# Protein S-guanylation by the biological signal 8-nitroguanosine 3′,5′-cyclic monophosphate

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Received 30 May; accepted 1 August; published online 30 September 2007;
doi:10.1038/nchembio.2007.33

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**The signaling pathway of nitric oxide (NO) depends mainly on guanosine 3′,5′-cyclic monophosphate (cGMP). Here we report the formation and chemical biology of a nitrated derivative of cGMP, 8-nitroguanosine 3′,5′-cyclic monophosphate (8-nitro-cGMP), in NO-mediated signal transduction. Immunocytochemistry demonstrated marked 8-nitro-cGMP production in various cultured cells in an NO-dependent manner. This finding was confirmed by HPLC plus electrochemical detection and tandem mass spectrometry. 8-Nitro-cGMP activated cGMP-dependent protein kinase and showed unique redox-active properties independent of cGMP activity. Formation of protein Cys-cGMP adducts by 8-nitro-cGMP was identified as a new post-translational modification, which we call protein S-guanylation. 8-Nitro-cGMP seems to regulate the redox-sensor signaling protein Keap1, via S-guanylation of the highly nucleophilic cysteine sulfhydryls of Keap1. This study reveals 8-nitro-cGMP to be a second messenger of NO and sheds**

**light on new areas of the physiology and chemical biology of signal transduction by NO.**

NO has diverse physiological functions, such as vascular and neuronal signal transduction, host defense and cell death regulation. The main NO pathway is mediated by activation of soluble guanylate cyclase (sGC), which leads to production of cGMP[1]. The neuronal and vascular signaling mediated by NO is typically driven by the sGC-cGMP signaling pathway[2,3]. However, the existence of NO-related biological phenomena that are not necessarily affected by cGMP has been suggested. For example, NO function seems to be modified by different chemical reactions, such as S-nitrosylation and nitration of amino acids and proteins, which are induced by NO-derived reactive nitrogen oxides such as nitrogen dioxide ($NO_2$) and peroxynitrite[4–6].

We previously reported that during microbial infections, guanine nitration of nucleic acids occurs in a manner that is dependent on production of NO by the inducible isoform of NO synthase (iNOS)[7]. More importantly, we found that, of various nitrated guanine derivatives, 8-nitroguanosine has a highly redox-active property—that is, the potential to generate superoxide anion via activation by NADPH reductase-like enzymes including three NOS isoforms and P450 reductase[7,8]. This finding indicates that guanine nitration may not be a simple chemical modification but may instead have a biologically significant effect, particularly on cell functions.

Nitration of biological molecules such as L-tyrosine and L-tryptophan likely occurs as a result of iNOS expression: a large amount of NO generated by iNOS, in the presence of reactive oxygen species, can effectively produce reactive nitrogen oxides, which in turn induce nitration[5,6,9,10]. In view of the fact that the endothelial isoform of NOS (eNOS) is associated with guanine nitration, as evidenced by our recent immunochemical analysis[11], guanine nitration may be related to the cell signaling functions of NO rather than its cytotoxic effects. Although the biological consequence of guanine nitration remains unclear, a large pool of various nucleotides exists in the cytosol of cells, the primary location of 8-nitroguanine)[7,11–13]. We can therefore envisage guanine nitration occurring in the nucleotide, which is associated with cell signaling involving guanine-based signal nucleotides such as cGMP and GTP[14].

In the more than 40 years since the discovery of cGMP in nature[15,16], no biologically functioning derivatives of cGMP have been identified in mammals. Thus, it is of great interest to investigate possible NO-induced cGMP nitration involving NO-mediated signal transduction. Our present results reveal that NO-dependent guanine nitration of cGMP does occur, particularly under pathophysiological conditions, because a unique derivative of cGMP, 8-nitro-cGMP, was indeed produced in cells expressing iNOS. This study is the first to clarify formation of 8-nitro-cGMP and its potent signaling function in biological systems. More importantly, this new derivative 8-nitro-cGMP not only behaves as a native cGMP but also mediates an NO signal independent of the classical cGMP pathway. In addition, we identified a unique chemical property of 8-nitro-cGMP: as a potent electrophile, this endogenous nitroheteroaromatic readily reacts with sulfhydryls of glutathione (GSH) and proteins in cells to form 8-thioalkoxy-cGMP (8-RS-cGMP) adducts. Similar NO-related chemistry has been well documented for S-nitrosylation of sulfhydryls of GSH and proteins[17–21]. This chemical reaction of 8-nitro-cGMP may (at least in part) account for NO-induced signal transduction via a completely novel chemical modification of GSH and proteins that we have named 'S-guanylation'.

## RESULTS

### Immunocytochemistry of 8-nitro-cGMP formed in cells

We first synthesized a series of 8-nitroguanine derivatives including 8-nitroguanosine and its phosphorylated forms (**Fig. 1**). Certain derivatives (for example, 8-nitroguanosine and 8-nitro-cGMP) were used for preparation of monoclonal antibodies and for analysis of chemical, biochemical and pharmacological activities. For immunocytochemistry, we used primarily three clones of monoclonal antibodies for 8-nitroguanine and 8-nitro-cGMP (see **Supplementary Methods** online), whose specificities appear in **Supplementary Figure 1a** online.

Cultured RAW 264.7 cells (a mouse macrophage cell line) were stimulated with interferon-γ (IFN-γ) and lipopolysaccharide (LPS) to induce expression of iNOS and high NO production. Guanine nitration in cells was then analyzed via immunocytochemistry with anti-8-nitroguanine (clone NO2-52) and anti-8-nitro-cGMP (clones 1G6 and 1H7) monoclonal antibodies (**Fig. 2a**). Strong immunostaining of activated RAW 264.7 cells

was evident with the anti-8-nitro-cGMP antibody (1G6); moderate but substantial staining was observed with anti-8-nitroguanine (NO2-52) and anti-8-nitro-cGMP (1H7) antibodies. Treatment with the NOS inhibitor $N^{\omega}$-monomethyl-L-arginine (L-NMMA) completely eliminated this positive immunostaining. The same cells without stimulation showed only marginal immunoreactivity. These results indicate that NO derived from iNOS produces 8-nitroguanine- and 8-nitroguanosine-related compounds (for example, 8-nitro-cGMP) in cells. Similar immunostaining results were obtained with mouse peritoneal exudate macrophages treated with IFN-γ plus LPS: wild-type macrophages (**Supplementary Fig. 2a** online) showed marked immunoreactivity with anti-8-nitro-cGMP antibodies, whereas iNOS-deficient cells (**Supplementary Fig. 2b**) showed negligible reactivity. Confocal laser scanning microscopy showed 8-nitro-cGMP (1G6) immunostaining mainly in the cytosol of RAW 264.7 cells, and colocalization in part with mitochondria rather than with endoplasmic reticulum (**Fig. 2b**). Intracellular localization of 8-nitro-cGMP immunostaining was not appreciably affected, even after trypsin and RNase treatment of fixed cells, which suggests that 8-nitro-cGMP may remain near its sites of generation or may be associated with putative targets for 8-nitro-cGMP in the cells.

#### Chemical identification of 8-nitro-cGMP formed in cells

To clearly identify formation of 8-nitroguanine and 8-nitroguanosine derivatives (including 8-nitro-cGMP) in RAW 264.7 cells, 8-nitroguanosine derivatives present in cell lysate were first enriched by use of immunoaffinity column chromatography (anti-8-nitroguanine and anti-8-nitro-cGMP antibodies) and were then analyzed by means of HPLC coupled with electrochemical detection (ECD) (see **Supplementary Fig. 1b**–**d**). Of the derivatives, 8-nitro-cGMP was the major product of RAW 264.7 cells expressing iNOS; 8-nitroguanine and 8-nitroxanthine were minor nitrated products (**Fig. 3a**,**b**), and no appreciable peak of 8-nitroguanosine was observed with the cell lysates. To exclude the possibility that 8-nitro-cGMP was formed during sample preparation, we confirmed that adding cGMP to RAW 264.7 cell lysates does not affect the ECD peak for endogenous 8-nitro-cGMP. More than 90% of 8-nitro-cGMP was recovered in an ethanol-precipitated protein fraction digested by proteinase K, which suggests that 8-nitro-cGMP was mostly associated with intracellular proteins.

To further elucidate formation of 8-nitro-cGMP, we analyzed lysates via LC-MS/MS. MS/MS analysis of authentic 8-nitro-cGMP showed fragments at $m/z$ = 197, 179 and 151 (**Fig. 3c**). The MS/MS chromatogram ($m/z$: 391→197) of immunoaffinity-enriched cell lysate (**Fig. 3c**) was identical to the LC-MS/MS chromatogram data obtained for the authentic standard (**Fig. 3c**), which verified the chemical identification of 8-nitro-cGMP. To the best of our knowledge, this is the first unequivocal demonstration of biological 8-nitro-cGMP production in cells. With regard to the amounts of the nitrated compounds determined by immunoaffinity and HPLC plus ECD analyses, intracellular concentrations of the derivatives were 5.7 nM 8-nitroxanthine, 0.44 nM 8-nitroguanine and 67.2 nM 8-nitro-cGMP, as corrected for cell volume. It is notable that these three nitrated nucleotide derivatives were recovered from supernatants of cultures of activated RAW 264.7 cells (by the same analysis), although at much lower levels (30- to 80-fold) compared with the cellular content. In the same cells, the cGMP content analyzed using a commercial ELISA kit (GE Healthcare) was 8.4 ± 3.5 μM (**Supplementary Fig. 2d**). It is important to note that the anti-cGMP antibody used in the ELISA kit had strong cross-reactivity for 8-nitro-cGMP, and therefore we could not separate results for cGMP from those for nitrated and thiolated cGMP derivatives (for example, 8-nitro-cGMP and 8-RS-cGMP) via this traditional ELISA. However, 8-nitro-cGMP amounted to about 0.8% of the total cGMP as measured by ELISA.

#### Intracellular regulation of 8-nitro-cGMP formation

With NO-dependent 8-nitro-cGMP formation now clearly illustrated, we sought to clarify the regulation mechanism of 8-nitro-cGMP formation by focusing on the effect of intracellular sulfhydryls. We first examined the effect of GSH on production of 8-nitro-cGMP. Treatment with L-buthionine sulfoximine (BSO), a specific inhibitor of γ-glutamylcysteine synthetase (a key enzyme for GSH synthesis)[22], increased formation of 8-nitro-cGMP in RAW 264.7 cells by 8.1-fold (to 545.8 nM) (**Fig. 3a**,**b** and **Supplementary Fig. 2c**,**d**). This enhancing effect of BSO on 8-nitro-cGMP immunostaining was observed with glucose-starved cultured HepG2 cells treated with the NO donors *S*-nitrosoglutathione (GSNO) and *S*-nitroso-D,L-penicillamine (SNAP) (**Supplementary Fig. 3a** online). These results therefore suggest that GSH is critically involved in the regulation of 8-nitro-cGMP formation in these cells.

We then evaluated whether sGC is involved in biosynthesis of 8-nitro-cGMP. We treated RAW 264.7 cells with NS 2028, a potent inhibitor of sGC (ref. 23), during LPS plus IFN-γ stimulation. Both immunocytochemical and HPLC-ECD analyses showed that NS 2028 treatment almost completely suppressed formation of 8-nitro-cGMP in stimulated RAW 264.7 cells in the presence of BSO (**Supplementary Fig. 2d**). Concentrations of protein-bound 3-nitro-L-tyrosine, an index of reactive nitrogen species formation, increased after stimulation but were not affected by NS 2028 treatment. This NS 2028 treatment substantially reduced cGMP concentrations, which indicates that NS 2028 can indeed inhibit sGC activity under these conditions. We thus suggest that NS 2028 specifically inhibits 8-nitro-cGMP production without suppressing other nitration reactions in cells. In other words, 8-nitro-cGMP formation seems to depend on sGC activity expressed by RAW 264.7 cells.

#### Nucleophilic substitution in 8-nitro-cGMP by GSH

To clarify the mechanism of decrease in 8-nitro-cGMP formation caused by GSH, authentic 8-nitro-cGMP was reacted with GSH in a cell-free system. Analysis of the reaction via LC-MS (**Fig. 4a**) showed generation of a product with a molecular weight of 651, which indicates that an adduct of GSH and cGMP was formed via a chemical reaction between GSH and 8-nitro-cGMP. Extensive two-dimensional NMR experiments (see **Supplementary Methods**) with the isolated GSH adduct revealed that the adduct is an 8-thioalkoxy (8-RS) adduct of cGMP—that is, 8-*S*-glutathionyl-cGMP (8-GS-cGMP) (**Fig. 4a**). Thus we have named this process 'S-guanylation' of sulfhydryls.

This reaction seems to be brought about by nucleophilic replacement of the nitro group of 8-nitro-cGMP with a thiol of GSH. In fact, according to the equation GSH + 8-$NO_2$-cGMP $\rightarrow$ 8-GS-cGMP + $HNO_2$, we found stoichiometric production of nitrite ($NO_2^-$) via our HPLC-based Griess reagent flow reactor analysis (**Fig. 4b**). To confirm that intracellular GSH can indeed contribute to degradation of 8-nitro-cGMP and that $NO_2^-$ is derived from the nitro group, 8-$^{15}NO_2$-cGMP was incubated with lysate of RAW 264.7 cells with or without BSO treatment, followed by measurement of $NO_2^-$ produced in the reaction mixture. HPLC-Griess assay and electron spin resonance (ESR) analysis clearly revealed $^{15}NO_2^-$ production, in a manner dependent on the GSH concentration in the cells (**Fig. 4c**). Because of effective $NO_2^-$ release from 8-nitro-cGMP caused by GSH

and concomitant 8-GS-cGMP formation, the overall reaction involves denitration of 8-nitro-cGMP with simultaneous nucleophilic replacement of the nitro group of 8-nitro-cGMP with the sulfhydryl of GSH. A similar reaction was found for L-cysteine and 8-nitro-cGMP (**Fig. 4b**), and for L-Cys-Gly dipeptide and *N*-acetyl-L-cysteine with 8-nitro-cGMP, with different rate constants, which seemed to depend on the degree of ionic dissociation of sulfhydryls to form thiolate anion, determined by the p$K_a$ of each compound (**Supplementary Table 1** online). Because the thiolate anion is more nucleophilic than the corresponding thiol, the nucleophilicity of cysteine residues is thought to be an important determinant governing S-guanylation by 8-nitro-cGMP; this reaction is greatly influenced by the p$K_a$ of cysteine sulfhydryls, which may be affected by an ionic interaction with surrounding amino acid residues located near the cysteines of proteins.

These data suggest that 8-nitro-cGMP is susceptible to denitration and could, with sulfhydryls, generate 8-RS-cGMPs in general, although reaction efficiencies may differ, as indicated by the rate constants for reactions of 8-nitro-cGMP with different sulfhydryls (**Supplementary Table 1**).

#### Protein S-guanylation induced by 8-nitro-cGMP

Protein–8-RS-cGMP adducts can be formed after nucleophilic replacement in 8-nitro-cGMP by sulfhydryls of proteins. Western blotting with an anti-8-RS-cGMP antibody (see **Supplementary Methods** and **Supplementary Fig. 4a** online) clearly showed expression of protein–8-RS-cGMP adducts in RAW 264.7 cells stimulated with LPS and IFN-γ in the presence or absence of BSO (**Fig. 5a**), with an intense band observed in the presence of BSO (see the arrowhead in **Figure 5a**). We then performed a preliminary study to probe for S-guanylated proteins generated by NO. We first focused on the thiol-rich, redox-sensor signaling protein Kelch-like ECH-associated protein 1 (Keap1), because it has a number of highly nucleophilic cysteine residues[24,25] that we expected could be targets for S-guanylation by 8-nitro-cGMP. Indeed, Keap1 protein in RAW 264.7 cells was substantially S-guanylated under conditions of NO production, especially in the presence of BSO, compared with Keap1 in control cells (without NO treatment), as verified by Keap1 immunoprecipitation and subsequent western blotting with anti-8-RS-cGMP antibody (**Fig. 5a**). Similar results were obtained from western

blotting analyses for S-guanylated Keap1 using a monoclonal anti-8-RS-cGMP antibody that we recently developed (**Supplementary Methods** and **Supplementary Fig. 4b**). In addition, western blotting with anti-8-RS-cGMP antibody demonstrated more extensive protein S-guanylation in wild-type macrophages than in iNOS-deficient macrophages after *Salmonella enterica* serovar Typhimurium infection (**Fig. 5b**). Mouse peritoneal exudate macrophages infected with *S. enterica* Typhimurium expressed iNOS and induced 8-nitro-cGMP formation, as suggested by strong immunostaining with anti-8-nitro-cGMP antibodies. A more important finding was clear NO-dependent S-guanylation of Keap1 in *S.* Typhimurium–infected macrophages (**Fig. 5b**), which was much greater in wild-type cells than in iNOS-deficient cells. S-Guanylated protein expression was also observed in HepG2 cells treated with exogenous NO (**Supplementary Fig. 3b**).

To further assess S-guanylation of Keap1 in cells, we developed an HPLC-ECD system coupled with immunoaffinity enrichment to evaluate formation of 8-*S*-cysteinyl-cGMP (8-Cys-cGMP) in proteins (see **Supplementary Methods** and **Supplementary Fig. 4**). Keap1 obtained from RAW 264.7 cells by immunoprecipitation was subjected to pronase digestion, followed by immunoaffinity enrichment of 8-RS-cGMP adducts with the use of monoclonal anti-8-RS-cGMP antibody. We identified the 8-Cys-cGMP adduct formed endogenously with Keap1, as evidenced by the presence of a peak identical to that for authentic 8-Cys-cGMP in HPLC-ECD analysis (**Supplementary Fig. 4d**).

*In vitro* experiments with recombinant Keap1 protein revealed that Keap1 is highly susceptible to S-guanylation induced by 8-nitro-cGMP. Even in the presence of excess GSH (more than ~3,100-fold relative to Keap1, on the basis of the SH unit), a clear immunoreactive band was detected for S-guanylated Keap1 (**Fig. 5c**). For comparison, we used human $\alpha_1$-protease inhibitor ($\alpha_1$-PI), which has a single cysteine residue that is susceptible to S-nitrosylation induced by NO and nitrosothiols, as we previously reported[26]. No appreciable level of S-guanylated $\alpha_1$-PI was detected in the presence of GSH, however (**Fig. 5c**). Thus, Keap1 may be a target protein for 8-nitro-cGMP–induced S-guanylation in cells.

These results may indicate that not only GSH but also intracellular proteins having sulfhydryls that are highly reactive for electrophiles could react with 8-nitro-cGMP to form 8-RS-cGMP adducts.

#### Effects of 8-nitro-cGMP on vascular signal regulation

One of the most important functions of the NO-cGMP signaling pathway concerns vasorelaxation of vascular smooth muscle cells. We therefore investigated the pharmacological activity of 8-nitro-cGMP, specifically its regulation of vascular tone, in a vascular organ bath study. We analyzed the effect of 8-nitro-cGMP on vasoconstriction of rat carotid arteries precontracted with phenylephrine and compared this effect with that of 8-bromo-cGMP, a widely used cGMP analog. 8-Nitro-cGMP had a biphasic effect (**Fig. 6a** and **Supplementary Fig. 5a** online): a concentration higher than 10 μM produced relaxation of the precontracted vascular strip; this effect was similar to that of 8-bromo-cGMP except that vasorelaxation induced by 8-nitro-cGMP was three times stronger (**Fig. 6a**). In addition, 8-nitro-cGMP at concentrations less than 10 μM substantially enhanced vasoconstriction (**Fig. 6a**); this effect was noted even at submicromolar concentrations (**Fig. 6b** and **Supplementary Fig. 5b**) but was absent in vessels without endothelium. Because no such vasoconstriction was observed with 8-bromo-cGMP, endothelium-dependent vasoconstriction is unique for 8-nitro-cGMP.

We analyzed activation of cGMP-dependent protein kinase (PKG) via western blotting by assessing phosphorylation of vasodilator-stimulated phosphoprotein (VASP) of human uterine smooth muscle cells (**Fig. 6c**,**d**). Much like the vasorelaxation of rat vessels just described, both 8-nitro-cGMP and 8-bromo-cGMP had strong PKG-activating potential, as shown by VASP phosphorylation of smooth muscle cells, which was inhibited by the PKG-specific inhibitor Rp-8-CPT-cGMPS (8-(4-chlorophenylthio)-guanosine 3′,5′-cyclic monophosphorothioate, Rp isomer)[27] (**Fig. 6d**).

To confirm that cGMP derivatives added exogenously to cell cultures were delivered into the cells, in a separate experiment we examined the membrane permeability of cultured cells to cGMP and its derivatives. The degree of permeability was assessed via values obtained by the following formula: ((intracellular concentration of cGMPs increased after incubation)/(concentration of cGMPs added to the cell culture))

× 100 (%). Calculated values were 3.9%, 15.0% and 41.9% for cGMP, 8-nitro-cGMP and 8-bromo-cGMP, respectively. This result indicates that, although 8-nitro-cGMP could penetrate cell membranes more efficiently than did native cGMP, it was less efficient than 8-bromo-cGMP. We believe that the amounts of the various cGMP derivatives used in the present study could produce the physiologically relevant cellular responses that we observed.

We also examined 8-nitro-cGMP–dependent vasoconstriction as related to redox activity—that is, production of superoxide anion radical by accelerating the uncoupling reaction of various isoforms of NOS and P450 reductase—as we previously proposed for 8-nitroguanosine[7,8]. Enhanced vasoconstriction was completely nullified by treatment with superoxide dismutase (SOD) and tiron (1,2-dihydroxy-3,5-benzenedisulfonic acid), a low-molecular-weight SOD mimic (**Supplementary Fig. 5c**). More notably, 8-nitro-cGMP caused no apparent endothelium-dependent vasoconstriction of aortas from eNOS-deficient mice (**Fig. 6e** and **Supplementary Fig. 5d**), which suggests a contribution of eNOS to 8-nitro-cGMP vasoconstriction.

Our previously published reports describe the redox-active property of 8-nitroguanosine[7,8], but here we observed that it has a very weak vasoconstricting potential compared with 8-nitro-cGMP (it was more than 100 times less potent; **Fig. 6b** and **Supplementary Fig. 5b**). Because 8-nitroguanosine was barely detected in RAW 264.7 cells (**Fig. 3a**,**b**), and because among 8-nitroguanine–related compounds only 8-nitro-cGMP produced a significant vascular response, 8-nitro-cGMP may be a major vasoactive signaling molecule.

Pharmacological actions of cGMP are known to be strictly regulated by various isoforms of phosphodiesterases (PDEs)[28], which readily degrade cGMP to guanosine 5′-monophosphate (GMP) and nullify its effects—for example, vasodilation. 8-Nitro-cGMP, however, like 8-bromo-cGMP, was markedly resistant to PDE1 and PDE5 (**Supplementary Fig. 6a** online). In fact, treatment with the PDE inhibitor zaprinast did not appreciably affect the concentration-dependent curve for 8-nitro-cGMP–induced vasorelaxation in vessels without endothelium that we observed in the present organ bath study. This resistance to PDE may contribute to the potent pharmacological properties of 8-nitro-cGMP, not only as a cGMP analog but also as a

nitrated nucleotide having unique redox and electrophilic characteristics, as revealed by our present work.

Because 8-GS-cGMP was thought to be formed in cells producing 8-nitro-cGMP, as just described, we tested the effect of 8-GS-cGMP and its related 8-thioalkoxy adducts on the vasculature. We found native cGMP activity that induced vasorelaxation (**Supplementary Fig. 6b**). In addition, as with 8-nitro-cGMP, we observed PDE resistance for all 8-thioalkoxy adducts of cGMP tested, that is, 8-GS-cGMP, 8-Cys-cGMP and 8-*S*-cysteinyl-glycyl-cGMP (8-Cys-Gly-cGMP) (**Supplementary Fig. 6a**).

#### Redox-active property of 8-nitro-cGMP as electrophile

To clarify the redox activity of 8-nitro-cGMP, we performed ESR spectroscopy with a reaction mixture containing cytochrome P450 reductase and various isoforms of NOS. Superoxide formation was identified by means of ESR spin trapping using 5-(2,2-dimethyl-1,3-propoxycyclophosphoryl)-5-methyl-1-pyrroline *N*-oxide (CYPMPO) as a spin trap. 8-Nitro-cGMP, in a concentration-dependent fashion, markedly enhanced generation of superoxide from P450 reductase and all isoforms of NOS (**Fig. 7a**). This enhancement was evident with as low a concentration as 0.1 μM 8-nitro-cGMP, which indicates strong redox activity of this electrophilic compound (**Fig. 7b**). Such activity may contribute to the vasoconstriction induced by this compound, as mentioned. Clear formation of 8-nitro-cGMP anion radical was noted under anaerobic conditions via direct ESR analysis without the use of spin traps (**Fig. 7c**), which confirms redox recycling of 8-nitro-cGMP for effective superoxide generation. We previously reported a similar redox-active property of 8-nitroguanosine, but 8-nitroguanosine was more than 10 times less active compared with 8-nitro-cGMP (**Fig. 7d**). This reduced superoxide-generating potential of 8-nitroguanosine may explain its weak vasoconstriction compared with the vascular effect of 8-nitro-cGMP shown in **Figure 6b**. Also, the concentrations of 8-nitro-cGMP (around 100 nM) at which substantial superoxide formation was observed are likely to occur in cells expressing iNOS, as illustrated by the data report here.

These results suggest that the potent redox activity of 8-nitro-cGMP is biologically relevant and may permit diverse functions of cGMP—for example, by

modulating NO-dependent vascular signal transduction (for instance, endothelium-dependent relaxing factor (EDRF) activity), as the schematic in **Figure 7e** illustrates.

## DISCUSSION

Although more than 40 years has passed since cGMP was first discovered in nature[15,16], no bioactive compounds related to cGMP have been identified in biological materials from mammals. To our knowledge, our report is the first clarification of NO-dependent formation of 8-nitro-cGMP, a nitrated derivative of cGMP, and the potential role of this derivative as a second messenger in cells. Among the nitrated guanine derivatives examined in the present work, 8-nitro-cGMP had unique chemical, biological and pharmacological properties, which may mediate multiple signaling functions of NO and regulate cellular physiology in critical ways.

An important chemical action of this novel cyclic nucleotide is sulfhydryl modification of low-molecular-weight thiols (for example, GSH) and proteins via S-guanylation. Because of the electrophilic nature of 8-nitro-cGMP, sulfhydryls interact exclusively with its nitro group, which causes denitration and nucleophilic replacement and results in 8-RS-cGMP adduct formation and S-guanylation. Formation of 8-GS-cGMP may have physiological implications for cGMP-dependent signal transduction induced by NO. Even if 8-nitro-cGMP formed via NO is degraded by GSH, the resultant 8-GS-cGMP may still function biologically (in vasorelaxation) (**Supplementary Fig. 6b**), and acquisition of resistance to PDEs after 8-thiolation of cGMP may potentiate this effect (**Supplementary Fig. 6a**). However, it is also important to note that various exogenous electrophiles can undergo conjugation with GSH and that these GSH conjugates are transported extracellularly and metabolized to remove L-glutamate and glycine residues[29]. Indeed, our preliminary data suggest that more than 90% of 8-RS-cGMP adducts including protein adducts formed in cells are metabolized and excreted extracellularly within hours, via a yet-unidentified mechanism, so that they are recovered as low-molecular-weight 8-RS-cGMP derivatives such as 8-Cys-cGMP and 8-GS-cGMP. Therefore, intracellular signal transduction of 8-nitro-cGMP is regulated by thiol-containing compounds (for example, GSH) in cells, and the 8-RS-cGMP adducts formed may be further metabolized extracellularly to terminate the

intracellular signaling by 8-nitro-cGMP and even 8-RS-cGMP. Meanwhile, sulfhydryls may have another important regulatory function for 8-nitro-cGMP production in cells. Specifically, intracellular GSH and low-molecular-weight thiols not only contribute to 8-nitro-cGMP degradation, but may also suppress its formation by preventing the nitration of its precursor, because of their potent scavenging activities for reactive nitrogen oxides[30].

In addition, protein S-guanylation induced by 8-nitro-cGMP may be regarded as a new type of NO-dependent post-translational modification, which may be closely related to NO-induced signaling pathways. For example, the transcriptional factor NF-E2–related factor 2 (Nrf2) upregulates phase 2 detoxification enzymes and antioxidant systems, whereas Keap1 blocks its transcriptional activity via cytoplasmic sequestration of Nrf2—that is, Keap1 attaches Nrf2 to actin filaments in the cytoplasm[24,25]. The Keap1 molecule has abundant cysteine residues and is thus readily modified chemically by various electrophilic substances and reactive oxygen species. Because chemically modified Keap1 loses its capacity to bind to Nrf2, which allows nuclear export of Nrf2 and activation of transcriptional activity of Nrf2, it can serve as a redox sensor for induction of phase 2 and antioxidant enzymes such as heme oxygenase 1 (HO-1)[24,25]. In fact, our present preliminary analysis showed that Keap1 expressed by various cultured cells was highly susceptible to S-guanylation induced by NO-dependent 8-nitro-cGMP production (**Fig. 5** and **Supplementary Fig. 4d**). It is therefore highly plausible that 8-nitro-cGMP can act as an endogenous electrophilic ligand and affect Keap1 sulfhydryls via S-guanylation, which would lead to antioxidant signaling. In other words, 8-nitro-cGMP may be a signaling molecule that mediates an adaptive response to oxidative and nitrative stress. Further study is required to verify this hypothesis concerning such an 8-nitro-cGMP–dependent Keap1-Nrf2 pathway.

Researchers recently provided data showing that nitrated fatty acids (for example, nitrated linoleic acid and oleic acid) functioning as anti-inflammatory mediators can undergo formation of adducts with GSH and proteins[31,32]. Because these endogenous nitroalkene derivatives also induced potent HO-1 upregulation in RAW 264.7 cells, the signaling mechanism mediated by nitrated fatty acids may resemble that mediated by 8-nitro-cGMP. Unique NO-related signal transduction may therefore exist that depends on nitration of various biological molecules, including nucleic acids and fatty acids.

Another unique chemical characteristic of 8-nitro-cGMP is its redox activity as an electrophile. We previously identified similar redox activity of 8-nitroguanosine[7,8]. It became clear from the current analysis, however, that 8-nitro-cGMP shows much greater electrophilic potential than does 8-nitroguanosine in terms of regulation of vascular tone—that is, eNOS-dependent EDRF inhibition—and also that 8-nitro-cGMP has potentially strong vasorelaxing activity via PKG activation (**Fig. 6**). This paradoxical effect actually results from 8-nitro-cGMP–enhanced production of superoxide from eNOS, via electron uncoupling induced by 8-nitro-cGMP (**Fig. 7**). The antagonistic action of 8-nitro-cGMP with EDRF may be beneficial in compensating for excessive vasorelaxation, particularly during NO overproduction.

The dependence of the major signaling pathway of NO on cGMP is now well established[1]. cGMP is generated from GTP via sGC activated by NO and is degraded by PDEs[14,28]. The action of cGMP is frequently mediated by PKG, although cGMP can also regulate other intracellular targets, such as cGMP-gated ion channels[33]. However, the existence of biological phenomena related to NO but not necessarily mediated by cGMP alone has been suggested[34]. One important chemical modification of biological molecules caused by NO is nitration caused by reactive nitrogen oxides such as $NO_2$ formed via NO and reactive oxygen species, or, the most common example, 3-nitro-L-tyrosine formed from L-tyrosine[4,5]. Nitration of specific tyrosine residues of proteins affects several cell functions, by modulating cytoskeletal and mitochondrial activities, for example[4,5,34].

In the present study, we examined whether NS 2028 treatment has any effect on 3-nitro-L-tyrosine production in activated RAW 264.7 cells. The results (**Supplementary Fig. 2d**) indicated that 1.0 μM NS 2028 abolishes 8-nitro-cGMP formation but not tyrosine nitration occurring in RAW 264.7 cells. These data therefore suggest that NS 2028 specifically inhibits 8-nitro-cGMP production by preventing formation of cGMP and thus lowering the steady state concentration of the major low-molecular-weight target of nitration, rather than by suppressing general nitration reactions in these cells. It is therefore most likely that 8-nitro-cGMP production occurs in a manner that is dependent on sGC.

NO-dependent nitration of various biological molecules now attracts considerable attention not only because of pathophysiological consequences of nonspecific nitration of amino acids and nucleotides but also because of physiological processes regulated by

nitration reactions not yet identified. In fact, as we verified here, 8-nitro-cGMP has potent redox-active and electrophilic properties, which seem to be critically involved in its unique signaling potential. 8-Nitro-cGMP had the highest redox activity among 8-nitroguanosine derivatives tested, with redox activity decreasing in the following order: 8-nitro-cGMP > 8-nitroguanosine > 8-nitroguanosine 5′-monophosphate (8-nitro-GMP) ≅ 8-nitroguanosine 5′-triphosphate (8-nitro-GTP) (only negligible redox activity was observed with 8-nitroguanine) (refs. 7,8 plus our present observations). Moreover, our studies report novel sulfhydryl and post-translational modifications of proteins, which correlate well with the redox activity of each 8-nitroguanine nucleoside-nucleotide derivative. It is therefore logical to expect that 8-nitro-cGMP that forms physiologically in cells has new biological functions achieved by nitration, in addition to the inherent pharmacological and signaling potential of cGMP.

In conclusion, the present discovery of 8-nitro-cGMP and its unique chemical properties now sheds light on an as-yet unrecognized area of NO and cGMP signal transduction, which should have important implications in NO-related physiology and pathology.

## METHODS

**Cell treatment.** RAW 264.7 cells were cultured at 37 °C in a humidified 5% $CO_2$ atmosphere in DMEM (4.5 g $l^{-1}$ glucose; GibcoBRL Life Technologies) supplemented with 1% penicillin-streptomycin (GibcoBRL), 1% MEM nonessential amino acid solution (GibcoBRL) and 10% fetal bovine serum (FBS). Cells were plated at densities of $2 \times 10^7$ cells per dish in 100-mm culture dishes for preparation of cell lysate and $3 \times 10^5$ cells per chamber in BD Falcon CultureSlides (BD Biosciences) for immunocytochemistry. To study formation of 8-nitro-cGMP by endogenous NO from iNOS, cells were stimulated with 10 μg $ml^{-1}$ LPS (Sigma) and 100 U $ml^{-1}$ IFN-γ (R&D Systems) for 11 h. In some experiments, L-NMMA (1 mM, Sigma) was added to the cell culture to inhibit NOS activity during the stimulation period. To determine the effect of depletion of cellular GSH on elimination of 8-nitro-cGMP, 1 mM BSO (Sigma) was added to the medium before (12 h) and during the stimulation period (11 h). We also examined the effect of an sGC inhibitor on formation of 8-nitro-cGMP. RAW 264.7 cells were treated with 1 μM NS 2028 (Cayman Chemical) during the stimulation period. For

studies of the effect of *S.* Typhimurium infection, mouse peritoneal exudate macrophages were isolated from wild-type and iNOS-deficient mice, as reported recently[35]. Cells were then plated at a density of $5 \times 10^5$ cells per chamber in CultureSlides and were infected with *S enterica* Typhimurium LT2 at a multiplicity of infection of 10. For preparation of cell lysates, cells were plated at a density of $1 \times 10^6$ cells per well in 12-well plates.

**HPLC-ECD of intracellular 8-nitro-cGMP.** We measured intracellular levels of 8-nitro-cGMP and related compounds by means of HPLC-ECD. RAW 264.7 cells were plated in 100-mm culture dishes at a density of $2 \times 10^7$ cells per dish. After stimulation with IFN-γ (100 U $ml^{-1}$) and LPS (10 μg $ml^{-1}$), cells were washed with phosphate-buffered saline (PBS) and were solubilized in 1 ml of RIPA buffer (10 mM Tris-HCl, 1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, pH 7.4) containing 1 mM *N*-ethylmaleimide to block sulfhydryl-mediated decomposition of 8-nitro-cGMP during sample preparation. Cell lysates from five dishes were combined and used for one analysis. The lysates were treated with 0.8 mg $ml^{-1}$ of proteinase K (Takara Bio) at 56 °C for 3 h, followed by filtration with a 5,000-Da cutoff membrane to remove high-molecular-weight compounds. In some experiments, cells were treated with 1 ml of 80% ethanol instead of direct solubilization in RIPA buffer. The cell suspension was then centrifuged to obtain supernatant (soluble fraction) and precipitate (protein fraction). The soluble fraction was dried under vacuo and was reconstituted with 10 mM Tris-HCl (pH 7.4). The protein fraction was resuspended in 10 mM Tris-HCl (pH 7.4) and was treated with 0.8 mg $ml^{-1}$ proteinase K at 56 °C for 3 h, followed by filtration with a 5,000-Da cutoff membrane. These samples were then subjected to immunoaffinity enrichment, as reported recently[36]. Briefly, the mouse monoclonal anti-8-nitroguanine (NO2-52) and anti-8-nitro-cGMP (1G6) antibodies were immobilized to protein A–sepharose 4B gels (GE Healthcare)[36]. The antibody-immobilized gels (1 ml) were then packed in a polystyrene column (Pierce). Cell lysates were applied to the column, which was washed with PBS (10 ml) and water (10 ml). 8-Nitro-cGMP and related compounds were eluted from the column with 5 ml of 85% methanol. Samples were dried under vacuo and were redissolved in 0.3 ml of water, followed by HPLC separation with a reverse-phase column (150 mm long, 3.0 mm inner diameter; Eicom Pak SC-5 ODS, Eicom) eluted with 0.4 ml $min^{-1}$ of 200 mM sodium phosphate buffer (pH 3.0) plus 8% acetonitrile. 8-Nitro-cGMP derivatives were detected electrochemically via an online

reductive activation method[36]. Electrode settings were −500 mV (first cell for reduction) and +250 mV (second cell for oxidation) (HTEC-500 and PEC-510; Eicom). With the current electrode setting, 8-nitroguanine derivatives were specifically converted by the first electrode (−500 mV) to electrochemically active compounds that could be detected by the second electrode (+250 mV), whereas normal and other 8-substituted guanine derivatives such as 8-oxo-2′-deoxyguanosine were not subject to redox conversion (**Supplementary Fig. 1b**–**d**) and hence could not be detected by the second electrode.

**S-Guanylation of recombinant Keap1 by 8-nitro-cGMP.** His-tagged mouse recombinant Keap1 was expressed in *Escherichia coli* and purified as described elsewhere[37]. Human $\alpha_1$-PI (Chemo-Sero-Therapeutic Institute) was pretreated with Blue Sepharose CL-6B (Amersham Biosciences) to remove the contaminant albumin. Keap1 and $\alpha_1$-PI (each at 3.2 μM as cysteine residue) were incubated with 8-nitro-cGMP (1–100 μM) in 100 mM sodium phosphate buffer (pH 7.4) with 0.1 mM diethylenetriaminepentaacetic acid and 0.5 mM dithiothreitol, with or without 10 mM GSH, at 37 °C for 3 h. In a separate experiment, $\alpha_1$-PI was incubated with 1,000 μM 8-nitro-cGMP in the absence of GSH at 37 °C for 20 h. All of these samples were electrophoresed in a single SDS-PAGE gel, followed by western blotting with polyclonal anti-8-RS-cGMP antibody (**Supplementary Methods** and **Supplementary Fig. 4a**). Therefore, all images were obtained under the same conditions including the antibody incubation period and exposure time, and immunoreactive bands were detected by using a chemiluminescence reagent (ECL Plus; GE Healthcare, Amersham Biosciences) with a luminescent image analyzer (LAS1000UV mini, Fuji Photo Film). Band intensities were quantified densitometrically by means of NIH Image (http://rsb.info.nih.gov/nih-image/index.html).

**Immunoprecipitation.** To identify formation of S-guanylated Keap1 in cells, we performed immunoprecipitation with anti-Keap1 antibody (goat polyclonal, E-20; Santa Cruz Biotechnology), followed by western blotting with the use of anti-Keap1 (rat monoclonal; see **Supplementary Methods**) and anti-8-RS-cGMP antibodies. RAW 264.7 cells were stimulated with LPS and IFN-γ in the presence or absence of BSO as mentioned earlier. Mouse peritoneal exudate macrophages, obtained from wild-type and iNOS-deficient mice, were infected with *S. enterica* Typhimurium LT2 strain (multiplicity of infection, 10). Cell lysates obtained were incubated at 4 °C for 12 h with

anti-Keap1 polyclonal antibody in RIPA buffer with protease inhibitors. After an additional 2 h of incubation with protein A/G plus agarose (Santa Cruz Biotechnology), the immune complex was retrieved and subjected to western blot analysis (100 μg per lane).

**Other methods.** Detailed information is available in **Supplementary Methods**.

*Note: Supplementary information and chemical compound information is available on the Nature Chemical Biology website.*

## ACKNOWLEDGMENTS

We thank J.B. Gandy for her excellent editing of the manuscript. Thanks are also due to K. Kaneko and Y. Ishima (Kumamoto University), and Y. Unno (University of Shizuoka) for their technical assistance; T. Honda and Z. Liu (Nagoya University) for 2'-*O*-succinyl-8-nitro-cGMP preparation; H. Taguchi (Tohoku University) for technical assistance with NMR experiments; K. Ichimori (AIST Kansai) and D.J. Stuehr (The Cleveland Clinic) for providing various recombinant NOSs; K.I. Tong (University of Tsukuba) for preparation of recombinant Keap1; and T. Yoshimura, K. Yoshida and A. Molla for reading our paper to evaluate its concepts and interdisciplinary accessibility. This work was supported in part by grants-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) and from the Ministry of Health, Labor and Welfare of Japan.

## AUTHOR CONTRIBUTIONS

T.S. performed chemical and biochemical analyses. M.H.Z., T.O. and T.A. performed immunocytochemistry. Y.T. and S.K.-M. performed organ bath experiments. H.I. performed tandem MS experiments. A.K. and M.Y. produced recombinant Keap1 and its antibody. S.F. performed western blot analyses. H.A. synthesized 8-nitro-cGMP and related compounds. T.A. designed and analyzed experiments and wrote the paper. All authors discussed the results and commented on the manuscript.

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**Figure 1** Chemical structures of 8-nitro-cGMP and related compounds.

**Figure 2** Immunocytochemical analysis of endogenous guanine nitration in mouse macrophages. (**a**) RAW 264.7 cells were stimulated with IFN-γ (100 U $ml^{-1}$) plus LPS (10 μg $ml^{-1}$) for 11 h. We performed analyses with three monoclonal antibodies: anti-8-nitroguanine (NO2-52) and anti-8-nitro-cGMP (1G6 and 1H7) antibodies. (**b**) Localization of 8-nitro-cGMP (1G6) immunostaining in RAW 264.7 cells stimulated with IFN-γ (100 U $ml^{-1}$) plus LPS (10 μg $ml^{-1}$) for 11 h. Anti-histone H1 antibody, anti-Bip antibody and anti-Hsp60 antibody were used to stain specifically for the nucleus, endoplasmic reticulum (ER) and mitochondria, respectively. These antibodies were labeled with Alexa Fluor 488 (green); 1G6 was labeled with Alexa Fluor 555 (red). Merged images acquired via confocal laser scanning microscopy are shown. Scale bars are 50 μm in **a** and 25 μm in **b**.

**Figure 3** Identification of 8-nitro-cGMP and related compounds formed in activated RAW 264.7 cells. (**a**,**b**) HPLC-ECD analyses of 8-nitro-cGMP and related compounds formed in RAW 264.7 cells stimulated with IFN-γ (100 U $ml^{-1}$) plus LPS (10 μg $ml^{-1}$) for 11 h without (**a**) or with (**b**) 1 mM BSO treatment. The solid line corresponds to a detector response obtained by applying voltages of −500 mV (first cell) and +250 mV (second cell); the dotted line corresponds to a detector response obtained by applying voltages of +100 mV (first cell) and +250 mV (second cell). (**c**) LC-MS/MS (*m*/*z*: 391→197) analysis of authentic 8-nitro-cGMP (upper panel) and 8-nitro-cGMP formed in RAW 264.7 cells (lower panel). Results shown in the bottom panel were obtained with the same

sample as that used for **Figure 3b**. Insets show the fragmentation pattern and mass spectrum of authentic 8-nitro-cGMP. c.p.s., counts per second.

**Figure 4** Reactions of 8-nitro-cGMP with sulfhydryls of GSH. (**a**) HPLC analysis of products of the reaction between 8-nitro-cGMP (10 μM) and GSH (10 mM) (left panels). The upper right panel shows an MS peak eluted at 20.9 min by HPLC. NMR studies established the chemical structure of the reaction product 8-GS-cGMP (lower right panel). (**b**) $NO_2^-$ release during reaction of 8-nitro-cGMP (10 μM) with 10 mM GSH or L-cysteine. Data represent means ± s.d. ($n$ = 3). (**c**) $NO_2^-$ release from 8-nitro-cGMP (50 μM) with RAW 264.7 cell lysates treated with BSO (left panel). NO obtained from $NO_2^-$ was captured by a complex of iron and *N*-(dithiocarboxy)sarcosine $(DTCS)_2$ for ESR analysis. The standard $^{14}NO\text{-}Fe^{2+}\text{-}(DTCS)_2$ complex provided a triplet signal, whereas the $^{15}NO\text{-}Fe^{2+}\text{-}(DTCS)_2$ complex gave a doublet signal (upper panels). A doublet signal was also detected when $^{15}NO_2$-cGMP was incubated with cell lysates (lower panel, on the right).

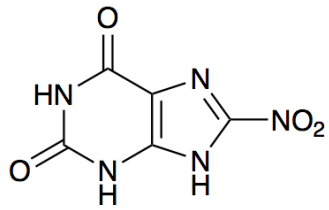
**Figure 5** Protein S-guanylation in cells and Keap1 induced by 8-nitro-cGMP. (**a**) Formation of protein–8-RS-cGMP adducts in RAW 264.7 cells. Western blotting (WB) results are given for 8-RS-cGMP adducts (left panel) and for Keap1 and β-actin (upper right panels). Lower right panels show western blotting results for 8-RS-cGMP and Keap1 with immunoprecipitated (IP) proteins from RAW 264.7 cells. Relative band intensities of S-guanylated Keap1 (Keap1–8-RS-cGMP) versus total Keap1 are given at the bottom. (**b**) Formation of protein–8-RS-cGMP adducts in macrophages, obtained from wild-type (WT) and iNOS-deficient (KO) mice, during *S.* Typhimurium infection. IP and WB analyses were performed as for the data in **a**. (**c**) Keap1 S-guanylation *in vitro*. Keap1 or $\alpha_1$-PI was treated with 8-nitro-cGMP in the presence or absence of GSH and subjected to WB analyses with anti-8-RS-cGMP antibody.

**Figure 6** Modulation of vascular tone by 8-nitro-cGMP. (**a**) 8-Nitro-cGMP concentration-response curves for rat carotid artery rings with or without endothelium (left panel), and relaxation of artery rings without endothelium in response to 8-nitro-cGMP or 8-bromo-cGMP (right panel). (**b**) Modulation of vascular tone by 8-nitro-cGMP and 8-nitroguanosine. Rat carotid artery rings precontracted with 0.1 μM phenylephrine were incubated with 8-nitro-cGMP or 8-nitroguanosine. (**c**) Phosphorylation of 46/50 kDa VASPin human uterine smooth muscle cells treated with

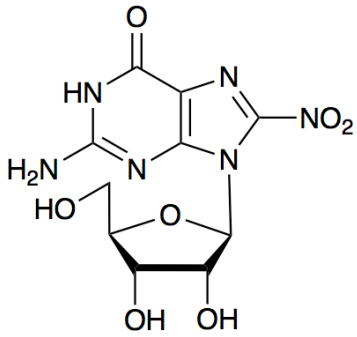
10 μM 8-nitro-cGMP or 8-bromo-cGMP. Total VASP and phosphorylated VASP were quantified by use of western blotting. (**d**) Effect of a PKG inhibitor (Rp-8-CPT-cGMPS, 10 μM) on 8-nitro-cGMP–induced phosphorylation of VASP in human uterine smooth muscle cells was examined in the same manner as in **c**. (**e**) Effects of 8-nitro-cGMP on vascular tone of aortas obtained from wild-type and eNOS-deficient ($eNOS^{-/-}$) mice. Data represent means ± s.d. ($n$ = 3–5).

**Figure 7** Generation of 8-nitro-cGMP–stimulated superoxide from P450 reductase and NOS isoforms. (**a**) ESR signals of CYPMPO-OOH adducts formed in the reaction mixture containing enzyme (0.2 μM), NADPH (0.2 mM), diethylenetriaminepentaacetic acid (0.1 mM) and CYPMPO (2 mM) with or without 10 μM 8-nitro-cGMP. The chemical structure of the CYPMPO-OOH adduct is shown at the top. (**b**) Increased CYPMPO-OOH formation in the P450 reductase/NADPH system after addition of various concentrations of 8-nitro-cGMP. (**c**) Direct ESR measurements of 8-nitro-cGMP anion radical. Hyperfine splitting constants (mT) of each signal for $^{14}NO_2$-cGMP ($\alpha_N(NO_2)$, 1.221; $\alpha_N(N^7)$, 0.323; $\alpha_N(N^9)$, 0.082) and for $^{15}NO_2$-cGMP ($\alpha_N(NO_2)$, 1.700; $\alpha_N(N^7)$, 0.323; $\alpha_N(N^9)$, 0.082), with a $g$ value of 2.0046. (**d**) Comparison of superoxide (CYPMPO-OOH adduct) formation by 8-nitro-cGMP and 8-nitroguanosine (1 μM each) in the P450 reductase/NADPH system. (**e**) Hypothetical scheme for 8-nitro-cGMP–mediated vascular contraction involving eNOS uncoupling leading to superoxide generation. Data represent means ± s.d. ($n$ = 4).

Fig. 1




8-Nitroxanthine

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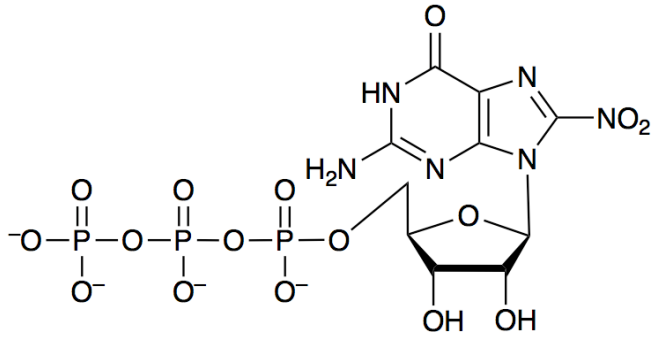


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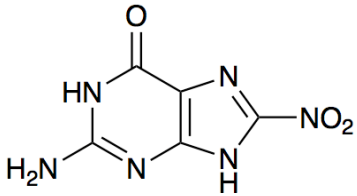


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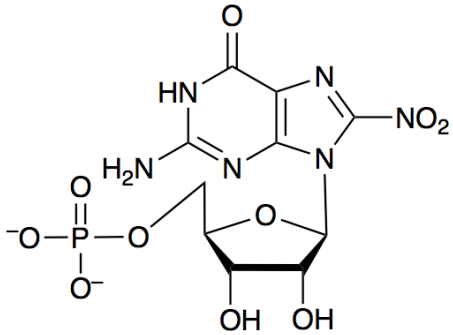
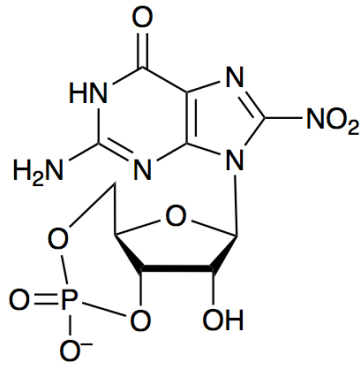



8-Nitro-GMP




8-Nitro-cGMP

Fig. 2

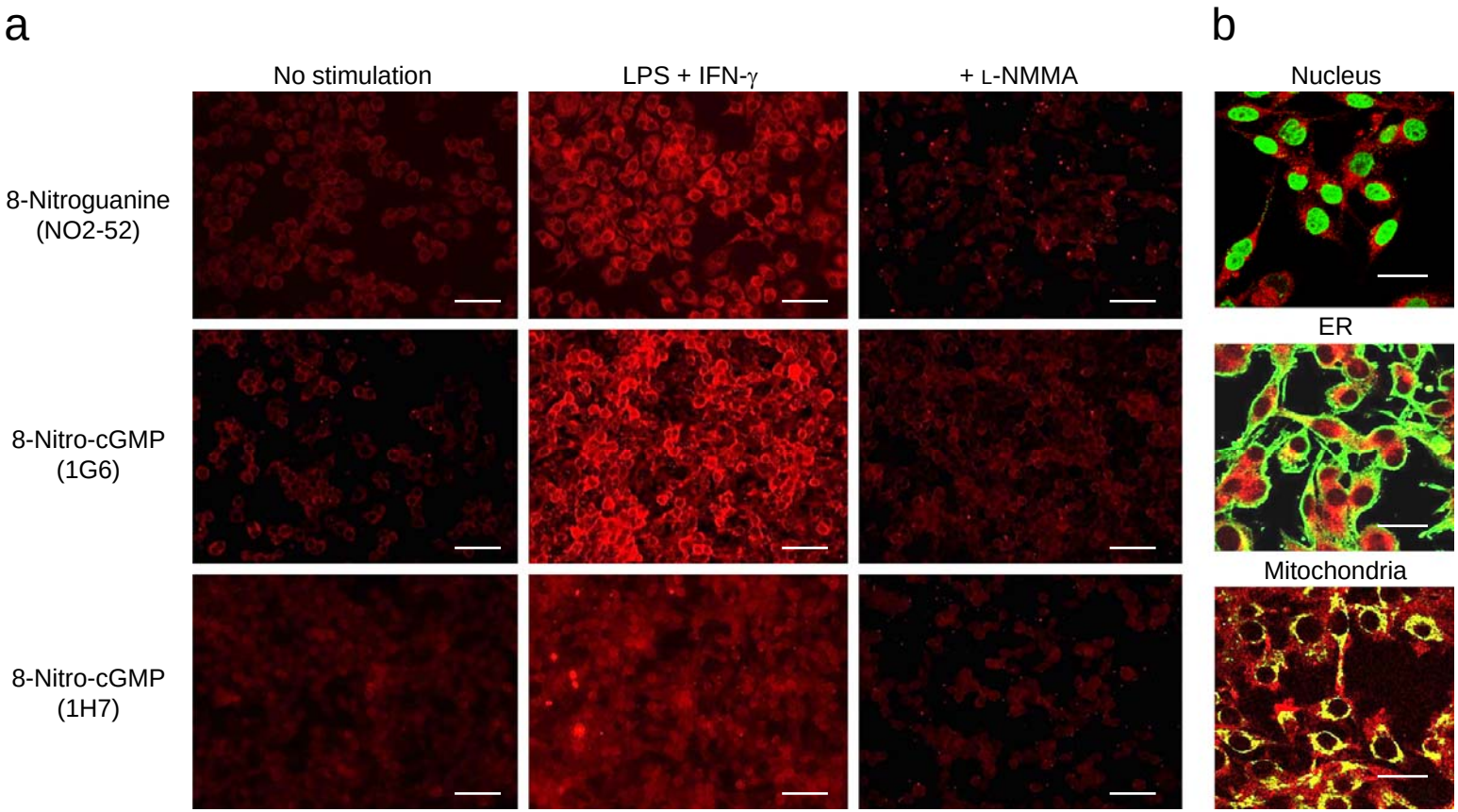

a
No stimulation
LPS + IFN-γ
+ L-NMMA
8-Nitroguanine
(NO2-52)
8-Nitro-cGMP
(1G6)
8-Nitro-cGMP
(1H7)
b
Nucleus
ER
Mitochondria

Fig. 3

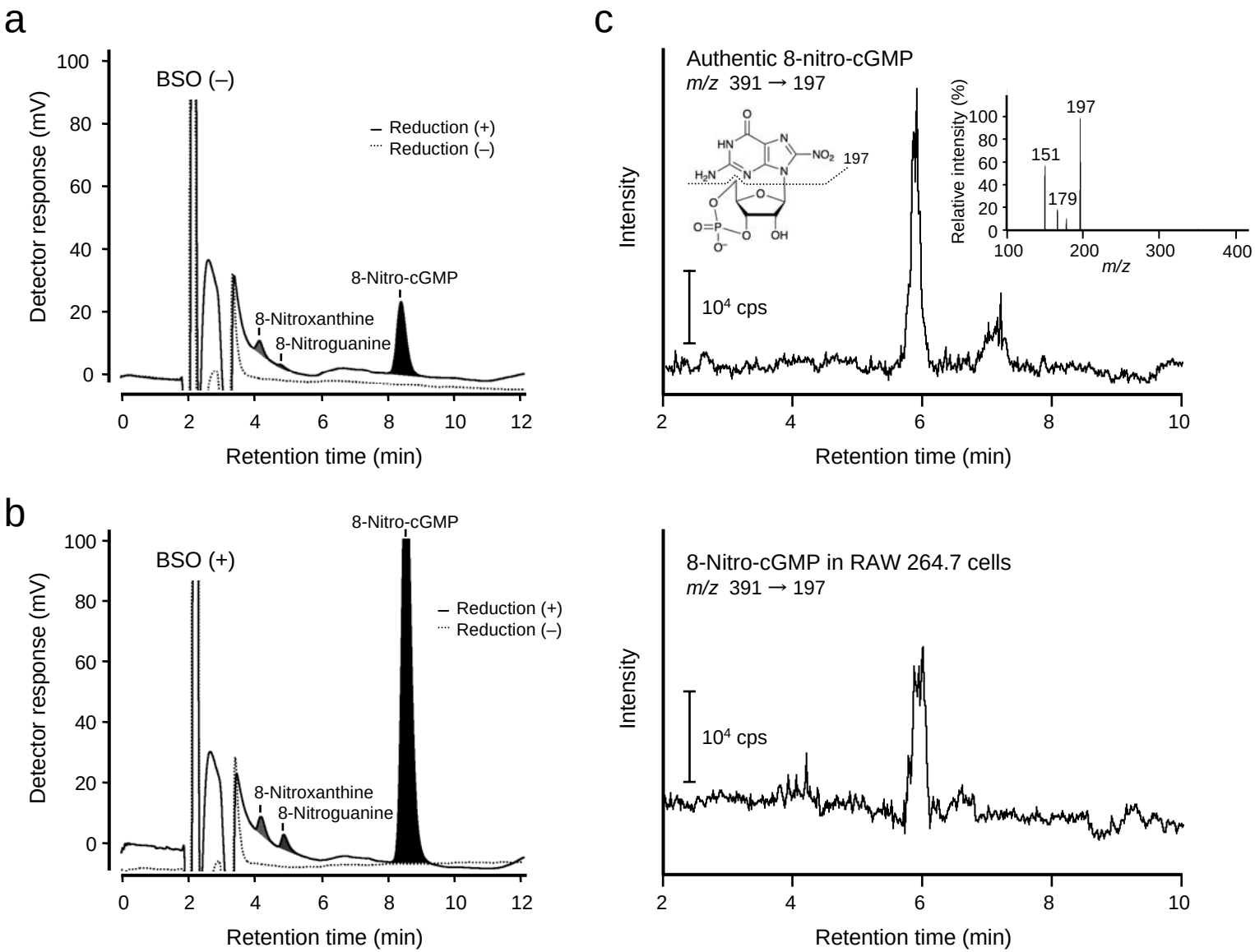

a
BSO (–)
Reduction (+)
Reduction (–)
8-Nitro-cGMP
8-Nitroxanthine
8-Nitroguanine
Detector response (mV)
Retention time (min)
b
BSO (+)
8-Nitro-cGMP
Reduction (+)
Reduction (–)
8-Nitroxanthine
8-Nitroguanine
Detector response (mV)
Retention time (min)
c
Authentic 8-nitro-cGMP
m/z 391 → 197
197
Relative intensity (%)
151
179
m/z
Intensity
10⁴ cps
Retention time (min)
8-Nitro-cGMP in RAW 264.7 cells
m/z 391 → 197
Intensity
10⁴ cps
Retention time (min)

Fig. 4

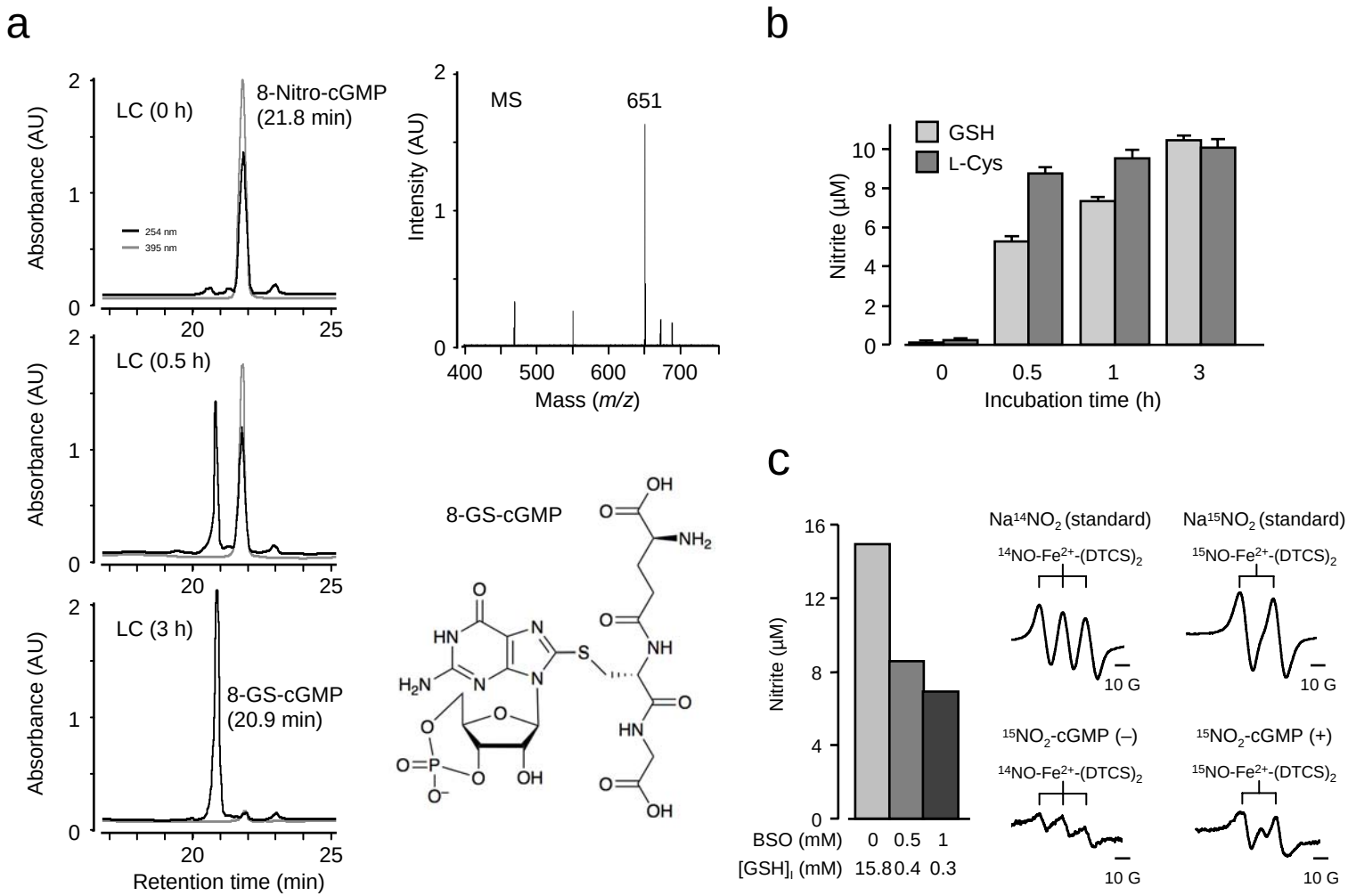

a
LC (0 h)
8-Nitro-cGMP
(21.8 min)
254 nm
395 nm
Absorbance (AU)
LC (0.5 h)
LC (3 h)
8-GS-cGMP
(20.9 min)
Retention time (min)
MS
651
Intensity (AU)
Mass (m/z)
8-GS-cGMP
b
GSH
L-Cys
Nitrite (μM)
Incubation time (h)
c
Nitrite (μM)
BSO (mM) 0 0.5 1
[GSH]i (mM) 15.8 0.4 0.3
Na14NO2 (standard)
Na15NO2 (standard)
14NO-Fe2+-(DTCS)2
15NO-Fe2+-(DTCS)2
15NO2-cGMP (–)
15NO2-cGMP (+)
10 G

Fig. 5

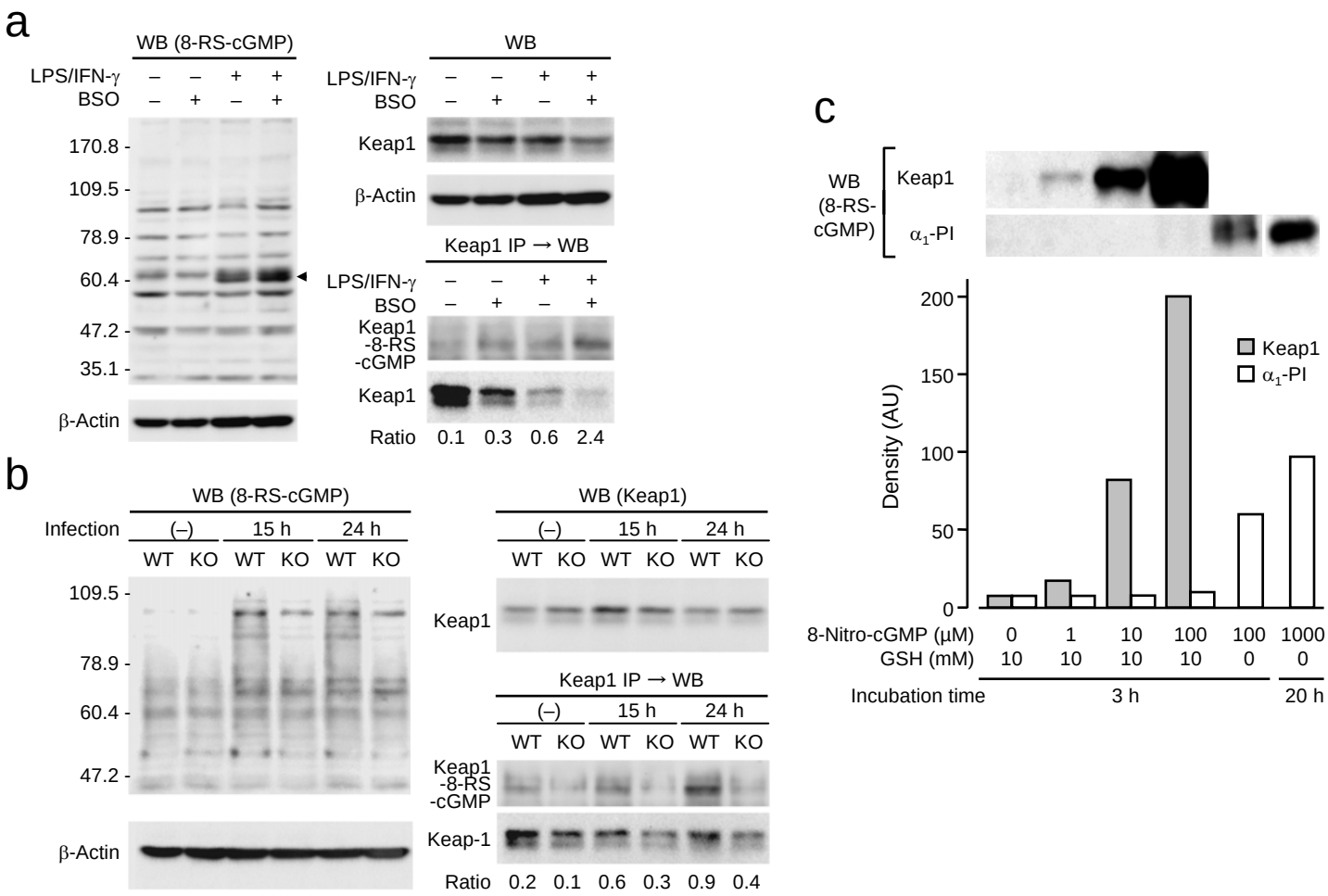
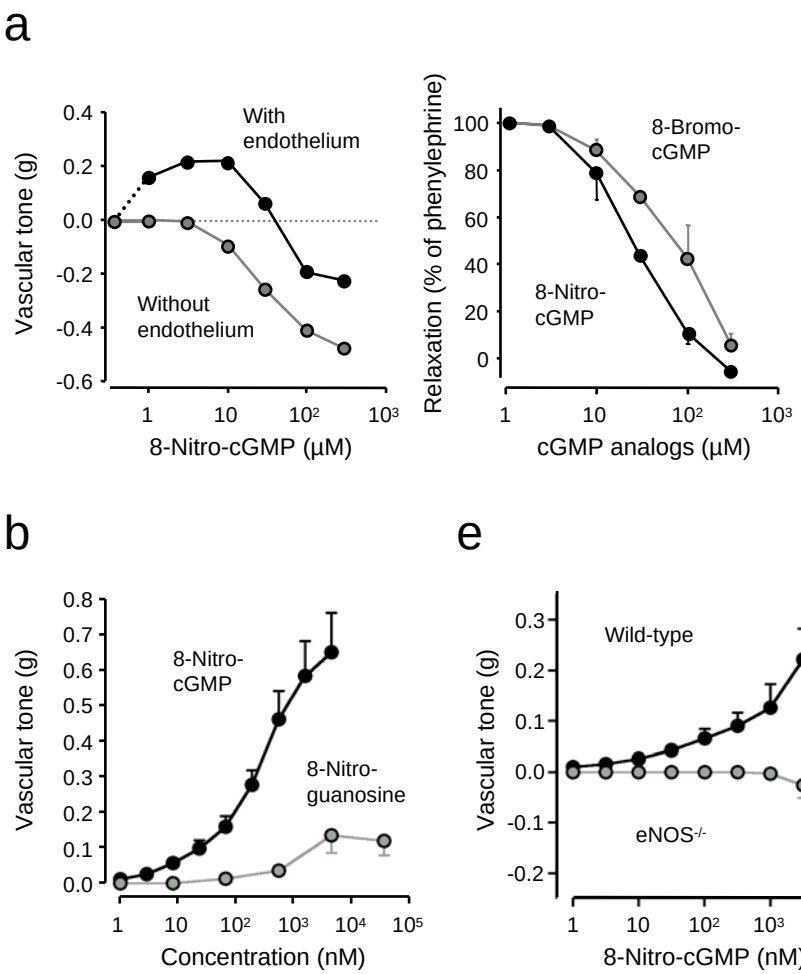

a
WB (8-RS-cGMP)
LPS/IFN-γ – – + +
BSO – + – +
170.8
109.5
78.9
60.4
47.2
35.1
β-Actin
WB
LPS/IFN-γ – – + +
BSO – + – +
Keap1
β-Actin
Keap1 IP → WB
LPS/IFN-γ – – + +
BSO – + – +
Keap1 -8-RS -cGMP
Keap1
Ratio 0.1 0.3 0.6 2.4
b
WB (8-RS-cGMP)
Infection (–) 15 h 24 h
WT KO WT KO WT KO
109.5
78.9
60.4
47.2
β-Actin
WB (Keap1)
(–) 15 h 24 h
WT KO WT KO WT KO
Keap1
Keap1 IP → WB
(–) 15 h 24 h
WT KO WT KO WT KO
Keap1 -8-RS -cGMP
Keap-1
Ratio 0.2 0.1 0.6 0.3 0.9 0.4
c
WB (8-RS-cGMP)
Keap1
α1-PI
Density (AU)
200
150
100
50
0
Keap1
α1-PI
8-Nitro-cGMP (μM) 0 1 10 100 100 1000
GSH (mM) 10 10 10 10 0 0
Incubation time 3 h 20 h

Fig. 6


a
Vascular tone (g)
With endothelium
Without endothelium
8-Nitro-cGMP (µM)
Relaxation (% of phenylephrine)
8-Bromo-cGMP
8-Nitro-cGMP
cGMP analogs (µM)
b
Vascular tone (g)
8-Nitro-cGMP
8-Nitro-guanosine
Concentration (nM)
e
Vascular tone (g)
Wild-type
eNOS-/-
8-Nitro-cGMP (nM)

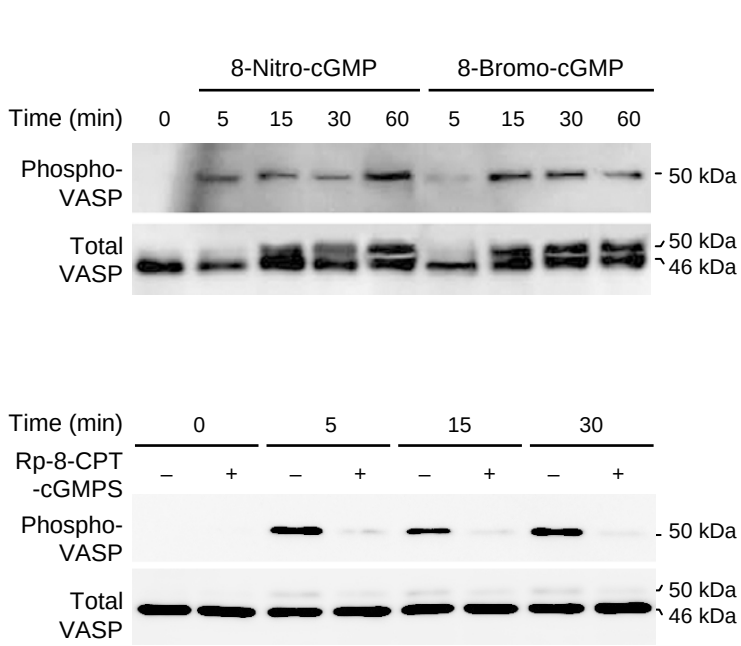
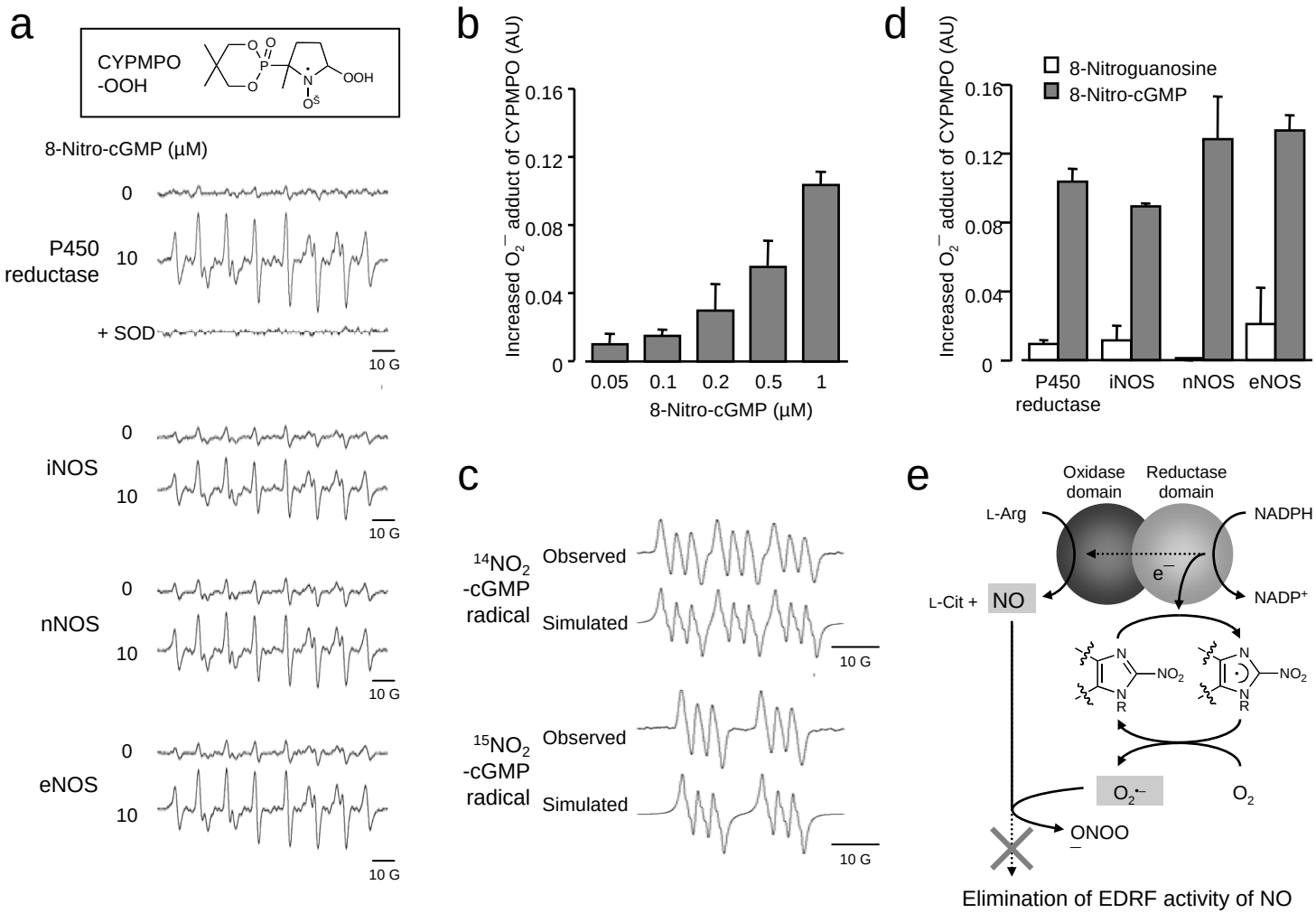

c
8-Nitro-cGMP
8-Bromo-cGMP
Time (min)
0 5 15 30 60 5 15 30 60
Phospho-VASP
50 kDa
Total VASP
50 kDa
46 kDa
d
Time (min)
0 5 15 30
Rp-8-CPT-cGMPS
– + – + – + – +
Phospho-VASP
50 kDa
Total VASP
50 kDa
46 kDa

Fig. 7




Elimination of EDRF activity of NO

# Graphical Abstract