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Cell signaling mediated by nitrated cyclic guanine nucleotide

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Abstract
We recently clarified the physiological formation of 8-nitroguanosine 3',5'-cyclic monophosphate (8-nitro-cGMP) and its critical roles in nitric oxide (NO) signal transductions. This discovery of 8-nitro-cGMP is the first demonstration of a nitrated cyclic nucleotide functioning as a new second messenger in mammals since the identification of cGMP more than 40 years ago. By means of chemical analyses, e.g., liquid chromatography-tandem mass spectrometry, we unequivocally identified 8-nitro-cGMP formation, which depended on NO production, in several types of cultured cells, including macrophages and glial cells. Most important, we previously showed that 8-nitro-cGMP as an electrophile reacted with particular sulfhydryls of proteins to generate a unique post-translational modification that we called protein S-guanylation. In fact, certain specific intracellular proteins, such as the redox-sensor protein Keap1, readily underwent S-guanylation induced by 8-nitro-cGMP.
8-Nitro-cGMP activated the Nrf2 signaling pathway by triggering dissociation of Keap1, via S-guanylation of its highly nucleophilic cysteine sulfhydryls. We also determined that S-guanylation of Keap1 was involved in cytoprotective actions of NO and 8-nitro-cGMP by inducing oxidative stress response genes such as heme oxygenase-1. Such unique chemical properties of 8-nitro-cGMP shed light on new areas of NO and cGMP signal transduction. Protein S-guanylation induced by 8-nitro-cGMP may thus have important implications in NO-related physiology and pathology, pharmaceutical chemistry, and development of therapeutics for many diseases.
Keywords: Cell signaling, Nitric oxide, Nitrated cyclic nucleotide, 8-Nitro-cGMP, S-Guanylation
Introduction

 Reactive oxygen species (ROS) and reactive nitrogen oxide species (RNOS) formed during infection and inflammation or by chemical means cause oxidative stress and contribute to the development of various diseases [1-3]. Diseases involving oxidative stress-related pathogenesis include inflammation, cancer, ischemia-reperfusion injuries such as cerebral and cardiac infarctions, diabetes, and atherosclerosis among others [4-7]. Organisms may, however, respond successfully to oxidative stress by inducing antioxidant and detoxifying enzymes, cellular reactions known as antioxidant and adaptive responses to oxidative stress. For some time, we have been investigating the mechanism of such cellular responses, with a focus on guanine nitration induced by RNOS produced from nitric oxide (NO) and ROS, which is being recognized as contributing to an important cytoprotective signaling mechanism against oxidative stress (Fig. 1) [8-12]. In 2003, for example, we first reported the in vivo formation of 8-nitroguanosine and 8-nitroguanine, which depended on NO generated from inducible NO synthase (iNOS), in airway epithelial cells in mice infected with influenza virus [13].

 NO was initially discovered as a signaling molecule regulating vascular tone and neuronal systems [14,15]. These functions are mainly mediated through a cGMP-dependent mechanism, but other pathways that are not directly related to cGMP appear to be responsible for many aspects of NO signaling [16-19]. Such a cGMP-independent pathway may be driven by nitrosylation and nitration of amino acids, proteins, lipids, and nucleotides, these processes being induced by RNOS (which are derived from NO and ROS), such as peroxynitrite (ONOO⁻) and nitrogen dioxide (NO₂) [3,4,8,18-20].

 In fact, nitration of nucleic acids is known to be caused by RNOS. For several years, the presence of nitrated guanine derivatives such as 8-nitroguanine and 8-nitroguanosine and their formation were observed in various cultured cells and specimens of tissues with viral pneumonia and human lung disease, and even in human
urine [13,21-24]. Of particular importance is the redox activity of 8-nitroguanosine, which suggests that guanine nitration may have a biological effect [13, 25]. In this regard, our recent discovery of a novel nitrated cyclic nucleotide, 8-nitroguanosine 3’,5’-cyclic monophosphate (8-nitro-cGMP), has many implications in that 8-nitro-cGMP, while remaining as a potential activator of cGMP-dependent protein kinase (protein kinase G [PKG]), shows the strongest redox-active property among nitrated guanine derivatives [8,11]. Related to this redox activity is the characteristic of 8-nitro-cGMP acting as an electrophile so that it can react with particularly reactive sulfhydryl groups of cysteine residues to form a protein-(8-S-Cys)-cGMP adduct, which is a novel post-translational modification (PTM) of proteins that we called protein S-guanylation [8].

In this review article, we describe cutting edge concepts of ROS/RNOS signaling arising from the unique chemical and physiological properties of the nitrated cyclic nucleotide 8-nitro-cGMP, specifically with regard to its potent signaling functions via protein S-guanylation that contribute to the adaptive cellular responses to oxidative stress (Fig. 1).

**Formation of nitrated nucleotides in cells**

To analyze the biological formation of nitroguanine and its derivatives, we first synthesized various derivatives, including 8-nitroxanthine and 8-nitroguanine, 8-nitroguanosine, and the monophosphate, triphosphate, and cyclic monophosphate of 8-nitroguanosine, as standard authentic compounds to be used for the chemical and immunochemical determination of guanine nitration occurring *in vivo* (Fig. 2) [8]. We expected that guanine nucleotides stored in cells could become targets of nitration by RNOS. We then prepared a series of polyclonal and monoclonal antibodies for these nitrated guanine nucleotides. For various analyses, we mainly used, from the series of successfully obtained antibodies, a monoclonal antibody, clone 1G6, that reacted strongly with 8-nitro-cGMP plus another clone, NO2-52, that exclusively recognized
8-nitropurine-containing structures. These antibodies were indeed useful not only for immunochemical analyses but also for chemical identification of 8-nitroguanine-related compounds that were to be separated from crude cell and tissue extracts and even biological samples such as urine. We first utilized the antibodies for chemical identification of nitrated purines generated in cells, as reported earlier [8]. For example, we confirmed the chemical identity of 8-nitro-cGMP and other nitrated purines by means of high performance liquid chromatography (HPLC) with electrochemical detection (ECD) (Fig. 3). We applied the lysate of RAW 264.7 cells, a murine macrophage cell line, which had been stimulated with lipopolysaccharide (LPS) and cytokines to express iNOS, to an immunoaffinity column containing the anti-nitroguanine antibodies (clones 1G6 and NO2-52) to separate and enrich the 8-nitroguanine derivatives, followed by HPLC-ECD analysis. The elution profile in Fig. 3 clearly contains a large peak for 8-nitro-cGMP in addition to small peaks for 8-nitrooxanthine and 8-nitroguanine. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) confirmed that the large peak was 8-nitro-cGMP, which indicated that 8-nitro-cGMP is the major nitration product among various nitrated guanine derivatives, as we documented earlier [8].

It is also critically important to provide proof of the biological relevance of endogenous 8-nitro-cGMP formation. We therefore performed extensive LC-MS/MS analyses utilizing the stable isotope-dilution technique with C6 cells, a rat glial cell line expressing iNOS, so that we could quantify the exact amount of 8-nitro-cGMP produced in cells [12]. We thus paid particular attention to cell production of not only 8-nitro-cGMP but also cGMP, because clarification of distinctive production kinetics (quantity and time-dependent profile), and thus differentiation between these two compounds, may lead to better understanding of their physiological and pathological significance. In fact, assessment of the exact quantity of endogenous 8-nitro-cGMP became possible via our stable isotope-dilution technique and LC-MS/MS analysis applied in a spike-and-recovery study with control nucleotides synthesized with stable...
$^{15}$N isotopes. A surprising finding was a greatly improved recovery of 8-nitro-cGMP by means of methanol cell extraction: recovery efficiency increased up to 100-fold after spiking with exogenous $^{15}$N-labeled 8-nitro-cGMP (i.e., 8-$^{15}$NO$_2$-cGMP and 8-$^{14}$NO$_2$-c$[^{15}$N$_5$]GMP), whereas recovery of cGMP was not appreciably affected by the same c$[^{15}$N$_5$]GMP spiking (Fig. 4A). This finding may indicate that a large part of 8-nitro-cGMP formed in the cells remained as a cellular component during methanol cell extraction, but that it could be efficiently removed and stabilized for recovery in the cell extract by adding the same authentic $^{15}$N-labeled compound, which would permit effective quantification of 8-nitro-cGMP via LC-MS/MS analysis.

Figure 4B illustrates that unexpectedly high levels of 8-nitro-cGMP were formed in the cells, a much greater amount than that of cGMP detected simultaneously in the same cells. Such a large amount of 8-nitro-cGMP leads us to suggest that GTP rather than cGMP may first be nitrated and thereby converted to 8-nitro-GTP, which would then become a substrate for soluble guanylate cyclase (sGC), as we proposed in our recent publication [12].

Moreover, because we now know that an appreciable fraction of 8-nitro-cGMP that formed in cells remained there even after methanol cell extraction, a measurable amount of 8-nitro-cGMP could conceivably be identified by means of immunocytochemical analysis (albeit not strictly quantitative), even after various fixative treatments of cells and tissues. Immunostaining is a conventional tool that allows analysis of clear and apparently dynamic expression profiles of various biological molecules produced in cells, as long as they remain intracellular. One successful immunocytochemical analysis with the anti-8-nitro-cGMP antibody 1G6 revealed strong immunostaining for 8-nitro-cGMP in the murine macrophage cell line RAW 264.7 and rat glial C6 cells that had been stimulated with interferon-γ (IFN-γ) and LPS to produce NO via iNOS [8,10,12]. As an intriguing result, intracellular 8-nitro-cGMP immunostaining colocalized with mitochondria rather than endoplasmic reticulum [8,24]. This intracellular localization may have certain implications for the mechanism and
physiological effects of 8-nitro-cGMP formation. Specifically, guanine nitration may occur in mitochondria or their related compartments, possibly by means of RNOS generated from NO and ROS in the vicinity of this particular cellular organelle, as evidenced by our recent preliminary data [12]. Localized 8-nitro-cGMP production is further supported by our earlier observation of guanine nitration in human lung tissues [24].

These robust experimental data now provide evidence of a new cyclic nucleotide entity, i.e., 8-nitro-cGMP, that is physiologically generated in cells in a manner depending on NO and possibly ROS, as they both form RNOS.

**Protein cysteine modification by 8-nitro-cGMP**

That 8-nitro-cGMP as an electrophile has unique chemical reactivity for protein cysteine residues was also revealed. In other words, 8-nitro-cGMP undergoes nucleophilic substitution with sulfhydryls to release the nitro group and form a cysteine-cGMP adduct. We called this new PTM protein S-guanylation (Fig. 5). Indeed, endogenous protein S-guanylation occurring in cells is currently a major focus of our studies, which may stimulate a new era of NO and ROS research.

One of the most important target proteins for S-guanylation is Keap1, which is being increasingly recognized as a potent redox-sensing protein (Fig. 5). Keap1 is a negative regulator of Nrf2, a transcription factor regulating phase 2-detoxifying and antioxidant enzymes for electrophiles and ROSs [26,27]. Binding of Keap1 to Nrf2 maintains cytosolic localization of Nrf2 and mediates rapid degradation of Nrf2 by proteasomes (Fig. 5). Because Keap1 has highly reactive Cys residues, chemical modification of the sulfhydryl group of Cys residues by electrophiles and ROSs has been proposed to trigger dissociation of Nrf2, which leads to its stabilization and nuclear translocation. Activated Nrf2 then binds to the antioxidant responsive element (ARE) to induce expression of various cytoprotective enzymes, which contribute to the adaptive response to oxidative stress [28,29].
The exact physiological function and structural characterization of Keap1 S-guanylation remained to be explored, however. We thus examined whether Keap1 could indeed be modified by NO and oxidative stress in cells. In one study, cultured murine macrophages were infected with a pathogenic bacterium, *Salmonella*, and Keap1 protein was analyzed for its S-guanylation by means of Western blotting using anti-S-guanylation antibody after Keap1 isolation via immunoprecipitation [10]. With wild-type macrophages after *Salmonella* infection, when 8-nitro-cGMP formation was clearly observed, strong S-guanylation of Keap1 occurred, as evidenced by Western blotting. Similar Keap1 S-guanylation was clearly illustrated with C6 cells in culture via the same immunoprecipitation technique used with Western blotting [12]. Indeed, the general proteomic profiling of S-guanylated proteins, which we recently clarified, indicated that Keap1 is the major target that is S-guanylated after NO exposure, and its S-guanylated structure derives solely from RS-cGMP adducts [12] (Fig. 5). Other chemical modifications, such as sulfhydryl oxidation and S-nitrosylation, that are often caused by NO and ROS [19,30,31] were not observed in Keap1 Cys residues in cells treated with the NO donor S-nitroso- N-acetylpenicillamine (SNAP) [12]. That Keap1 may play a predominant role in the NO signaling pathway that depends on 8-nitro-cGMP produced in cells is therefore highly plausible. The structural basis of Keap1 modification leading to Nrf2 activation is discussed in greater detail below.

**Characterization of 8-nitro-cGMP electrophilicity and its biological significance**

Because several electrophiles are known to form endogenously, it is important to compare their chemical properties, e.g., reactivity with sulfhydryls, reversibility of electrophilic adduction (S-alkylation), compartmentalization, and biological stability. In general, electrophiles react readily with various sulfhydryl-containing peptides and proteins because of their high reaction rate constants with sulfhydryls. Most highly reactive electrophiles appear to undergo reversible electrophilic sulfhydryl adduction, so the stability of such an adduction produced in cells may be greatly affected by
glutathione (GSH), which is a major cellular low-molecular-weight sulfhydryl. Because of the redox-recycling potential of abundant GSH present in cells, the C-S bonds of S-alkylation protein adducts will be effectively dissociated, and released electrophiles are again bound covalently with GSH to form low-molecular-weight S-alkylation adducts (Fig. 6).

Specific intracellular factors may therefore be required for electrophiles to have notable biologically relevant effects on particular cellular proteins, enzymes, and signaling molecules. For example, even reactive electrophiles readily undergoing reversible protein S-alkylation may participate in intracellular signaling, if it occurs in a molecular compartment in which low-molecular-weight sulfhydryl compounds such as GSH are scarce. In this situation, the electrophiles could react specifically with particular protein sulfhydryl residues that would serve as biologically functioning acceptors to form stable S-alkylation Cys adducts involved in cell signaling [11,32].

In contrast, irreversible S-alkyl adductions with electrophiles may cause permanent loss or activation of protein functions and thus be regarded as pathological rather than physiological reactions. An exceptional case of apparently irreversible protein S-alkylation occurs endogenously, however, for which such a simplistic explanation cannot apply. This situation is illustrated by 8-nitro-cGMP-induced protein S-guanylation, which is a unique, irreversible PTM of protein Cys residues [11]. The best characterized of the chemical and pharmacological features of 8-nitro-cGMP is its relative stability in cells, so that it can maintain its electrophilicity and PKG-activating potential. The electrophilicity of 8-nitro-cGMP is generally lower than that of other endogenous electrophiles, as shown by the second-order rate constant for the reaction of the sulfhydryl of GSH: 0.03 M$^{-1}$s$^{-1}$ at pH 7.4, 37 °C [8]. 8-Nitro-cGMP is thought to be much less reactive compared with several other electrophiles such as α,β-unsaturated aldehydes, ω-6 and ω-3 unsaturated fatty acids, and nitroalkene fatty acids, which include, e.g., 4-hydroxynonenal, 15-deoxy-$\Delta^{12,14}$-prostaglandin J$_2$, and nitrolinoleic and nitrooleic acids. The reaction rate constants with GSH are 1.3 M$^{-1}$s$^{-1}$
(4-hydroxynonenal), 0.7 M$^{-1}$s$^{-1}$ (15-deoxy-$\Delta^{12,14}$-prostaglandin J$_2$), 355 M$^{-1}$s$^{-1}$ (nitrolinoleic acid), and 183 M$^{-1}$s$^{-1}$ (nitrooleic acid) at pH 7.4, 37 °C [11,33,34]. Therefore, these compounds seem to react with the sulfhydryls of GSH more than 20-10,000 times more rapidly than does 8-nitro-cGMP [11].

Such stability of 8-nitro-cGMP as an electrophile may support the considerable biological relevance of 8-nitro-cGMP formed in cells. As discussed above, we recently used an isotope dilution-LC-MS/MS analysis to extensively evaluate and precisely determine the quantity of 8-nitro-cGMP formed. The fact that a large amount of 8-nitro-cGMP remained in cells may not be consistent with its electrophilic nature, because electrophilic compounds are generally thought to react readily with sulfhydryls and thus be degraded. 8-Nitro-cGMP undergoes, during its reaction with sulfhydryls, denitration to release its nitro moiety, so that it in turn loses its unique electrophilicity. However, in view of the reaction rate constants for sulfhydryls just noted, we now know that 8-nitro-cGMP, compared with other biological electrophiles, is inert in terms of electrophilicity. For example, the reaction rate constant for 8-nitro-cGMP and GSH is orders of magnitude lower than the rate constants of most fatty acid-derived electrophiles, as just mentioned. This inert electrophilic property appears to agree well with our recent finding that 8-nitro-cGMP generation was not greatly affected after GSH was eliminated by administration of buthionine sulfoximine to SNAP-treated and LPS-cytokine-treated cells [12].

Furthermore, for highly reactive electrophiles, nucleophilic amino acids other than Cys, especially histidine and lysine, also become targets during their electrophilic reactions. Because of the very high electron-withdrawing potential of these electrophiles, they undergo unstable, reversible $S$-alkylation, which eventually can be transferred to other sulfhydryls of Cys of different proteins, a process known as transalkylation (Fig. 6). This fact indicates that many endogenous electrophiles, except 8-nitro-cGMP, may not necessarily produce site-specific PTMs, which can transduce signaling (Fig. 6). These electrophiles may need, therefore, specific reaction
conditions or compartmentalization in which they can serve as signaling molecules and have significant effects on sulfhydryls of acceptor proteins. For example, certain unique structural characteristics, although yet not identified, may be prerequisites for much of the stable covalent binding of electrophilic protein S-alkylation maintained near PTM sites.

It is also important to emphasize that 8-nitro-cGMP can produce a unique electrophilic PTM. The major distinction between S-guanylation and other S-alkylations is that S-guanylation is apparently a quite stable and irreversible sulfhydryl modification, because the nucleophilic nitro moiety of its purine structure is lost during adduct formation with protein Cys residues. Another important property of 8-nitro-cGMP is that, because of its relatively inert chemical reactivity as an electrophile compared with other electrophiles as just discussed, S-guanylation occurs almost exclusively with sulfhydryls possessing high nucleophilicity, as indicated by the pKₐ of Cys moiety sulfhydryls (Fig. 7). This finding is strongly supported by the finding that Keap1 Cys is highly susceptible to 8-nitro-cGMP-induced S-guanylation [8-12].

Another interesting characteristic of 8-nitro-cGMP is its possible molecular interaction with cyclic nucleotide (cGMP) receptor proteins such as PKG and other cGMP-binding proteins. The function of S-guanylated proteins may therefore be affected by the structural characteristics of parental cGMP. Also, of great importance is that cGMP-protein adducts acquire resistance to phosphodiesterases, which may confirm the strong, steady stimulation conferred during signal transduction via S-guanylation [8]. All these unique characteristics of S-guanylation together indicate that despite the irreversibility of protein S-guanylation, which is quite different from the situation for most electrophilic PTMs among various sulfhydryls, 8-nitro-cGMP may serve as an effective electrophilic regulator of intracellular signaling by virtue of its elegant chemistry via ingenious electrophilic reactions with protein sulfhydryls and S-guanylation structures thereby generated in cells.
Signal transduction via protein S-guanylation

Involvement of S-guanylation in signal transduction caused by NO and ROS was clearly verified in terms of Nrf2-related gene expression, which led to activation of downstream enzymes including heme oxygenase-1 (HO-1), a well-known antioxidant and cytoprotective enzyme [35]. As discussed above, we determined that the redox sensor protein Keap1 is a major target of S-guanylation. Keap1 is now widely accepted as a negative regulator of Nrf2, as described earlier. Activated Nrf2 then binds to the ARE to induce expression of various cytoprotective enzymes, thereby contributing to the phase 2 detoxifying and adaptive response to toxic electrophiles and ROS-induced oxidative stress [26,27].

In this context, considerable evidence from our recent work shows that Keap1 S-guanylation and subsequent Nrf2 activation are mediated by 8-nitro-cGMP formed via NO derived from NO donors or generated by iNOS [8-12]. It is intriguing that proteomic analysis for endogenously modified Keap1 with matrix-assisted laser desorption/ionization time-of-flight-MS/MS analysis revealed that 8-nitro-cGMP S-guanylated the Cys434 residue of Keap1 in a site-specific manner [12]. In recombinant Keap1 reacted with 8-nitro-cGMP in an in vitro cell-free system, however, 18 of 25 total cysteine residues were S-guanylated, so that Cys434 S-guanylation was not dominant. Therefore, Cys434 of Keap1 may have structural or chemical properties that are related to its specific S-guanylation and that are distinct from those of other cysteine residues of Keap1. A number of studies have examined Keap1-electrophile adducts generated in vitro in the presence of high concentrations of electrophilic reagents [28,30,36-39]. Yamamoto’s group reported the in vivo significance of Cys151, Cys273, and Cys288 for Keap1 functions in a transgenic complementation rescue experiment in mice [40]. Although verification of the in vivo requirement for Cys434 is yet to come, a recent study demonstrated that Cys434 in Keap1 is one of the cysteine residues that are most sensitive to S-glutathionylation and that disulfide adduct
formation of Cys434 with GSH causes marked structural changes in the Nrf2-binding surface of the Keap1 molecule [41]. Thus, the specific S-guanylation of Cys434 by 8-nitro-cGMP may play an important role in a molecular sensing system operated by Keap1 for RNOS and/or ROS [12].

Yamamoto’s group also determined that the Keap1-Nrf2 complex consists of one molecule of Nrf2 and two molecules of Keap1, possibly as a result of high- and low-affinity binding between Keap1 and Nrf2 [42-45]. The BTB domains at the N-terminal regions of two molecules of Keap1 bind each other directly to maintain a homodimer, and the DC domains of Keap1 located at the C-terminal region form a spherical shape at both ends of the whole dimer complex by virtue of their tertiary structure [45]. A co-crystallization study showed that two motifs of Nrf2, i.e., DLG and ETGE, interact with the β-propeller structure at the bottom of the sphere of the DC domain [43,44]. Structural alterations in the integrity of the Keap1-Nrf2 complex may disrupt the low-affinity binding between the ETGE/DLG motifs and the DC domains, which would then distort or dissociate this tertiary Keap1-Nrf2 complex, stabilize Nrf2, and trigger translocation of Nrf2 to nuclei for transcriptional activation. X-ray crystallographic analysis showed that Cys434 is located at blade 3 and is exposed to the outer surface of the β-propeller structure of the Keap1 DC domain [44,46]. S-Guanylation of Keap1 Cys434 may therefore cause Nrf2 activation, via two possible mechanisms: S-guanylation of Cys434 may weaken Keap1 binding to the ETGE and DLG motifs of Nrf2, because Cys434 lies close to the Nrf2-binding region of the DC domain; or the Cys434 modification may affect the integrity of the entire Keap1-Nrf2 complex.

In fact, 8-nitro-cGMP can mediate the cytoprotective response through S-guanylation of Keap1, as evidenced by our finding that treatment of C6 cells with 8-nitro-cGMP reduced cell death induced by oxidative stress related to H2O2 exposure [12]. We also found that 8-nitro-cGMP increased the nuclear accumulation of Nrf2 and expression of HO-1 in C6 cells [12]. NO-induced expression of HO-1 reportedly
contributes to cell survival in solid tumor models and during bacterial infection [10,47-49]. Therefore, the cytoprotection against oxidative stress that 8-nitro-cGMP confers is associated, at least in part, with increased HO-1 expression. That 8-nitro-cGMP participates in the major NO signaling pathway for cytoprotection or adaptive responses to ROS and oxidative stress is thus conceivable (Fig. 8).

**Concluding remarks**

In summary, we have clarified the NO-dependent formation of 8-nitro-cGMP and simultaneous S-guanylation of Keap1 induced by 8-nitro-cGMP in cells in culture. This NO- and 8-nitro-cGMP-mediated signaling pathway leads to Nrf2 activation and cytoprotective gene expression, including functioning of HO-1, which seems to be involved in the general adaptive response to oxidative stress. Most important, chemical identification of specific Keap1 S-guanylation unequivocally indicated a remarkable structural alteration of Keap1, which can maintain activation of Nrf2 for its ARE transcriptional regulation. These findings therefore suggest that protein S-guanylation induced by 8-nitro-cGMP is a unique pathway for modulating various cellular functions mediated by NO and ROS signaling. Present evidence of protein S-guanylation induced by 8-nitro-cGMP may thus warrant further extensive study that would reveal new aspects of NO-related physiology and pathology, pharmaceutical chemistry, and development of therapeutics for diseases.
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FIGURE LEGENDS

Fig. 1
A schematic drawing of potential involvement of nitrated nucleotide signaling molecules during the antioxidant adaptive response of cells to oxidative stress caused by ROS and RNOS.

Fig. 2
Chemical structures of 8-nitroxanthine and 8-nitroguanine derivatives including several nucleotides such as 8-nitro-cGMP.

Fig. 3
Chemical identification of 8-nitro-cGMP formed in RAW 264.7 cells. Cells were stimulated with a mixture of lipopolysaccharide (LPS) (10 µg/ml) and interferon-γ (IFN-γ) (100 U/ml) for 11 h. 8-Nitro-cGMP formed in cells was detected by means of HPLC-ECD analysis (upper panel), whose principle is illustrated by the diagram (lower panel). Modified from Sawa et al. [8].

Fig. 4
Quantitative LC-MS/MS analysis for measurement of cGMP and 8-nitro-cGMP formed in cells stimulated with LPS plus cytokines. (A) Recovery efficiency of cGMP and 8-nitro-cGMP as determined in the spike-and-recovery study [12]. Recovery was evaluated by using rat glial C6 cells stimulated with a mixture of LPS (10 µg/ml), IFN-γ (200 U/ml), tumor necrosis factor α (500 U/ml), and interleukin-1β (10 ng/ml) for 27 h, and by using stable isotope-labeled nucleotides (right panel). Although the amount of endogenous cGMP recovered from cells was not affected by different amounts of exogenous c[^15]N₅GMP, recovery of endogenous 8-nitro-cGMP improved greatly with increasing concentrations of 8[^15]NO₂-cGMP exogenously spiked to the cell extract (left
(B) C6 cells were stimulated in the same manner as in (A). The amounts of endogenous cGMP (c\(^{14}\text{N}\)GMP) and 8-nitro-cGMP (8-\(^{14}\text{NO}_2\)-cGMP) were quantified by the stable isotope dilution-LC-MS/MS technique [12]. Upper panel, representative LC-MS/MS chromatograms of cGMP and 8-nitro-cGMP. Lower panel, time profiles of intracellular 8-nitro-cGMP and cGMP concentrations as determined with LC-MS/MS. Data represent means ± SEM (n = 3). Modified from Fujii et al. [12].

**Fig. 5**
A scheme illustrating the mechanism of Nrf2-Keap1 transcriptional regulation via S-guanylation of the Cys sulfhydryls of Keap1 and activation of subsequent antioxidant enzymes induced by ROS and electrophiles. The model of activation of Nrf2-dependent downstream antioxidant signaling pathways, resulting from Keap1 modifications caused by ROS and various electrophiles, was originally proposed by Dr. Masayuki Yamamoto’s group [28,29]. The upper right panel shows a reaction scheme for protein S-guanylation caused by 8-nitro-cGMP. Note that the nitro moiety of 8-nitro-cGMP is lost as nitrite that is released during nucleophilic substitution of the Cys sulfhydryl of the protein by 8-nitro-cGMP. ARE, antioxidant responsive element. Modified from Sawa et al. [8] and Fujii et al. [12].

**Fig. 6**
A schematic illustration showing transalkylation of electrophilic residues of S-alkylation adducts among various Cys sulfhydryls. Transalkylation confers reversibility of protein S-alkylation induced by reactive endogenous electrophiles.

**Fig. 7**
A hypothetical scheme for protein S-guanylation as an irreversible but selective PTM contributing to precisely regulated electrophilic signaling mediated by 8-nitro-cGMP
formed in cells. Although highly reactive electrophiles produce reversible protein S-alkylation, which readily undergoes nonselective transalkylation with protein Cys sulfhydryls, a much less reactive 8-nitro-cGMP may affect only Cys residues with high nucleophilicity. The fate of S-guanylated proteins remains unclear, but they may be processed via a type of proteolytic degradation in cells.

**Fig. 8**
Overview of unique cell signaling regulated by a nitrated cyclic nucleotide (8-nitro-cGMP) generated through a classical NO-soluble guanylate cyclase (sGC) pathway coupled with a nitration reaction dependent on NO or ROS (possibly RNOS). Various nucleotides, which are abundant in cells, may serve as redox (ROS/NO) sensors and evoke many downstream signaling pathways.
Figure 1

Akaike et al.

Infection, inflammation, chemicals (xenobiotics), etc

Reactive oxygen and nitrogen oxide species
($\text{H}_2\text{O}_2$, $\text{O}_2^-$, $\cdot\text{OH}$, $\text{ONOO}^-$, $\cdot\text{NO}_2^-$, etc)

Signaling function

Cellular response

Oxidative stress

Cytoprotection

Disease development
(atherosclerosis, diabetes,
neurodegenerative disorders,
cancer, etc)
Figure 2

Akaike et al.

8-Nitro-GMP
8-Nitro-GTP

8-Nitro-cGMP
8-Nitro-GTP

8-Nitroxanthine
8-Nitroguanine
8-Nitroguanosine
Figure 3

Akaike et al.

RAW 264.7 cells stimulated with IFN-γ/LPS

Cell lysate → Immunoaffinity column (mix of NO2-52 and 1G6)

HPLC

Reducing cell → Oxidizing cell

Detector

Retention time (min)

Detector response (mV)

8-Nitro-cGMP

+ Reduction

– Reduction

8-Nitroxanthine

8-Nitroguanine

0 2 4 6 8 10 12

Retention time (min)

Detector response (mV)
Figure 4

A

- cGMP
- 8-Nitro-cGMP

Amount recovered (pmol/mg protein)

B

<table>
<thead>
<tr>
<th></th>
<th>cGMP</th>
<th>8-Nitro-cGMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>m/z 346 → 152</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endogenous</td>
<td></td>
<td></td>
</tr>
<tr>
<td>m/z 351 → 157</td>
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<tr>
<td>Spiked c[15N5]GMP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>m/z 391 → 197</td>
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<tr>
<td>Endogenous</td>
<td></td>
<td></td>
</tr>
<tr>
<td>m/z 392 → 198</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spiked 8-15NO2-cGMP</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

0 time

- 4 x 10^9 count
- 1.5 x 10^9 count
- 8 x 10^7 count
- 3 x 10^6 count

36 h

Retention (min)

Concentration (µM)

Time (h)
Figure 5

Akaike et al.

Proteasomal degradation

Antioxidant/cytoprotective enzymes
Heme oxygenase-1 (HO-1)
Thioredoxin reductase, etc

Phase II enzymes
Glutathione S-transferase
NADPH:quinone oxidoreductase
Aldo-keto reductases
Glutathione biosynthesis
γ-Glutamylcysteiny1 synthase, etc

8-Nitro-cGMP

Protein Cys-SH
8-S-Cys-cGMP adduct

Protein S-guanylation

Nrf2

Keap1

Keap1

Keap1

Keap1

8-Nitro-cGMP

S-Guanylation

sMaf

Nrf2

ARE

Gene

Nrf2

Nrf2

Nrf2

Nrf2

Nrf2
Reversible protein S-alkylation

GS-CH₂-R

Protein-S-CH₂-R

GSH

Cell signaling

Intracellular degradation

Trans-alkylation

Extracellular excretion
Figure 7

Akaike et al.

Transalkylation
Nonselective Cys targets

S-Alkylation

Electrophiles

High
Electrophilicity

RSH
GSH
Protein-SH

8-Nitro-cGMP
(unique structure as cGMP)

Low

S-Guanylation

Selective Cys targets with high nucleophilicity
(e.g., Keap1)

Protein degradation?
Figure 8

Akaike et al.

NO

sGC

ROS/oxidative stress

2nd messenger

PKG

Protein S-guanylation

8-Nitro-cGMP

Redox sensors (e.g., Keap1)

Effectors (e.g., Nrf2 → HO-1)

Adaptive and cytoprotective responses

Phosphorylation signaling in vascular and neuronal systems, etc

sGC: soluble guanylyl cyclase

PKG: protein kinase G

8-Nitro-cGMP: 8-nitroguanylyl-cyclic monophosphate

NO: nitric oxide

ROS: reactive oxygen species

Nrf2: nuclear factor erythroid 2-related factor 2

HO-1: heme oxygenase-1