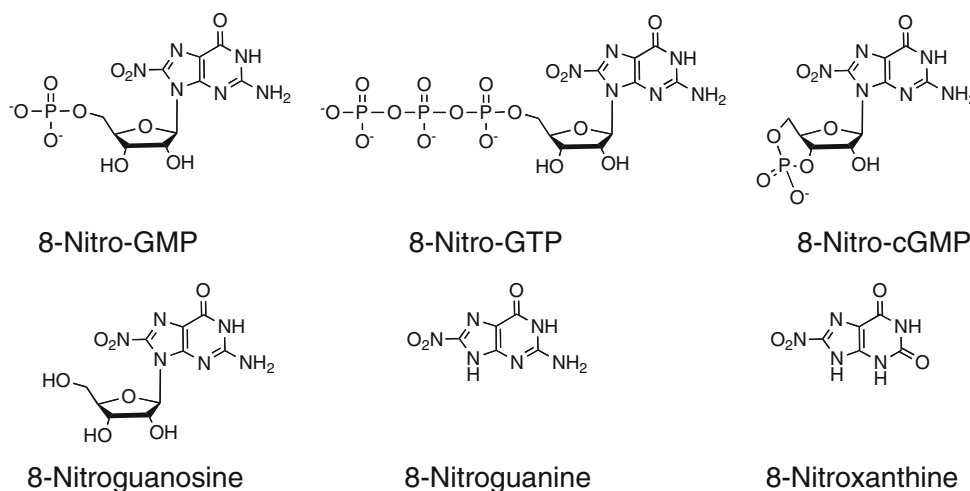
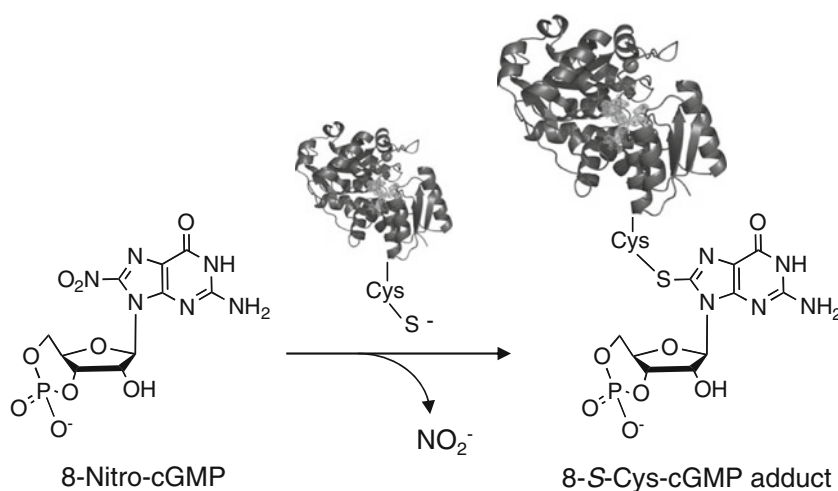


Fig. 2 Schematic representation of protein S-guanylation. Nucleophilic protein thiolates attack the C8 carbon of 8-nitro-cGMP, which results in adduction of cGMP moieties to Cys residues in proteins, with concomitant release of nitrite anion



some thiols (i.e., those with low pK_a values) were deprotonated to the thiolate anion state more readily because of their surrounding environment (Eaton 2006). This deprotonation makes these thiols more reactive toward NO, RNOS, and other related electrophiles (Eaton 2006; Winterbourn and Hampton 2008). The pK_a value of the thiol of free Cys is 8.33, and the pK_a values of protein-associated thiols usually stays between 8.2 and 8.5 (Winterbourn and Hampton 2008). The characteristic of the chemical milieu that lowers the pK_a values of Cys thiols is proximity to (a) basic amino acids such as histidine, lysine, and arginine; (b) aromatic amino acids such as tyrosine and tryptophan; or (c) metal centers such as those in heme-metal complexes (Hess et al. 2005; Winterbourn and Hampton 2008). Moreover, protein allostery may affect the accessibility and reactivity of the Cys thiols (Hess et al. 2005; Ishima et al. 2008; Winterbourn and Hampton 2008).

Protein Cys modifications by NO, RNOS, ROS, and related electrophiles include S-nitrosylation, S-oxidation, and S-alkylation. S-Nitrosylation is one of the best characterized PTMs associated with NO signaling. A number of proteins have been identified as targets for S-nitrosylation, and their functional impact has been extensively studied (Forrester et al. 2009; Hess et al. 2005; Miyamoto et al. 2000). RNOS also react directly with thiol groups. For example, peroxynitrite, a coupling product of NO and superoxide (Szabo et al. 2007), reacts with the thiol group, which results in oxidation of the thiol to form sulfenic acid (RSOH) (Radi et al. 1991). RSOH then reacts with neighboring thiol groups to form intra- or inter-molecular disulfide bonds (Poole et al. 2004). RSOH also plays an important role in glutathionylation, a covalent attachment of glutathione (GSH) to protein thiols via a mixed disulfide bond. S-Alkylation results from a reaction with electrophilic secondary metabolites of RNOS. The reaction patterns can be additions or substitutions depending on the structure of the electrophile. Examples of such electrophiles include nitrated

fatty acids and nitrated cyclic nucleotides. A number of nitrated fatty acid residues have been found in biological systems and proved as potent as Cys-alkylating electrophilic agents (Baker et al. 2005; Balazy et al. 2001; Batthyany et al. 2006; Freeman et al. 2008; Trostchansky and Rubbo 2006). The nitrated fatty acids have conjugated nitroalkene functions, and the electron-withdrawing nature of the nitro group facilitates the Michael addition of the thiols to the alkenes, which is known as S-nitroalkylation (Batthyany et al. 2006).

Cys thiol modification by NO, RNOS, ROS, and RNOS- and ROS-derived electrophiles has been shown to regulate the activity of numerous metabolic enzymes, such as oxidoreductases, proteases, protein kinases, and phosphatases; receptors; ion channels and transporters; and transcription factors. Important regulations of thiol modification-mediated protein functions include (a) inhibition of enzymes by modification of active center thiols; (b) thiol modification-induced functional and structural modulation (allosteric regulation); and (c) regulation of protein-protein interactions (Hess et al. 2005; Jones 2008).

Later in this review, we will provide more details about protein Cys modification by nitro-nucleotides as a novel PTM and its impact on redox signal transduction.

Biological formation of nitrated nucleotides

In vitro and in vivo experiments have identified nitration of nucleic acids (Fig. 1), more specifically guanine derivatives, as associated with various inflammatory conditions. Yermilov et al. (1995) found that peroxynitrite reacts with the guanine base of nucleic acids to form 8-nitroguanine in vitro. Masuda et al. (2002) demonstrated peroxynitrite-mediated 8-nitroguanosine formation from RNA in vitro. Our group first reported in vivo evidence of guanine nitration: we found marked guanine nitration in the lungs of influenza virus-infected mice and human patients with

idiopathic pulmonary fibrosis and lung cancer that depended on production of NO by iNOS (Akaike et al. 2003; Sawa et al. 2006; Terasaki et al. 2006). We also observed formation of 8-nitroguanosine in mice with infections with bacteria such as *Salmonella typhimurium* (Zaki et al. 2009). In addition, Hoki et al. (2007) detected 8-nitroguanine formation in malignant fibrous histiocytoma. Recently, 8-nitroguanine was linked with diabetic retinopathy, which is a major cause of blindness (Yuasa et al. 2008).

Our chemical analyses using high-performance chromatography-based electrochemical detection and tandem mass spectrometry (LC–MS/MS) revealed that a nitrated derivative of cGMP, 8-nitro-cGMP, was generated in significant amounts in cell culture models with different types of cells (Sawa et al. 2007; unpublished observation). For example, 8-nitro-cGMP was identified in the mouse macrophage cell line RAW 264.7 stimulated with cytokines such as interferon- γ and lipopolysaccharide (LPS) to produce NO via iNOS. More important, LC–MS/MS clearly revealed quantitative formation of 8-nitro-cGMP in a rat glioma cell line stimulated with an exogenous NO donor or with LPS plus cytokines (unpublished observation). Infection with the gram-negative bacterium *Salmonella* also facilitated formation of 8-nitro-cGMP in mouse macrophages (Sawa et al. 2007; Zaki et al. 2009). Formation of 8-nitro-cGMP was easily analyzed via immunocytochemistry with the use of anti-8-nitro-cGMP monoclonal antibodies. It was intriguing that immunostaining of intracellular 8-nitro-cGMP colocalized with mitochondria rather than endoplasmic reticulum (Sawa et al. 2007). This intracellular localization may have implications for the mechanism and physiological effects of 8-nitro-cGMP formation. Moreover, this mitochondria-related nitration suggests that 8-nitro-cGMP may have a role as a regulator of mitochondrial functions such as energy metabolism and cell death. 8-Nitro-cGMP formation was also detected with other cultured cells, such as human hepatocellular carcinoma (HepG2) cells, adipocytes, and endothelial cells. Thus, clear evidence now exists that nitrated derivatives of nucleotides are generated under various physiological and pathophysiological conditions, so that they may serve not only as oxidative stress markers but also as biologically functioning electrophilic signal transducers.

Redox signaling property of nitro-nucleotides

8-Nitroguanosine and its derivatives have significant redox activity. In the presence of certain oxidoreductases and electron donors such as NADPH, 8-nitroguanosine derivatives readily reduced to form their anion radicals, which then transferred a single electron to molecular oxygen to form

superoxide radical (Akaike et al. 2003; Sawa et al. 2003). 8-Nitro-cGMP had the highest redox activity among the 8-nitroguanosine derivatives tested, with redox activity decreasing in the following order: 8-nitro-cGMP > 8-nitroguanosine > 8-nitroguanosine 5'-monophosphate (8-nitro-GMP) $\approx$ 8-nitroguanosine 5'-triphosphate (8-nitro-GTP). 8-Nitroguanine had only negligible redox activity (Akaike et al. 2003; Sawa et al. 2003, 2007). Oxidoreductases that can reduce 8-nitroguanosines include cytochrome P450 reductase and all three NOS isoforms (Akaike et al. 2003; Sawa et al. 2003).

In view of this redox activity, 8-nitroguanosines may serve to regulate vascular tone. In fact, 8-nitro-cGMP can induce vasoconstriction, possibly through formation of superoxide, as determined in organ bath experiments (Sawa et al. 2007). 8-Nitro-cGMP-induced vasoconstriction depended completely on endothelium, and more specifically on eNOS, which indicates the occurrence of eNOS-dependent reduction of 8-nitro-cGMP to form superoxide, which in turn antagonizes NO-dependent vasorelaxation. At higher concentrations, however, 8-nitro-cGMP induced vasorelaxation, through activation of cGMP-dependent kinase (PKG) in vascular smooth muscle cells.

Nitro-nucleotides: endogenously formed electrophiles

Electrophilicity is another unique property of nitro-nucleotides. Because of their electrophilicity, nitro-nucleotides readily react with nucleophilic thiol compounds of low and high molecular weight to form 8-thioalkoxy-guanosine adducts. We named this unique electrophilic reaction *S*-guanylation of sulfhydryls (Saito et al. 2008; Sawa et al. 2007). This reaction seems to occur via nucleophilic attack by the thiol group of a protein Cys or GSH on the C8 carbon of 8-nitro-cGMP so that the nitro moiety is released and the 8-RS-cGMP adduct is formed (Fig. 2). The major distinction between *S*-guanylation and other *S*-alkylations is that *S*-guanylation is apparently quite stable and produces irreversible sulfhydryl modifications, because the nitro moiety of the purine structure is lost during formation of adducts with protein Cys residues. It is noteworthy that 8-nitro-cGMP is the first known nitrated derivative of a cyclic nucleotide that possesses electrophilicity.

Among the nitroguanine derivatives examined (Fig. 1), 8-nitro-cGMP showed the highest reactivity with thiol compounds. The second-order rate constant for the reaction of 8-nitro-cGMP with the sulfhydryl of GSH was determined to be $0.03 \text{ M}^{-1} \text{ s}^{-1}$ at pH 7.4 and 37°C (Sawa et al. 2007). This value is much smaller than values for other electrophiles such as 4-hydroxynonenal, 15-deoxy- $\Delta^{12,14}$ -prostaglandin J_2 , and nitro-linoleic and -oleic acids; i.e., those compounds have reaction rate constants with GSH of

1.3, 0.7, 355, 183 $\text{M}^{-1} \text{s}^{-1}$, respectively, at pH 7.4 and 37°C. This comparatively lower second-order rate constant may account for the stable nature of this novel compound in the cellular compartment where GSH is abundant (at $\sim \text{mM}$ levels) and may be responsible for the fact that 8-nitro-cGMP causes very selective S-guanylation with sulfhydryls possessing high nucleophilicity, as determined, at least in part, by low pK_a values of sulfhydryls of the Cys moiety.

Protein S-guanylation in electrophilic signaling

Because of its electrophilicity, 8-nitro-cGMP may mediate electrophilic signaling via induction of S-guanylation of redox sensor proteins. Among this class of proteins, Kelch-like ECH-associated protein 1 (Keap1) was identified as a highly sensitive S-guanylation target (Sawa et al. 2007). Keap1 is a negative regulator of NF-E2-related factor 2 (Nrf2), a transcription factor that regulates phase-2 detoxifying and antioxidant enzymes for electrophiles and ROS (Dinkova-Kostova et al. 2005; Motohashi and Yamamoto 2004). The binding of Keap1 to Nrf2 maintains the cytosolic localization of Nrf2 and brings about rapid degradation of Nrf2 by proteasomes. Because Keap1 has highly reactive Cys residues, chemical modification of the sulfhydryl group of Cys residues by electrophiles and ROS has been proposed to decline the ubiquitin ligase activity of Keap1, which would lead to the stabilization and nuclear translocation of Nrf2. Once translocated to nuclei, activated Nrf2 would thus form a heterodimer with small musculoaponeurotic fibrosarcoma (sMaf) and bind to the antioxidant responsive element (ARE) (Fig. 3) to induce expression of various cytoprotective enzymes, which are involved in adaptive responses to oxidative stress (Dinkova-Kostova et al. 2002; Itoh et al. 2004).

Our recent chemical analyses for Keap1 revealed that Keap1 expressed by various cultured cells was highly susceptible to S-guanylation induced by NO-dependent 8-nitro-cGMP production (Sawa et al. 2007). In fact, we determined that NO and RNOS can modify Keap1 in macrophages during bacterial infections and in rat glial cells in culture after proinflammatory stimuli. 8-Nitro-cGMP formation was also clearly observed in macrophages infected with the pathogenic bacterium *Salmonella*. S-Guanylated Keap1 was then detected in cultured murine macrophages after *Salmonella* infection. These findings are further confirmed by the fact that Keap1 can be exclusively S-guanylated by 8-nitro-cGMP produced in vivo in cultured cells (unpublished observation). It is therefore highly plausible that 8-nitro-cGMP may act as an endogenous electrophilic ligand and affect Keap1 sulfhydryls via S-guanylation, which would lead to antioxidant signaling (Fig. 3).

We now understand that the Keap1-Nrf2 system can be an important cytoprotective mechanism against electrophiles and ROS (Wakabayashi et al. 2004). Noticeably, cytoprotection and host defense conferred by 8-nitro-cGMP were clearly associated with increased expression of heme oxygenase 1 (HO-1) in macrophages and in vivo during *Salmonella* infection (Zaki et al. 2009). HO-1 is an enzyme with various physiological roles including vasoregulation (Mottetlini et al. 1998), cytoprotection (Zuckerbraun et al. 2004), and anti-inflammatory effects (Otterbein et al. 2000). We also reported earlier that HO-1 expression induced by NO contributed to cell survival in certain solid tumor models (Doi et al. 1999; Tanaka et al. 2003). Our present belief that 8-nitro-cGMP may mediate electrophilic signaling via induction of S-guanylation was unambiguously supported by our significant data showing that 8-nitro-cGMP directly caused site-specific S-guanylation of Keap1, which led to subsequent Nrf2-dependent HO-1 induction with its potent antioxidant effect

Fig. 3 8-Nitro-cGMP-mediated cytoprotection by S-guanylation of Keap1. After S-guanylation of Keap1, Nrf2 dissociates from Keap1, is translocated to the nucleus, and acts as a transcription factor in the expression of cytoprotective genes including heme oxygenase-1 (HO-1). ARE antioxidant responsive element

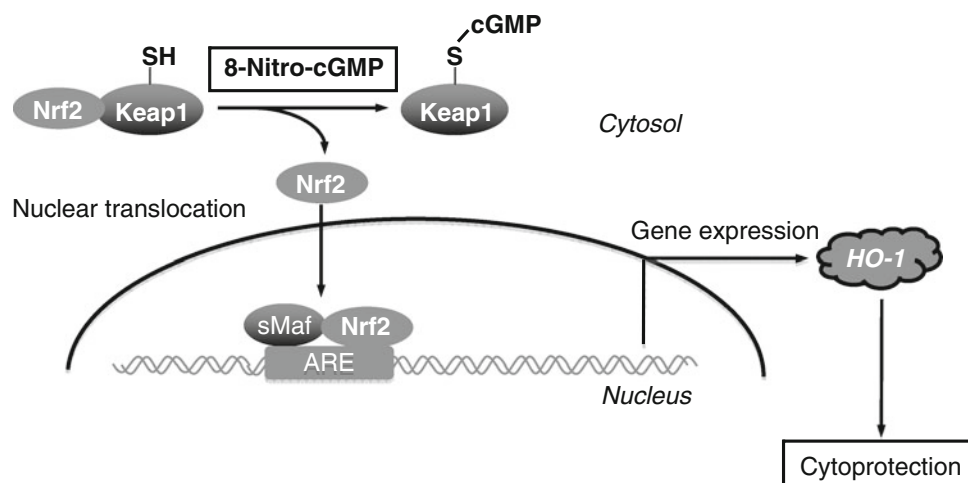
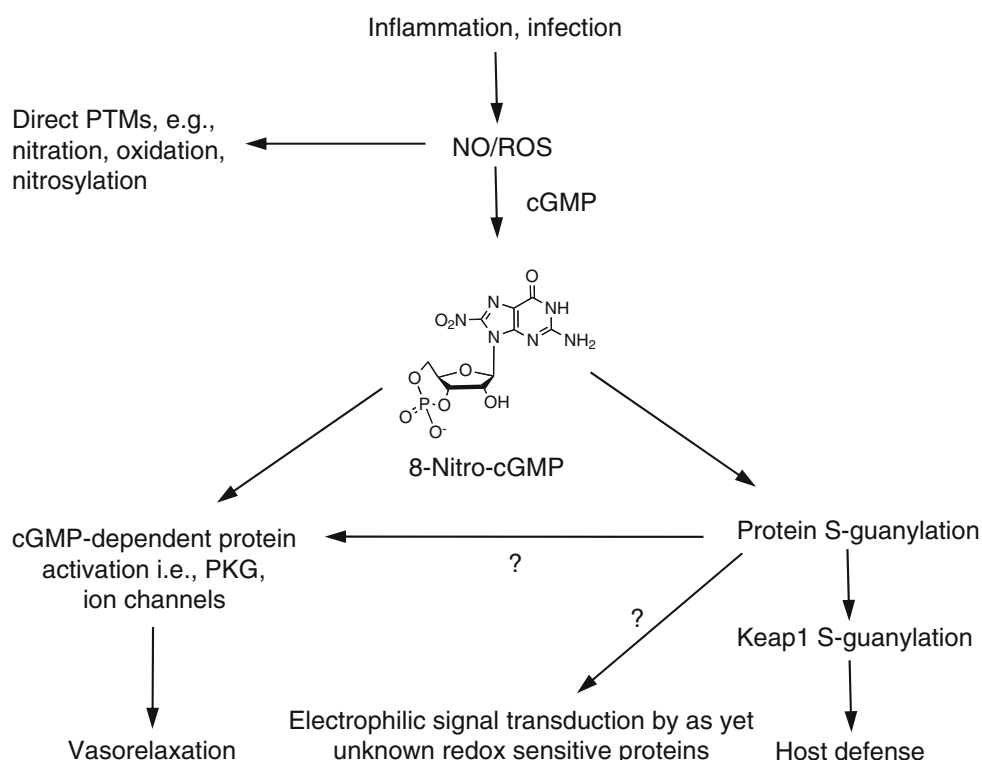


Fig. 4 Signal transduction by 8-nitro-cGMP via protein S-guanylation



(unpublished observation). That 8-nitro-cGMP-mediated electrophilic signal transduction may have various effects in the physiology and pathophysiology of NO- and RNOS-related phenomena is therefore conceivable (Sawa et al. 2010) (Fig. 4).

Conclusions

Cys thiol modification constitutes the most important PTM in redox signaling. Inasmuch as Cys thiol modification by an array of RNOS and ROS has been sufficiently explained and researched, that Cys thiol modification plays critical roles in redox signaling is well accepted. S-Guanylation is a pretty unique mechanism of protein Cys thiol modification that occurs via electrophilic attack by the recently discovered nitro-nucleotide 8-nitro-cGMP. It is noteworthy that, because RNOS such as ONOO^- has very short half life in physiological condition (less than a second) (Squadrito and Pryor 1998), it can induce direct PTMs including nitration, oxidation, and nitrosylation of various amino acids in the vicinity of its formation. In contrast, 8-nitro-cGMP is relatively stable, and may transduce ROS/RNOS signaling via indirect PTMs (S-guanylation) specific to redox-active cysteines. In fact, this electrophilic compound can modify the Cys moiety of redox-sensitive proteins including Keap1, thereby contributing in a critical fashion to redox signaling involving NO-dependent cellular adaptive response mechanisms.

Among approximately 214,000 total Cys residues encoded in the human genome, the redox-sensitive Cys is reported to reach 10–20% (Jones 2008). Thus, a number of proteins containing redox-sensitive thiols remain to be investigated in terms of the 8-nitro-cGMP-mediated PTM S-guanylation and their roles in regulating redox signaling. In fact, dozens of proteins that undergo S-nitrosylation and S-glutathionylation have been described to date (Forrester et al. 2009; Martinez-Ruiz and Lamas 2007). Further analysis is now warranted to achieve comprehensive understanding of target proteins for S-guanylation. The recent development of mass spectroscopy-based proteomics may provide a powerful tool for identification of novel redox-sensitive protein targets and the regulation of thiol modification in the cellular milieu.

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