

Supplementary Information for:

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

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Supplementary Methods

Synthesis of 8-nitroguanosine 3',5'-cyclic monophosphate (8-nitro-cGMP). We synthesized 8-nitro-cGMP by two reactions: (i) bromination at the C8 position of the guanosine moiety, and (ii) nucleophilic substitution of the bromo group with NO_2^- . In brief, guanosine 3',5'-cyclic monophosphate (cGMP, 150 mg, 0.4 mmol, ICN Biomedicals) was reacted with bromine (0.5 ml; Wako Pure Chemical Industries) in 5 ml of formamide on ice for 30 min. The reaction was terminated by adding 2 ml of aniline. 8-Bromoguanosine 3',5'-cyclic monophosphate (8-bromo-cGMP) was extracted with water and purified by means of HPLC with a preparative reverse-phase column (TSKgel ODS-80Ts, 300 mm long \times 21.5 mm inner diameter; Tosoh) eluted with 3.5 ml min^{-1} of 0.02% trifluoroacetic acid plus 20% methanol monitored at 254 nm. The yield of 8-bromo-cGMP was 53.4 mg (35.6%). 8-Bromo-cGMP (35 mg, 0.12 mmol) thus obtained was reacted with sodium nitrite (34.5 mg, 0.5 mmol) in 1.5 ml of dimethyl sulfoxide containing 23 mM HCl at 70 °C for 5 days. The pH of the solution was adjusted to be 9 by adding 1 M NaOH, after which 2 volumes of butanol was added to the solution. 8-Nitro-cGMP was extracted from this solution with water and was purified via HPLC, in a manner similar to that used for 8-bromo-cGMP. The yield of 8-nitro-cGMP was 11.1 mg (20.8%): $^1\text{H NMR}$ (400 MHz, $\text{DMSO-}d_6$): δ : 4.06 (1H, ddd, $j=4.9, 10, 10$ Hz), 4.28 (1H, ddd, $J=1.7, 10, 10$ Hz), 4.43 (1H, ddd, $J=20, 10, 4.9$ Hz), 4.83 (1H, d, $J=5.4$ Hz), 5.02 (1H, ddd, $J=10, 5.4, 1.7$ Hz), 6.00 (1H, br s), 6.33 (1H, s), 7.05 (2H, br s), 11.3 (1H, s); UV/Vis: λ_{max} 253, 275, 390 nm (solvent: methanol); MS (ESI, negative): calcd. for $\text{C}_{10}\text{H}_{11}\text{N}_6\text{O}_9\text{P}$ ($[\text{M-H}]^-$), 389.02; found, 389.10.

^{15}N -labeled 8-nitro-cGMP (8- $^{15}\text{NO}_2$ -cGMP) was synthesized with ^{15}N -labeled sodium nitrite (Shoko).

Syntheses of 8-nitroguanosine derivatives. 8-Nitroguanosine, 8-nitroguanosine 5'-monophosphate (8-nitro-GMP), 8-nitroguanosine 5'-diphosphate (8-nitro-GDP, **33**), and 8-nitroguanosine 5'-triphosphate (8-nitro-GTP) were synthesized as reported previously^{1,2}. 8-Nitroguanine and 8-nitroxanthine were obtained as byproducts during syntheses of 8-nitroguanosine and 8-nitroxanthosine (**34**). RNA containing 8-nitroguanine was prepared by reacting yeast RNA (2 mg ml⁻¹ in 0.1 M sodium phosphate buffer, pH 7.4, Wako Pure Chemical Industries) with 1.5 mM peroxyxynitrite. The purified compounds were identified by means of their UV absorption spectra, molecular mass, and NMR spectra. Note that, during the synthesis of these compounds, we found 8-nitro-cGMP to be very stable in solution even at low pH, whereas 8-nitroguanosine and its phosphorylated derivatives (e.g., 8-nitro-GMP, 8-nitro-GDP and 8-nitro-GTP) decayed spontaneously in solution, particularly at acidic pH, within a few hours. 8-Nitro-2'-deoxyguanosine (**35**) and its phosphorylated forms were extremely unstable: they decomposed readily via spontaneous depurination within 1 h, even at neutral pH.

Synthesis of 2'-O-succinyl-8-nitroguanosine-3', 5'-cyclic monophosphate

(2'-O-succinyl-8-nitro-cGMP, 36). Aqueous solution of 8-nitro-cGMP (15.7 mg, 0.04 mmol in 1.5 ml H₂O) was added triethylamine (0.177 ml, 1.28 mmol) and succinic anhydride (64.0 mg, 0.644 mmol) and stirred at room temperature for 1 h. The reaction mixture was concentrated and lyophilized. The product was purified by HPLC column (Develosil RP AQUEOUS, 250 mm long × 20 mm inner diameter, Nomura Chemical Co., Ltd.) eluted with methanol: 0.1% trifluoroacetic acid (30:70) to yield 11.5 mg (59%) of a yellow powder: ¹H NMR (400 MHz, DMSO-*d*₆): δ: 2.50-2.67 (4H, m), 4.09 (1H, ddd, J=4.6, 9.5 Hz), 4.27 (1H, t, J=9.5 Hz), 4.49 (1H, m), 5.21 (1H, dd, J=5.9, 9.5 Hz), 5.93 (1H, d, J=5.9 Hz), 6.49 (1H, s), 7.11 (2H, br s), 11.4 (1H, s); MS (ESI, negative): calcd. for C₁₄H₁₅N₆O₁₂P ([M-H]⁻), 489.04;

found, 489.74.

Antibody generation. Mouse monoclonal antibodies that specifically recognized the 8-nitro-cGMP structure were generated by immunizing BDF1 mice (SLC) with 8-nitro-cGMP-conjugated bovine serum albumin (BSA, Sigma). We conjugated 8-nitro-cGMP to BSA through succinyl coupling between the 2'-OH group of 8-nitro-cGMP and lysine residues in BSA. BSA (3 mg) dissolved in 0.75 ml of 0.1 M morpholinoethanesulfonic acid (pH 5.5) was reacted with 2'-*O*-succinyl-8-nitro-cGMP (1.4 mg, 2.8 μ mol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (**37**) (1.5 mg, 7.8 μ mol, Dojindo Laboratories) at room temperature for 16 h in the dark. Reaction mixture was then dialyzed against phosphate-buffered saline (PBS) overnight at 4 °C. We began immunization by intraperitoneal injection of an 8-nitro-cGMP-BSA conjugate plus Freund's complete adjuvant, followed by injection of antigen with Freund's incomplete adjuvant. After the antibody titer in the blood increased, the spleen was harvested for preparation of splenocytes, which were fused with murine myeloma cells (SP2/0) for production of hybridomas. Cells were fused via polyethylene glycol, and hybridomas were selected by incubation in hypoxanthine, aminopterin, and thymidine medium. Hybridomas were screened with an ELISA and then cloned by limited dilution.

Among antibody clones generated in this study, we used two clones of monoclonal anti-8-nitro-cGMP antibodies, clones 1G6 and 1H7, for immunocytochemistry and preparation of immunoaffinity columns. We also used monoclonal anti-8-nitroguanine antibody (clone NO2-52) prepared recently^{3,4}. Specificities of these antibodies are shown in **Supplementary Fig. 1a** online.

The polyclonal anti-8-thioalkoxy (RS)-cGMP antibody was raised in rabbits by subcutaneous injection of the 8-RS-cGMP adduct of BSA, which was prepared by reacting

8-nitro-cGMP with BSA, plus Freund's complete adjuvant. A booster dose of the same antigen plus Freund's incomplete adjuvant was given four times every 2 weeks. Putative contamination with anti-BSA antibody was eliminated by using a BSA-conjugated HiTrap NHS-activated HP column (Amersham Biosciences). Finally, the IgG fraction was purified from antiserum by using Protein A-Sepharose CL-4B gel (Amersham Biosciences).

Similar to the protocol used for obtaining anti-8-nitro-cGMP antibodies described above, the mouse monoclonal anti-8-RS-cGMP antibody was generated by immunizing BDF1 mice with the 8-RS-cGMP adduct of BSA.

The specificity of these antibodies was confirmed by means of a competitive ELISA, as described below.

Competitive ELISA. To determine the specificity of the antibodies produced, we used competitive ELISA as described previously¹. Briefly, each well of a 96-well microtiter plate was coated with 100 μ l of the 8-nitro-cGMP-BSA conjugate ($5 \mu\text{g ml}^{-1}$) or 8-RS-cGMP-BSA conjugate ($0.5 \mu\text{g ml}^{-1}$) in PBS, blocked with 0.5% gelatin, and washed three times with PBS containing 0.05% Tween 20 (washing buffer). Wells were incubated at room temperature for 1 h with 0.1 ml of antibodies ($0.1 \mu\text{g ml}^{-1}$) in the presence or absence of various bases and nucleosides dissolved in washing buffer. The wells were then washed with washing buffer three times and reacted with horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody, followed by reaction with *o*-phenylenediamine dihydrochloride (**38**). The reaction was terminated by addition of 0.05 ml of 2.0 M sulfuric acid, and absorbance at 490 nm was read by using a micro-ELISA plate reader.

HepG2 cell culture. We used cultured human hepatoma cells (HepG2) to study the effect of exogenous NO exposure on 8-nitro-cGMP formation. Our preliminary study showed that

HepG2 cells cultured under conditions of glucose starvation had more intense immunostaining for 8-nitro-cGMP when treated with NO donors such as *S*-nitrosoglutathione (GSNO) and *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP) than did HepG2 cells that had not been glucose starved. HepG2 cells were cultured, at 37 °C in a humidified 5% CO₂ atmosphere, in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% penicillin-streptomycin, 1% nonessential amino acid, and 10% fetal bovine serum (FBS). Cells were plated at densities of 3×10^5 cells/chamber in CultureSlides (BD Biosciences). For glucose starvation, cells were cultured in DMEM without glucose. To investigate formation of 8-nitro-cGMP via exogenous NO donors, cells were treated for 1 h with 500 μM SNAP or GSNO in DMEM without glucose supplemented with 1% nonessential amino acid and 10% FBS (glucose-free medium). FBS, that was dialyzed against 150 mM NaCl, was used for glucose-free medium. Cells were then washed with glucose-free medium, followed by further incubation for 2 or 11 h in glucose-free medium. In some experiments, cells were treated with 1 mM L-buthionine sulfoximine (BSO, Sigma) before (12 h), during, and after SNAP or GSNO treatment. Formation of 8-nitro-cGMP was determined immunocytochemically as described for RAW 264.7 cell culture.

Immunocytochemistry. To examine the generation and localization of 8-nitro-cGMP and related compounds in the cells, we performed immunocytochemistry with the use of anti-8-nitro-cGMP monoclonal antibodies (1G6 or 1H7) prepared as described above, and anti-8-nitroguanine antibody (NO2-52)^{3,4}. Cells plated in CultureSlides and treated as described in **Methods** were washed with PBS and fixed with Zamboni solution (4% paraformaldehyde and 10 mM picric acid (**39**) in 0.1 M phosphate buffer, pH 7.4) at 4 °C for 7 h. After three washes with PBS, cells were permeabilized with 0.5% Triton X-100 at room temperature for 15 min and washed with PBS. In some experiments, fixed cells were

treated with trypsin (0.1%, GibcoBRL) or RNaseA (0.1 mg ml⁻¹, Sigma) for 30 min at room temperature. Cells were then washed 3 times with PBS. To block nonspecific antigenic sites, cells were incubated at 4 °C overnight with BlockAce (Dainippon Pharmaceutical). Cells were then incubated overnight at 4 °C with the monoclonal antibody (10 µg ml⁻¹) in PBS with 10% goat serum, after which they were rinsed three times with PBS and incubated for 1 h at room temperature with Cy3-labeled goat anti-mouse IgG antibody (PA43002, 10 µg ml⁻¹; Amersham Biosciences) in PBS with 10% goat serum. Cells were washed with PBS, covered with SlowFade Light Antifade Kit (S-7461; Molecular Probes), and examined under a fluorescence microscope (excitation at 568 nm; model Eclipse E1000, Nikon). Images were digitized and stored in PICT format by using a Color Chilled 3CCD Camera C5810 (Hamamatsu Photonics KK). Image processing and quantification were performed by using Adobe Photoshop Elements v.2.0 (Adobe Systems). To localize anti-8-nitro-cGMP (1G6) immunostaining in RAW 264.7 cells, anti-histone H1 antibody (MS-628-P0, NeoMarkers), anti-Bip antibody (SPA-827, Stressgene), and anti-Hsp antibody (SC-13966, Santa Cruz Biotechnology) were used to stain specifically for the nucleus, ER, and mitochondria, respectively. These antibodies were labeled with ZenonTM Alexa Fluor 488 mouse IgG_{2a} labeling kit (green, Invitrogen); 1G6 was labeled with ZenonTM Alexa Fluor 555 mouse IgG₁ labeling kit (red, Invitrogen).

Determination of cellular glutathione (GSH). We determined cellular GSH levels by means of HPLC with a reverse-phase column as described previously⁵. Cells were plated in 24-well cell culture plates at a density of 3×10^5 cells/well and incubated as described above. Cells were treated with 70% ethanol, and supernatants obtained after centrifugation ($8,000 \times g$ for 5 min) were mixed with an equal volume of THIOLYTE monobromobimane reagent (Calbiochem, EMD Biosciences) (4 mM in 50 mM sodium *N*-ethylmorpholine, pH 8.0) and

incubated for 5 min at room temperature in the dark. Proteins were precipitated with 5% trichloroacetic acid and were removed by centrifugation at $6,000 \times g$ for 15 min; supernatants were injected onto a reverse-phase column (TSKgel ODS-80Ts, 150 mm long \times 4.6 mm diameter; Tosoh Co.) and eluted with 10% acetonitrile in 0.25% acetic acid, at a flow rate of 1 ml min⁻¹. GSH was detected by using fluorescence (excitation, 394 nm; emission, 480 nm) (model 821-FP; JASCO Co.) and was quantified by comparison with external standards.

3-Nitro-L-tyrosine analysis. Protein-bound 3-nitro-L-tyrosine was measured as an index of reactive nitrogen species formation in cells by means of HPLC coupled with electrochemical detection (ECD) according to our previous paper¹, with modification to improve the specificity and sensitivity of detection⁶. In brief, RAW 264.7 cells, cultured at a density of 2×10^7 cells in 100-mm dishes, were stimulated in the absence or presence of 1 mM BSO as described in **Methods**. In some experiments, cells were treated with 1 μ M NS 2028 (Cayman Chemicals) during stimulation in the presence of BSO. After stimulation, cells were washed three times with PBS and were lysed in RIPA buffer (10 mM Tris-HCl, 1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, pH 7.4) (0.5 ml/dish). Low-molecular-weight compounds including nitrite and nitrate were almost entirely removed from the lysates by repeating centrifugation three times, using an ultrafiltration membrane (5,000-Da cutoff membrane), with nitrite/nitrate-free RIPA buffer added after each centrifugation. High-molecular-weight fractions were then treated with Pronase (Calbiochem) at 56 °C for 24 h to liberate free 3-nitro-L-tyrosine, followed by filtration with a 5,000-Da cutoff membrane and then HPLC-ECD analysis.

HPLC separation was carried out 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 3.5) plus 2% acetonitrile and 5 μ g ml⁻¹ ethylenediaminetetraacetic acid.

3-Nitro-L-tyrosine was detected electrochemically via an online reductive activation method with electrode settings of -600 mV (first cell for reduction) and $+100$ mV (second cell for oxidation) (HTEC-500 and PEC-510; Eicom)¹. In separate experiments, tyrosine levels were determined by HPLC with a UV detector (280 nm) to normalize the 3-nitro-L-tyrosine level.

cGMP measurement. The intracellular cGMP concentration was determined by using a commercial ELISA kit (cGMP-EIA Biotrak System, GE Healthcare Bioscience). We first examined whether the ELISA kit had cross-reactivity for 8-nitro-cGMP and 8-RS-cGMP derivatives. Authentic 8-nitro-cGMP and 8-RS-cGMPs such as 8-Cys-cGMP and 8-GS-cGMP had dose-response curves comparable to that obtained for authentic cGMP. Thus, we could determine the total cGMP levels by using the kit but we could not differentiate cGMP from nitrated and 8-RS-cGMP derivatives. To avoid degradation of cGMP during lysate preparation, we added a PDE inhibitor (10 μ M zaprinast, Alexis Biochemicals) to the RIPA buffer. cGMP in the RAW 264.7 cell lysates was then measured according to the manufacturer's instruction.

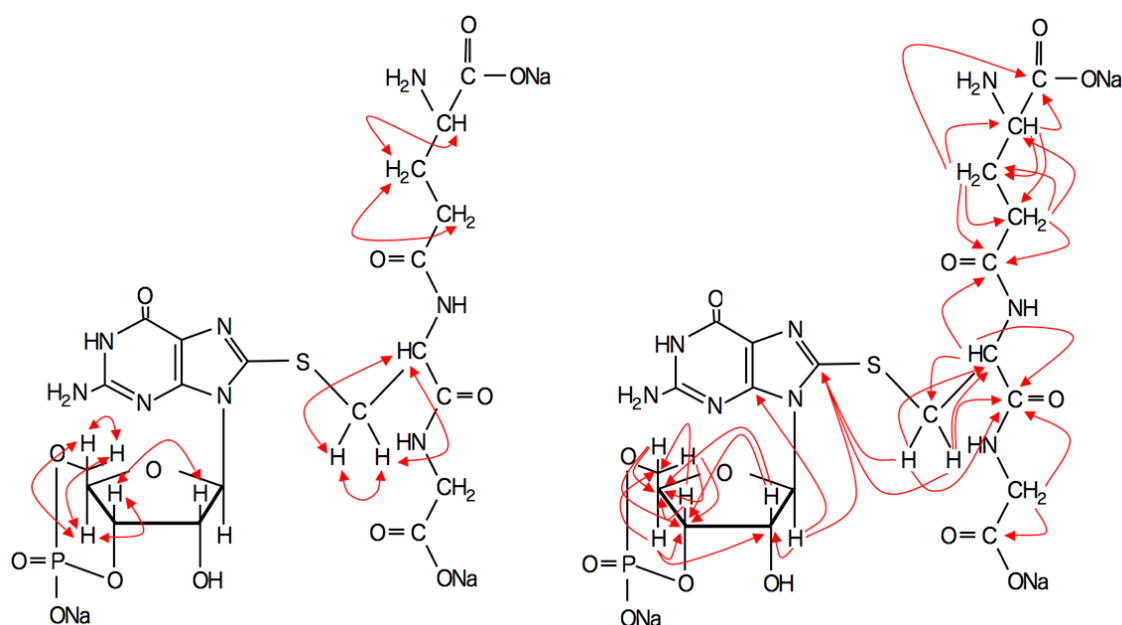
Permeability of membranes to cGMP derivatives. Membrane permeability of cGMP derivatives was determined by measuring cellular uptake of the derivatives. RAW 264.7 cells were harvested by trypsinization and were resuspended in DMEM plus 10% FBS with 10 μ M zaprinast in the absence or presence of 10 or 50 μ M cGMP derivatives including authentic cGMP, 8-nitro-cGMP, and 8-bromo-cGMP. Cells (6×10^6 cells/tube) were then incubated at room temperature for 30 min with gentle mixing. After incubation, cells were washed twice with PBS containing 10 μ M zaprinast, and cell pellets were lysed in 0.2 ml of RIPA buffer containing 10 μ M zaprinast. Concentrations in cell lysates were determined by

using a commercial ELISA kit as mentioned above. The degree of permeability was assessed via values obtained by the formula: [(intracellular concentration of cGMPs increased after incubation)/(concentration of cGMPs added to the cell culture)] × 100 (%).

Reactions of 8-nitro-cGMP with low-molecular-weight sulfhydryls. We performed liquid chromatography-mass spectrometry (LC-MS) analyses to identify products formed in the reaction of 8-nitro-cGMP with low-molecular-weight sulfhydryls including GSH, L-cysteine and L-Cys-Gly dipeptide (Cosmo Bio Co.). 8-Nitro-cGMP (10 μM) was incubated with sulfhydryls (10 mM) in 100 mM sodium phosphate buffer (pH 7.4) at 37 °C. After certain incubation periods, an aliquot of the reaction solution was taken and analyzed via electrospray ionization (ESI)-LC-MS using an LCMS-QP8000α mass spectrometer (Shimadzu). A reverse-phase column (5 μm, 150 mm long, 2 mm inner diameter, STR ODS-II; Shinwa Chemical Industries) was eluted with solvent A [0.02% trifluoroacetic acid (TFA) plus 2% acetonitrile] and solvent B (0.02% TFA plus 15% acetonitrile) in a linear gradient from solvent A to B for 20 min. Electrospray voltage was 2 kV; capillary temperature was 250 °C. Reaction products were detected in the positive mode with a mass range of 400-750. HPLC separations were monitored simultaneously with a UV-VIS spectrometer at 254 and 395 nm for detection of the cGMP moiety and the 8-nitro-cGMP moiety, respectively. In a separate experiment, 8-nitro-cGMP (1 mM) was reacted with GSH (100 mM) in 0.5 M Tris-HCl (pH 8.0) at 37 °C for 1 h. 8-S-Glutathionyl-cGMP (8-GS-cGMP) was purified by means of HPLC with a preparative reverse-phase column (TSKgel ODS-80Ts, 300 mm long × 21.5 mm inner diameter; Tosoh) eluted with 0.02% trifluoroacetic acid plus 18% methanol monitored at 254 nm.

8-GS-cGMP characterization. ¹H NMR (600 MHz, D₂O) δ: 2.10 (1H, dt, *J* = 7.2, 7.2 Hz,

Glu H^β), 2.46 (1H, t, $J = 7.2$ Hz, Glu H^γ), 3.36 (1H, m: Cys H^β), 3.65 (1H, m: Cys H^β), 4.00 (1H, t, $J = 7.2$ Hz, Glu H^α), 3.91 (2H, s: Gly H^α), 4.12 (1H, m: H4'), 4.25 (1H, dd, $J = 9.6, 9.6$ Hz, H5'), 4.43 (1H, ddd, $J = 4.2, 9.6, 21.6$ Hz), 4.67 (1H, dd, $J = 4.8, 7.2$ Hz: Cys H^α), 4.79 (overlapped with residual H₂O: H2'), 5.17 (1H, m: H3'), 5.98 (1H, s, H1'); ¹³C NMR (150 MHz, D₂O) δ : 25.5 (Glu C^β), 31.0 (Glu C^γ), 35.1 (Cys C^β), 41.2 (Gly C^α), 52.2 (Glu C^α), 53.1 (Cys C^α), 67.3 (d, $J_{CP} = 6.75$ Hz, C5'), 71.4 (d, $J_{CP} = 7.95$ Hz, C2'), 72.0 (d, $J_{CP} = 3.75$ Hz, C4'), 77.0 (d, $J_{CP} = 4.20$ Hz, C3'), 92.3 (C1'), 116.5 (C5), 143.9 (C8), 152.8 (C4), 153.8 (C2), 157.8 (C6), 171.5 (Glu carboxylate), 171.9 (Cys amide-carbonyl), 172.9 (Gly carboxylate), 174.1 (Glu amide-carbonyl); MS (ESI, positive): calcd. for C₂₀H₂₇N₈O₁₃PS ([M+H]⁺), 651; found, 651. The figure below shows correlation of protons by two-dimensional correlated spectroscopy experiments (left panel) and correlations found via heteronuclear multiple bond correlation experiments (D₂O) (right panel).



8-S-Cysteinyl-cGMP (8-Cys-cGMP) characterization. MS (ESI, positive): calcd. for C₁₃H₁₇N₆O₉PS ([M+H]⁺), 465; found, 465.

8-S-Cysteinyl-glycyl-cGMP (8-Cys-Gly-cGMP) characterization. MS (ESI, positive): calcd. for C₁₅H₂₀N₇O₁₀PS ([M+H]⁺), 522; found, 522.

LC-ESI/MS/MS analysis. LC-ESI/MS/MS was performed with a Varian 1200L triple-quadrupole mass spectrometer (Varian) after reverse-phase HPLC on a 50 × 2.1-mm inner diameter 2 μm X-PressPak C18S column (Jasco) with a linear 0–100% acetonitrile gradient for 5 min in 0.1% aqueous acetic acid at 40 °C. The total flow rate was 0.15 ml min⁻¹; injection volume was 10 μl. The column effluent was introduced directly into the mass spectrometer operated in positive mode under following conditions: collision gas 2.2 mT argon, drying gas 19 psi nitrogen at 300 °C, nebulizing gas 54 psi nitrogen, scan time 1 s, needle voltage 5,000 V, shield voltage 600 V, capillary voltage 30 V, detector voltage 1,800 V. The mass spectrometer was operated in the multiple reaction monitoring (MRM) mode. Scan parameters are shown in **Table 1**. These parameters were determined with the Automated MS/MS Breakdown software (Varian) using a stock solution of 8-nitro-cGMP and 8-Cys-cGMP (10 μg ml⁻¹) in acetonitrile/0.1% aqueous acetic acid (1:1 v/v). Authentic 8-nitro-cGMP (1 pmol) or 8-nitro-cGMP formed in the cell lysate (600 fmol equivalent) was analyzed by this LC-MS/MS system (results appear in **Fig. 3c**).

Table 1 Scan parameters for LC-MS/MS

Analyte	Precursor ion, <i>m/z</i>	Product ion, <i>m/z</i>	Collision energy (V)
8-Nitro-cGMP	391	197	-14
8-Cys-cGMP	465	184	-32

Nitrite (NO₂⁻) measurement. We quantified NO₂⁻ released from 8-nitro-cGMP during denitration by means of an HPLC-flow reactor system, as reported previously⁷.

8-Nitro-cGMP (10 μM) was incubated with 10 mM GSH or L-cysteine in 100 mM sodium phosphate buffer (pH7.4) at 37 °C. The reaction solution was applied to a reverse-phase HPLC column (4.6 × 30 mm, CA-ODS; Eicom Co.) to remove peptides and proteins in solution. NO₂⁻ was eluted with 0.55 ml min⁻¹ of 10 mM sodium acetate buffer (pH 5.5) containing 0.1 M NaCl and 0.5 mM diethylenetriaminepentaacetic acid, and Griess reagent (0.1 ml min⁻¹) was added via a flow-reactor system. The diazo compound thus formed was detected at 540 nm by using a visible light detector (Eicom Co.) and an integrator (System Instruments Co.).

We also investigated the release of NO₂⁻ from 8-nitro-cGMP incubated with cell lysates. RAW 264.7 cells were plated in 6-well plates at a density of 5 × 10⁶ cells/well and were incubated at 37 °C for 12 h with DMEM plus 2% FBS and various concentrations of BSO (0, 0.5, and 1 mM), followed by stimulation for 12 h with lipopolysaccharide (LPS, 10 μg ml⁻¹, Sigma) and interferon-γ (IFN-γ, 100 U ml⁻¹, R&D Systems) in DMEM plus 2% FBS with or without BSO (0.5 or 1 mM). Cells were then solubilized in 1 ml of RIPA buffer to obtain cell lysates. We incubated 0.1 mM 8-nitro-cGMP dissolved in 50 μl of reaction buffer (50 mM Tris-HCl, pH 7.6, 3 mM *N*⁰-monomethyl-L-arginine, Sigma) with 50 μl of the cell lysate at 37 °C for 6 h. The NO₂⁻ concentration in the reaction solution was determined by means of the HPLC-Griess assay mentioned above. Simultaneously, electron spin resonance (ESR) spin trapping was employed to confirm the origin of the NO₂⁻ released: 8-¹⁵NO₂-cGMP was used for this purpose. A 100-μl aliquot of the same reaction solution containing either ¹⁵NO₂-cGMP or ¹⁴NO₂-cGMP was incubated for 30 min at 37 °C with a complex of iron with *N*-(dithiocarboxy)sarcosine [Fe²⁺-(DTCS)₂] that was prepared by mixing 10 mM iron(II) sulfate heptahydrate (Wako Pure Chemical Industries) and 40 mM DTCS disodium salt

(Dojindo Laboratories) in 125 mM sodium acetate buffer (pH 4.0) containing 10 mM ascorbic acid. Under these conditions, NO_2^- was converted to NO, which was trapped by Fe^{2+} -(DTCS)₂ to give NO- Fe^{2+} -(DTCS)₂⁸. The formation of NO- Fe^{2+} -(DTCS)₂ was identified by means of X-band ESR spectroscopy (JES-RE1X; JEOL) using a quartz flat cell (inner size, 60 × 10 × 0.31 mm) at room temperature. Conditions for ESR measurements were as follows: modulation amplitude, 0.2 mT; time constant, 0.3 s; sweep time, 4 min; microwave power, 20 mW; scanning field, 336 ± 5 mT; receiver gain, 500; microwave frequency, 9.454 GHz. With the present conditions, the ¹⁴NO- Fe^{2+} -(DTCS)₂ complex provided a triplet signal with a *g* value of 2.045, whereas the ¹⁵NO- Fe^{2+} -(DTCS)₂ complex gave a doublet signal with a *g* value of 2.045.

Western blotting. We performed Western blotting analysis to assess expression levels of various proteins in cells. Antibodies used included anti-8-RS-cGMP antibody prepared as described earlier; anti-Keap1 (rat monoclonal; ref. 9); anti-activated-VASP (46/50-kDa vasodilator-stimulated phosphoprotein) (mouse monoclonal, specific for phosphoserine 239; Alexis Biochemicals); anti-VASP (rabbit polyclonal; Alexis Biochemicals); and anti-iNOS (rabbit polyclonal; Santa Cruz Biotechnology). 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 Co.).

HPLC-ECD measurement of 8-RS-cGMP adducts. We developed an HPLC-ECD system coupled with immunoaffinity enrichment to determine the formation of 8-RS-cGMP adducts. We first analyzed 8-RS-cGMP adduct formation in cells treated with authentic 8-nitro-cGMP. In brief, RAW 264.7 cells (2×10^7 cells/100-mm dish) were incubated with 10 μM 8-nitro-cGMP for 11 h. Cells were washed twice with PBS and were lysed in 0.5 ml of

RIPA buffer. The lysate was then treated with Pronase at 56 °C for 24 h, followed by filtration with a 5,000-Da cutoff membrane as reported previously¹. In another experiment, we performed pronase digestion of Keap1 protein obtained from RAW 264.7 cells (2×10^7 cells/100-mm dish, 5 dishes) stimulated with LPS and IFN- γ in the presence of BSO by means of immunoprecipitation with anti-Keap1 antibody (cf. **Methods**). These samples were then subjected to immunoaffinity enrichment.

Immunoaffinity columns were prepared by using monoclonal anti-8-RS-cGMP antibody in a manner similar to that used for preparation of immunoaffinity columns for enrichment of 8-nitroguanine derivatives as described in **Methods**.

HPLC separation of 8-RS-cGMP adducts was achieved with a reverse-phase column (150 mm long, 3.0 mm inner diameter; Eicom Pak SC-5 ODS, Eicom) eluted with 0.5 ml min⁻¹ of 200 mM sodium phosphate buffer (pH 5.0) plus 8% acetonitrile. 8-RS-cGMP derivatives (e.g., 8-Cys-cGMP and 8-GS-cGMP) were detected electrochemically with electrode settings of +700 mV (first cell) and +850 mV (second cell). The second cell was used as a detection cell. Under these conditions, detection limits were 50 and 100 fmol/injection for 8-Cys-cGMP and 8-GS-cGMP, respectively.

Animal experiments. We performed all animal experiments according to the ethical guideline and protocol No. O18-138, approved by the Animal Use and Care Administrative Advisory Committee of Kumamoto University.

Vascular organ bath study. Carotid artery rings (4 mm) from male Sprague Dawley rats (350 g body weight; Charles River Laboratories Japan) were connected to a force transducer (model AG-621G; Nihon Kohden Co.) for recording isometric force and were placed in organ baths at 37 °C filled with 3 ml of Krebs buffer (1.2 mM NaHPO₄, 120 mM NaCl, 5.9 mM

KCl, 2.5 mM CaCl₂, 15.5 mM NaHCO₃, 11.5 mM glucose, pH 7.4) (95% O₂ and 5% CO₂). Concentration-response curves to 8-nitro-cGMP derivatives were obtained for arteries precontracted with 0.1 μM phenylephrine. In some experiments, the endothelium was mechanically removed. Superoxide dismutase (SOD, from bovine erythrocytes; Sigma) or tiron (Dojindo Laboratories) was added to the organ baths to investigate the effect of superoxide. We performed similar experiments with aorta rings (4 mm) from wild-type and eNOS-deficient mice. eNOS-deficient mice used in the present study were produced and maintained as described elsewhere¹⁰.

Phosphorylation of VASP. To study whether 8-nitro-cGMP can activate cGMP-dependent protein kinase (PKG), we analyzed phosphorylation of VASP, a target of PKG, by means of Western blotting. Human uterine smooth muscle cells (Cambrex Bio Science Walkersville, Inc) were exposed to 10 μM 8-nitro-cGMP or 8-bromo-cGMP. In some experiments, cells were pretreated with 10 μM 8-(4-chlorophenylthio)-guanosine 3',5'-cyclic monophosphorothioate, Rp isomer (Sigma), a PKG inhibitor, for 30 min before exposure to 8-nitro-cGMP. Cells were then harvested in lysis buffer (20 mM Tris-HCl pH 8.0, 0.15 M NaCl, 1 mM ethylenediaminetetraacetic acid, 1 mM ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM Na₃VO₄, and 1 μM phenylmethanesulfonyl fluoride). Aliquots (10 μg of protein each) were subjected to SDS-PAGE. After size separation, proteins were blotted onto PVDF membranes (Millipore), which were first probed with anti-activated-VASP antibody (specific for phosphoserine 239) and then with HRP-conjugated sheep anti-mouse IgG (Amersham Biosciences). Immunoreactive bands were visualized via chemiluminescence with ECL Plus. After reprobing, anti-VASP was used as a primary antibody, after which HRP-conjugated goat

anti-rabbit IgG (Amersham Biosciences) was used as a secondary antibody.

Phosphodiesterase (PDE) assay. We studied susceptibility of 8-substituted cGMP derivatives against PDE activity in vitro. cGMP (10 μM) or its 8-substituted derivatives (10 μM) were incubated with PDE1 (1 mU ml^{-1} , from bovine brain; Sigma) in 20 mM Tris-HCl (pH 7.4) containing 3 mM magnesium chloride, 15 mM magnesium acetate, 0.03 mM calcium chloride, and 1 unit ml^{-1} calmodulin at 30 °C for 30 min, or with PDE5 (0.2 mU ml^{-1} ; Chemicon International) in 40 mM Tris-HCl (pH 7.4) containing 15 mM magnesium acetate and 0.2 mg ml^{-1} BSA at 30 °C for 60 min. After reaction of PDEs with 8-substituted derivatives of cGMP, enzymes were inactivated by incubation at 95 °C for 2 min. cGMP derivatives remaining in the reaction buffer were quantified by HPLC using a reverse-phase column (TSKgel ODS-80Ts, 150 mm long \times 4.6 mm diameter; Tosoh Co.) eluted with 1 ml min^{-1} 0.02% TFA plus 10% or 15% methanol. cGMP derivatives were detected via a UV detector at 254 nm.

Superoxide generation measured by ESR spectroscopy. We used ESR spin trapping to examine 8-nitro-cGMP-mediated enhancement of superoxide generation from cytochrome P450 reductase and all three isoforms of NOS. P450 reductase and human recombinant NOS isoforms were prepared and purified according to the literature^{11,12}. Each preparation was confirmed as fully active on the basis of NADPH oxidation and L-arginine oxidation to form L-citrulline¹³.

We used 5-(2,2-dimethyl-1,3-propoxy cyclophosphoryl)-5-methyl-1-pyrroline *N*-oxide (CYPMPO, Radical Research Co.) as the spin trap¹⁴. ESR spectroscopy data, from an X-band ESR spectrometer (JES-RE1X), were acquired after 1 min of incubation of 8-nitro-cGMP with 0.2 μM enzyme in the presence of 0.2 mM NADPH and 2 mM CYPMPO

in 100 mM sodium phosphate buffer (pH 7.4) at room temperature. ESR measurement was repeated five times, and these spectra were averaged to obtain the signal of CYPMPO-OOH (a superoxide adduct). ESR spectra were recorded at room temperature under the following conditions: modulation frequency, 100 kHz; modulation amplitude, 0.1 mT; scanning field, 336 ± 5 mT; receiver gain, 500; response time, 0.3 s; sweep time, 2 min; microwave power, 10 mW; and microwave frequency, 9.421 GHz. CYPMPO-OOH was identified according to its hyperfine splitting constants (hfc, mT): a_N, a_H, a_P : 1.37, 1.37, 4.88; 1.35, 1.23, 4.70¹⁴ (diastereomers), and its complete disappearance in the presence of SOD. We further determined formation of the radical intermediate of 8-nitro-cGMP by direct ESR spectroscopy without spin trapping. 8-Nitro-cGMP (0.2 mM) was incubated with 0.2 μ M P450 reductase and 1 mM NADPH in 100 mM potassium phosphate buffer (pH 8.8) that had been purged with argon gas before use. After 7 min of incubation at room temperature, ESR spectra were recorded.

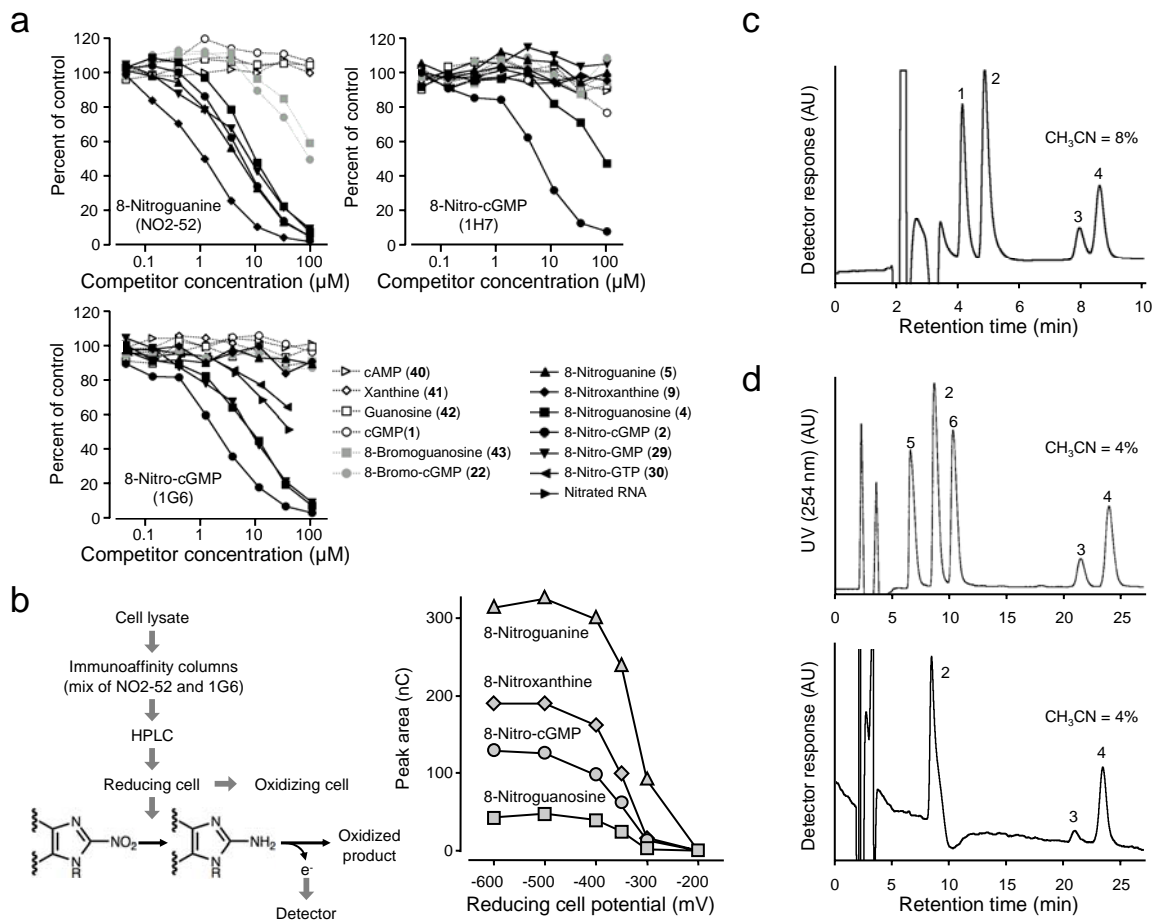
Statistical analysis. All data are expressed as means \pm s.d. Data for each condition were acquired from at least three independent experiments. Statistical analyses were performed by using Student's *t*-test.

Supplementary References

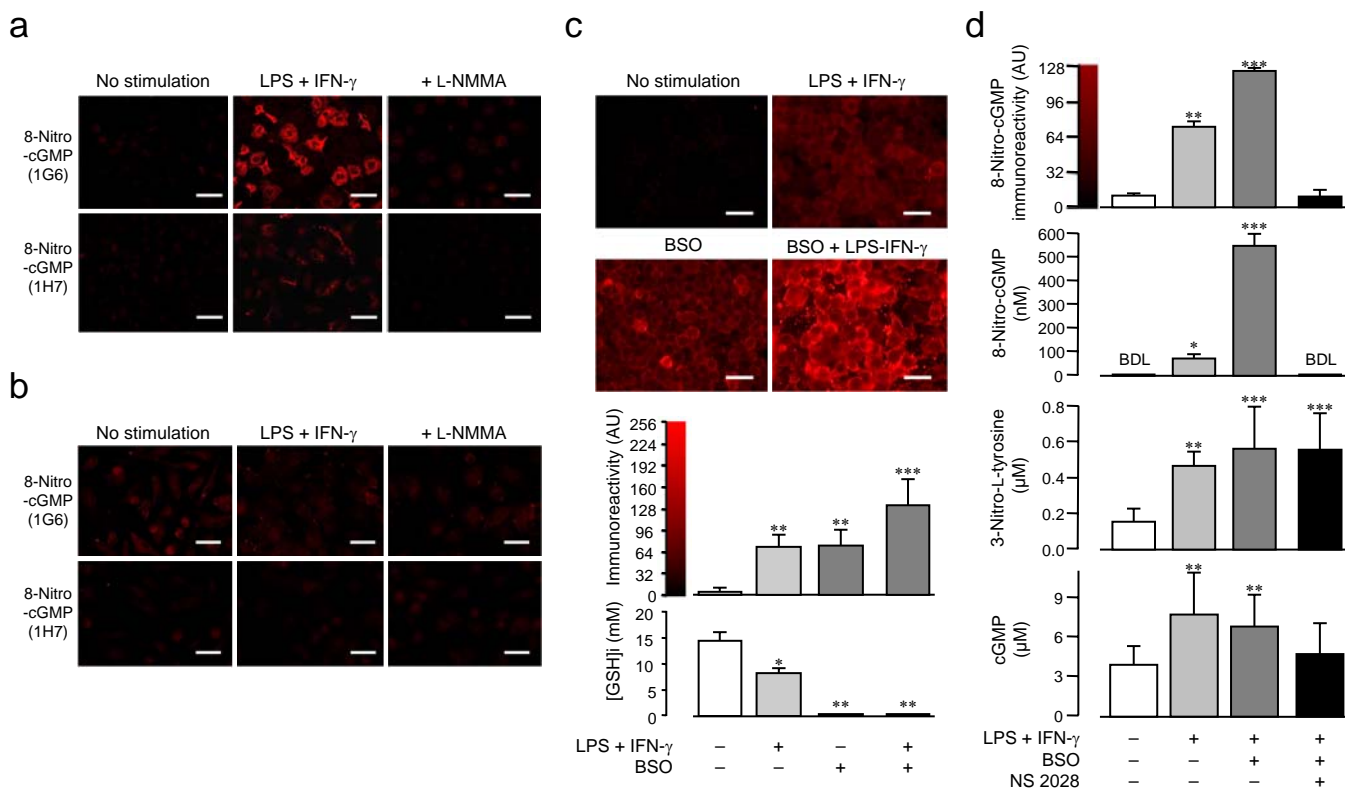
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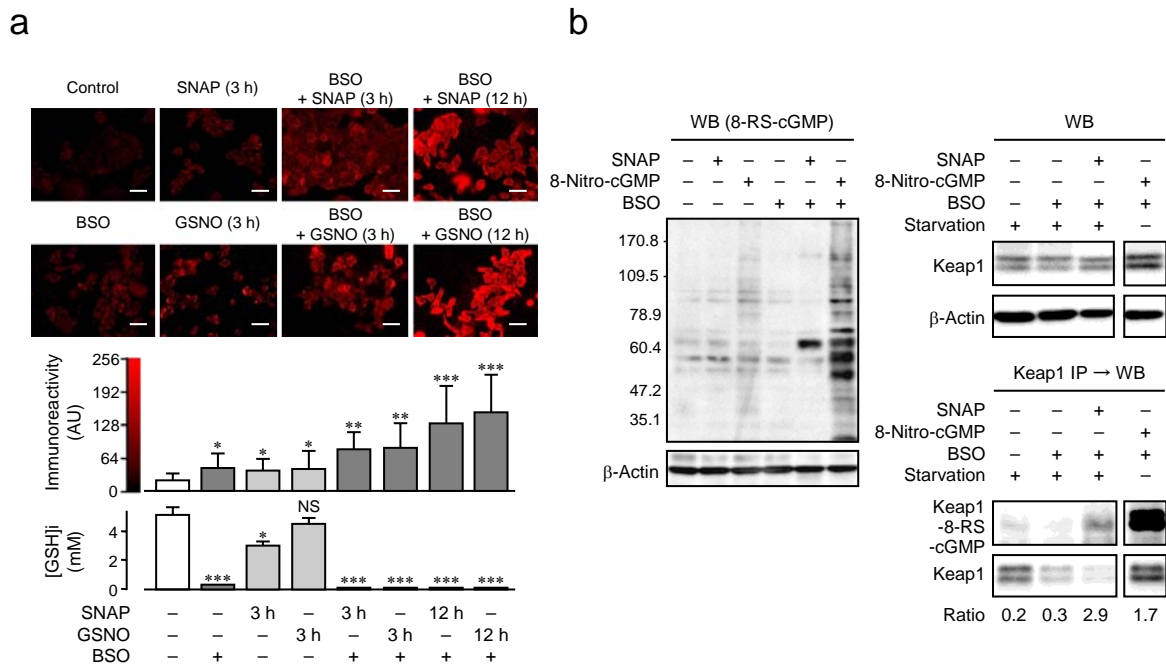
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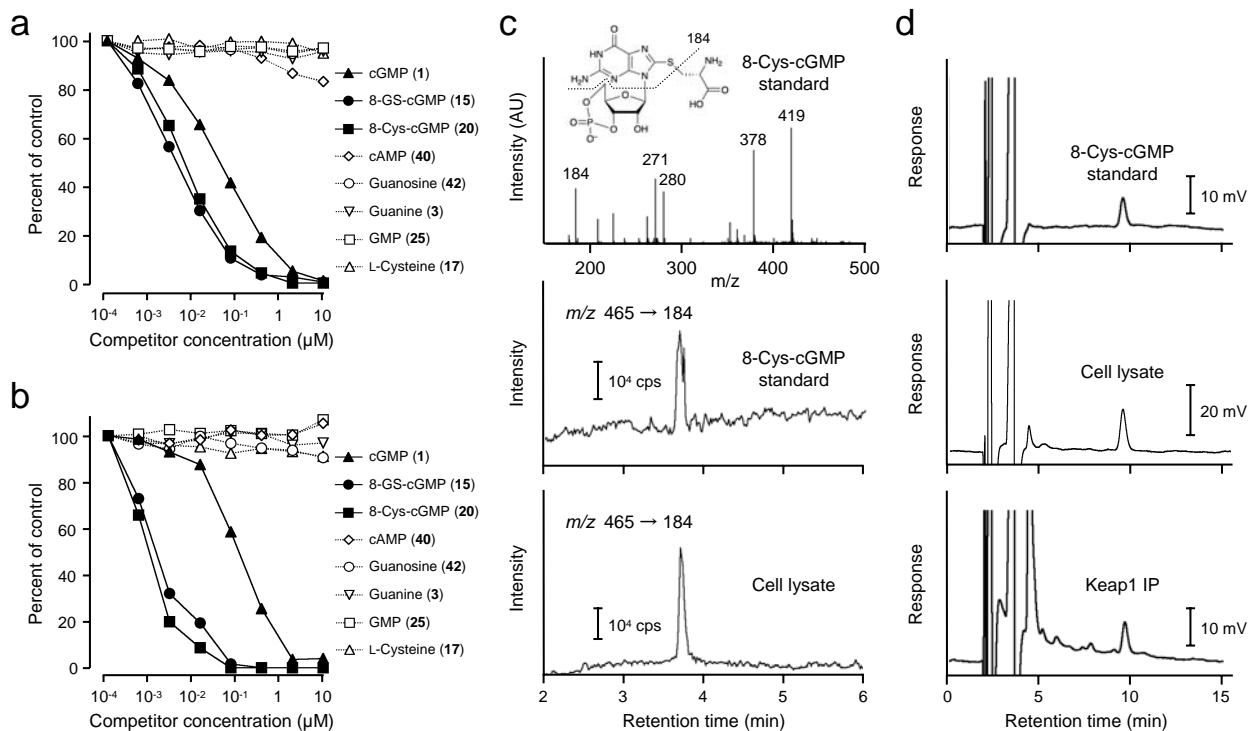
Supplementary Figure 1 Competitive ELISA (**a**) and HPLC-ECD analysis (**b-d**) of 8-nitro-cGMP and related compounds. (**a**) Competitive ELISA for reaction of three clones of monoclonal antibodies (NO2-52, 1G6, and 1H7) with the 8-nitro-cGMP-BSA conjugate. Clone NO2-52 specifically recognized the 8-nitro moiety of the purine base; clone 1G6 was relatively specific for 8-nitro-cGMP and had no cross-reactivity with nitrated purines including 8-nitroxanthine and 8-nitroguanine. The third antibody used was clone 1H7, which was specific for 8-nitro-cGMP, although a weak cross-reaction was observed with 8-nitroguanosine. RNA containing 8-nitroguanine was very weakly reactive with 1G6, but 1H7 had no cross-reactivity with this RNA. (**b**) General analytical procedure for HPLC-ECD. Cell lysates were subjected to immunoaffinity enrichment, followed by HPLC separation. 8-Nitroguanine derivatives were reduced at the first cell to form electrochemically active 8-aminoguanine derivatives, which were detected at the second cell via the oxidation mode (+250 mV). The electrochemical response at the second cell was monitored. Right panel shows relationship between reducing cell potential and peak response. The potential of the reducing cell was varied to obtain a reduction potential peak response of 8-nitroguanine derivatives. (**c**) HPLC-ECD chromatogram of authentic samples eluted in the presence of 8% acetonitrile (CH₃CN). Peak 1, 8-nitroxanthine; peak 2, 8-nitroguanine; peak 3, 8-nitroguanosine; peak 4, 8-nitro-cGMP. (**d**) HPLC-UV (upper panel) and HPLC-ECD (lower panel) chromatograms of authentic samples eluted in the presence of 4% acetonitrile. UV detection at 254 nm revealed cGMP (peak 5) and 8-oxo-2'-deoxyguanosine (peak 6) in addition to 8-nitroguanine (peak 2), 8-nitroguanosine (peak 3), and 8-nitro-cGMP (peak 4). However, only 8-nitroguanine derivatives could be detected via HPLC-ECD under the current conditions.



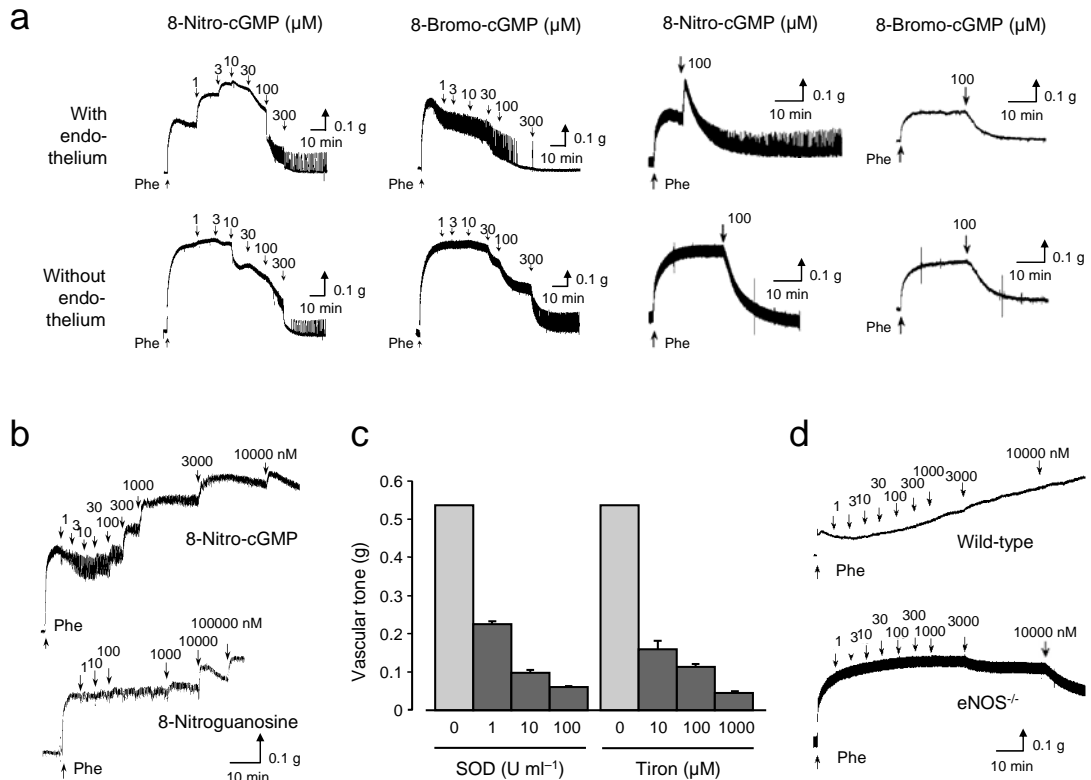
Supplementary Figure 2 Formation of 8-nitro-cGMP in macrophages and its regulation by GSH and sGC. **(a, b)** Formation of 8-nitro-cGMP-related compounds in peritoneal exudate macrophages obtained from wild-type mice **(a)** and iNOS-deficient mice **(b)** after stimulation of cells with IFN- γ (100 U ml⁻¹) plus LPS (10 μ g ml⁻¹) for 11 h. **(c)** Formation of 8-nitro-cGMP in RAW 264.7 cells. Cells were treated with 1 mM BSO 12 h before and during (11 h) stimulation [IFN- γ (100 U ml⁻¹) plus LPS (10 μ g ml⁻¹)]. The intensity of immunostaining with anti-8-nitro-cGMP antibody (1G6) was determined as described in **Methods**. Levels of intracellular GSH ([GSH]i), quantified by HPLC, are given in the lower panel. **(d)** Intracellular levels of 8-nitro-cGMP in RAW 264.7 cells determined by immunocytochemistry with the anti-8-nitro-cGMP antibody (top panel) and by HPLC-ECD (second panel); levels of protein-bound 3-nitro-L-tyrosine determined by HPLC-ECD (third panel); and levels of cGMP determined by using a commercial ELISA kit (bottom panel). NS 2028 (1 μ M), an sGC inhibitor, was added to the culture medium during stimulation. Details of experimental procedures are provided in **Supplementary Methods** online. BDL, below the detection limit (25 fmol/injection). Scale bars, 25 μ m **(a, b, c)**. Data are expressed as mean \pm s.d. (n=3-5). * P < 0.05, ** P < 0.01, and *** P < 0.001, compared with controls.



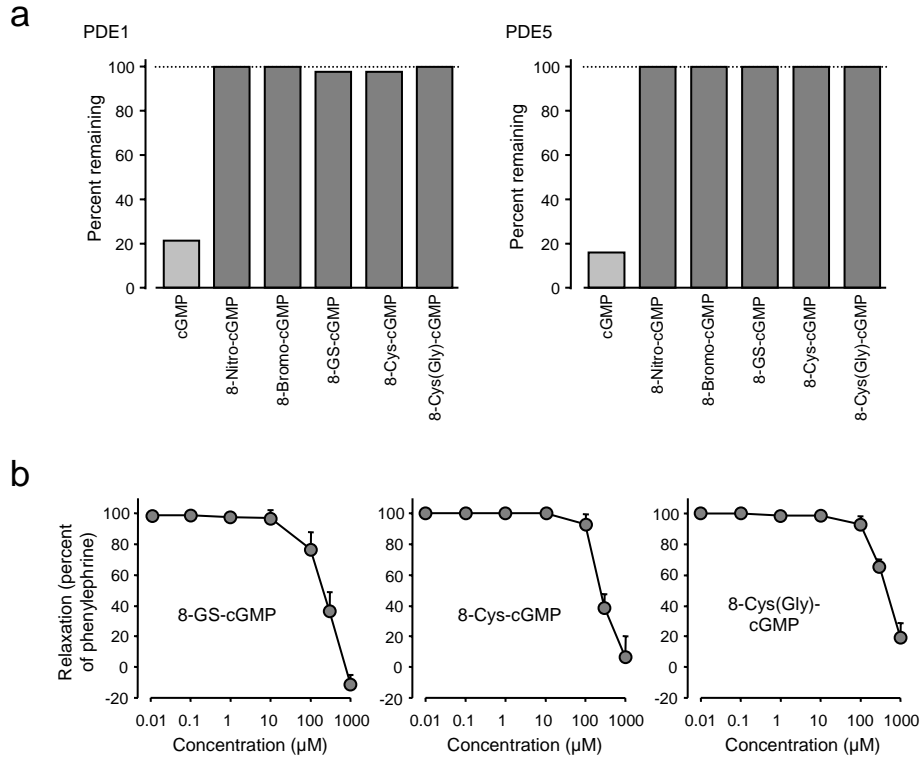
Supplementary Figure 3 Formation of 8-nitro-cGMP (**a**) and protein-8-RS-cGMP adducts (**b**) in HepG2 cells. HepG2 cells were pretreated with BSO (1 mM) for 12 h or had no pretreatment, followed by incubation in glucose-free medium (glucose starvation) with or without 500 μ M SNAP or GSNO (1 h). Cells were washed with glucose-free medium and further incubated in glucose-free medium for 2 h (total 3 h of glucose starvation) or 11 h (total 12 h of glucose starvation). (**a**) Immunostaining with anti-8-nitro-cGMP antibody (1G6). Intracellular GSH concentration ([GSH]i) is provided in the bottom panel. Scale bars, 25 μ m. Data are expressed as mean \pm s.d. (n=3). * P < 0.05, ** P < 0.01, and *** P < 0.001, compared with controls. (**b**) Formation of protein-8-RS-cGMP adducts in HepG2 cells. Cells were pretreated with 1 mM BSO for 12 h or were untreated, followed by a 1-h treatment with SNAP (500 μ M) or 8-nitro-cGMP (500 μ M) in the absence or presence of BSO (1 mM) under glucose starvation. Cells were washed with glucose-free medium and further incubated in the absence or presence of BSO (1 mM) under glucose starvation for 11 h (total 12 h of glucose starvation). Cell lysates were subjected to Western blotting (WB) for 8-RS-cGMP (left panel). S-Guanylated Keap1 was detected in HepG2 cells. HepG2 cells were treated with various reagents in the same manner as in **a**, except for 8-nitro-cGMP treatment. In that case, unstarved cells (no glucose starvation) were incubated with 8-nitro-cGMP for 12 h before cell lysate preparation. Western blotting data for Keap1 and β -actin are shown. Whole-cell extracts were also subjected to immunoprecipitation (IP) with anti-Keap1 antibody. Immunoprecipitated proteins were further analyzed for 8-RS-cGMP and Keap1 by means of Western blotting. Relative band intensities of S-guanylated Keap1 (Keap1-8-RS-cGMP) vs. total Keap1 are given at the bottom.



Supplementary Figure 4 Identification of 8-Cys-cGMP adducts in RAW 264.7 cells. Specificities of polyclonal (**a**) and monoclonal (**b**) anti-8-RS-cGMP antibodies were determined by means of competitive ELISA. The 8-RS-cGMP adduct of BSA was used as an antigen. (**c**) LC-MS/MS determination of 8-Cys-cGMP. Fragmentation mass spectrum of the 8-Cys-cGMP adduct (top panel). The parent pseudo-molecular ion $[M + H]^+$ was at $m/z = 465$. LC-MS/MS chromatograms (m/z : 465 \rightarrow 184) of authentic 8-Cys-cGMP (2.5 pmol, middle panel) and of RAW 264.7 cell lysate (2.5 mg of protein) (bottom panel) treated with 10 μM 8-nitro-cGMP for 11 h, followed by immunoaffinity enrichment, are shown. (**d**) HPLC-ECD identification of 8-Cys-cGMP adduct. Authentic 8-Cys-cGMP (500 fmol) was detected at a retention time of 9.5 min under the present conditions (top panel). 8-Cys-cGMP in cell lysate (0.5 mg of protein; same sample as that used for **c**) could be detected in this HPLC-ECD system (middle panel). Endogenous *S*-guanylation of Keap1 in stimulated RAW 264.7 cells (bottom panel). Keap1 protein was obtained from stimulated RAW 264.7 cells (5 mg of protein) by means of immunoprecipitation with the use of anti-Keap1 antibody. The protein was then digested by pronase, and the digested sample was subjected to immunoaffinity enrichment with monoclonal anti-8-RS-cGMP antibody before HPLC-ECD analysis.



Supplementary Figure 5 Modulation of vascular tone by 8-nitro-cGMP. **(a)** Effects of 8-nitro-cGMP on responses of rat carotid artery rings, with or without endothelium, precontracted by phenylephrine. 8-Bromo-cGMP was used as the cGMP analogue. The left panels show results for cumulative addition of 8-nitro-cGMP or 8-bromo-cGMP to organ baths. The right panels show vascular responses obtained for one administration of 8-nitro-cGMP or 8-bromo-cGMP. **(b)** Effects of 8-nitro-cGMP and 8-nitroguanosine on vascular tone of rat carotid artery rings. **(c)** Effects of superoxide scavengers on 8-nitro-cGMP-mediated vasoconstriction. Rat carotid artery rings pretreated with indicated concentrations of SOD or tiron were precontracted with phenylephrine, followed by addition of 1 μM 8-nitro-cGMP. Data are expressed as mean \pm s.d. ($n=3$). **(d)** Effects of 8-nitro-cGMP on vascular tone of aortas obtained from wild-type and eNOS-deficient mice.



Supplementary Figure 6 Resistance of 8-substituted cGMP derivatives to PDE1 and PDE5 (**a**) and vasorelaxation activities of 8-RS-cGMP adducts (**b**). In **a**, cGMP (10 μM) or its 8-substituted derivatives (10 μM) were incubated with PDE1 (1 mU ml^{-1}) at 30 $^{\circ}\text{C}$ for 30 min (left panel), or with PDE5 (0.2 mU ml^{-1}) at 30 $^{\circ}\text{C}$ for 60 min (right panel). cGMP derivatives remaining in the reaction buffer were quantified by means of HPLC. In **b**, rat carotid arteries precontracted with phenylephrine (0.1 μM) were incubated with indicated concentrations of 8-RS-cGMP adducts. Changes in vascular tone were measured as described in **Supplementary Methods** online. Data are expressed as mean \pm s.d. (n=3).

Supplementary Table 1 Rate constants for the reaction of 8-nitro-cGMP with sulfhydryls ^a

Sulfhydryl	pKa ^b	Reaction pH	k_2 (M ⁻¹ min ⁻¹) ^c
L-Cysteine	8.30	6.5	1.54
		7.4	5.40
		9.0	8.14
GSH	8.75	6.5	0.56
		7.4	1.70
		9.0	2.15
α_1 -PI		7.4	0.43

^a8-Nitro-cGMP (20 μ M) was reacted with sulfhydryls (10 mM), except for α_1 -PI, in 200 mM sodium phosphate buffer (pH 6.5 and 7.4) or in 200 mM Tris-HCl buffer (pH 9.0) at 37 °C. For α_1 -PI, 8-nitro-cGMP (100 μ M) was reacted with 100 μ M α_1 -PI in 200 mM sodium phosphate buffer (pH 7.4) at 37 °C.

^bData reported by Jencks, W.P. & Regenstein, J. in *The Handbook of Biochemistry—Selected Data for Molecular Biology* (ed. Sober, H.A.) 186 (The Chemical Rubber Co., Cleveland, 1968).

^cApparent second-order rate constants were determined from the slope of the plot of incubation time vs $\log(A - A_0)$, where A_0 is initial absorbance.