SUPPLEMENTAL DATA

Supplemental Methods

Preparation of Antibodies for 8-Nitro-cGMP and *S*-Guanylated Proteins. Mouse monoclonal antibodies that specifically recognized the 8-nitro-cGMP structure were generated by immunizing BDF1 mice with 8-nitro-cGMP-conjugated bovine serum albumin (BSA; Sigma-Aldrich). Briefly, 8-nitro-cGMP was conjugated to BSA through succinyl coupling between the 2'-OH group of 8-nitro-cGMP and the lysine residues in BSA. 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 were then cloned by means of limited dilution. From the antibody clones generated in this study, we chose the clone of monoclonal anti-8-nitro-cGMP antibody 1G6 for immunocytochemical assays. The clone 1G6 is specific for 8-nitro-cGMP, although some cross-reaction is observed with other nitrated nucleotides including 8-nitro-GMP and 8-nitro-GTP, and has no cross-reactivity with native cGMP and nitrated purines including 8-nitroguanine and 8-nitroxanthine. A polyclonal anti-*S*-guanylated protein (8-RS-cGMP) antibody was raised in rabbits by subcutaneous injection of *S*-guanylated BSA, which was prepared by reacting 8-nitro-cGMP with BSA, plus Freund's complete adjuvant. After contamination with anti-BSA antibody was eliminated by using a BSA-conjugated HiTrap NHS-activated HP column (GE Healthcare), the antibody IgG fraction was purified from antiserum by using Protein A-Sepharose CL-4B gel (GE Healthcare). Mouse monoclonal anti-*S*-guanylated (8-RS-cGMP) antibody was generated, as in the protocol just described that was used to obtain anti-8-nitro-cGMP antibody, by immunizing BDF1 mice with *S*-guanylated BSA. The specificity of these antibodies was confirmed by means of a competitive ELISA. To study protein thiol modifications caused by different 8-nitroguanosine derivatives, another polyclonal anti-*S*-guanylated (8-RS-Guo) antibody that

recognizes the 8-thioalkoxy-guanosine moiety was generated. The antigen used was BSA reacted with 8-nitroguanosine. As in the protocol for preparation of 8-RS-cGMP antibody, antiserum was obtained from rabbits immunized with 8-RS-Guo-BSA adducts, followed by removal of the anti-BSA antibody fraction. This 8-RS-Guo antibody obtained showed reactivity against proteins *S*-adducted with various 8-nitroguanosine derivatives including 8-nitroguanosine, 8-nitro-cGMP, 8-nitroguanosine monophosphate, and 8-nitroguanosine triphosphate, as assessed via Western blotting analysis (data not shown) and as illustrated in supplemental Fig. S9.

Determination of Intracellular Concentrations of Various Nucleotides. Amounts of various nucleotides measured by LC-MS/MS were corrected by the cellular protein content measured simultaneously with cell lysates (methanol precipitates). Intracellular nucleotide concentrations were then determined from these corrected values based on a single protein content calculated separately and a single cell volume of C6 cells reported by Rouzaire-Dubois et al. (Glia 45: 249-257, 2004).

Supplemental Figure Legends

Supplemental Fig. S1. **Chemical structures of various cyclic nucleotides labeled with stable isotope or unlabeled and their characteristic fragmentation patterns found as a result of ionization during MS analysis.** The cyclic guanine nucleotides used in this study include cGMP (c[^{14}N]GMP), c[$^{15}N_5$]GMP (c[U-$^{15}N_5$]GMP), 8-$^{14}NO_2$-cGMP (8-nitro-cGMP), 8-$^{15}NO_2$-cGMP, and 8-$^{14}NO_2$-c[$^{15}N_5$]GMP (8-nitro-c[U-$^{15}N_5$]GMP).

Supplemental Fig. S2. **Recovery efficacy of cGMP and 8-nitro-cGMP determined in the spike-and-recovery study.** The amount of each endogenous nucleotide varies depending on its efficacy of recovery from cells. Recovery was evaluated in this study by using C6 cells stimulated with LPS and cytokines for 27 h, and using stable isotope-labeled nucleotides, as described in Experimental Procedures. *A*, recovery of authentic isotope ^{15}N-labeled nucleotide derivatives added exogenously during cell extraction was measured by using LC-MS/MS analysis. Recovery after use of different concentrations of authentic compounds

did not vary greatly. *B*, the amounts of endogenous cGMP (c[^{14}N]GMP) and 8-nitro-cGMP (8-$^{14}NO_2$-cGMP) were quantified in the same manner as in *A*. Although the amount of endogenous cGMP recovered from cells was not affected by different amounts of exogenous c[$^{15}N_5$]GMP, recovery of endogenous 8-nitro-cGMP improved greatly with increasing concentrations of 8-$^{15}NO_2$-cGMP exogenously spiked to the cell extract. *C*, the same improved yield of endogenous 8-nitro-cGMP was obtained when 8-NO_2-c[$^{15}N_5$]GMP was used instead of 8-$^{15}NO_2$-cGMP. *D*, the table summarizes the mean values illustrated in *B*. Because the increased recovery of 8-nitro-cGMP reached a plateau at about 100 pmol/dish of ^{15}N-labeled 8-nitro-cGMP and cGMP as well, we chose this concentration for the spike-and-recovery analysis. Data represent means ± S.E.M. (n = 3).

Supplemental Fig. S3. **Dose-dependent effect of SNAP on 8-nitro-cGMP formation.** C6 cells were treated with various concentrations of SNAP for different times in the presence of 1 mM BSO, and cell extracts were prepared as described in Experimental Procedures. cGMP (*A*) and 8-nitro-cGMP (*B*) concentrations in cells are shown at 10 min and 24 h after SNAP treatment. In the presence of BSO, a relatively low concentration of SNAP was sufficient to result in an appreciable amount of 8-nitro-cGMP, and 8-nitro-cGMP levels (24 h) were comparable to or higher than cGMP levels (10 min). Data represent means ± S.E.M. (n = 3).

Supplemental Fig. S4. **Elution profiles of 8-nitro-cGMP obtained by HPLC-ECD analysis (*A*) and correlation of HPLC-ECD results with those from LC-ESI-MS/MS analysis (*B*).** *A*, 8-nitro-cGMP (2 nM) (*left panel*); extract of nontreated control C6 cells (*middle panel*); extract of SNAP-treated C6 cells (*right panel*). C6 cells were treated with 50 μM SNAP for 12 h in the presence of 1 mM BSO. Cell extracts were prepared as described in Experimental Procedures and were subjected to HPLC-ECD. HPLC separations were carried out with a reverse-phase column (150 mm long, 3.0 mm inner diameter; Eicom Pak SC-5 ODS; Eicom, Kyoto, Japan) eluted with 0.4 ml/min of 0.2 M sodium phosphate buffer (pH. 3.0) plus 8% CH_3CN. 8-Nitro-cGMP was detected electrochemically via an online reductive activation method with -500 mV for reduction and +250 mV for oxidation (HTEC-500 and PEC-510; Eicom). The injection volume was 50 μl. The 8-nitro-cGMP

peaks are shown in red. *B*, each value obtained via HPLC-ECD analysis was correlated with that from LC-MS/MS. Note that data were obtained with the same lysate derived from C6 cells treated with 50 μM SNAP for various time periods, but these analyses were carried out without utilizing the spike-and-recovery protocol with stable isotope-labeled compounds.

Supplemental Fig. S5. **iNOS expression and nitrite production in C6 cells stimulated with LPS plus cytokines.** *A*, expression of iNOS after treatment with LPS and cytokines. Cells were pretreated with BSO (1 mM) for 8 h, after which they were stimulated with a mixture of LPS (1 or 10 μg/ml), IFN-γ (100 U/ml), TNFα (100 U/ml), and IL-1β (5 ng/ml) for the indicated time periods in the presence of 1 mM BSO. Cell lysates (10 μg of protein) were analyzed via Western blotting with an anti-iNOS antibody (Santa Cruz Biotechnology). *B*, immunocytochemistry of iNOS. Cells were stimulated with LPS (1 μg/ml) and cytokines (as in *A*) for 12 h in the presence of 1 mM BSO. iNOS (green) was detected. Nuclei were stained with Hoechst 33258 (blue). Scale bars indicate 25 μm. *C*, nitrite concentrations in culture supernatants of C6 cells. Cells were treated with the same mixture of LPS and cytokines as in *A*. Nitrite concentrations in culture supernatants were analyzed by means of the Griess reagent assay. Data represent means ± S.E.M. (n = 3).

Supplemental Fig. S6. **Immunocytochemical analysis of NO-dependent 8-nitro-cGMP formation in C6 cells.** *A*, cells were pretreated with 1 mM BSO for 8 h or were untreated, followed by treatment with 50 μM SNAP for the times indicated. Immunostaining was performed with anti-8-nitro-cGMP monoclonal antibody 1G6. *B*, cells were pretreated with BSO (1 mM) for 8 h or were left untreated, after which they were treated with various SNAP concentrations for 12 h in the absence or presence of 1 mM BSO. Cells were stained with anti-8-nitro-cGMP monoclonal antibody 1G6. *C*, cells pretreated with 1 mM BSO were stimulated with a mixture of LPS (1 μg/ml) and the cytokines IFN-γ (100 U/ml), TNFα (50 U/ml), and IL-1β (30 ng/ml) for 12 or 24 h in the absence or presence of 1 mM L-NMMA. Cells were stained with 1G6. Scale bars indicate 25 μm.

Supplemental Fig. S7. **Evaluation of *S*-guanylation levels in Keap1 of C6 cells.** Cells

overexpressing FLAG-Keap1 were treated with 8-nitro-cGMP, LPS plus cytokines, or SNAP for 12 h as described in Experimental Procedures. FLAG-Keap1 of the cells was then immunoprecipitated with anti-FLAG M2 affinity gel (Sigma-Aldrich), followed by Western blotting for *S*-guanylation. To obtain fully *S*-guanylated Keap1, an immunoprecipitated fraction of FLAG-Keap1 was first reduced with 0.2 mM DTT for 15 min at room temperature and then treated with an excessive amount of 8-nitro-cGMP (1 mM) for 3 h *in vitro*. The extent of *S*-guanylation of Keap1 from C6 cells having had various treatments was determined by comparing their immunoreactive intensities with that of fully *S*-guanylated FLAG-Keap1 serially diluted in Western blotting. *A*, Western blot analysis for *S*-guanylation of fully *S*-guanylated FLAG-Keap1 and FLAG-Keap1 from C6 cells after treatment with authentic 8-nitro-cGMP, LPS plus cytokines, or SNAP. The absolute protein content of each sample after treatments was compared with that of the fully *S*-guanylated FLAG-Keap1 (relative content 1.00) (*leftmost lane*, *A*). *B*, the immunoreactive band intensity of serially diluted, fully *S*-guanylated Keap1 and its relative protein content showed a linear correlation. The *S*-guanylation levels of Keap1 obtained from cells after the various treatments, as determined from this correlation curve (*B*), appear in *A* as the relative content given above the right three lanes: 0.22 (22%), 0.14 (14%), and 0.12 (12%) for 8-nitro-cGMP, LPS plus cytokines, and SNAP, respectively.

Supplemental Fig. S8. **Protein *S*-nitrosylation in SNAP-treated C6 cells.** Cells overexpressing FLAG-Keap1 were treated with 5 mM SNAP for 6 h. Cell lysates were subjected to immunoprecipitation analysis for *S*-nitrosylation, as described in Experimental Procedures, and to Western blotting analysis using FLAG M2 antibody. *A*, *S*-nitrosylation (*left panel*) and Western blotting for FLAG-Keap1 (*right panel*) with whole-cell lysates (2 μg of protein). No apparent bands of *S*-nitrosylated proteins were observed at the electrophoretic position of FLAG-Keap1 (*arrowhead*). *B*, *S*-nitrosylation of FLAG-Keap1. Lysates (200 μg of protein), prepared from cells overexpressing FLAG-Keap1 and treated with 5 mM SNAP for 6 h (*SNAP*) or not treated (*Control*), were subjected to immunoprecipitation analysis for *S*-nitrosylation (*C6 cell Keap1 IP*). FLAG-Keap1 detected with FLAG M2 antibody appears at the bottom (*FLAG*). HSA and SNO-HSA (2 μg) were used as negative and positive

controls, respectively.

Supplemental Fig. S9. **Reactivity of *S*-guanylated antibodies against cysteine-modified proteins.** Native rabbit serum albumin, 8-RS-Guo-albumin adduct, and 8-RS-cGMP-albumin adduct were immobilized onto ELISA plates at a concentration of 0.1 μg/well. Plates were washed and subjected to blocking with 0.5% gelatin. *S*-Guanylated (8-RS-cGMP and 8-RS-Guo) antibodies having the same titers with the dilutions as shown were added to the ELISA plates and incubated at room temperature for 1 h. After incubation, the ELISA plates were washed and reacted with anti-rabbit IgG antibody conjugated with horseradish peroxidase, followed by reaction with *o*-phenylenediamine dihydrochloride. The reaction was terminated by adding 0.05 ml of 2 M sulfuric acid, and absorbance at 490 nm was read by using a microplate reader. The epitope structure of the *S*-guanylated adducts that each antibody can recognize is illustrated at the top.

Supplemental Fig. S10. **Mitochondrial ROS formation in C6 cells treated with SNAP.** ROS formation was analyzed after SNAP stimulation by use of a mitochondria-specific ROS fluorescence probe, MitoSOX Red. After cells were treated with various SNAP concentrations for different time periods, they were stained with 4 μM MitoSOX Red. *A*, fluorescence images of cells obtained at 24 h after treatment with different concentrations of SNAP. *B*, similar images for cells at various time points after treatment with 50 μM SNAP. *C*, a typical confocal laser scanning image of cells treated with 50 μM SNAP for 24 h. *D*, semiquantitative illustration of fluorescence intensities observed for SNAP-treated cells determined by fluorometric analysis. See the text (Experimental Procedures) for details. Scale bars: 20 μm in *A*, *B*; 50 μm in *C*.

Supplemental Figure S1

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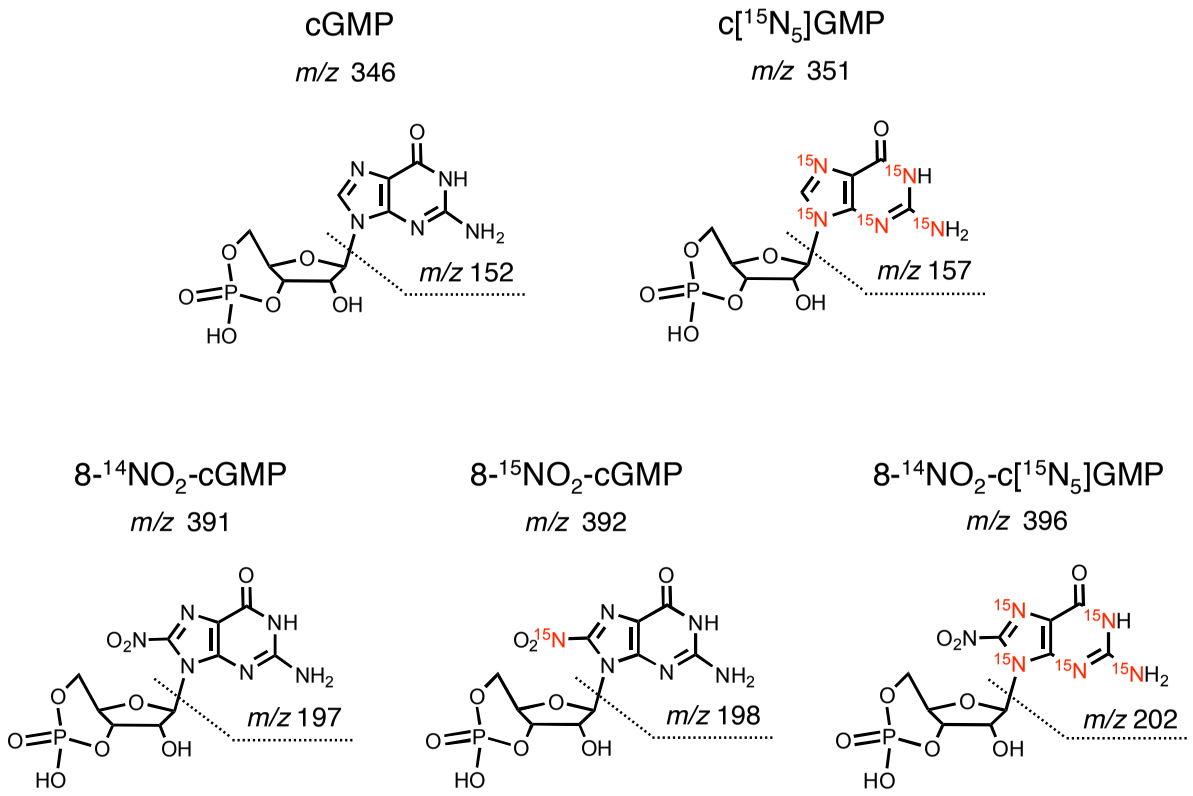

Supplemental Figure S2

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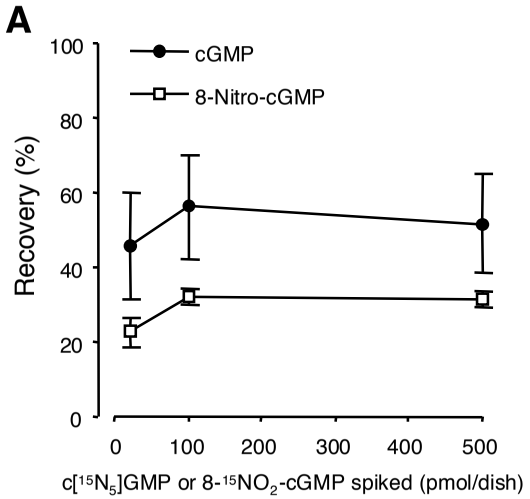

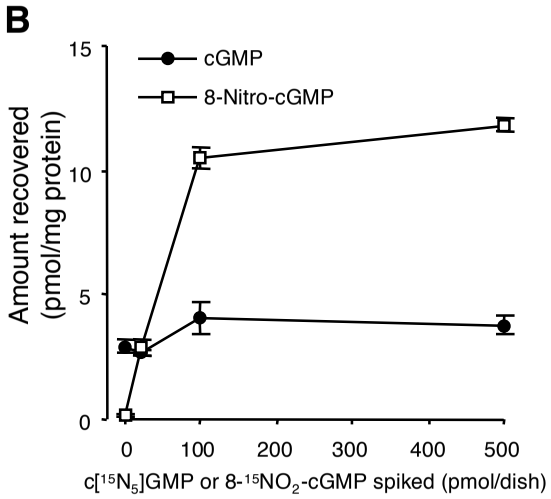

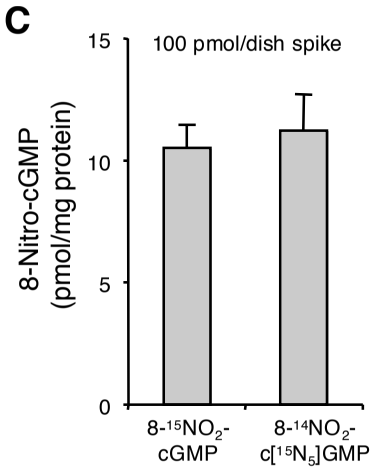

D

Endogenous cyclic nucleotides recovered

	Mean amounts of nucleotides recovered (pmol/mg protein) for c[$^{15}N_5$]GMP or 8-$^{15}NO_2$-cGMP spiked			
	0 pmol/dish	20 pmol/dish	100 pmol/dish	500 pmol/dish
cGMP	2.92	2.67	4.05	3.80
8-Nitro-cGMP	0.17	2.90	10.53	11.82

Supplemental Figure S3

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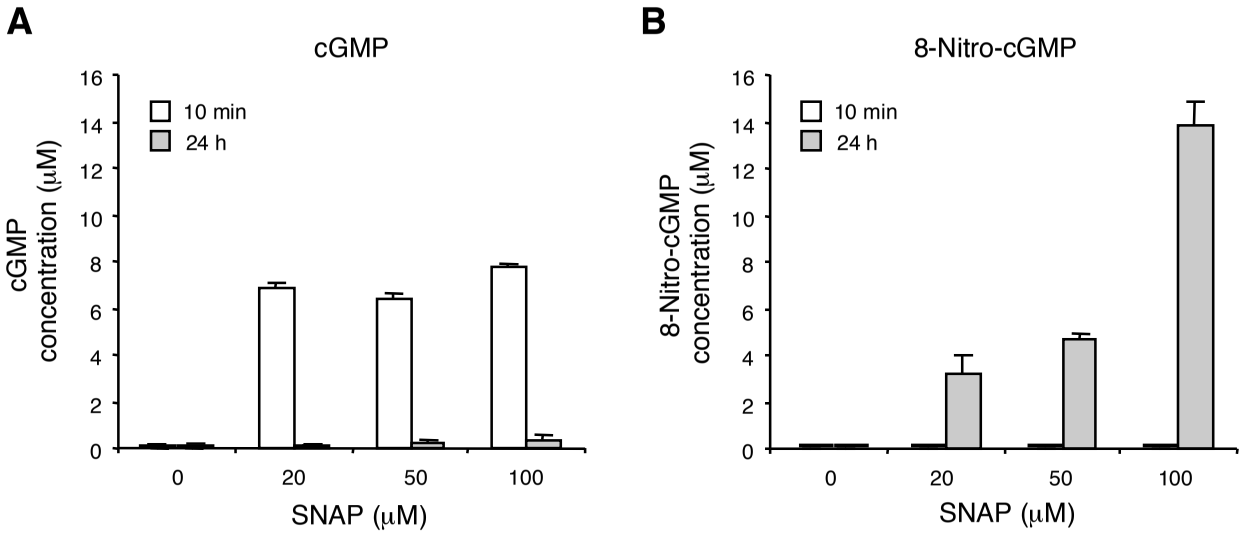

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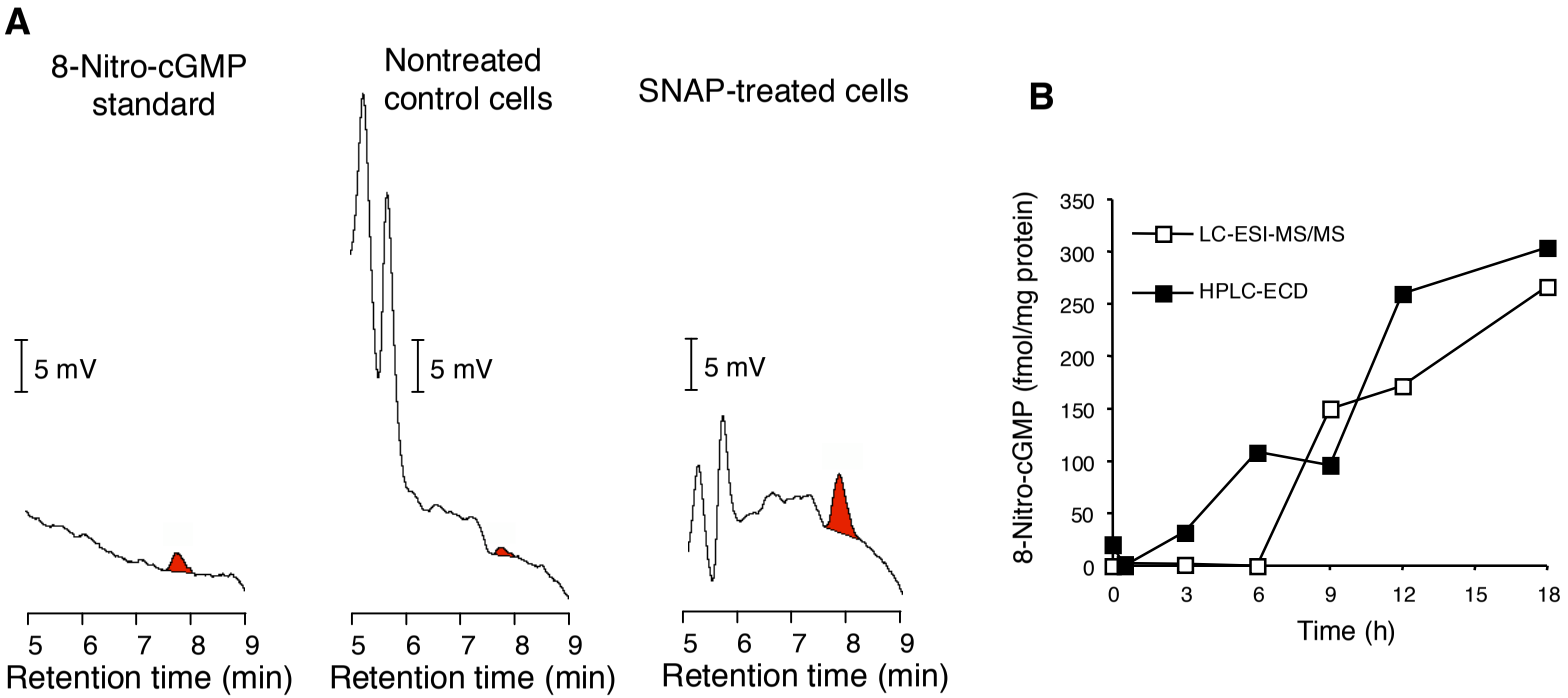
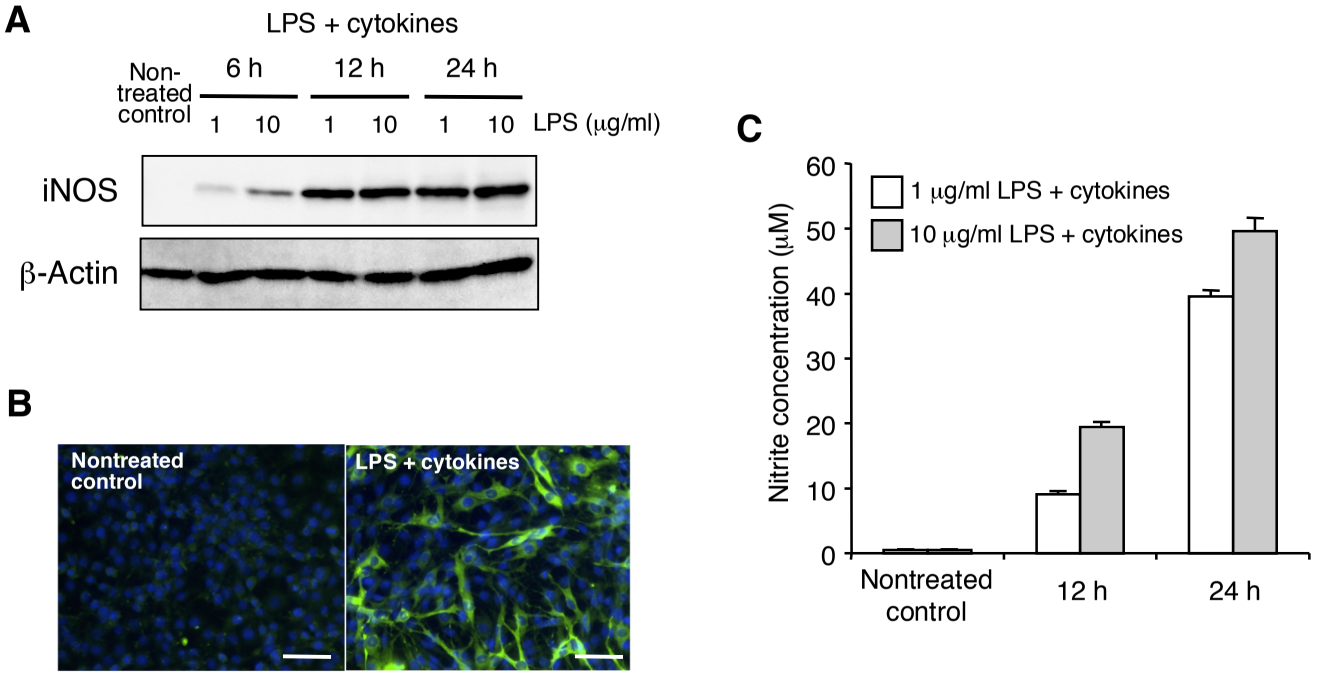


Supplemental Figure S5 **Fujii et al.**



Supplemental Figure S6

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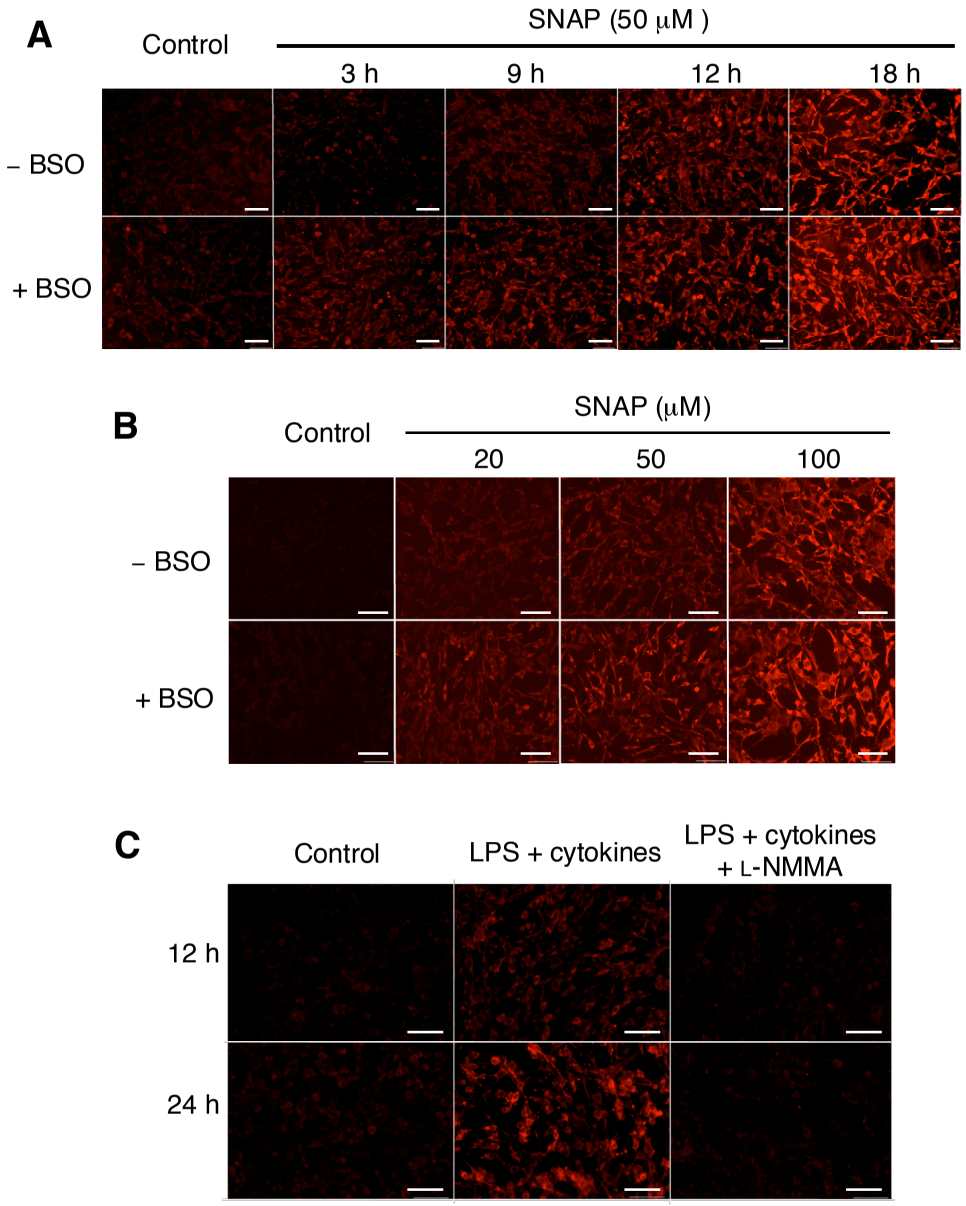
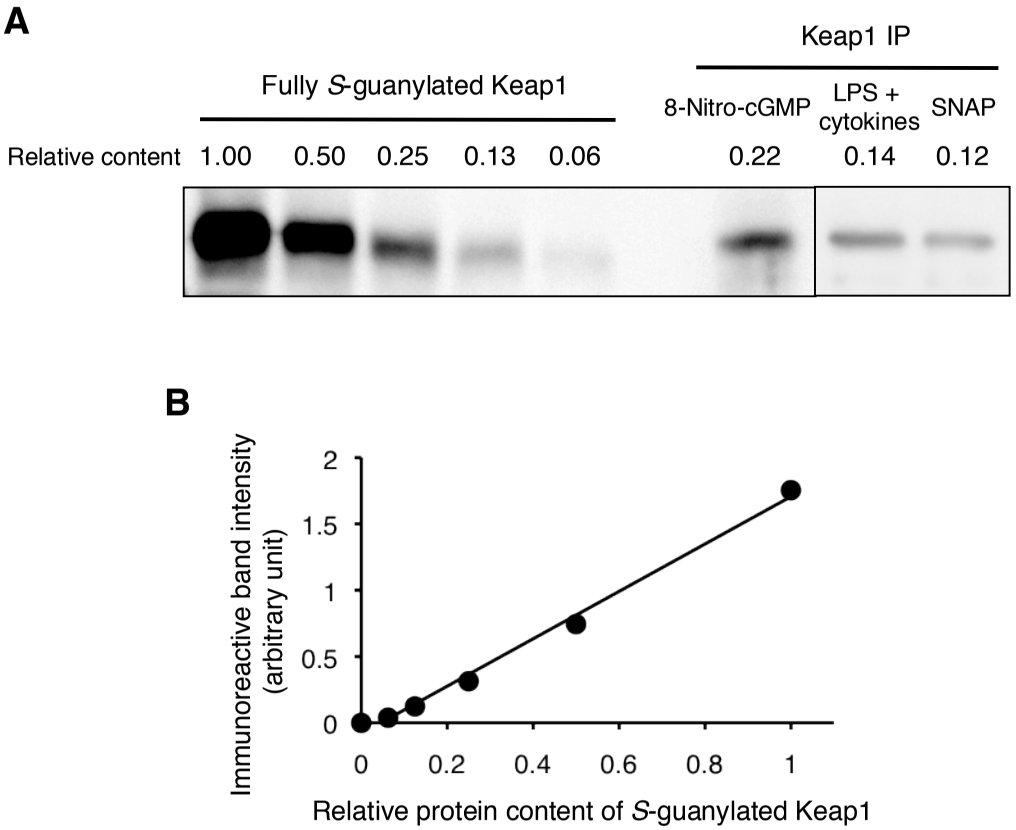


Supplemental Figure S7 **Fujii et al.**



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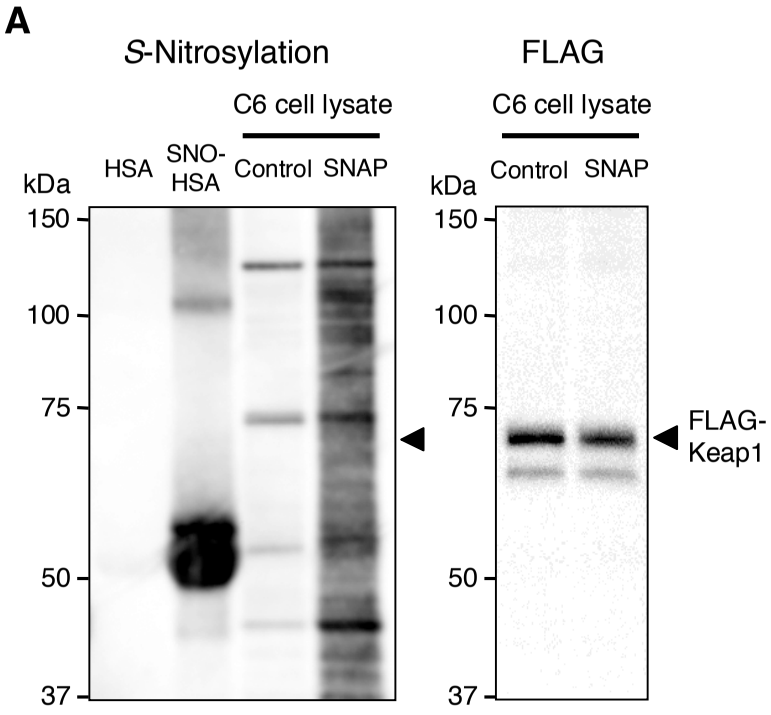
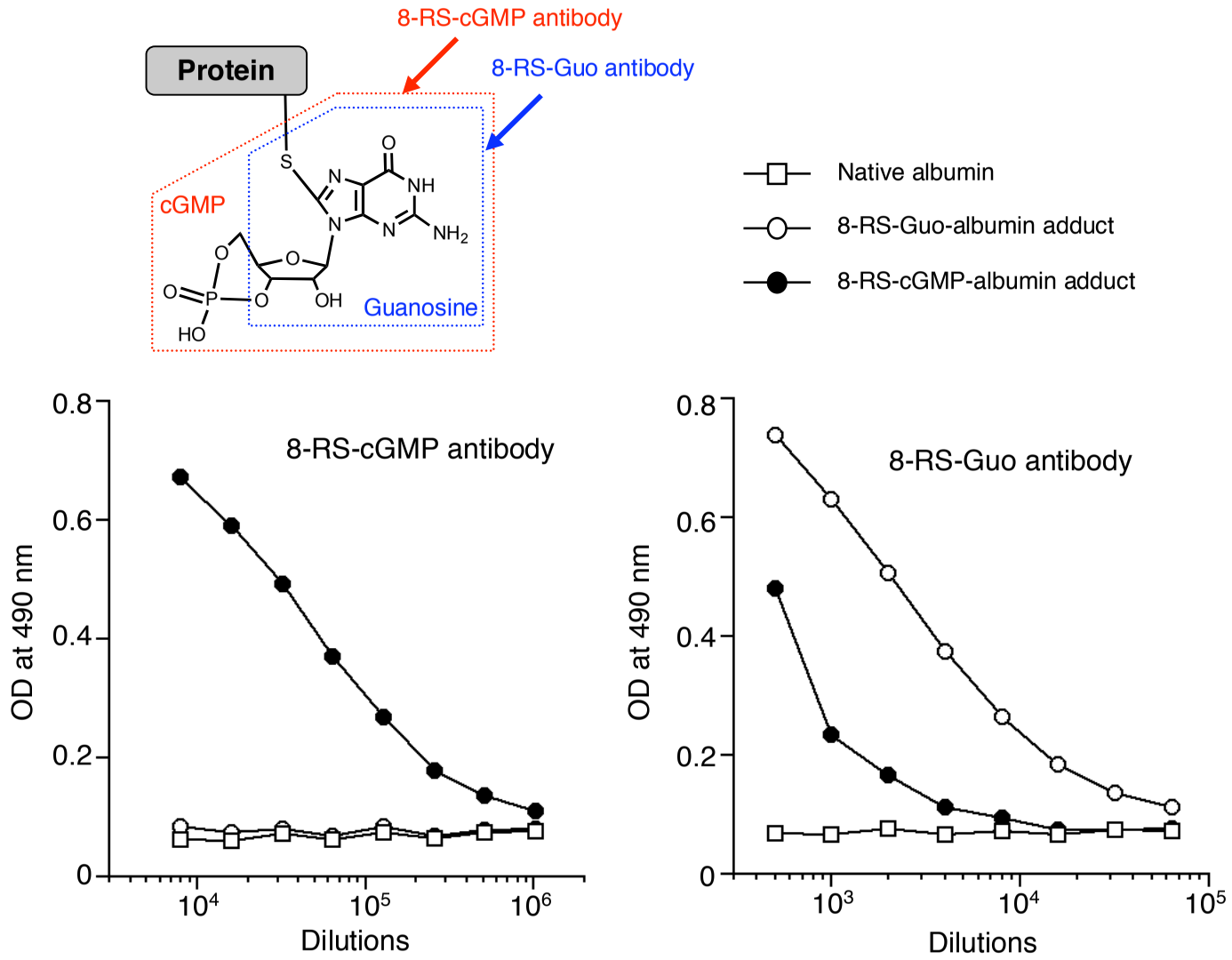


Supplemental Figure S9 **Fujii et al.**



Supplemental Figure S10 **Fujii et al.**