a nitrated cyclic nucleotide, 8-nitro-cGMP, as a completely new derivative of cGMP (Fig. 1) [*22*]. *In vitro* experiments showed that reaction of cGMP with authentic peroxynitrite and with the nitrite/H_2O_2-peroxidase system generated 8-nitro-cGMP, but NO alone did not, which indicates that RNS are involved in nitration of cGMP (unpublished observation). We developed an anti-8-nitro-cGMP monoclonal antibody and immunostained cytokine-stimulated macrophage-like cells (RAW264.7 cells) to demonstrate that 8-nitro-cGMP formation depended on production of both NO and ROS [*22*]. Moreover, *Salmonella* infection in cultured murine macrophages generated 8-nitro-cGMP in wild-type mice but not in iNOS$^{-/-}$ mice. Furthermore, immunostaining analysis of liver tissue from *Salmonella*-infected mice demonstrated abundant 8-nitro-cGMP generation in wild-type mice but not in iNOS$^{-/-}$ mice [*23*]. This NO-dependent 8-nitro-cGMP formation was also observed in lung tissue from influenza virus-infected mice (unpublished data), which indicated that 8-nitro-cGMP was produced in an iNOS-dependent manner *in vivo*.

NO-mediated Induction of the Cytoprotective Molecule HO-1

During infection, various cytoprotective and antioxidant systems are induced to protect cells and tissues from pathogenic microbes [*24–26*]. Heme oxygenase (HO)-1 is known as a factor that is rapidly induced during infection and that contributes to the host defense mechanism [*27*]. HO-1 degrades free heme, used as a substrate, into biliverdin, iron ions, and carbon monoxide (CO). Both biliverdin and iron ions carry out antioxidant activity by means of reduction to bilirubin and production of ferritin, respectively [*28*, *29*]. CO is known to exhibit cytoprotection via suppressing production of inflammatory cytokines and apoptosis [*30*]. HO-1 is reportedly induced by various stresses and by multiple transcription factors, e.g., heat shock factor 1 (HSF1), nuclear factor-κB (NF-κB), activator protein-1 (AP-1), and nuclear factor-erythroid 2-related factor 2 (Nrf2) (Fig. 2) [*27*]. Modulators that upregulate these transcription factors include heat shock or intracellular accumulation of abnormal proteins (HSF1), infection or inflammatory responses (NF-κB), abnormal cell growth (AP-1), and electrophiles or oxidants (Nrf2).

Analyses of HO-1 levels in *Salmonella*-infected mice demonstrated that both the amount of HO-1 protein and activity of HO-1 (as evidenced by blood CO levels) as well as its mRNA levels increased during infection, with induction seen mainly in macrophages [*23*]. Pharmacological inhibition of HO-1 activity increased bacterial growth (by approximately 10-fold) and apoptosis in liver tissues [*23*]. Therefore, HO-1 was suggested to function in host defense by suppressing macrophage apoptosis essential for elimination of *Salmonella* (Fig. 1). An important finding was lower levels of protein, mRNA, and activity of HO-1 in iNOS$^{-/-}$ mice compared with wild-type mice, which suggests the possible involvement of NO in HO-1 induction [*3*, *23*]. NO- and ROS-mediated induction of HO-1 has been reported from other groups [*10*, *31*].

The Unique Signaling Function of 8-Nitro-cGMP via Protein *S*-Guanylation

To further clarify the mechanisms of NO-mediated induction of HO-1, we used cultured macrophages from iNOS$^{-/-}$ mice to analyze the effect of authentic 8-nitro-cGMP. Treatment of iNOS$^{-/-}$ macrophages with 8-nitro-cGMP resulted in increased HO-1 levels in a manner that depended on time and concentration of 8-nitro-cGMP. In addition, HO-1 levels in *Salmonella*-infected macrophages were lower in iNOS$^{-/-}$ cells than in wild-type cells, but addition of 8-nitro-cGMP restored these lower levels to values comparable to those found in wild-type macrophages. Moreover, 8-nitro-cGMP treatment also markedly suppressed apoptosis associated with infection [*23*]. These findings suggest the possible involvement of 8-nitro-cGMP that is generated during infection in the signaling pathway of HO-1 induction (Fig. 1).

We further analyzed the molecular mechanisms governing 8-nitro-cGMP signaling functions. Because of its electrophilicity, 8-nitro-cGMP adducted with a sulfhydryl group of proteins via nucleophilic substitution with the nitro moiety of 8-nitro-cGMP to form a protein-*S*-cGMP adduct, which is a novel post-translational modification called protein *S*-guanylation (Fig. 2A) [*22*]. This protein adduct formation accompanies the denitration of 8-nitro-cGMP, with release of nitrite. Because 8-nitro-cGMP loses electrophilicity after S-guanylation, this electrophilic modification seems to be irreversible, at least under physiological conditions without specific catalysts yet to be identified. We found that one of the most important target proteins for *S*-guanylation is Kelch-like ECH-associated protein 1 (Keap1), which is now increasingly recognized as a potent redox-sensing protein. Keap1 is a negative regulator of Nrf2, which is a transcription factor regulating antioxidant enzymes for electrophiles and ROS [*32*, *33*]. Binding of Keap1 to Nrf2 inhibits Nrf2 transcriptional activity via sustaining rapid degradation of Nrf2 by proteasomes in the cytosolic compartment of cells. Keap1 is proposed to be a sensor protein for oxidative stress (Fig. 2B). Because Keap1 has highly reactive Cys residues, chemical modification of the Cys residue sulfhydryl groups by electrophiles and ROS is considered to trigger dissociation of Nrf2. Activated Nrf2 then translocates to nuclei to induce expression of various antioxidant and cytoprotective enzymes including HO-1, which contributes to the adaptive response to oxidative stress [*34–36*].

We thus examined whether Keap1 can indeed be modified

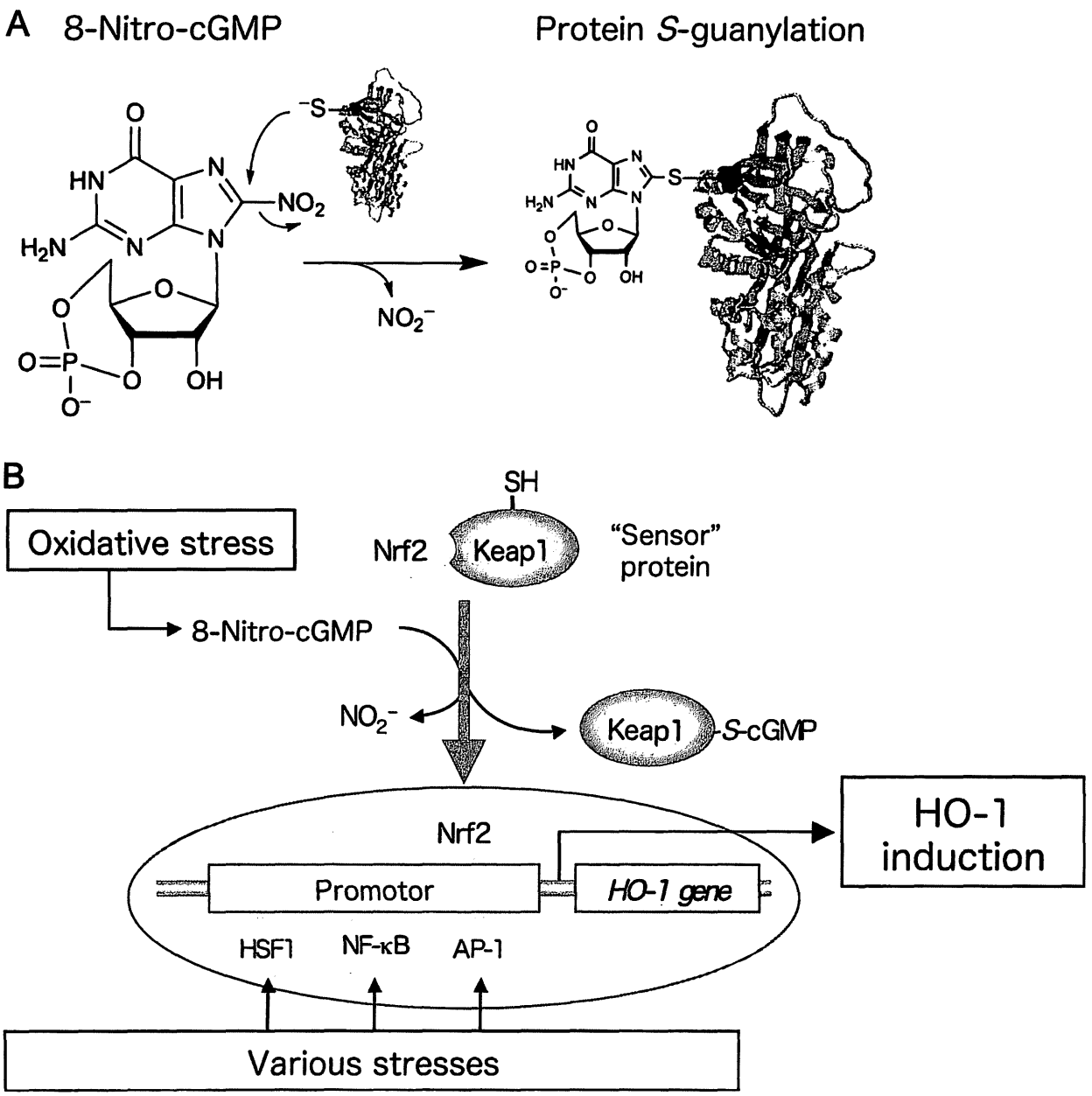

Fig. 2. New post-translational modification of proteins and HO-1 induction by 8-nitro-cGMP. (A) 8-Nitro-cGMP, a novel nitrated cyclic nucleotide generated from NO and ROS in cells, possesses an electrophilic property and causes *S*-guanylation of proteins, i.e., adduction of cGMP to protein sulfhydryls. (B) 8-Nitro-cGMP activates the Nrf2 pathway via S-guanylation of a sensor protein, Keap1, which leads to HO-1 induction.

by 8-nitro-cGMP produced via NO in cells. We infected cultured murine macrophages with *Salmonella*, and we found clear evidence of Keap1 *S*-guanylation by means of Western blotting using anti-*S*-guanylation antibody after isolation by immunoprecipitation [*22*, *23*]. We interpret these results to mean that 8-nitro-cGMP is involved in the major NO signaling pathway for cytoprotection and adaptive responses to ROS and oxidative stress through *S*-guanylation of Keap1. Strong support for this interpretation comes from the finding that cytoprotection and host defense conferred by 8-nitro-cGMP were clearly associated with increased HO-1 expression during *Salmonella* infection in macrophages and *in vivo*, as mentioned above [*23*] (Fig. 2B).

Beneficial and Pathological Effects of HO-1 in Various Microbial Infections

Analysis of the *Salmonella* infection model demonstrated that the NO-dependent and 8-nitro-cGMP-mediated signal pathway leads to HO-1 expression, and suppression of

apoptosis of infected macrophages potentiates microbial clearance—a host defense function against infection. However, some types of intracellular pathogens survive and multiply in macrophages because they escape the macrophage bactericidal system. One possible survival mechanism of these bacteria is, however, suppression of apoptosis of infected macrophages [*37*]. That is to say, HO-1 overexpression and resultant suppression of apoptosis are presumed to provide pathogens with a favorable intracellular environment so that they survive and grow. Similarly, inhibition of virus elimination by suppression of apoptosis of infected respiratory epithelial cells has been suggested as a potential pathogenic mechanism for the harmful effects of NO observed in a murine influenza virus-infected pneumonia model [*6*, *8*]. Although HO-1 induction via 8-nitro-cGMP is believed to contribute to host defense against infection [*23*, *38*, *39*], at the same time it may promote survival of particular pathogens (typically viruses). Various factors, such as the type of cells infected and the timing of HO-1 expression during infections, may lead to completely opposite biological effects of HO-1.

Conclusions

In summary, we have here clarified NO-dependent formation and cell signaling functions of 8-nitro-cGMP, which lead to expression of HO-1 and consequent cytoprotection in infected hosts. The 8-nitro-cGMP-mediated signaling pathway may protect host cells from apoptosis and support the antimicrobial effects of macrophages that are critically involved in innate immunity. Now in progress in our laboratory are further investigations of the antimicrobial functions of NO and 8-nitro-cGMP, with a special focus on potential target proteins of *S*-guanylation that is mediated by 8-nitro-cGMP, which may help establish a new understanding of host defense and microbial pathogenesis.

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Abbreviations

8-nitro-cGMP, 8-nitroguanosine 3',5'-cyclic monophosphate; AP-1, activator protein-1; cGMP, guanosine 3',5'-cyclic monophosphate; HSF1, heat shock factor 1; HO, heme oxygenase; iNOS, inducible nitric oxide synthase; $iNOS^{-/-}$, iNOS-deficient; Keap1, Kelch-like ECH-associated protein 1; Nrf2, nuclear factor-erythroid 2-related factor 2; NF-κB, nuclear factor-κB; RNS, reactive nitrogen species; ROS, reactive oxygen species.

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Review

Nucleotides function as endogenous chemical sensors for oxidative stress signaling

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Oxidized and nitrated nucleotides including 8-oxoguanine and 8-nitroguanine derivatives such as 8-nitroguanosine 3',5'-cyclic monophosphate were generated by reactive nitrogen oxides and reactive oxygen species in cultured cells and in tissues. 8-oxoguanine and 8-nitroguanine in DNA and RNA are potentially mutagenic, and the former also induces cell death. Some derivative, 8-nitroguanosine 3',5'-cyclic monophosphate a major nitrated guanine nucleotide, was identified as a novel second messenger. Surprisingly, the amount of 8-nitroguanosine 3',5'-cyclic monophosphate generated was found to be higher than that of guanosine 3',5'-cyclic monophosphate in cells expressing inducible nitric oxide synthase. More important, 8-nitroguanosine 3',5'-cyclic monophosphate is electrophilic and reacted efficiently with sulfhydryls of proteins to produce a novel posttranslational modification (named *S*-guanylation) *via* guanosine 3',5'-cyclic monophosphate adduction. For example, 8-nitroguanosine 3',5'-cyclic monophosphate-induced *S*-guanylation of Kelch-like ECH-associated protein 1 led to NF-E2-related factor activation and induction of antioxidant enzymes. 8-nitroguanosine 3',5'-cyclic monophosphate may thus protect cells against oxidative stress-related cytotoxicity. Therefore, although chemically modified nucleotides produced *via* oxidative and nitrative stress are regarded simply as endogenous *mutagens*, the endogenous nucleotides stored in cells *per se* may serve functionally as a sensing mechanism for reactive nitrogen oxides and oxygen species to induce cellular adaptive responses to oxidative stress.

Key Words: **oxidative stress, adaptive response, ROS signaling, nucleotide sensing, electrophilic signaling**

Nucleotides stored in the cells not only serve as substrates for nucleic acid biosynthesis but also participate in the energy metabolism and signal transduction. Nitric oxide (NO) is a gaseous free radical that is synthesized by nitric oxide synthases (NOSs).[1] NO plays important roles in the regulation of diverse physiological phenomena such as vascular and neuronal signal transduction, host defense, and cell death regulation.[2–5] Signal transduction by NO primarily involves a nucleotide signal molecule, guanosine 3',5'-cyclic monophosphate (cGMP), generated by soluble guanylate cyclase (sGC) from guanosine triphosphate (GTP).[6] cGMP thus formed binds to allosteric regulatory domains of target proteins, including protein kinases, ion channels, and phosphodiesterases, with various downstream biological consequences that allow cells to adapt to changes and stresses occurring under different environmental conditions and metabolic demands.[6]

Excess production of NO has been suggested to be a cause of diverse pathophysiological conditions, such as inflammation, neurodegenerative and cardiovascular diseases, and cancer.[7–12] These detrimental effects of NO are attributed to reactive nitrogen oxide species (RNOS), including nitrogen dioxide (NO_2) and peroxynitrite ($ONOO^-$), which are formed by the reaction of NO with molecular oxygen[13] and reactive oxygen species (ROS) such as the superoxide anion radical $O_2^{\bullet-}$.[14] Peroxynitrite is thought to act as a strong oxidizing and nitrating agent in different pathophysiological situations.[15–20] Other RNOS may also contribute to pathophysiological conditions. Such RNOS include nitryl chloride (NO_2Cl), which is formed from nitrite (NO_2^-) and hypochlorous acid (HOCl),[21,22] and NO_2, which is generated by oxidation of NO with molecular oxygen[23] or by catalysis of peroxidases such as myeloperoxidase (MPO) and eosinophil peroxidase using hydrogen peroxide (H_2O_2) and nitrite as substrates.[24,25] When production of RNOS exceeds the cellular antioxidant capacity, these molecules can cause nitrative and oxidative damage of nucleic acids, proteins, lipids, and carbohydrates by nitrosation, nitration, and oxidation reactions. RNOS are known to have a strong potential to oxidize and nitrate nucleic acids at the level of their base structures, e.g., guanine and adenine. During the past several years, oxidized and nitrated guanine derivatives, including 8-oxoguanine and 8-nitroguanine, were identified in diverse cultured cells, in tissues and organs from humans with cancer or degenerative diseases, and in different organisms with viral pneumonia, cancer, and other inflammatory conditions.[11,26–36] Not only the mutagenic potential but also the redox-active property of 8-nitroguanine derivatives suggested that guanine nitration may have significant biological effects yet to be identified.[26,34] In fact, we recently discovered a nitrated cyclic nucleotide, 8-nitroguanosine 3',5'-cyclic monophosphate (8-nitro-cGMP), that was produced in cells expressing inducible NOS (iNOS).[35] 8-nitro-cGMP is an extremely potent signaling molecule in biological systems because of its dual nature in signal transduction, i.e., in the canonical NO/cGMP pathway and in noncanonical electrophilic signaling.[35] Among the nitrated guanine derivatives studied, 8-nitro-cGMP possessed the strongest redox-active and electrophilic properties.[35,37] Because of its electrophilic behavior, 8-nitro-cGMP reacts effectively with highly nucleophilic sulfhydryl groups of certain cysteine (Cys) residues and formed a protein-*S*-cGMP adduct *via* a unique posttranslational modification named *S*-guanylation. In addition, some particular biological effects, e.g., cell death induction, rather than mutagenic potential are now well recognized to be caused by 8-oxoguanine accumulated in the nucleotide pool in the cells.[38] Here, we will review current knowledge about endogenous nucleotides chemically modified *via* oxidative/nitrative stress with respect to their formation and biological significance.

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Biological Formation of Oxidized and Nitrated Nucleotides

There is now ample evidence from a number of data indicating relatively frequent formation of 8-oxoguanine in various cells and tissues under oxidative stress.[36] ROS derived from both endogenous origins such as mitochondria, leukocytes (oxidative burst), peroxisomes (degradation of fatty acids) and cytochrome P450 system (mixed function oxidative system), as well as exogenous origins such as cigarette smoking, UV radiation, and ionizing radiation can contribute to the formation of 8-oxoguanine.[39] Epidemiological studies showed the increased formation of 8-oxoguanine as a risk factor for cancer, atherosclerosis, diabetes[40] and neurodegenerative disorders.[41] There are two pathways for the accumulation of 8-oxoguanine in DNA or RNA: one is a result of the incorporation of oxidized (deoxy)guanosine triphosphate (8-oxo-dGTP) generated in nucleotide pools while the other is a result of the direct oxidation of guanine in DNA or RNA. Recent progress in studies of the sanitization of nucleotide pools, as well as DNA repair, has revealed that the impact of oxidation of free nucleotides such as dGTP is unexpectedly large, in comparison with the direct oxidation of DNA.[38]

Similarly, *in vitro* and *in vivo* experiments have shown possible nitration of nucleic acids, more specifically guanine derivatives, that have been associated with various inflammatory conditions.[11,12,26–35] Yermilov *et al.*[42] found that peroxynitrite reacted with the guanine base of nucleic acids *in vitro* to form 8-nitroguanine. Masuda *et al.*[43] demonstrated that peroxynitrite mediated 8-nitroguanosine formation from RNA *in vitro*. Our group was the first to report *in vivo* evidence of guanine nitration: we found marked guanine nitration in the lungs of influenza virus-infected mice and in the lungs of patients with idiopathic pulmonary fibrosis and lung cancer, with the nitration depending on production of NO by iNOS.[26,28,30] We also observed formation of 8-nitroguanosine in mice infected with bacteria such as *Salmonella typhimurium*.[44] In addition, Hoki *et al.*[45] detected 8-nitroguanine formation in malignant fibrous histiocytoma specimens from patients. It is also interesting that formation of 8-nitroguanine was recently suggested to be linked to diabetic retinopathy, which is a major cause of blindness.[46]

Our chemical analyses using high-performance liquid chromatography-based electrochemical detection and tandem mass spectrometry (LC-MS/MS) revealed that, from among a series of 8-nitroguanine derivatives and related compounds, only a nitrated derivative of cGMP, 8-nitro-cGMP, was generated in significant amounts in cell culture models with different types of cells.[35,47] For example, by using LC-MS/MS, we identified 8-nitro-cGMP in murine macrophage RAW 264.7 cells that had been stimulated with interferon-γ and lipopolysaccharide to produce NO *via* iNOS. As just mentioned, infection of murine macrophages with the gram-negative bacterium *Salmonella* also facilitated formation of 8-nitro-cGMP, which was reported to be involved in host defense against infection.[12,35,44] Formation of 8-nitro-cGMP and 8-nitroguanine derivatives can be easily detected by means of conventional immunocytochemistry with the use of anti-8-nitro-cGMP monoclonal antibodies. It was intriguing that intracellular 8-nitro-cGMP formation and 8-nitroguanine formation had similar immunostaining profiles for time and location.[26,30,44] This may suggest that a major nitrated guanine derivatives formed in the cells is likely to be 8-nitro-cGMP rather than other nitrated nucleotides and DNA/RNA.

We recently precisely quantified the NO-dependent formation of 8-nitro-cGMP in C6 glioma cells *via* LC-MS/MS.[47] Treatment of cultured rat C6 glial cells with the NO donor *S*-nitroso-*N*-acetylpenicillamine (SNAP) led to a rapid and transient increase in cGMP, but the level of 8-nitro-cGMP gradually increased linearly up to a peak value comparable to that of cGMP at 24 h and declined thereafter. Markedly high levels (reaching up to 100 μM) of 8-nitro-cGMP were also evident in C6 cells that had been stimulated to express iNOS and produce excessive NO. The amount of 8-nitro-cGMP generated was much higher than that of cGMP, whose production profile slightly preceded 8-nitro-cGMP formation in the activated iNOS-expressing cells. Because of these unexpectedly large amounts of 8-nitro-cGMP, we suspected that GTP (a substrate of cGMP biosynthesis), rather than cGMP *per se*, may undergo guanine nitration. This idea was indeed supported by the fact that 8-nitroguanosine 5'-triphosphate (8-nitro-GTP), produced in a cell-free chemical reaction of GTP with peroxynitrite, served as an effective substrate for sGC.[47]

Mutagenesis Caused by Guanine Derivatives Chemically Modified *via* Oxidative and Nitrative Stress

Among the pathological effects associated with oxidative and nitrative stress, the mutagenic potential of ROS and RNOS is of great interest. RNOS such as peroxynitrite that commonly generated during infection and inflammation nonselectively affect a host's cells and tissues. Obviously, such host defense molecules are produced to kill invading pathogens, which then suffer oxidative stress because of the host's antimicrobial attack. It may therefore be logical to expect that mutagenesis of various microbial pathogens occurs during infections in biological systems as a result of host defense.[48]

Evidence of this mutagenesis includes the finding that human leukocytes producing $O_2^{\cdot-}$, but not leukocytes from patients with chronic granulomatous disease, were shown to be mutagenic for *S. typhimurium* TA100.[49] Our earlier study also confirmed that oxidative and nitrative stress induced by a high output of NO and ROS accelerated mutation of the RNA virus.[50] Related to this *in vivo* RNA virus mutation, our investigations also found that 8-nitroguanine formed by RNOS in the viral genome led to an increased frequency of mutations in an RNA virus (Fig. 1).[32] In addition, authentic 8-nitroguanosine added exogenously to an RNA virus-infected cells caused a dose-dependent increase in the frequency of viral mutations, especially C to U transitions.

An earlier study by Wogan's group documented that a high NO output induced mutations in an endogenous hypoxanthine-guanine phosphoribosyltransferase gene (*hprt*) in murine macrophages expressing iNOS.[51] Genetic analysis of the NO-induced mutated gene indicated that the NO-associated mutational spectrum was similar to that arising spontaneously, but small deletions and insertions were found in the NO-induced mutants. The same group showed that mutagenicity was enhanced by NO overproduction *in vivo*, as assessed by mutation of an exogenously expressed *lacZ* gene in *lacZ*-containing pUR288 plasmid-transgenic mice.[52] Excess production of NO by iNOS induced by inflammatory cytokines, possibly through RNOS (particularly peroxynitrite), caused DNA damage and impaired DNA repair in human cholangiocarcinoma cells, as assessed by the comet assay, which suggests an NO-dependent development and progression of cholangiocarcinoma.[53]

It is important to note that guanine nitration appears to cause DNA mutagenesis as well, with a mutation spectrum similar to that induced by peroxynitrite.[54] Thus, 8-nitroguanine in DNA may be rapidly depurinated from DNA *in vitro*, within 1–4 h under physiological conditions, the result being the formation of mutagenic abasic sites and release of free 8-nitroguanine.[27] Formation of 8-nitroguanine in DNA may therefore facilitate G to T transversion *via* abasic site formation.[42] In addition, Suzuki *et al.*[55] used photochemical synthesis to obtain an oligodeoxynucleotide containing a single 8-nitrodeoxyguanosine at a specific position[56] and utilized the oligodeoxynucleotide as a template in primer extension reactions catalyzed by mammalian DNA polymerases. This finding suggests that 8-nitrodeoxyguanosine in DNA can mispair with adenine, thereby directly inducing a G to T transversion in mammalian cells. Consistent with those *in vitro* findings, 8-nitroguanosine exhibited mutagenic activity in mammalian

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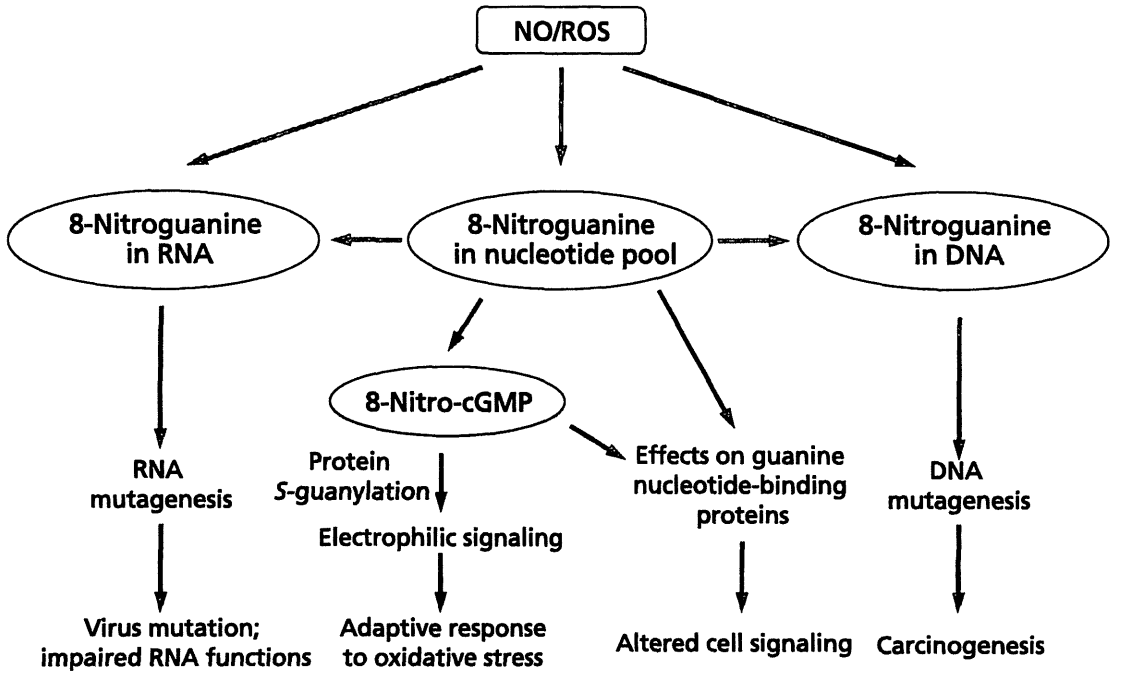

Fig. 1. Biological significance of nitration of guanine nucleotides. NO, nitric oxide; ROS, reactive oxygen species.

cells.[57] Treatment of Chinese hamster ovary AS52 cells with 8-nitroguanosine significantly increased the mutation frequency of the xanthine-guanine phosphoribosyltranserase gene, with G to T transversion at the gene locus. Concomitant increase of abasic sites in DNA further supports the notion that depurination-dependent mutagenesis may be operative for 8-nitroguanosine-induced mutation.

A similar mutation spectrum is observed for oxidative damaged nucleic acids caused by peroxynitrite and ROS. Specifically, 8-oxoguanine can pair with both cytosine and adenine during DNA synthesis, and this base mismatching could contribute significantly to spontaneous mutations in genomic DNA.[58] Direct oxidation in G:C pair can result in G to T transversion during replication, whereas 8-oxo-dGTP can be incorporated in DNA opposite to the adenine, leading to the A to C transversion.[59] Mammalian cells are equipped multiple enzyme systems to suppress 8-oxoguanine accumulation in DNA.[60] For example, 8-oxoguanine DNA glycosylase1 (OGG1) excises 8-oxoguanine from 8-oxo-G:C pairs in DNA, thus initiating base excision repair. MutT homolog-1 (MTH1) is an enzyme that hydrolyzes 8-oxo-dGTP to the monophosphate form, thus preventing incorporation of 8-oxo-dGTP into DNA during replication.

Immunohistochemical studies demonstrated that 8-oxoguanine and 8-nitroguanine are also present in the cytosol of various cells and tissues suffering from oxidative/nitrative stress,[26,30,35,61,62] which suggests that not only DNA but also the nucleotide pool, RNA, and other cytosolic compartments are modified chemically *via* oxidative/nitrative stress. For example, because certain enzymes such as MPO are unlikely to nitrate DNA directly, nitrated nucleic acids may be formed in the nucleotide pool or in RNA by reactive nitrogen species that are generated by such enzyme systems. As shown for certain oxidized and halogenated nucleosides,[63–65] 8-nitroguanine and related nucleosides and nucleotides may be misincorporated into DNA, which can result in mutations. Similarly, 8-nitroguanine and related nucleosides and nucleotides, like oxidized and halogenated nucleosides, may be incorporated into RNA and interfere with RNA function and metabolism.[66–68] The studies summarized here therefore clearly show that oxidative and nitrative DNA damage, as evidenced by increased formation of 8-oxoguanine and 8-nitroguanine, can be induced under various inflammatory conditions. In fact, chronic inflammation induced by various biological, chemical, and physical factors has also been associated with an increased risk of human cancer at many locations.[10,11,69] However, to establish a causal relationship between this type of DNA damage and human cancer, additional studies that utilize a molecular epidemiological approach in a large human population are required.

Redox and Electrophilic Signaling Property of Nitrated Guanine Nucleotides

8-nitro-cGMP has the potential to activate cGMP-dependent protein kinase (PKG) in vascular smooth muscle cells; in addition, 8-nitroguanosine and its derivatives possess a significant redox activity. 8-nitro-cGMP had the highest redox activity among the 8-nitroguanosine derivatives we tested, with redox activity decreasing in the following order: 8-nitro-cGMP > 8-nitroguanosine > 8-nitroguanosine 5'-monophosphate (8-nitro-GMP) ≈ 8-nitro-GTP. 8-nitroguanine had only negligible redox activity.[26,34,35] In the presence of certain oxidoreductases and electron donors such as NADPH, 8-nitroguanosine derivatives were readily reduced to form their anion radicals, after which a single electron was transferred to molecular oxygen to form superoxide anion radical.[26,34]

Electrophilicity is another unique redox 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 (8-RS-cGMP) adducts (Fig. 2). We named this unique electrophilic reaction *S*-guanylation of sulfhydryls.[35,37,70] This reaction seems to occur *via* a nucleophilic attack by the thiol group of a protein Cys or GSH on C8 of 8-nitro-cGMP, the results being release of the nitro moiety and formation of the 8-RS-cGMP adduct (Fig. 2). The second-order rate constant for the reaction of 8-nitro-cGMP with the sulfhydryl of GSH was determined to be 0.03 M^{-1} s^{-1} at pH 7.4 and 37°C.[35] This value is much smaller than values for

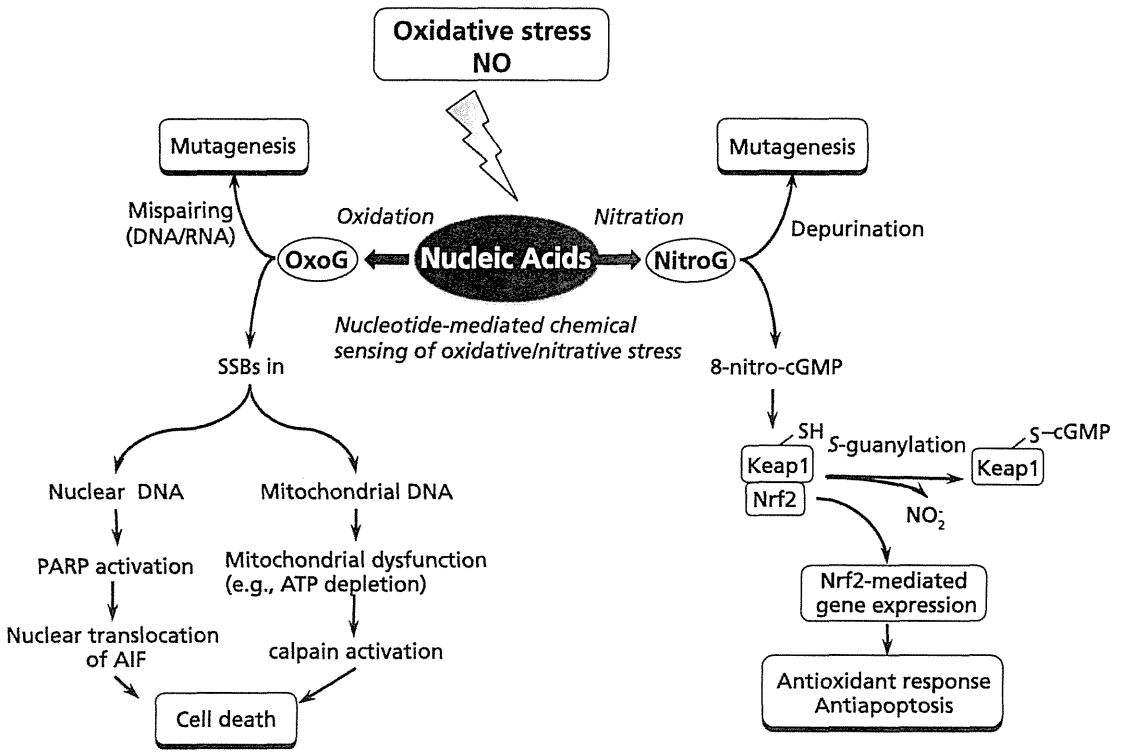

Fig. 2. Schematic representation for cell stress responses activated *via* two major nucleotide modifications, such as guanine nitration and oxidation, with subsequent completely different pathways for downstream signaling, leading to cell death and antioxidant cytoprotective effects. NO, nitric oxide; OxoG, 8-oxoguanine; NitroG, 8-nitroguanine; SSBs, single strand breaks; PARP, poly-ADP-ribose polymerase; AIF, apoptosis-inducing factor; cGMP, guanosine 3',5'-cyclic monophosphate; Keap1, Kelch-like ECH-associated protein 1; Nrf2, NF-E2-related factor 2.

other electrophiles such as 4-hydroxynonenal, 15-deoxy-$\Delta^{(12,14)}$-prostaglandin J_2, and nitrolinoleic and nitrooleic acids. Those compounds have reaction rate constants with GSH of 1.3, 0.7, 355, and 183 M^{-1} s^{-1}, respectively, at pH 7.4 and 37°C.[37] 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 ~ 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 p*K*a values of sulfhydryls of the Cys moiety.

Because of its electrophilicity, 8-nitro-cGMP may mediate electrophilic signaling by means of 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.[35] Keap1 is a negative regulator of NF-E2-related factor 2 (Nrf2), which is a transcription factor that regulates phase-2 detoxifying and antioxidant enzymes for electrophiles and ROS.[71,72] Our chemical analyses revealed that Keap1 expressed by various cultured cells was highly susceptible to *S*-guanylation induced by NO-dependent 8-nitro-cGMP.[35] In fact, we found that NO and RNOS could activate the Keap1-Nrf2 pathway in macrophages during bacterial infections and in rat C6 glial cells in culture after treatment with proinflammatory stimuli. That 8-nitro-cGMP may act as an endogenous electrophilic ligand and affect Keap1 sulfhydryls *via* *S*-guanylation, which would lead to antioxidant signaling, is therefore highly plausible. Cytoprotection and host defense conferred by 8-nitro-cGMP were clearly associated with increased expression of heme oxygenase 1 (HO-1) in cultured macrophages and *in vivo* during *Salmonella* infection.[11,35,44] HO-1 is an enzyme with various physiological roles including vasoregulation,[73] cytoprotection,[74] and anti-inflammatory effects.[75] We also reported earlier that HO-1 expression induced by NO contributed to cell survival in certain solid tumor models.[76,77] We recently found, in rat C6 glial cells, that Keap1 is the major target that is *S*-guanylated by NO exposure and that its *S*-guanylated structure derives primarily from 8-RS-cGMP adducts.[47] *S*-Guanylated Keap1 led to Nrf2 activation and subsequent induction of antioxidant enzymes including HO-1, so 8-nitro-cGMP protected cells against the cytotoxic effects of hydrogen peroxide. Proteomic analysis for endogenously modified Keap1 with matrix-assisted laser desorption/ionization time-of-flight-MS/MS revealed that 8-nitro-cGMP *S*-guanylated the Cys434 of Keap1 (Fig. 2). This finding is therefore the first compelling corroboration of the potential roles of 8-nitro-cGMP in the Nrf2-dependent antioxidant response.

Nucleotide Oxidation Caused by Oxidative Stress Triggering Cell Death Signaling

Recent studies have suggested that 8-oxoguanine formation not only can contribute to mutagenesis, but also may play an important role in the regulation of cell death.[78] Excessive formation of 8-oxoguanine was found to induce cell death *via* nuclear DNA-dependent and mitochondrial DNA-dependent mechanisms.[78] As mentioned above, 8-oxoguanine in DNA pairing with cytosine is removed by OGG1. In addition to OGG1, MutY homolog (MUTYH) excises adenine opposite 8-oxoguanine in template DNA, with concomitant formation of single strand breaks (SSBs) during the base excision repair. The accumulation of SSBs in nuclear DNA by the action of MUTYH leads to poly-ADP-ribose polymerase-dependent nuclear translocation of apoptosis-inducing factor, and triggers cell death. On the other hand, the accumulation of SSBs in mitochondrial DNA, which is also dependent on

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MUTYH, caused mitochondrial dysfunction and calcium ion release, thereby activating calpain, and finally triggers cell death. Recent study also demonstrated that excessive production of 8-oxo-dGTP in the nucleotide pool is a major source of oxidized bases, which may in turn cause accumulation of 8-oxoguanine in DNA,[38,79] leading to activate the cell death pathway as mentioned above. Taken together, 8-oxoguanine may function as a signaling molecule triggering the cell death, which appears to cause a completely opposite effect compared with the downstream cyto-protective effect of 8-nitro-cGMP signaling as described above (Fig. 2).

Conclusion

In this article, we overviewed available data on the physiology and pathophysiology of oxidized and nitrated guanine nucleotides, with emphasis on information on mutagenesis and cell signaling related to these nucleotides reported in the last decades. Different RNOS produced under various pathophysiological conditions may oxidize and nitrate guanine and its related nucleosides and nucleotides, which exist as part of DNA or RNA or in free form as an abundant component of the intracellular nucleotide pool. Not only do 8-oxoguaine and 8-nitroguanine function biologically as an endogenous mutagen but it may also serve as a biomarker for ROS- and RNOS-induced nucleic acid damage. More important, the major nitrated guanine nucleotide product, 8-nitro-cGMP, may play a critical role in signal transduction during cellular responses to oxidative stress that are primarily mediated by NO and ROS. This concept was unambiguously confirmed by our recent observation that NO-dependent formation of 8-nitro-cGMP is a potent contributor to activation of the antioxidant signaling pathway controlled by the Keap1-Nrf2 system *via* unique site-specific *S*-guanylation of Keap1 in cells. Also, structural evidence of Keap1 *S*-guanylation at Cys434 *in vivo* indicated that 8-nitro-cGMP, formed from the major nucleotide sensor GTP that exists in an intracellular pool, appears to act as a critical signaling molecule in the initial defense against nitrative and oxidative stress. Of importance is that, while guanine oxidation to form 8-oxoguanine may activate the cell death pathway, either dependent or independent of the p53 regulation, guanine nitration involving 8-nitro-cGMP-mediated antioxidant responses exhibits a completely opposite cellular protective effect (Fig. 2). Therefore, the nucleotides in the intracellular pool seem to be sensing the oxidative and nitrative stress occurring in cells, as being differentially recognized based on the altered chemical structures of nucleotides (e.g., oxo- and nitro-moieties) modified by ROS and RNOS. In other words, oxidative and nitrative nucleotide modifications may not be simple chemical damages, which mostly lose their biological functions because of the altered nucleotide structures, but may be physiologically relevant phenomena, which allow the cells to evoke the versatile cell signaling for adaptive responses to the various chemical stress. Further clarification of the signaling functions *via* oxidized and nitrated nucleotides may shed light on the chemical biology and mutation research and may support an emerging paradigm for oxidative stress-related chemical sensing and signaling of NO and ROS *via* cellular nucleotides functioning potentially as nitrative/oxidative stress sensors.

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JB Review

Regulation of redox signalling by an electrophilic cyclic nucleotide

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Reactive oxygen species (ROS) have been believed to be toxic substances that induce nonspecific damage in various biological molecules. ROS toxicology is now developing an emerging concept for physiological functions of ROS in the regulation of cell signal transductions. ROS signalling functions and their mechanisms are precisely regulated by several endogenous moderate electrophiles that are themselves generated from ROS during diverse physiological and pathophysiological cellular responses. The chemical biology of electrophiles is an emerging scientific area involving molecular mechanisms that conduct ROS cell signals through receptors to effector molecules at molecular, cellular and organism levels. The formation, signalling and metabolism of 8-nitroguanosine 3′,5′-cyclic monophosphate (8-nitro-cGMP) in cells are probably precisely regulated, and nonselective ROS reactions can be converted into stable, well-controlled electrophilic signal transduction via 8-nitro-cGMP. Modern redox biology is today advancing its frontier of basic research and clinical medicine, including infection, cancer biology, metabolic syndromes, ageing and even stem cell research. As one aspect of this advance, the 8-nitro-cGMP-mediated signalling that may be integrated into cells as a major redox signalling pathway may be a potential target in drug development and may lead to discovery of new therapeutic agents for various diseases.

Keywords: electrophilic signalling/8-nitro-cGMP/NO/ROS signalling/redox signalling.

Abbreviations: 15 d-PGJ$_2$, 15-deoxy-$\Delta^{12,14}$-prostaglandin J$_2$; Duox, dual oxidase; ERK, extracellular signal-regulated kinase; GSH, glutathione; GST, glutathione *S*-transferase; HNE, 4-hydroxy-2-nonenal; HS^-, hydrogen sulphide anion; iNOS, inducible type of NO synthase; LC-MS/MS, liquid chromatography tandem mass spectrometry; LPS, lipopolysaccharide; NO, nitric oxide; Nox, NADPH oxidase; 1,2-NQ, 1,2-naphthoquinone; 8-nitro-cGMP, 8-nitroguanosine 3′,5′-cyclic monophosphate; PTM, posttranslational modification; Rb, retinoblastoma gene product; RNAi, RNA interference; ROS, reactive oxygen species; sGC, soluble guanylate cyclase.

Aerobes conduct high-level vital activities during energy metabolism by using the chemical reactivity of molecular oxygen (oxidation–reduction or redox activity). Reactive oxygen species (ROS) are reduced derivatives of molecular oxygen (*e.g.* superoxide anion [O_2^-] and hydrogen peroxide [H_2O_2]), which are produced during energy metabolism or the defense process against infection in cells and tissues. ROS were thought to be harmful agents that mediate oxygen toxicity (ROS toxicity theory). Indeed, ROS may be involved in the pathogenesis of various diseases (*1–5*). These diseases include infections, inflammations, cancer, lifestyle-related diseases and metabolic diseases, such as arteriosclerosis and diabetes mellitus and neurological disorders such as Alzheimer's disease. However, the clinical application of antioxidant agents to prevent and treat these diseases has not yet achieved the hoped-for results.

Several isoforms of ROS-producing enzymes such as NADPH oxidase (Nox) and Dual oxidase (Duox), expressed by many types of cells (*6*, *7*), have been regarded as general antimicrobial effectors, but current reports indicate that they demonstrate physiological functions other than antimicrobial actions, in a wide range of cells and tissues including vasculature and in the phagocyte immune system, epithelial system and endocrine system. Also, H_2O_2 has been said to act as a potential signalling molecule that mediates vascular tone regulation (*8*, *9*). Furthermore, although the toxicity of nitric oxide (NO) is augmented by reactions with ROS, NO serves as a master cell signalling molecule involved in diverse biological phenomena (*10*). In view of these accumulated data, the concept of ROS toxicity causing nonspecific injury to biomolecules has changed drastically. Researchers in a wide variety of life science fields, therefore, have produced advances in the physiological cell signalling functions of ROS.

In this review article, we provide a brief overview of the signalling functions of ROS as a new paradigm arising from the conventional ROS toxicity theory. Thus, the emerging field of ROS signalling has

increasingly yielded the most innovative results in recent years and is a field that is now well integrated into chemistry and biology.

The ROS Signalling Process

Generation of ROS signals

Different from many other signalling molecules, ROS, which are simple, low-molecular-weight inorganic compounds, are chemically reactive and thus mostly unstable in biological systems. The specificity of ROS signalling functions depends on the ROS production system and chemical reactivity, especially a spatiotemporal property, which defines the molecular reaction environment. Therefore, identifying the ROS production system that contributes to signal ligand formation and clarifying the mechanisms of ROS signal production and sensing are extremely important (Fig. 1). The exact mechanisms of such spatiotemporal regulation of ROS signalling remain unknown, however.

Many researchers have focused on the structure and function of ROS-producing enzymes (*e.g.* Nox), which may be involved in signalling functions in various cells and tissues, and on the localization and spatiotemporal dynamics of ROS production in cells (*6*, *7*, *11*). For example, we elucidated an ROS production mechanism that involved cross-talk between Nox and mitochondria, which contributed to the formation of the second messenger 8-nitroguanosine 3′,5′-cyclic monophosphate (8-nitro-cGMP) in cells. This mechanism may explain, at least in part, how ROS can specifically transduce their cell signalling in a spatiotemporal manner (Fig. 2) (*11*). In fact, we recently identified a unique mode of the interaction of this 8-nitro-cGMP signal ligand and its sensor-effector molecules, not only on a cellular level but also on an *in vivo* organism level, by using our model of a redox-based and electrophile-mediated signalling system in cultured cells and *in vivo* (*12*), as discussed in detail later in this review.

Regulation of the sensing function

ROS signalling process generally begins with an unstable primary ROS signal that is subsequently transformed into a more stable secondary signal (Fig. 1). During this process, ubiquitous biological molecules (*e.g.* nucleic acids, nucleotides, lipids and reactive protein residues) serve as chemical sensors, which can effectively perceive ROS and are available in cells for a wide repertoire of reactions. For example, interaction of ROS and NO with various sensors, such as nucleic acids, lipids and sulfhydryls of proteins, produces stable secondary signalling molecules (*e.g.* 8-nitro-cGMP and nitro-fatty acids) (*13*, *14*). Also, a sensor protein with nucleophilic Cys sulfhydryls, because of high redox activity, directly or indirectly mediates receptor functions for ROS signalling. Identifying and analyzing these sensor molecules are therefore critical to understand the sensing specificity and selectivity of the ROS signalling system. Thus, we and other

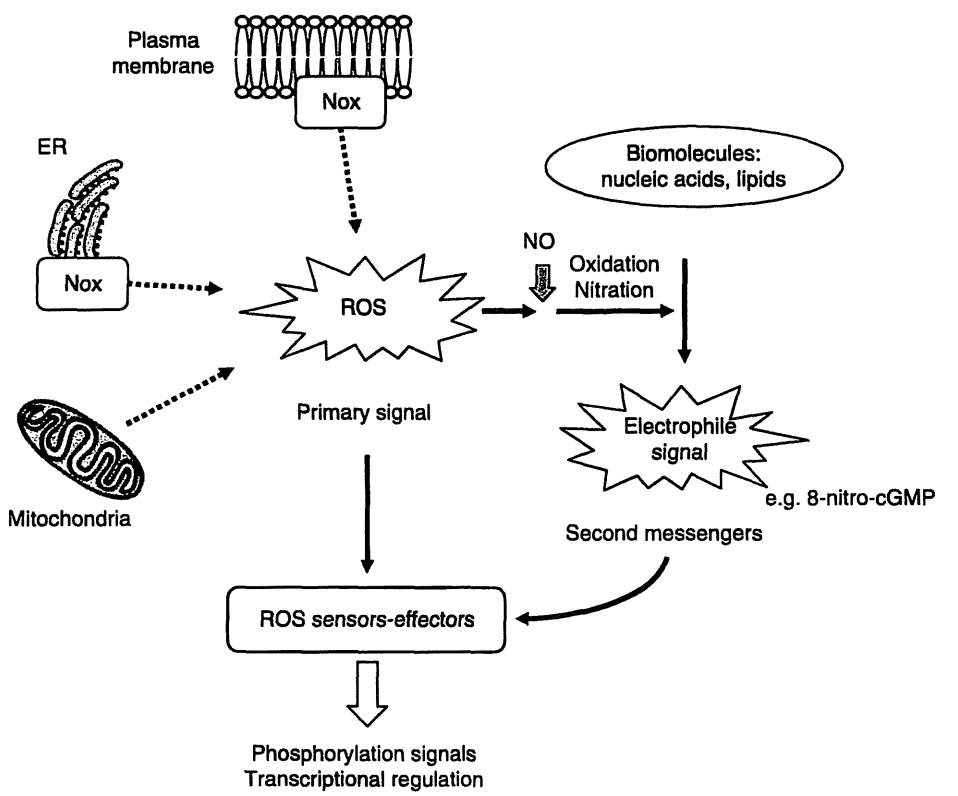

Fig. 1 Schematic illustration of the ROS signalling mechanism. ROS produced from different sources, such as Nox and mitochondria, are unstable primary signals. ROS themselves, or via their reaction with NO, react with various sensor molecules in cells and are converted to more stable second messengers such as 8-nitro-cGMP. These primary signals and second messengers are sensed by ROS sensor-effector molecules, which mediate different signal transduction and transcriptional pathways. ER, endoplasmic reticulum.

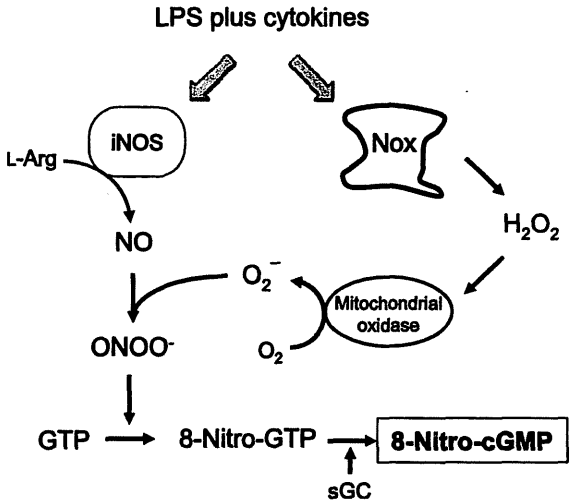

Fig. 2 The mechanism of ROS- and NO-dependent formation of 8-nitro-cGMP in cells. Various stress stimuli, especially proinflammatory substances, can induce cellular production of ROS and NO by Nox2 and iNOS, respectively. For example, mitochondria-derived O_2^-, as regulated by Nox2-produced H_2O_2, plays an important role in the nitration of guanine nucleotides. For cellular 8-nitro-cGMP formation, peroxynitrite ($ONOO^-$) derived from NO and O_2^- nitrates GTP to form 8-nitro-GTP, and 8-nitro-GTP is converted to 8-nitro-cGMP by catalysis with sGC. This mechanism is proposed on the basis of observations obtained with LPS plus cytokine-treated rat C6 glioma cells (*11*, *16*).

groups actively conducted many extensive studies to identify ROS sensor molecules and clarify their chemical sensing mechanisms in different cells, tissues and organs (*14–16*). We also analyzed the structures and functions of sensor and effector proteins that were modified by the ROS signal or its secondary signalling molecules (*e.g.* 8-nitro-cGMP) (*12*). More important, we recently investigated a still-unidentified new sensor for ROS (*12*). Recent discoveries of unique signal ligands and sensors will help us describe the whole picture of diverse ROS sensing and cell signal transmission pathways.

Regulation of the effector function

The biological functions of effectors, being directly affected by ROS or indirectly mediated by secondary signalling molecules of ROS, can be induced by ROS signal-generated structural changes of sensor proteins, which in some cases serve simultaneously as sensors and effectors (Fig. 1). For example, phosphorylation and transcription signalling pathways are regulated by means of structural changes of sensor-effector proteins (*i.e.* particular redox-sensitive protein kinases, phosphatases and transcription factors) that result from chemical modification by ROS and reactive nitrogen oxide species (*e.g.* oxidation, nitrosylation, alkylation and guanylation of Cys sulfhydryls) (*3*). Clarifying the molecular mechanisms of various sensor-effector relationships with ROS is an important theme of ROS research. Recent studies have reported advances in such research, such as a novel mechanism of ROS-dependent NF-κB activation (*17*), *S*-nitrosylation-mediated regulation of PI3 kinase-Akt signalling (*18*) and angiotensin type 1 receptor expression (*19*). Further investigation of redox-based signal effector regulation will provide greater understanding of cellular response mechanisms, including cell proliferation and cell death, mediated by the ROS signalling system, with a focus on sensor-effector proteins involved in intracellular phosphorylation signal transduction, transcriptional regulation, endoplasmic reticulum stress, and neuronal and vascular signal transduction (Fig. 1).

8-Nitro-cGMP: A Unique Second Messenger in ROS Signalling

Formation of 8-nitro-cGMP

We recently found that the nitrated guanine nucleotide 8-nitro-cGMP forms in cells (Fig. 1) and plays a pivotal role in ROS signalling via *S*-guanylation, a unique posttranslational modification (PTM) of Cys residues in proteins (*13*, *16*). Biologically relevant nitration is achieved via cellular expression of both O_2^- and NO to produce the potent nitrating agent peroxynitrite ($ONOO^-$), or via expression of nitrite plus H_2O_2 and myeloperoxidase to generate another nitrating agent, NO_2 (*20*). Chemical and cellular analyses conducted in our laboratory verified that $ONOO^-$ was the molecular species that was most likely responsible for nitration of guanine nucleotides and that nitrite plus H_2O_2 and myeloperoxidase may nitrate guanine nucleotides in a particular molecular environment in cells (*11*). To analyze the cellular formation of 8-nitro-cGMP, we used rat C6 glioma cells because these cells produce O_2^- and NO in response to stimulation with lipopolysaccharide (LPS) plus proinflammatory cytokines via activation of Nox2 and induction of the expression of the inducible type of NO synthase (iNOS), respectively (*11*, *16*). In these stimulated cells, we identified mitochondria-derived O_2^- as a direct determinant of 8-nitro-cGMP formation. This finding was the first demonstration that mitochondria-derived O_2^- together with concomitant NO generation plays an important role in the biological nitration of guanine nucleotides. Mitochondria-derived O_2^- production was itself regulated by H_2O_2 generated from Nox2, a result that suggests a critical link between Nox2-dependent H_2O_2 production and mitochondrial O_2^- production (Fig. 2). This finding may lend credence to our proposal that 8-nitro-cGMP may serve as a unique second messenger that forms downstream of NO and ROS generation.

Our rigorous liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis unequivocally proved the biological relevance of endogenous 8-nitro-cGMP formation. We carefully analyzed the exact amount of 8-nitro-cGMP formed in stimulated C6 cells by using our stable isotope dilution technique and quantitative LC-MS/MS analysis in a spike-and-recovery study with control nucleotides synthesized with stable ^{15}N isotopes (*16*). We measured not only 8-nitro-cGMP but also cGMP, to clarify their distinctive production kinetics (quantity- and time-dependent profiles), with the expectation that differentiation between the two compounds may lead to a better understanding of their physiological and

pathological significance. With this technique, we found that quite high levels (greater than 40 μM) of 8-nitro-cGMP formed in cells and that these levels were much higher than the cGMP levels (4.6 μM) formed in the same cells, which indicates that 8-nitro-cGMP is one of the major cyclic nucleotides generated in cells (*16*). In addition, our quantitative LC-MS/MS analysis provided an important insight into the 8-nitro-cGMP formation mechanism—that 8-nitro-cGMP is not formed by direct nitration of cGMP but that it is generated after nitration of the abundant GTP, to produce 8-nitro-GTP, which then reacts with guanylate cyclases to generate 8-nitro-cGMP (Fig. 2). Indeed, studies with *in vitro* systems revealed that of various guanine nucleotides including cGMP, GTP was the nucleotide that was most susceptible to $ONOO^-$-mediated nitration (*11*). Furthermore, the 8-nitro-GTP formed by the reaction of GTP with $ONOO^-$ appeared to be an efficient substrate for soluble guanylate cyclase (sGC) to form 8-nitro-cGMP (*16*). These findings suggest that nitration of GTP is a main pathway for 8-nitro-cGMP formation in cells.

8-Nitro-cGMP formation was also found in heart tissues of mice. Heart failure is a leading cause of morbidity and mortality in developed countries, and ROS and NO generated from Nox2 and iNOS, respectively, have been implicated in the pathogenesis of heart failure (*21*, *22*). In fact, 3-nitrotyrosine, an endogenous nitration product, is generated in mouse hearts after myocardial infarction, accompanied by dramatic increases in expression levels of Nox2 and iNOS proteins. By using LC-MS/MS analysis and immunohistochemistry with an anti-8-nitro-cGMP-specific antibody, we discovered that 8-nitro-cGMP was also produced in mouse hearts after myocardial infarction or pressure overload (*12*). The iNOS-derived NO may be essential for 8-nitro-cGMP formation in mouse hearts, because iNOS-deficient hearts evidenced no 8-nitro-cGMP formation after myocardial infarction. LPS and ATP were recently reported to generate NO through iNOS induction in heart cells (*19*). ATP- or LPS-activated cultured rat neonatal cardiac myocytes and fibroblasts to generate endogenous 8-nitro-cGMP, which coincided with *S*-guanylation as detected by immunocytochemistry. These results indicate that heart cells actively produce, in an iNOS-dependent manner, 8-nitro-cGMP in response to stimulation with different agonists.

Metabolism of 8-nitro-cGMP by glutathione

Oxidative stress induces production of various electrophiles including 8-nitro-cGMP. The best characterized chemical and pharmacological feature of 8-nitro-cGMP is its relative stability in cells, such that it maintains its signalling functions. In fact, the electrophilicity of 8-nitro-cGMP was much lower than that of other endogenous electrophiles. The second-order rate constant for the reaction of 8-nitro-cGMP with the glutathione (GSH) sulfhydryl at pH 7.4 and 37°C was $0.03\,M^{-1}s^{-1}$ (*13*, *23*). 8-Nitro-cGMP was much less reactive than other endogenous electrophiles such as α,β-unsaturated aldehydes, ω-6 and ω-3 unsaturated fatty acids, and nitroalkene fatty acids, which include *e.g.* 4-hydroxy-2-nonenal (HNE), 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15 d-PGJ_2), and nitrolinoleic and nitrooleic acids. The second-order rate constants for the reaction of those electrophiles with GSH at pH 7.4 and 37°C were $1.3\,M^{-1}s^{-1}$ (HNE), $0.7\,M^{-1}s^{-1}$ (15 d-PGJ_2), $355\,M^{-1}s^{-1}$ (nitrolinoleic acid) and $183\,M^{-1}s^{-1}$ (nitrooleic acid) (*23–25*). Therefore, these electrophiles reacted with the GSH sulfhydryl 20–10,000 times more rapidly than did 8-nitro-cGMP (*23*). 8-Nitro-cGMP is thus stable enough to remain at appreciable levels in cells even with excessive amounts of GSH.

The reactions of some electrophiles with GSH are facilitated by enzymes such as glutathione *S*-transferase (GST). Reactive electrophiles produced in cells during oxidative stress were detoxified by GST (*26*). During our investigations about whether 8-nitro-cGMP could serve as a substrate for GST, we observed no obvious degradation of 8-nitro-cGMP in the catalytic reaction with GST. Indeed, even with the use of several isoforms of GST, 8-nitro-cGMP was not metabolized by GSH (our unpublished data). This notable biochemical characteristic of 8-nitro-cGMP supports its marked stability and thus its availability for cellular redox signalling.

The stability of the endogenous electrophile 8-nitro-cGMP may therefore validate the major biological relevance of 8-nitro-cGMP formed in cells. That a large amount of 8-nitro-cGMP persisted in cells (*16*) may be consistent with its relatively poor electrophilicity, because electrophilic compounds are believed to react readily with sulfhydryl compounds such as GSH and be degraded, as just mentioned.

Metabolism of 8-nitro-cGMP by hydrogen sulphide anion

Endogenous formation of H_2S in mammalian cells and tissues has been reported in recent years (*27*, *28*). The chemical nature and truly physiological functions of hydrogen sulphide (H_2S) are as-yet unclear, however. Although H_2S was proposed to have anti-inflammatory and antioxidant effects, because of the modest rate constants for the reaction of H_2S with ROS and reactive nitrogen oxides such as H_2O_2 and $ONOO^-$ (*29*), H_2S *per se* would not be expected to directly scavenge ROS and reactive nitrogen oxides unless they are strong electrophiles, or unless reactive sulphur derivatives like sulphane sulphides (R-(S)*n*-SH) rather than H_2S could be formed endogenously.

Because GSH does not seem to be a major determinant of 8-nitro-cGMP metabolism, as described earlier, we investigated, by utilizing RNA interference (RNAi) screening and focusing on Cys metabolism and its redox-related metabolic pathways, other still unknown factors that may contribute to the regulatory and metabolic pathways of 8-nitro-cGMP signalling (*12*). Our RNAi screening study revealed substantial evidence of the effect of two key enzymes in sulphide biosynthesis on 8-nitro-cGMP metabolism. Specifically, cystathionine β-synthase and cystathionine γ-lyase appeared to mediate the metabolism of 8-nitro-cGMP in different cultured mammalian cells. This metabolic activity took place simultaneously with

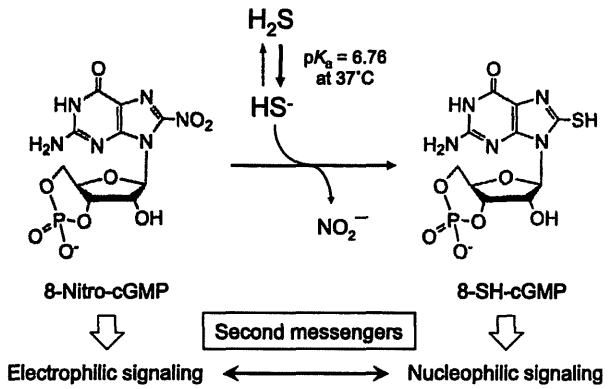

Fig. 3 Schematic representation of the reaction of 8-nitro-cGMP with HS^- and its unique redox signal regulation. In the reaction of 8-nitro-cGMP with HS^-, the electrophilic nitro moiety of 8-nitro-cGMP undergoes nucleophilic substitution to produce 8-SH-cGMP. 8-Nitro-cGMP and 8-SH-cGMP, as unique second messengers derived from ROS and NO, may mediate signal transduction in a manner that reflects the opposite of their redox biology, *i.e.* electrophilic signalling by 8-nitro-cGMP and nucleophilic signalling by 8-SH-cGMP.

the release of nitrite, which suggests a mechanism that depends on the nucleophilic qualities of H_2S (pK_a 6.76), especially the hydrogen sulphide anion (HS^-) that is the dominant form in neutral biological solutions (Fig. 3). To clarify how HS^- could undergo an addition reaction (*i.e.* electrophile sulfhydration), we used LC-MS/MS analysis to investigate the reaction of 8-nitro-cGMP with HS^- in a cell-free system. The electrophilic nitro moiety underwent nucleophilic substitution, with the yield being the new product 8-SH-cGMP (*12*). As Fig. 3 illustrates, two unique second messengers endogenously derived from ROS and NO as affected by HS^- may apparently mediate signal transduction in a manner that reflects the opposite of their redox properties, *i.e.* electrophilic signalling by 8-nitro-cGMP and nucleophilic signalling by 8-SH-cGMP (Fig. 3).

Such electrophile sulfhydration by HS^- commonly occurs with all biological electrophiles, including 15d-PGJ_2, HNE, acrolein and fatty acid nitroalkene derivatives. The metabolic fate of these sulfhydrated derivatives allows the sorting of the electrophiles into three groups (*12*). In the first group (including 8-nitro-cGMP and 15d-PGJ_2), the SH derivative is so stable that no additional reaction occurs, except for oxidative degradation of SH by ROS and other reactive species. With the second group (including nitrooleic acid, 1,2-naphthoquinone [1,2-NQ], 1,4-naphthoquinone, *tert*-butylbenzoquinone, *N*-ethylmaleimide, diethylmaleate and monobromobimane), a relatively stable bis-product forms. The third group may include other highly reactive electrophiles, such as HNE and acrolein, for which additional metabolism and secondary chemical reactions follow the SH addition. 8-SH-cGMP seems to be an extremely stable end product among sulfhydrated derivatives of the electrophiles. Endogenous HS^--dependent sulfhydration occurs in different cultured cells, as evidenced by 8-SH-cGMP formation after addition of exogenous 8-nitro-cGMP (*12*).

Physiological Cell Signalling Mediated by 8-Nitro-cGMP

Selective and specific signalling dependent on the moderate electrophilicity of 8-nitro-cGMP

That 8-nitro-cGMP as an electrophile has unique chemical reactivity with protein Cys residues is now clear. As one example of such reactivity, 8-nitro-cGMP undergoes nucleophilic substitution with a protein sulfhydryl, releases the nitro group and forms a protein Cys-cGMP adduct in a process called protein *S*-guanylation, which is also said to be a PTM (*13*). Endogenous protein *S*-guanylation occurring in cells is a major focus of our present studies, because its identification may stimulate new research on oxidative stress and redox signalling (*30*).

During physiological signal transduction via protein *S*-guanylation, the relatively low electrophilicity or redox reactivity of 8-nitro-cGMP may be a critical factor that would determine the specificity of 8-nitro-cGMP in protein *S*-guanylation. For highly reactive electrophiles, nucleophilic amino acids other than Cys, especially histidine and lysine, also become targets in electrophilic reactions, *i.e.* Michael additions (*10*). Because these electrophiles have an extremely high electron-withdrawing potential, they undergo unstable, reversible *S*-alkylation, with the alkyl group being transferred at some point to other sulfhydryls of Cys of different proteins, in a process called transalkylation (*31*). This finding indicates that many endogenous electrophiles, but not 8-nitro-cGMP, may not necessarily cause site-specific PTMs, which can transduce signalling. To act as signalling molecules and have significant effects on sulfhydryls of acceptor proteins, reactive electrophiles may require specific reaction conditions or compartmentalization. Certain unique structural characteristics, yet unidentified, may be requirements for much of the stable covalent binding of electrophilic protein *S*-alkylation occurring near PTM sites. Because of the inert chemical reactivity of 8-nitro-cGMP as a moderate electrophile, however, *S*-guanylation occurs almost solely with sulfhydryls having high nucleophilicity, as determined by the pK_a of the Cys moiety sulfhydryls. This idea is strongly supported by the finding that a particular Keap1 Cys residue was highly susceptible to 8-nitro-cGMP-induced *S*-guanylation at a specific site (*13*, *16*). We previously reviewed, in detail, these differences between *S*-guanylation and other *S*-alkylation reactions induced by highly reactive electrophiles, as well as the critical role of Keap1 *S*-guanylation in adaptive responses to oxidative stress (*30*, *32*). In this regard, another major distinction between *S*-guanylation and other *S*-alkylation reactions is that *S*-guanylation appears to be a quite stable, irreversible sulfhydryl modification, because the nucleophilic nitro moiety of the purine structure is lost during formation of adducts with protein Cys residues. Our preliminary investigation showed that *S*-guanylated proteins may be readily degraded via autophagy, so that this irreversible PTM can be effectively compensated for by a translationally regulated rapid protein turnover (*33*; our unpublished observation).

H-Ras activation by site-specific S-guanylation

We recently found that *S*-guanylation of H-Ras, in addition to Keap1 *S*-guanylation, was involved in ROS signalling mediated by 8-nitro-cGMP (*12*). The small GTP-binding protein H-Ras works as a molecular switch in different intracellular signalling pathways (*34*). Structural remodelling of the heart in chronic heart failure is one of the inflammatory responses of the heart, and an inherited disorder (*i.e.* constitutive activation) of the H-Ras-mediated signalling pathway caused hypertrophic cardiomyopathy or congenital heart defects (*35*). Recent investigations of oxidative inflammatory reactions suggest that ROS- or reactive nitrogen oxide species-derivatives and the electrophile 15d-PGJ_2 can induce an H-Ras oncogenic cellular response (*36*, *37*). We applied affinity capture analyses using the GTP-bound Ras-binding domain of Raf to verify H-Ras activation in the failing heart, and we found a marked increase in H-Ras activation and simultaneous *S*-guanylation of activated H-Ras protein pulled down from hypertrophic heart tissue after myocardial infarction or transverse aortic constriction. A critical downstream cellular response to Ras activation was mediated by extracellular signal-regulated kinase (ERK) and p38 MAPK, which play a critical role in cardiac hypertrophy. Sustained ERK and p38 MAPK activation then induced activation of p53 and the retinoblastoma gene product (Rb), which have an essential function in cardiac cellular senescence (*38*, *39*). We found that *S*-guanylation-mediated H-Ras activation participated in ERK and p38 MAPK activation after myocardial infarction, which led to induction of p53/Rb-mediated cardiac cellular senescence and resulted in the transition from hypertrophy to heart failure.

LC-MS/MS sequencing analysis with recombinant H-Ras protein revealed *S*-guanylation of only Cys-184 of the H-Ras protein (Fig. 4). 8-Nitro-cGMP caused senescence of cardiac fibroblasts only when cells expressed wild-type H-Ras, not the C184S H-Ras mutant (*12*). Thus, the H-Ras Cys-184 is a highly susceptible nucleophilic sensor for 8-nitro-cGMP-induced protein *S*-guanylation. Among Ras isoforms, only H-Ras possesses two palmitoylation sites (Cys-181 and Cys-184) located at its carboxy-terminal domain, and palmitoylation of Ras proteins plays a key role in Ras protein localization and activity (Fig. 5A and B) (*34*, *40*). Monopalmitoylation of Cys-181 was required and sufficient for efficient trafficking of H-Ras to the plasma membrane, and GDP-bound H-Ras was detected predominantly in lipid rafts. GTP loading of H-Ras released H-Ras from the rafts so that they became more diffusely distributed in the plasma membrane, an event necessary for efficient activation of Raf. Although Cys-184 is not essential for targeting of H-Ras to the plasma membrane, it is required for control of GTP-regulated lateral segmentation of H-Ras between lipid rafts and non-rafts, which is necessary for efficient activation of Raf. Inhibition of Cys-184 by 8-nitro-cGMP palmitoylation efficiently delivered H-Ras to the plasma membrane with little Golgi or endosome pooling, which indicates that *S*-guanylation of H-Ras at Cys-184 promotes plasma membrane localization of

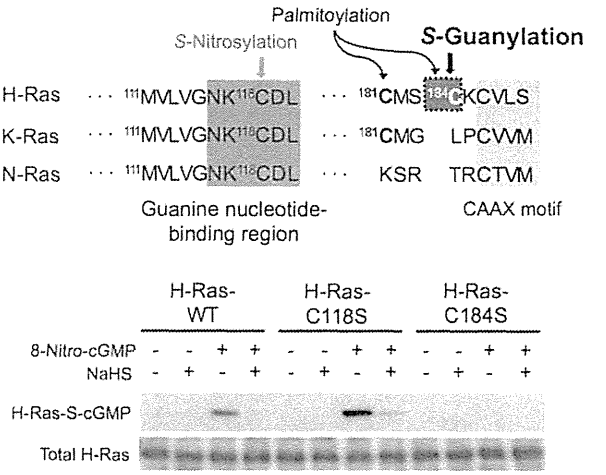

Fig. 4 Site-specific *S*-guanylation of H-Ras and its regulation by HS^-. 8-Nitro-cGMP treatment of cardiac fibroblasts induced *S*-guanylation of H-Ras when only wild-type (WT) H-Ras was expressed, not the C184S H-Ras mutant (lower panel, Western blotting), which indicates Cys-184-specific *S*-guanylation. NaHS (an H_2S donor) inhibited H-Ras *S*-guanylation. The upper panel shows the amino acid sequences of H-Ras (and K-Ras and N-Ras) around the *S*-guanylation target Cys-184 and other modification (palmitoylation and *S*-nitrosylation) sites. Schematic based on our recent finding (*12*), and modified from Nishida and Kitajima (*40*).

H-Ras and association with Raf by dissociating H-Ras from lipid rafts (Fig. 5A and B). Thus, our new findings provide evidence that electrophilic modification causes precise structural alterations so that H-Ras is accessible to the effector molecule Raf, which leads to transduction of electrophile-mediated signalling to downstream phosphorylation signalling pathways.

The Cys-184 of H-Ras may be chemically modified not only by 8-nitro-cGMP but also by HNE and 15d-PGJ_2, because HNE and 15d-PGJ_2 increased H-Ras activity in crude membrane preparations of rat cardiac fibroblasts. However, downstream signals of H-Ras, such as ERK and p38 MAP kinase, are not necessarily activated by reactive electrophiles such as HNE and 15d-PGJ_2 (our unpublished data), probably because these highly reactive electrophiles may nonspecifically affect signalling molecules other than H-Ras and may perturb specific signalling pathways. In contrast, another electrophile, 1,2-NQ, never modified Cys-184 of H-Ras and induced H-Ras activation, although 1,2-NQ strongly increased ERK activity in cardiomyocytes. In view of the much higher electrophilicity of HNE, 15d-PGJ_2, and other synthetic electrophiles such as 1,2-NQ compared with that of 8-nitro-cGMP, these findings strongly suggest that 8-nitro-cGMP acts as a specific physiological ligand for H-Ras activation.

Conclusion

ROS signalling is precisely regulated by various sensor and effector molecules. 8-Nitro-cGMP is a moderate electrophile compared with other electrophiles generated by ROS. This unique property of 8-nitro-cGMP

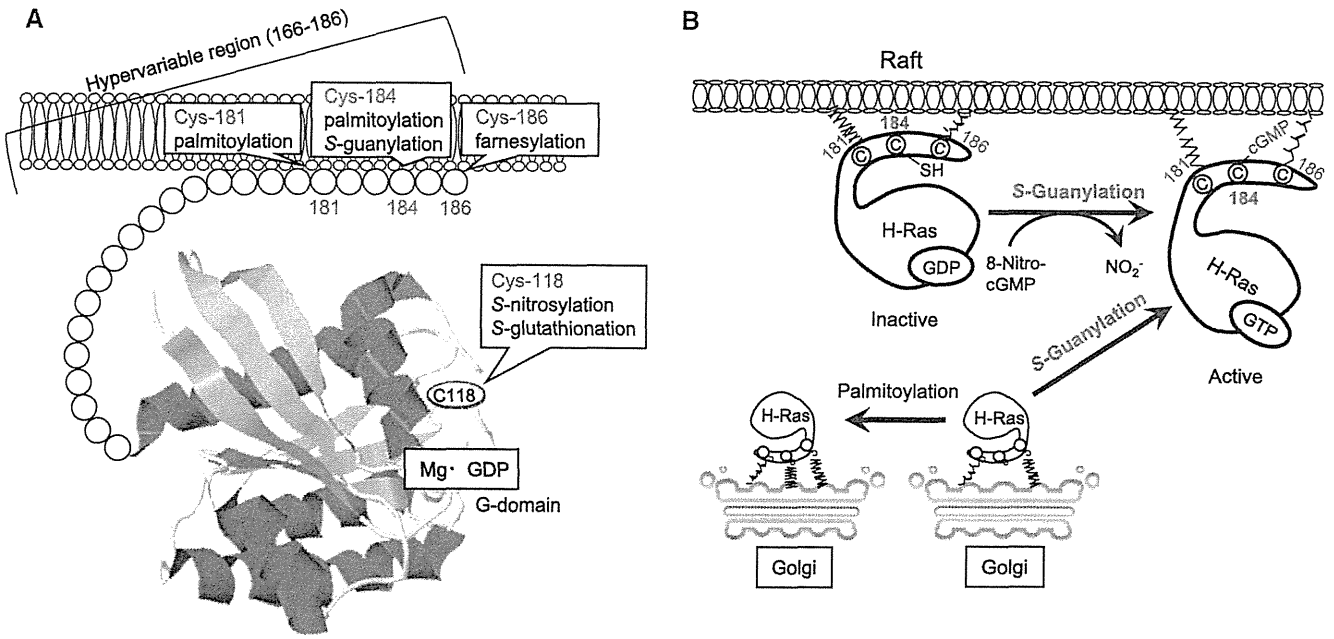

Fig. 5 Schematic illustration of the H-Ras structure (A) and the proposed mechanism of H-Ras activation via site-specific *S*-guanylation (B). (A) Locations of Cys residues susceptible to various modifications, including *S*-guanylation. (B) Membrane anchors of H-Ras in palmitoylation and farnesylation confer targeting specificity for membrane lipid rafts or Golgi membrane, and *S*-guanylation of Cys-184 would dissociate H-Ras from the lipid rafts or Golgi membrane to the non-raft membrane, which would cause increased interaction with effector proteins, including Raf, and activation of downstream signalling pathways. Schematic based on our recent finding (*12*), and modified from Nishida and Kitajima (*40*).

seems to contribute to its specificity and selectivity for ROS signal transduction. 8-Nitro-cGMP may thus be able to serve as a natural endogenous ROS ligand that can execute specific cellular responses through *S*-guanylation of particular Cys residues in redox sensor-effector proteins, such as Keap1 and H-Ras. The present evidence of protein *S*-guanylation induced by 8-nitro-cGMP may thus warrant further extensive study that may reveal new aspects of ROS- and NO-related redox chemical biology, physiology and pathophysiology, and pharmaceutical chemistry and may lead to development of therapeutics for oxidative stress-related diseases.

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Conflict of Interest

None declared.

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Hydrogen sulfide anion regulates redox signaling via electrophile sulfhydration

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An emerging aspect of redox signaling is the pathway mediated by electrophilic byproducts, such as nitrated cyclic nucleotide (for example, 8-nitroguanosine 3′,5′-cyclic monophosphate (8-nitro-cGMP)) and nitro or keto derivatives of unsaturated fatty acids, generated via reactions of inflammation-related enzymes, reactive oxygen species, nitric oxide and secondary products. Here we report that enzymatically generated hydrogen sulfide anion (HS^-) regulates the metabolism and signaling actions of various electrophiles. HS^- reacts with electrophiles, best represented by 8-nitro-cGMP, via direct sulfhydration and modulates cellular redox signaling. The relevance of this reaction is reinforced by the significant 8-nitro-cGMP formation in mouse cardiac tissue after myocardial infarction that is modulated by alterations in HS^- biosynthesis. Cardiac HS^-, in turn, suppresses electrophile-mediated H-Ras activation and cardiac cell senescence, contributing to the beneficial effects of HS^- on myocardial infarction-associated heart failure. Thus, this study reveals HS^--induced electrophile sulfhydration as a unique mechanism for regulating electrophile-mediated redox signaling.

Endogenous formation of the gaseous signaling mediator hydrogen sulfide (H_2S) has been demonstrated in mammalian cells and tissues[1,2], but its chemical nature and physiological functions remain poorly defined. Although reactive oxygen species (ROS) are typically viewed as toxic mediators of oxidative stress in aerobic organisms[3], ROS are now appreciated to mediate signal transduction events during both basal metabolism and inflammatory responses[4–6]. Electrophilic products can be formed via enzymatic oxidation reactions or reactions of ROS, nitric oxide and their secondary products[7–12]. Biological electrophiles can lend additional specificity to redox-dependent signal transduction via the nucleophilic substitution or Michael addition of electrophiles with cysteine sulfhydryls of various sensor or effector proteins to form their S-alkylation or S-arylation adducts[7,8]. Redox signal transduction reactions include those mediated by electrophilic byproducts of redox reactions[4,7], such as the electrophilic nucleotide 8-nitro-cGMP and multiple unsaturated fatty acid–derived oxo and nitro derivatives[7–12]. Aerobic cells rely on various oxidant-scavenging enzyme systems and low-molecular-weight scavengers to defend against the vicissitudes of oxidative stress[3]. Other than reactions with glutathione (GSH), however, the reactions modulating potential signaling and pathogenic actions of electrophilic species remain undefined.

Herein, we demonstrate that HS^-, rather than H_2S, regulates the metabolism and signaling functions of endogenous electrophiles. The nucleophilic properties of HS^- support a reaction with various electrophiles in cells via direct chemical sulfhydration (that is, electrophile sulfhydration). *In vivo* treatment of mice with HS^- ameliorated chronic heart failure after myocardial infarction, and improvement was partially a consequence of the sulfhydration of 8-nitro-cGMP generated in excess in cardiac tissues after myocardial infarction. These potent beneficial pharmacological effects stemmed from the sulfhydration of electrophilic 8-nitro-cGMP, which resulted in the suppression of a cellular senescence response induced by electrophile-dependent H-Ras activation in cardiomyocytes and cardiac tissues. This H-Ras activation involved downstream signaling pathways, including the Raf-extracellular signal–regulated kinase (ERK) and p38 mitogen-activated protein kinase (p38 MAPK) pathways, which led to activation of p53 and retinoblastoma protein (Rb). These data support that HS^--induced sulfhydration of electrophile species is a mechanism for terminating electrophile-mediated signaling and suggest a new therapeutic strategy for treating oxidative inflammation-related diseases[3,5–9].

RESULTS

HS^--producing enzymes involved in electrophile metabolism

To clarify the mechanisms regulating the metabolism and signaling actions of various electrophiles, we performed RNA interference (RNAi) screening that focused on cysteine metabolism and its redox-related metabolic pathways (**Supplementary Methods** and **Supplementary Results**, **Supplementary Table 1**). This method was

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