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Supplementary information

Nitric oxide promotes recycling of 8-nitro-cGMP, a cytoprotective mediator, into intact cGMP in cells

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Figures

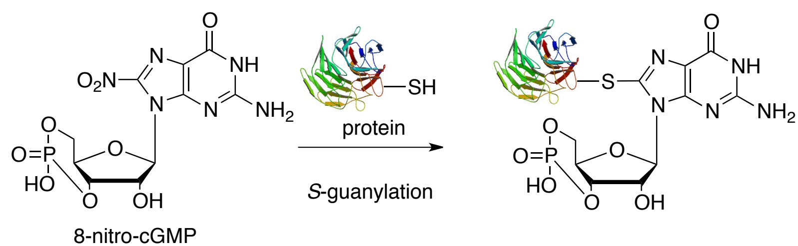

Figure S1. Protein *S*-guanylation

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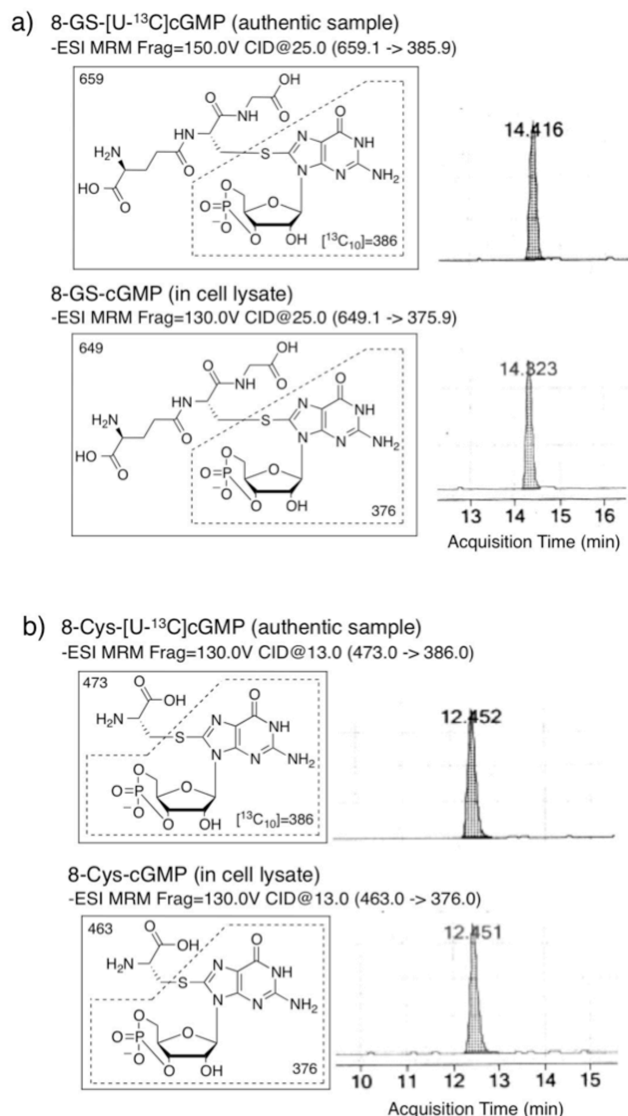

Figure S2 Identification of 8-GS-cGMP and 8-Cys-cGMP as metabolites of 8-nitro-cGMP by comparison with authentic 8-GS-[U-^{13}C]cGMP and 8-Cys-[U-^{13}C]cGMP using LC-MS/MS

a) LC-MS/MS chromatograms of 8-nitro-cGMP metabolite (8-GS-cGMP, *m/z*: 649-376; lower panel), and authentic 8-GS-[U-^{13}C]cGMP (*m/z*: 659-386; upper panel). HepG2 cells were treated with 50 μM 8-nitro-cGMP for 6 h. After addition of authentic 8-GS-[U-^{13}C]cGMP, metabolites in the cell lysate were analyzed by LC-MS/MS (negative mode). b) LC-MS/MS chromatograms of 8-nitro-cGMP metabolite (8-Cys-cGMP, *m/z*: 463-376; lower panel), and authentic 8-Cys-[U-^{13}C]cGMP (*m/z*: 473-386; upper panel). HepG2 cells were treated with 50 μM 8-nitro-cGMP for 6 h. After addition of authentic 8-GS-[U-^{13}C]cGMP, metabolites in the cell lysate were analyzed by LC-MS/MS (negative mode).

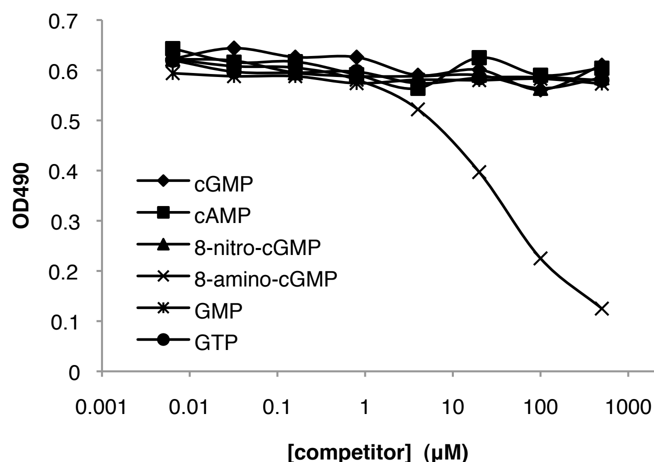

Figure S3. Competitive ELISA for anti-8-amino-cGMP polyclonal antibody

ELISA was conducted after incubation of the antibody with competitors for 30 min at room temperature.

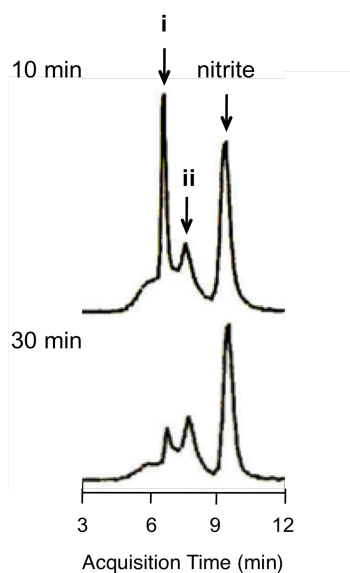

Figure S4. Conversion of 8-amino-cGMP to cGMP by an NO donor under aerobic conditions (pH 6.8)

8-amino-cGMP (0.2 mM) and DEA-NONOate sodium salt (Sigma, 1 mM) were incubated in 0.1 M sodium phosphate buffer (pH 6.8) at room temperature for the indicated time. The peaks **i** and **ii** in HPLC chromatogram correspond to 8-amino-cGMP and cGMP, respectively. The reaction mixture was analyzed by HPLC (Cosmosil 5C_{18}-AR-II, 150 mm long × 4.6 mm inner diameter, Nacalai Tesque) and eluted with methanol- 0.1% aqueous trifluoroacetic acid (5:95). Representative results of three experiments are shown.

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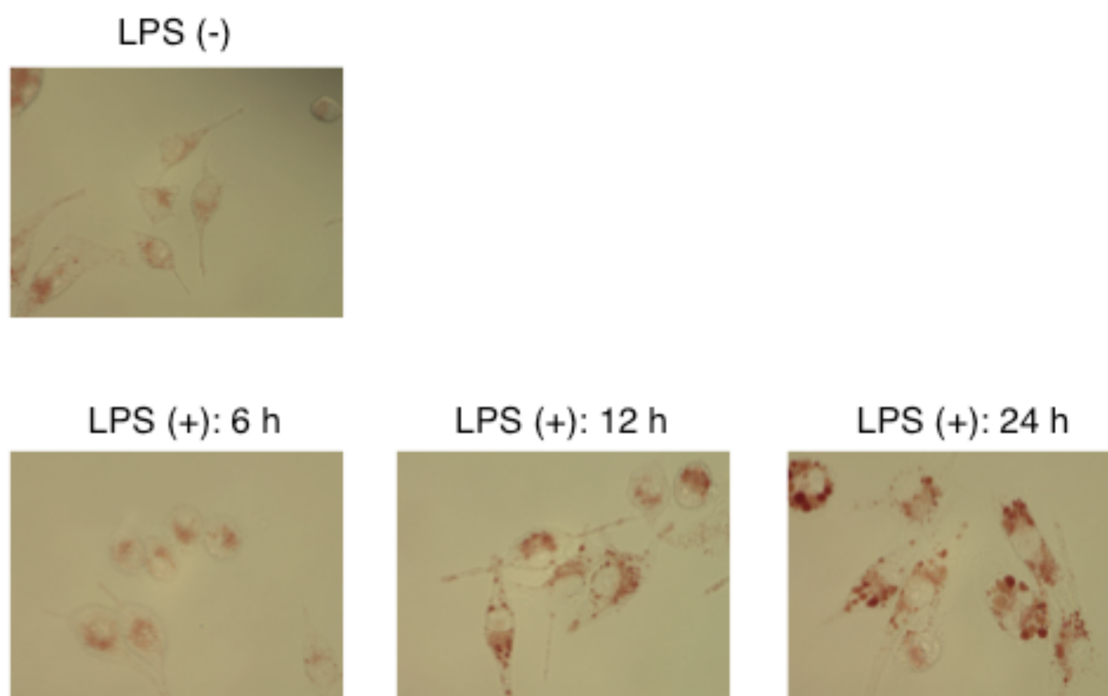

Figure S5. Intracellular acidification by LPS and IFN-γ stimulation

RAW 264.7 cells were stimulated with LPS (10 μg/ml) and IFN- γ (100 U/ml) for the indicated time. The cells were then treated with neutral red (100 μM) for 1 min and observed under a microscope. Neutral red turns red at a pH less than 6.8. Representative results of the three independent experiments are shown.

Methods

Synthesis of compounds

8-Cys-[U-^{13}C]cGMP

To a [U-^{13}C]GTP sodium salt solution (10 mM) in Tris HCl was added recombinant soluble guanylate cyclase, and the mixture was incubated at 37 °C. Crude [U-^{13}C]cGMP was purified by HPLC as described in the synthesis of compound **7**. Bromination and nitration of [U-^{13}C]cGMP were conducted as described in the synthesis of compounds **4** and **1**. The reaction with cysteine was conducted as described in our previous report.[5]

8-GS-[U-^{13}C]cGMP

Preparation of 8-GS-[U-^{13}C]cGMP was prepared as described in the synthesis of 8-Cys-[U-13C]cGMP. The reaction with glutathione was conducted as described in our previous report.[5]

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2'-*O*-succinylated-8-nitro-cGMP [5]

To a solution of 8-nitro-cGMP (5.0 mg, 13 μmol) in H_2O (0.12 mL) were added triethylamine (32 μl, 230 μmol) and succinic anhydride (19 mg, 190 μmol). After stirring for 5 h, the mixture was purified by HPLC (Develosil ODS-HG-5, 250 mm long × 20 mm inner diameter, Nomura Chemical Co., Ltd.) and eluted with acetonitrile-0.1% aqueous trifluoroacetic acid (10:90) to give 2'-*O*-succinylated-8-nitro-cGMP (3.8 mg, 60%) as a yellow solid. Structural data was consistent with the literature.[5]

Competitive ELISA

To determine the specificity of the antibody, we used competitive ELISA. Each well of a 96-well microtiter plate was coated with 100 μl of 8-amino-cGMP-BSA conjugate (1 μg mL^{-1}) in PBS, blocked with 0.5% gelatin, and washed three times with PBS containing 0.05% Tween 20 (washing buffer). The wells were incubated at room temperature for 1 h with 100 μl of antibody in the presence of various nucleotides 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. The reaction was terminated by addition of 50 μl of 2.0 M sulfuric acid, and absorbance at 490 nm was measured using a microplate reader (model 550, BIO-RAD).