SUPPLEMENTARY ONLINE DATA

Regulation by mitochondrial superoxide and NADPH oxidase of cellular formation of nitrated cyclic GMP: potential implications for ROS signaling

Khandaker Ahtesham Ahmed[*], Tomohiro Sawa[*†], Hideshi Ihara[‡], Shingo Kasamatsu[‡], Jun Yoshitake[*], Md. Mizanur Rahaman[*], Tatsuya Okamoto[*], Shigemoto Fujii[*], and Takaaki Akaike[*1]

[*]Department of Microbiology, Graduate School of Medical Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto 860-8556, Japan, [†]PRESTO, Japan Science and Technology Agency (JST), 4-1-8 Honcho Kawaguchi, Saitama 332-001, Japan, and [‡]Department of Biological Science, Graduate School of Science, Osaka Prefecture University, 1-1 Gakuen-cho, Sakai, Osaka 599-8531, Japan

Running title: ROS-dependent formation of nitrated cyclic GMP

[1]Address correspondence to: Takaaki Akaike, Department of Microbiology, Graduate School of Medical Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto 860-8556, Japan.
Tel.: +81-96-373-5100; Fax: +81-96-362-8362;
E-mail: takakaik@gpo.kumamoto-u.ac.jp

EXPERIMENTAL

Mitotracker Green staining

In order to confirm the mitochondrial origin of superoxide generated by LPS-cytokine stimulation, we performed double staining of cells with 50 nM MitoTracker Green FM dissolved in Hank's buffer [1] (excitation at 488 nm; green photomultiplier channel of the confocal microscope used for image acquisition) plus 2.5 μM MitoSOX Red dissolved in Hank's buffer, for 15 min.

Nitrite and nitrate measurement

C6 cells were seeded in 96-well plate at a density of 10^5 cells/well, followed by stimulated with LPS-cytokines in the absence or the presence of PEG-SOD (200 U/ml) or PEG-catalase (200 U/ml) for 36 h. We quantified NO_2^- and NO_3^- produced in C6 cells by means of an HPLC-flow reactor system, as reported previously [2].

Superoxide and H_2O_2 measurements

C6 cells were seeded in 12-well plate at a density of 2 x 10^5 cells/well, followed by treated with control siRNA or p47phox siRNA or untreated for 36 h. Cells were then stimulated with LPS-cytokines for further 36 h. Cells were then washed twice with PBS, followed by subjected for measurements of superoxide and H_2O_2. Superoxide production by C6 cells was measured by means of cytochrome c reduction assay as described previously [3]. Cytochrome c reduction was measured by reading absorbance at 550 nm. Superoxide production was calculated from SOD-inhibitable absorbance using coefficient for change of ferricytochrome c to ferrocytochrome c (i.e., 21. 0 mmol/L/cm). For H_2O_2 measurement, C6 cells was incubated in PBS at 37°C for 10 min, and H_2O_2 produced in supernatant was measured by using Hydrogen peroxide colorimetric detection kit (Catalog No. ADI-907-015, Enzo Life Sciences, PA, USA) according to the manufacture's protocol.

Cell viability assay

C6 cells were seeded in 96-well plate at a density of 10^3 cells/well, followed by stimulated with LPS-cytokines in the presence of tiron (0, 10, 100 μM) for 36 h. Cell viability was measured using the Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) according to the manufactures protocol.

REFERENCES

1. Pendergrass, W., Wolf, N. and Poot, M. (2004) Efficacy of MitoTracker Green and CMXrosamine to measure changes in mitochondrial membrane potentials in living cells and tissues. Cytometry A **61**, 162-169
2. Akaike, T., Inoue, K., Okamoto, T., Nishino, H., Otagiri, M., Fujii, S. and Maeda, H. (1997) Nanomolar quantification and identification of various nitrosothiols by high performance

liquid chromatography coupled with flow reactors of metals and Griess reagent. J. Biochem. **122**, 459-466

3. Li, J. M. and Shah, A. M. (2002) Intracellular localization and preassembly of the NADPH oxidase complex in cultured endothelial cells. J. Biol. Chem. **277**, 19952-19960

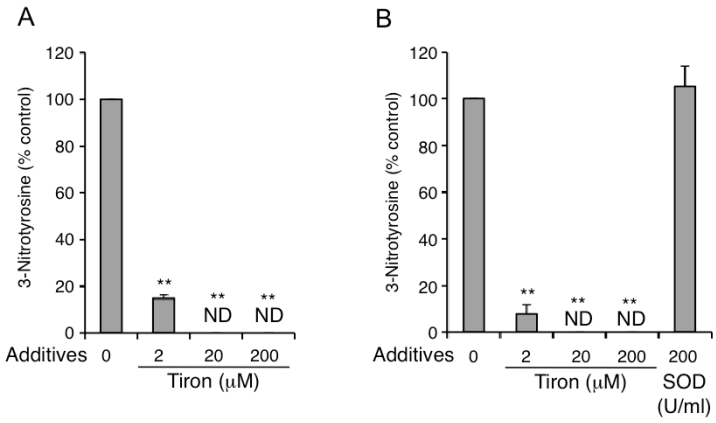

Figure S1 Effect of tiron on tyrosine nitration caused by RNOS

Tyrosine (100 μM) was reacted with $ONOO^-$ (5 μM) (A) or with P-NONOate (100 μM) (B) in 0.1 M sodium phosphate buffer (pH7.4) containing 0.1 mM DTPA in the presence of indicated concentrations of tiron. In B, effect of SOD (200 U/ml) was also examine. Formation of 3-nitrotyrosine was determined by HPLC-ECD. Data are expressed as means ± S.E. (n = 3). **, $p < 0.01$ vs control (without tiron). ND, not detected.

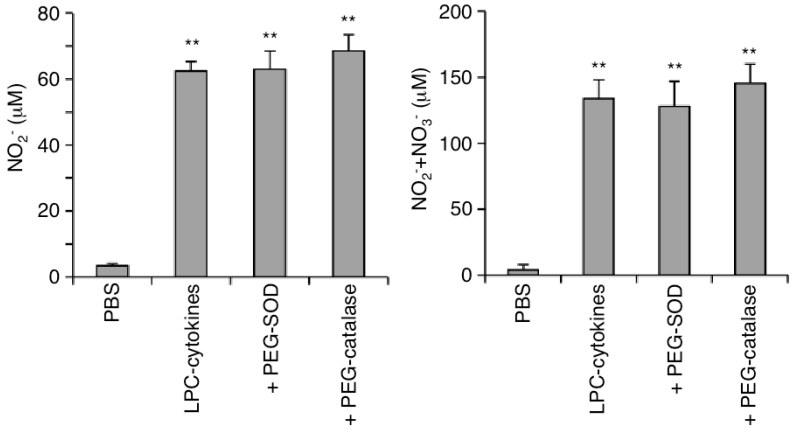

Figure S2 Effect of PEG-SOD and PEG-catalase on NO production from C6 cells

C6 cells were seeded in 96-well plate at a density of 10^5 cells/well, followed by stimulated with LPS plus pro-inflammatory cytokines in the presence of PEG-SOD (200 U/ml) or PEG-catalase (200 U/ml) for 36 h. NO metabolites (NO_2^-, NO_3^-) in culture supernatants were determined by Griess assay. Data are expressed as means ± S.E. (n = 3). **, $p < 0.01$ vs control (PBS).

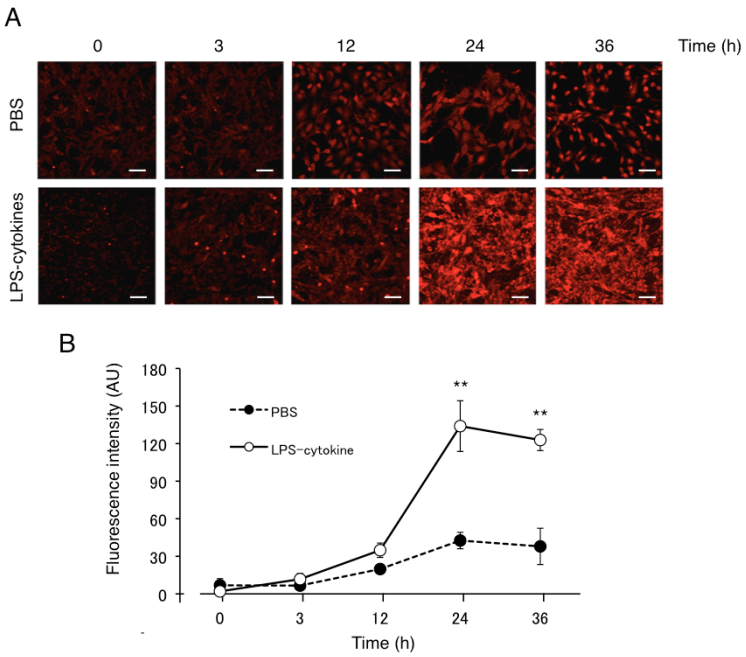

Figure S3 Time-dependent increase in mitochondrial superoxide in rat C6 glioma cells stimulated with LPS-cytokines

Cells were stimulated with a mixture of LPS (10 μg/ml), IFN-γ (200 U/ml), TNFα (500 U/ml), and IL-1β (10 ng/ml) for 0, 3, 12, 24, and 36 h. Cells were then analyzed for the presence of mitochondrial superoxide as described under "Experimental Procedures." (**A**) MitoSOX Red staining of PBS-treated cells (upper panels) and LPS-cytokine-treated cells (lower panels) as detected by the Nikon EZ-C1 confocal laser microscope (for MitoSOX Red: excitation at 420 nm and red photomultiplier channel; for DCDHF-DA: excitation at 488 nm and green photomultiplier channel). Scale bars indicate 50 μm. (**B**) Fluorescence intensity for MitoSOX Red staining. Data are expressed as means ± S.E. (n = 3). ** $p < 0.01$ vs PBS-treated cells.

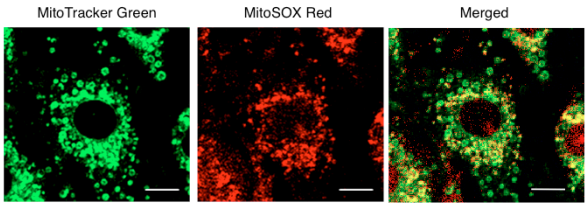

Figure S4 LPS-cytokine stimulation of rat C6 glioma cells produced mitochondrial superoxide

Cells were stimulated with a mixture of LPS (10 μg/ml), IFN-γ (200 U/ml), TNFα (500 U/ml), and IL-1β (10 ng/ml) for 36 h. Stimulated cells were then analyzed for co-localization of mitochondria and superoxide generated in the cells, as described under "Experimental Procedures." MitoTracker Green (left panel) and MitoSOX Red (middle panel) staining of stimulated cells, as detected by the Nikon EZ-C1 confocal laser microscope (for MitoTracker Green: excitation at 488 nm and green photomultiplier channel; for MitoSOX Red: excitation at 420 nm and red photomultiplier channel). Right panel, merged image. Scale bars indicate 7 μm.

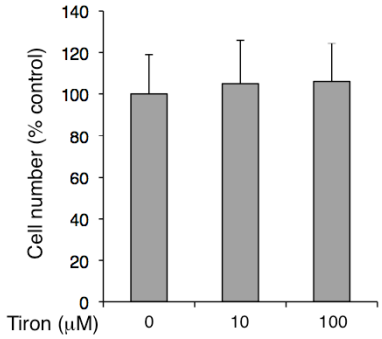

Figure S5 Effect of tiron on C6 cell viability

C6 cells were seeded in 96-well plate at a density of 10^3 cells/well, followed by stimulated with LPS plus pro-inflammatory cytokines in the presence of indicated concentrations of tiron for 36 h. Number of viable cells were then determined by cell counting kit. Data are expressed as means ± S.E. (n = 3).

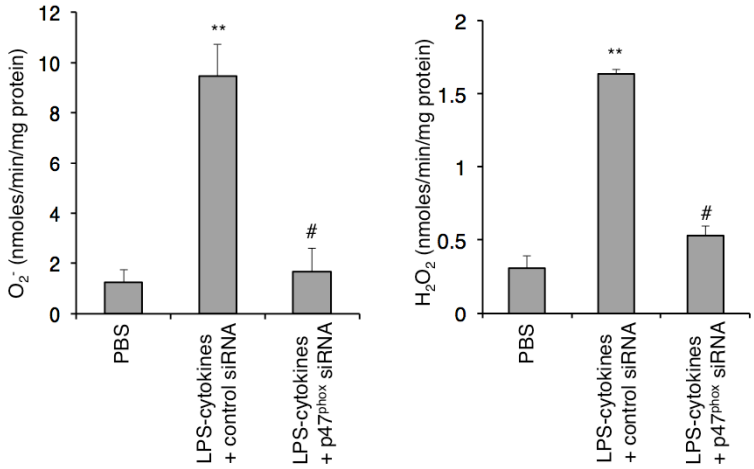

Figure S6 Extracellular concentrations of O_2^- and H_2O_2 and their modulation by p47^{phox} knockdown

C6 cells were seeded in 12-well plate at a density of 2x10^5 cells/well, followed by treated with control siRNA or p47^{phox} siRNA or untreated for 36 h. Cells were then stimulated with LPS plus pro-inflammatory cytokines for further 36 h. Supernatant obtained from each culture condition was then subjected for O_2^- measurement (cytochrome c assay) and H_2O_2 measurement (xyrenol orange assay). Data are expressed as means ± S.E. (n = 3). **, $p < 0.01$ vs control (PBS). #, $p < 0.01$ vs LPS-cytokines + control siRNA.

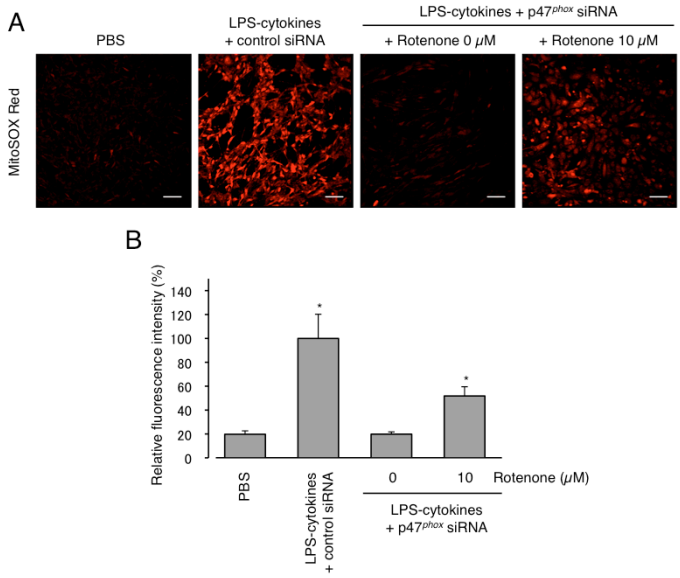

Figure S7 Rotenone induced mitochondrial superoxide in rat C6 glioma cells with Nox2 gene knockdown

Cells were transfected with control siRNA or p47^{phox}-specific siRNA as described under "Experimental Procedures," followed by stimulation with a mixture of LPS (10 μg/ml), IFN-γ (200 U/ml), TNFα (500 U/ml), and IL-1β (10 ng/ml) for 36 h. Cells transfected with p47^{phox}-specific siRNA were treated with 10 μM rotenone or were untreated before stimulation with the LPS-cytokine mixture. MitoSOX Red staining was used to detect mitochondrial superoxide as described under "Experimental Procedures." (**A**) Fluorescent staining of mitochondrial superoxide. Scale bars indicate 50 μm. (**B**) relative fluorescence intensity of C6 cells, treated as just described, for MitoSOX Red staining. Data are expressed as means ± S.E. (n = 3). *, $p < 0.01$, compared with the PBS-treated group.

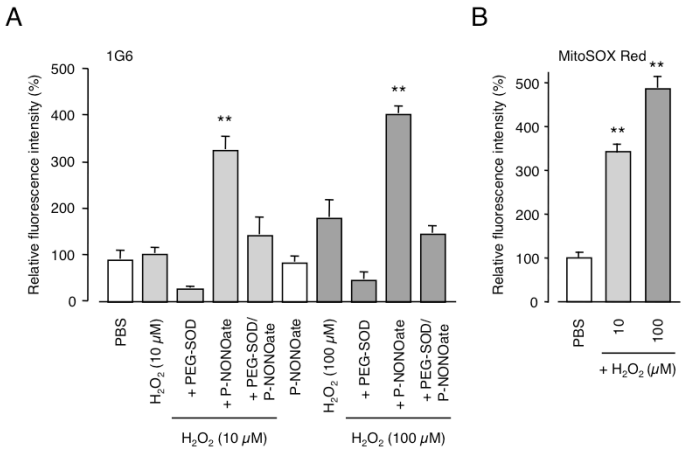

Figure S8 Increase in fluorescence intensity of 8-nitro-cGMP and mitochondrial superoxide in rat C6 glioma cells after H_2O_2 and NO treatment

(**A**) Cells were untreated or treated with 10 or 100 μM H_2O_2 for 36 h, plus PEG-SOD (200 U/ml), P-NONOate (100 μM), or PEG-SOD (200 U/ml) plus P-NONOate (100 μM), and the relative fluorescence intensity of 8-nitro-cGMP was measured with the use of 1G6 monoclonal antibody, as described under "Experimental Procedures." (**B**) In other experiments, cells were untreated or treated with only 10 or 100 μM H_2O_2, followed by determination of relative fluorescence intensity for MitoSOX Red staining compared with the fluorescence intensity of PBS-treated control cells. Data are expressed as means ± S.E. (n = 3). *, $p < 0.01$, compared with the PBS-treated control group.