Molecular basis for cellular formation of a nitrated cyclic nucleotide, a second messenger for ROS signaling

Doctor's Thesis by

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学位論文
Doctoral Thesis

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（活性酸素シグナルのセカンドメッセンジャー、ニトロ化環状ヌクレオチドの細胞内生成の分子基盤）

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Abstract

8-Nitroguanosine 3′,5′-cyclic monophosphate (8-nitro-cGMP) is a novel nitrated derivative of guanosine 3′,5′-cyclic monophosphate (cGMP) of which endogenous formation has recently been identified in mammalian cells. 8-Nitro-cGMP can function as a unique electrophilic second messenger that induces antioxidant adaptive response to cells via cGMP adduction to sulfhydryls of redox sensor proteins, the process is called protein *S*-guanylation. I here studied chemical and biochemical regulatory mechanisms involved in the formation of 8-nitro-cGMP, with particular focus on the roles of reactive oxygen species (ROS).

In vitro formation of nitrated guanine derivatives was analyzed by means of reverse-phase high performance liquid chromatography equipped with photodiode array detector or with electrochemical detector. Cellular formation of 8-nitro-cGMP in rat glioma C6 cells was analyzed by means of immunocytochemistry with the use of anti-8-nitro-cGMP monoclonal antibody and by means of liquid chromatography-tandem mass spectrometry (LC-MS/MS). Cellular production of ROS was determined by means of fluorescent microspectrometry using chemical probes that become fluorescent upon reaction with ROS.

Chemical analyses demonstrated that peroxynitrite ($ONOO^-$) and myeloperoxidase-dependent oxidation of nitrite in the presence of hydrogen peroxide (H_2O_2) were two major pathways for guanine nucleotide nitration. Among guanine nucleotides examined, guanosine 5′-triphosphate was the most sensitive against

$ONOO^-$-mediated nitration. Immunocytochemical as well as LC-MS/MS analyses revealed that formation of 8-nitro-cGMP in rat glioma C6 cells stimulated with lipopolysaccharide (LPS) plus pro-inflammatory cytokines (LPS-cytokines) was highly dependent on the production of both superoxide (O_2^-) and H_2O_2. By using mitochondria-targeted chemical probe MitoSOX^{TM} Red, I found that mitochondria-derived O_2^- can act as a direct determinant for 8-nitro-cGMP formation. This is the first demonstration that mitochondria-derived O_2^- plays an essential role in biological nitration of guanine nucleotides. Furthermore, I also clarified that mitochondria-derived O_2^- production was regulated by NADPH oxidase 2 (Nox2)-generated H_2O_2, suggesting the importance of cross-talk between Nox2-dependent H_2O_2 production and mitochondrial O_2^- production. These data suggest that 8-nitro-cGMP can serve as a unique second messenger that may implicate in regulation of ROS signaling in the presence of nitric oxide (NO).

In the second part of this study, I examined the methodological proof of immunocytochemistry for specific identification of 8-nitro-cGMP in cultured cells. I performed immunocytochemistry with monoclonal antibodies specific for 8-nitroguanine (clone NO2-52) and 8-nitro-cGMP (clone 1G6) in rat C6 glioma cells and rat primary cultured astrocytes. Immunocytochemistry utilizing the anti-8-nitro-cGMP monoclonal antibody (1G6) indicated that immunostaining increased markedly in C6 cells expressing increased amounts of inducible NO synthase (iNOS) after treatment with LPS-cytokines. Treatment of C6 cells with inhibitors for iNOS

and soluble guanylate cyclase (sGC) completely nullified the elevated 1G6 immunoreactivity. These results were consistent with the LC-MS/MS analyses. Immunocytochemistry performed using NO2-52 also showed that treatment of cells with inhibitors for iNOS and sGC completely nullified the elevated immunoreactivity; this indicated that 8-nitro-cGMP is a major component of 8-nitroguanine derivatives produced in cells. Similar results were obtained in the primary astrocytes stimulated with LPS-cytokines. Because immunocytochemistry is a conventional, powerful, and fairly straightforward method for determining the presence, localization, and relative abundance of an antigen of interest in cultured cells, anti-8-nitroguanine (NO2-52) and anti-8-nitro-cGMP (1G6) antibodies could be useful tools for analyzing nitrated guanine nucleotides, especially 8-nitro-cGMP, by means of immunocytochemistry.

In summary, I herein verified that ROS play a pivotal role in the formation of 8-nitro-cGMP in C6 cells stimulated with LPS-cytokines. O_2^-, most likely derived from mitochondria, is directly involved in formation of 8-nitro-cGMP, whereas H_2O_2 generated by Nox2 also has an important role by increasing mitochondrial O_2^- production. The present study thus suggests that 8-nitro-cGMP may serve as a unique second messenger for ROS signaling in the presence of NO. Greater understanding of 8-nitro-cGMP formation in relation to mitochondrial functions and Nox2 regulation may help us develop new diagnostic methods and treatment of diseases related to dysregulation of NO and ROS.

Keywords Nitric oxide · Reactive oxygen species · Oxidative stress ·

Posttranslational modification · Redox signal · ROS signal · Electrophilic signal · Adaptive response

List of Publications

1. **Ahmed KA**, Sawa T, Ihara H, Kasamatsu S, Yoshitake J, Okamoto T, Fujii S, Akaike T. Regulation by mitochondrial superoxide and NADPH oxidase of cellular formation of nitrated cyclic GMP: potential implication in ROS signalling. ***Biochemical Journal*** (2011 in press)
2. Ihara H*, **Ahmed KA***, Ida T, Kasamatsu S, Kunieda K, Okamoto T, Sawa T, Akaike T. Methodological proof of immunochemistry for specific identification of 8-nitroguanosine 3′,5′-cyclic monophosphate formed in glia cells. ***Nitric Oxide*** 25:169-75 (2011) *** equal contribution**
3. **Ahmed KA**, Sawa T, Akaike T. Protein cysteine *S*-guanylation and electrophilic signal transduction by endogenous nitro-nucleotides. ***Amino Acids*** 41: 123-130 (2011)
4. Tokutomi Y, Kataoka K, Nakamura T, Fukuda M, Nako H, Toyama K, Dong Yi, **Ahmed KA**, Sawa T, Akaike T, Kim-Mitsuyama S. Vascular responses to 8-nitro-cyclic GMP in nondiabetic and diabetic mice. ***British Journal of Pharmacology*** 162: 1884–1893 (2011)
5. Fujii S, Sawa T, Ihara H, Tong KI, Ida T, Okamoto T, **Ahmed KA**, Ishima Y, Motohashi H, Yamamoto M, Akaike T. The critical role of nitric oxide signaling via protein *S*-guanylation and nitrated cyclic GMP, in the antioxidant adaptive response. ***Journal of Biological Chemistry*** 285: 23970-84 (2010)
6. Zaki MH, Fujii S, Okamoto T, Islam S, Khan S, **Ahmed KA**, Sawa T, and Akaike T. Cytoprotective function of heme oxygenase-1 induced by a nitrated cyclic nucleotide formed during murine salmonellosis. ***Journal of Immunology*** 182: 3746-3756 (2009)
7. Alam MS, Zaki MH, Sawa T, Islam S, **Ahmed KA**, Fujii S, Okamoto T, and Akaike T. Nitric oxide produced in Peyer's patches exhibits antiapoptotic activity contributing to an antimicrobial effect in murine salmonellosis. ***Microbiology and Immunology*** 52: 197-208 (2008)

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Abbreviations

ARE	Antioxidant responsive element
cGMP	Guanosine 3′,5′-cyclic monophosphate
DCDHF-DA	2′,7′-Dichlorodihydrofluorescein diacetate
DMEM	Dulbecco's modified Eagle's medium
DNase-1	Deoxyribonuclease I
DTPA	Diethylenetriamine-N,N,N′,N″,N″-pentaacetic acid
EDTA	Ethylenediamine-N,N,N′,N′-tetraacetic acid
eNOS	Endothelial NO synthase
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDP	Guanosine 5′-diphosphate
GMP	Guanosine 5′-monophosphate
GTP	Guanosine 5′-triphosphate
HO-1	Hemeoxygenase 1
HPLC	High performance liquid chromatography
HPLC-ECD	High performance liquid chromatography-electrochemical detection
HPLC-PDA	High performance liquid chromatography-photodiode array detection
HRP	Horseradish peroxidase
IFN-γ	Interferon-γ
iNOS	Inducible NO synthase

IL-1β	Interleukin-1β
Keap1	Kelch-like ECH-associated protein 1
L-NMMA	N^{ω}-monomethyl-L-arginine
LPS	Lipopolysaccharide
mETC	Mitochondrial electron-transport chain
MPO	Myeloperoxidase
MS/MS	Tandem mass spectrometry
NADPH	Nicotinamide adenine dinucleotide phosphate
NEM	*N*-ethylmaleimide
8-Nitro-cGMP	8-Nitroguanosine 3′,5′-cyclic monophosphate
8-Nitro-GDP	8-nitroguanosine 5′-diphosphate
8-Nitro-GMP	8-nitroguanosine 5′-monophosphate
8-Nitro-GTP	8-nitroguanosine 5′-triphosphate
nNOS	Neuronal NO synthase
NOS	Nitric oxide synthase
Nox2	NADPH oxidase 2
Nrf2	NF-E2-related factor 2
PBS	Phosphate-buffered saline
PEG	Polyethylene glycol
PEG-catalase	Pegylated catalase
PEG-SOD	Pegylated superoxide dismutase

P-NONOate	propylamine NONOate ($CH_3N[N(O)NO]^-(CH_2)_3NH_2^+CH_3$, 1-hydroxy-2-oxo-3-(*N*-methyl-3-aminopropyl)-3-methyl-1-triazene)
RNOS	Reactive nitrogen oxide species
ROS	Reactive oxygen species
RP	Reverse phase
RP-HPLC	Reverse phase high performance liquid chromatography
Tiron	1,2-dihydroxy-3,5-benzene-disulfonic acid
TNFα	Tumor necrosis factor α
siRNA	Small interfering RNA
sGC	Soluble guanylate cyclase
SOD	Cu, Zn-superoxide dismutase

INTRODUCTION

1.1. Biology of Nitric Oxide (NO)

Nitric oxide (NO) is one of the smallest known bioregulatory molecules. It modulates a number of different cellular functions in prokaryotes, plants, and animals. In stark contrast to the apparent simplicity of this diatomic molecule, its biological chemistry is surprisingly complex, which makes it one of the most versatile signaling molecules known. In the past 20 years, NO has been established as a gaseous free radical with critical and unforeseen roles in quite varied biological functions and organisms (1), including regulation of vascular and neuronal signal transduction, host defense, and cell death regulation (2-6). In mammals, NO is synthesized by three different NO synthases (NOSs): neuronal NO synthase (nNOS), inducible NO synthase (iNOS), and endothelial NO synthase (eNOS). These NOSs catalyze the oxidation of L-arginine to form NO and L-citrulline (7). Signal transduction of NO can be classified into guanosine 3′,5′-cyclic monophosphate (cGMP)-dependent (canonical NO/cGMP pathway) and cGMP-independent (noncanonical: NO-induced posttranslational modifications) (1, 8). The canonical NO/cGMP signaling paradigm involves NO-dependent activation of soluble guanylate cyclase (sGC), which results in formation of cGMP, which itself activates protein kinases, cyclic nucleotide-gated ion channels, and phosphodiesterases (8, 9).

NO readily reacts with oxygen radicals and metals to produce chemically reactive compounds named collectively reactive nitrogen oxide species (RNOS), such as peroxynitrite ($ONOO^-$) and nitrogen dioxide (NO_2). Recent studies suggested that

biomolecules such as fatty acids and nucleotides react with NO and RNOS to form electrophilic metabolites. Several groups reported the biological formation of nitrated fatty acids (10-12), which has been found to cause thiol modification via *S*-nitroalkylation (13). In addition to fatty acid nitration RNOS can induce nitration of nucleic acids (Fig. 1). During the past several years, nitrated guanine derivatives such as 8-nitroguanine and 8-nitroguanosine were identified in diverse cultured cells and in tissues from humans with lung diseases and different organisms with viral pneumonia, cancer, and other inflammatory conditions (14-22). The redox activity of 8-nitroguanosine in particular suggests that guanine nitration may have potent biological effects (23). We recently discovered that another nitrated cyclic nucleotide, 8-nitroguanosine 3′,5′-cyclic monophosphate (8-nitro-cGMP), is produced in cells expressing iNOS (24). 8-Nitro-cGMP has an extremely potent signaling function in biological systems because of its dual nature in signal transduction, i.e., the canonical NO/cGMP pathway and noncanonical electrophilic signaling; among the nitrated guanine derivatives studied, it possessed the strongest redox-active property (24). Being an electrophile, 8-nitro-cGMP effectively reacted with sulfhydryl groups of cysteine (Cys) residues and formed a protein-*S*-cGMP adduct, via a post-translational modification named protein *S*-guanylation (24) (Fig. 2).

1.2 Biological Formation of Nitro-nucleotides

In vitro and in vivo experiments have identified nitration of nucleic acids (Fig.

1), more specifically guanine derivatives, as associated with various inflammatory conditions. Yermilov et al. (1995) found that $ONOO^-$ reacts with the guanine base of nucleic acids to form 8-nitroguanine in vitro (26). Masuda et al. (2002) demonstrated $ONOO^-$-mediated 8-nitroguanosine formation from RNA in vitro (27). Our group first reported in vivo evidence of guanine nitration: we found marked guanine nitration in the lungs of influenza virus-infected mice and human patients with idiopathic pulmonary fibrosis and lung cancer that depended on production of NO by iNOS (14, 17, 19). We also observed formation of 8-nitroguanosine in mice with infections with bacteria such as *Salmonella typhimurium* (28). In addition, Yoko et al. (2007) detected 8-nitroguanine formation in malignant fibrous histiocytoma (29). Recently, 8-nitroguanine was linked with diabetic retinopathy, which is a major cause of blindness (30).

Our chemical analyses using high-performance chromatography-based electrochemical detection (HPLC-ECD) and tandem mass spectrometry (MS/MS) revealed that a nitrated derivative of cGMP, 8-nitro-cGMP, was generated in significant amounts in cell culture models with different types of cells (24, 25). For example, 8-nitro-cGMP was identified in the mouse macrophage cell line RAW 264.7 stimulated with cytokines such as interferon-γ (IFN-γ) and lipopolysaccharide (LPS) to produce NO via iNOS. More important, liquid chromatography-tandem mass spectrometry (LC-MS/MS) clearly revealed quantitative formation of 8-nitro-cGMP in C6 cells, a rat glioma cell line, stimulated with an exogenous NO donor or with LPS plus cytokines

(LPS-cytokines) (25). Infection with the gram-negative bacterium *Salmonella* also facilitated formation of 8-nitro-cGMP in mouse macrophages (24, 28). Formation of 8-nitro-cGMP was easily analyzed via immunocytochemistry with the use of anti-8-nitro-cGMP monoclonal antibodies. It was intriguing that immunostaining of intracellular 8-nitro-cGMP colocalized with mitochondria rather than endoplasmic reticulum (24). This intracellular localization may have implications for the mechanism and physiological effects of 8-nitro-cGMP formation. Moreover, this mitochondria-related nitration suggests that 8-nitro-cGMP may have a role as a regulator of mitochondrial functions such as energy metabolism and cell death. 8-Nitro-cGMP formation was also detected with other cultured cells, such as human hepatocellular carcinoma (HepG2) cells, adipocytes, and endothelial cells. Thus, clear evidence now exists that nitrated derivatives of nucleotides are generated under various physiological and pathophysiological conditions, so that they may serve not only as oxidative stress markers but also as biologically functioning electrophilic signal transducers.

1.3 Redox Signaling Property of Nitro-nucleotides

8-Nitroguanosine and it's derivatives have significant redox activity. In the presence of certain oxidoreductases and electron donors such as nicotinamide adenine dinucleotide phosphate (NADPH), 8-nitroguanosine derivatives readily reduced to form their anion radicals, which then transferred a single electron to molecular oxygen to

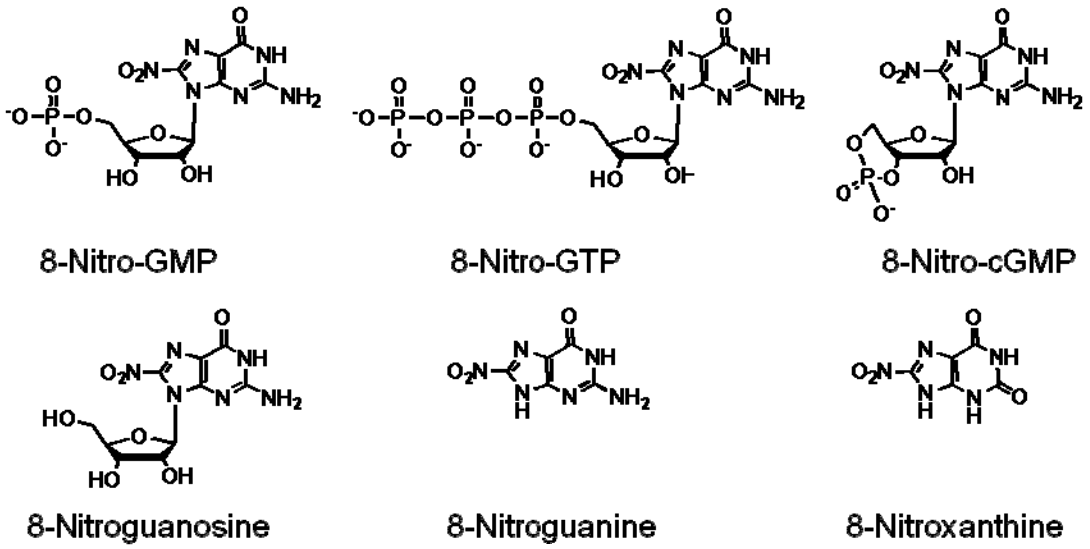

Fig. 1 Chemical structures of 8-nitroguanine-related compounds including various 8-nitroguanine nucleotides

form superoxide (O_2^-) radical (14, 23). 8-Nitro-cGMP had the highest redox activity among the 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). 8-Nitroguanine had only negligible redox activity (14, 23, 24). Oxidoreductases that can reduce 8-nitroguanosines include cytochrome P450 reductase and all three NOS isoforms (14, 23).

In view of this redox activity, 8-nitroguanosines may serve to regulate vascular tone. In fact, 8-nitro-cGMP can induce vasoconstriction, possibly through formation of O_2^-, as determined in organ bath experiments (24). 8-Nitro-cGMP-induced vasoconstriction depended completely on endothelium, and more specifically on eNOS, which indicates the occurrence of eNOS-dependent reduction of 8-nitro-cGMP to form O_2^-, which in turn antagonizes NO-dependent vasorelaxation. At higher concentrations, however, 8-nitro-cGMP induced vasorelaxation, through activation of cGMP-dependent kinase (PKG) in vascular smooth muscle cells.

1.4 Nitro-nucleotides: Endogenously Formed Electrophiles

Electrophilicity is another unique property of nitro-nucleotides. Because of their electrophilicity, nitro-nucleotides readily react with nucleophilic thiol compounds of low and high molecular weight to form 8-thioalkoxy-guanosine adducts. We named this unique electrophilic reaction *S*-guanylation of sulfhydryls (24, 31). This reaction

seems to occur via nucleophilic attack by the thiol group of a protein Cys or glutathione (GSH) on the C8 carbon of 8-nitro-cGMP so that the nitro moiety is released and the 8-RS-cGMP adduct is formed (Fig. 2). The major distinction between *S*-guanylation and other *S*-alkylations is that *S*-guanylation is apparently quite stable and produces irreversible sulfhydryl modifications, because the nitro moiety of the purine structure is lost during formation of adducts with protein Cys residues. It is noteworthy that 8-nitro-cGMP is the first known nitrated derivative of a cyclic nucleotide that possesses electrophilicity.

Among the nitroguanine derivatives examined (Fig. 1), 8-nitro-cGMP showed the highest reactivity with thiol compounds. The second-order rate constant for the reaction of 8-nitro-cGMP with the sulfhydryl of GSH was determined to be 0.03 M^{-1} s^{-1} at pH 7.4 and 37^{o}C (24). This value is much smaller than values for other electrophiles such as 4-hydroxynonenal, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2, and nitro-linoleic and -oleic acids; i.e., those compounds have reaction rate constants with GSH of 1.3, 0.7, 355, 183 M^{-1} s^{-1}, respectively, at pH 7.4 and 37^{o}C. This comparatively lower second-order rate constant may account for the stable nature of this novel compound in the cellular compartment where GSH is abundant (at ~ mM levels) and may be responsible for the fact that 8-nitro-cGMP causes very selective *S*-guanylation with sulfhydryls possessing high nucleophilicity, as determined, at least in part, by low pK_a values of sulfhydryls of the Cys moiety.

1.5 Protein *S*-guanylation in Electrophilic Signaling

Because of its electrophilicity, 8-nitro-cGMP may mediate electrophilic signaling via induction of *S*-guanylation of redox sensor proteins. Among this class of proteins, Kelch-like ECH-associated protein 1 (Keap1) was identified as a highly sensitive *S*-guanylation target (24, 25). Keap1 is a negative regulator of NF-E2-related factor 2 (Nrf2), a transcription factor that regulates phase-2 detoxifying and antioxidant enzymes for electrophiles and reactive oxygen species (ROS) (32, 33). The binding of Keap1 to Nrf2 maintains the cytosolic localization of Nrf2 and brings about rapid degradation of Nrf2 by proteasomes. Because Keap1 has highly reactive Cys residues, chemical modification of the sulfhydryl group of Cys residues by electrophiles and ROS has been proposed to trigger dissociation of Nrf2, which would lead to its stabilization and nuclear translocation. Activated Nrf2 would thus bind to the antioxidant responsive element (ARE) to induce expression of various cytoprotective enzymes, which are involved in adaptive responses to oxidative stress (34, 35).

Our recent chemical analyses for Keap1 revealed that Keap1 expressed by various cultured cells was highly susceptible to *S*-guanylation induced by NO-dependent 8-nitro-cGMP production (24, 25). In fact, we determined that NO and RNOS can modify Keap1 in macrophages during bacterial infections and in C6 cells in culture after proinflammatory stimuli. 8-Nitro-cGMP formation was also clearly observed in macrophages infected with the pathogenic bacterium *Salmonella*. *S*-Guanylated Keap1 was then detected in cultured murine macrophages

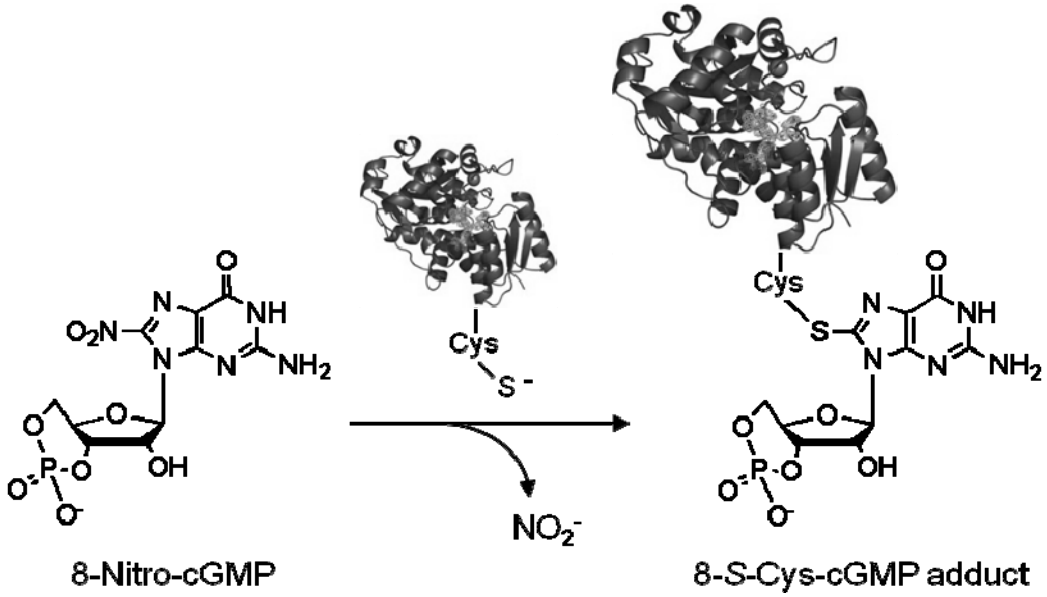

Fig. 2 Schematic representation of protein *S*-guanylation. Nucleophilic protein thiolates attack the C8 carbon of 8-nitro-cGMP, which results in adduction of cGMP moieties to Cys residues in proteins, with concomitant release of nitrite anion

after *Salmonella* infection. These findings are further confirmed by the fact that Keap1 can be exclusively *S*-guanylated by 8-nitro-cGMP produced in vivo in cultured cells (25). It is therefore highly plausible that 8-nitro-cGMP may act as an endogenous electrophilic ligand and affect Keap1 sulfhydryls via *S*-guanylation, which would lead to antioxidant signaling (Fig. 3).

We now understand that the Keap1-Nrf2 system can be an important cytoprotective mechanism against electrophiles and ROS (36). Noticeably, cytoprotection and host defense conferred by 8-nitro-cGMP were clearly associated with increased expression of heme oxygenase 1 (HO-1) in macrophages and in vivo during *Salmonella* infection (28). HO-1 is an enzyme with various physiological roles including vasoregulation (37, 38), cytoprotection (39, 40), and anti-inflammatory effects (41, 42). We also reported earlier that HO-1 expression induced by NO contributed to cell survival in certain solid tumor models (43, 44).

Our present belief that 8-nitro-cGMP may mediate electrophilic signaling via induction of *S*-guanylation was unambigously supported by our significant data showing that 8-nitro-cGMP directly caused site-specific *S*-guanylation of Keap1, which led to subsequent Nrf2-dependent HO-1 induction with its potent antioxidant effect (25). That 8-nitro-cGMP-mediated electrophilic signal transduction may have various effects in the physiology and pathophysiology of NO- and ROS-related phenomena is, therefore, conceivable (45) (Fig. 4).

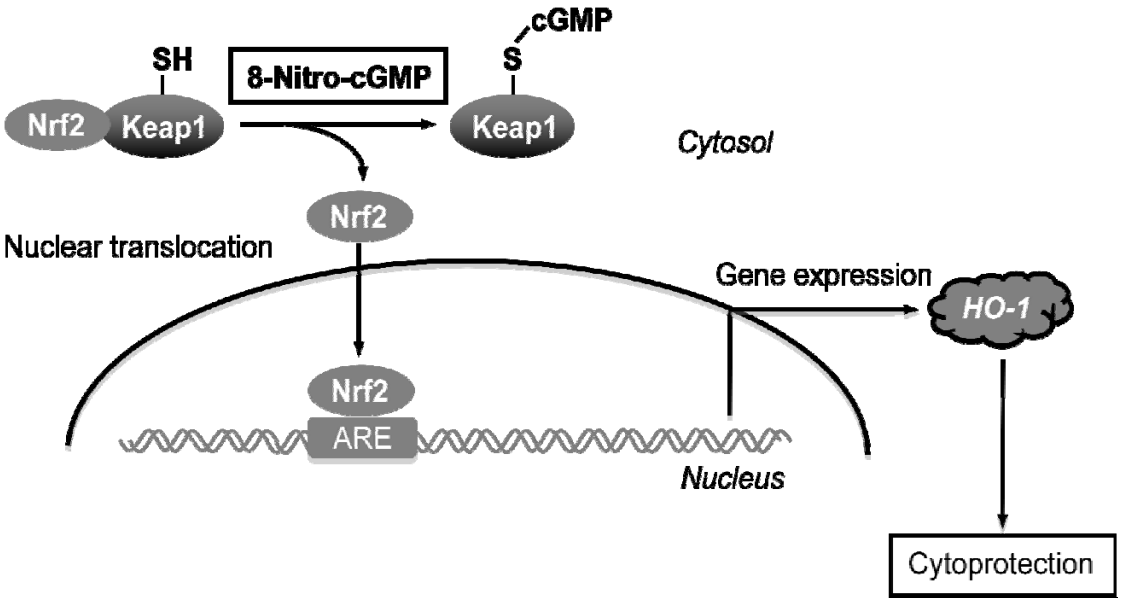

Fig. 3 8-Nitro-cGMP-mediated cytoprotection by *S*-guanylation of Keap1. After *S*-guanylation of Keap1, Nrf2 dissociates from Keap1, is translocated to the nucleus, and acts as a transcription factor in the expression of cytoprotective genes including heme oxygenase-1 (HO-1). *ARE* antioxidant responsive element

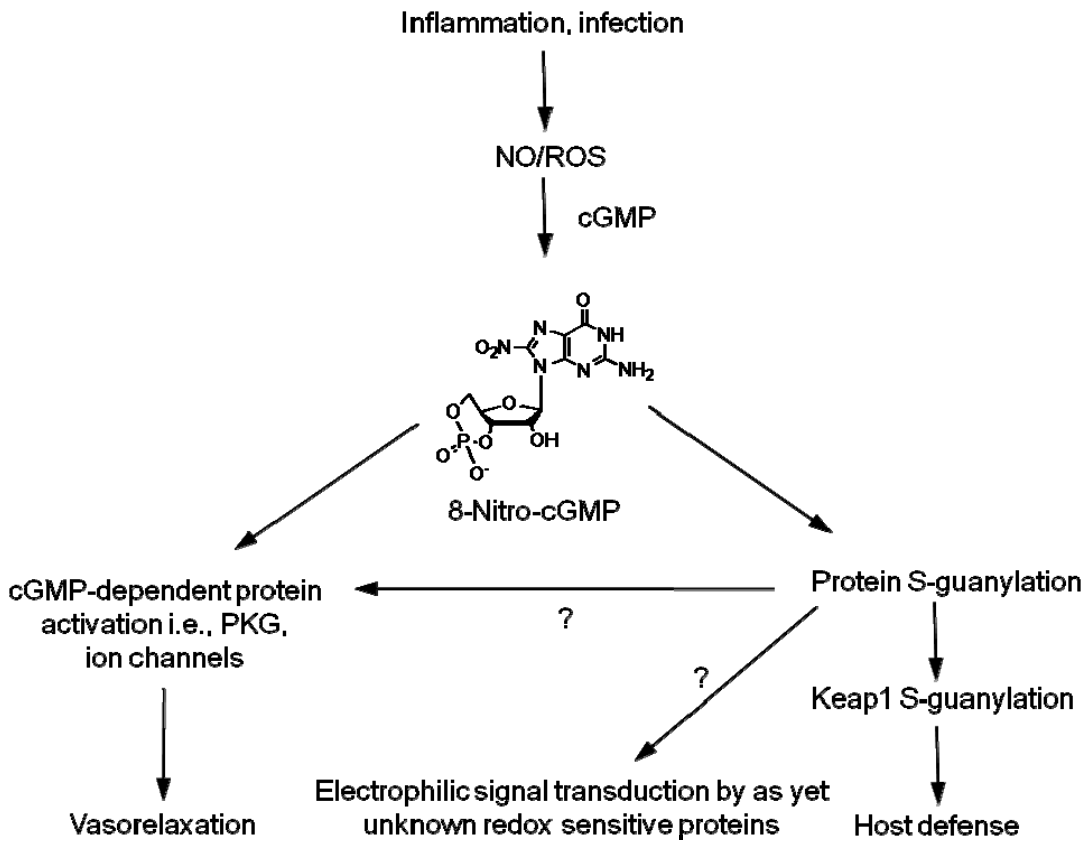

Fig. 4 Signal transduction by 8-nitro-cGMP via protein *S*-guanylation

1.6 Objective of the Study

To explore how and when 8-nitro-cGMP is involved in regulating cell physiology via its unique electrophilic property, understanding of the molecular mechanisms regulating 8-nitro-cGMP formation in cells is essential. Nitration of the guanine moiety is a crucial step for production of nitrated nucleotides including 8-nitro-cGMP. Previous studies suggested that, RNOS, formed from the reaction of NO and ROS, can nitrate guanine derivatives under biologically relevant conditions (15, 16, 46). Examples of RNOS include $ONOO^-$, which is a potent oxidizing and nitrating agent formed from the reaction of NO and O_2^-. It is therefore hypothesized that, formation of 8-nitro-cGMP might be regulated by cell production of ROS to form RNOS.

In the first part of this study (Part A), I investigated the roles of ROS in 8-nitro-cGMP formation both in vitro and in cells. Chemical analyses revealed that $ONOO^-$ was a potent agent for nitration of guanine nucleotides. In addition to $ONOO^-$, nitrite in the presence of hydrogen peroxide (H_2O_2) and myeloperoxidase (MPO) may nitrate guanine nucleotides. Rat C6 glioma cells were used to study cell formation of 8-nitro-cGMP, because the cells produced a significant amount of 8-nitro-cGMP in response to stimulation with LPS-cytokines via expression of the iNOS.

In the second part of the study (Part B), I evaluated the use of immunocytochemical approaches to semiquantitatively assess 8-nitro-cGMP levels in cells. Immunocytochemistry is a conventional method for detecting an antigen of

interest in cultured cells, and provides information not only about relative abundance but also about localization of an antigen in cells, which is complementary to alternative analytical methods including mass-spectrometry. We previously produced monoclonal antibodies specific for 8-nitroguanine (clone NO2-52) and 8-nitro-cGMP (clone 1G6) (24). In the present study, I performed immunocytochemistry with monoclonal antibodies NO2-52 and 1G6 in rat C6 glioma cells and rat primary cultured astrocyte. The results were compared with chemical identification and quantification by LC-MS/MS analysis.

MATERIALS AND METHODS

2.1 Materials

cGMP, guanosine 5′-triphosphate (GTP), guanosine 5′-diphosphate (GDP), guanosine 5′-monophosphate (GMP), and rotenone (mitochondrial electron transport inhibitor) were obtained from Sigma-Aldrich Corporation, St. Louis, MO, USA. The NO-liberating agent propylamine NONOate ($CH_3N[N(O)NO]^-(CH_2)_3NH_2^+CH_3$, 1-hydroxy-2-oxo-3-(*N*-methyl-3-aminopropyl)-3-methyl-1-triazene) (P-NONOate), which has a half-life of 7.6 min to release NO in aqueous solutions at a neutral pH under these experimental conditions, was obtained from Dojindo Laboratories, Kumamoto, Japan. 3-Morpholinosydnonimine (SIN-1), diethylenetriamine-N,N,N′,N′′,N′′-pentaacetic acid (DTPA), ethylenediamine-N,N,N′,N′-tetraacetic acid (EDTA), and 1,2-dihydroxy-3,5-benzene-disulfonic acid (tiron) were obtained from Dojindo Laboratories. Tyrosine was obtained from Kyowa Hakko Co. Ltd, Tokyo, Japan. MPO was purchased from Alexis Biochemicals, Plymouth Meeting, PA, USA. Horseradish peroxidase (HRP) was obtained from Wako Pure Chemical Industries, Osaka, Japan. Bovine Cu, Zn-superoxide dismutase (SOD) was purchased from Sigma-Aldrich Corporation, St. Louis, MO, USA. Catalase was purchased from Boehringer Mannheim GmbH, Mannheim, Germany. The succinimidyl derivative of polyethylene glycol (PEG) propionic acid, which has an average molecular mass of M_r 5000, was obtained from NOF Corporation, Tokyo, Japan. NADPH oxidase 2 (Nox2) $p47^{phox}$ small interfering RNA (siRNA) was purchased from Invitrogen, Carlsbad, CA, USA. 2′,7′-Dichlorodihydrofluorescein diacetate (DCDHF-DA) and MitoSOX^{TM} Red

were purchased from Invitrogen Corporation, Molecular Probes, Inc., Eugene, OR, USA. For effective delivery of proteinaceous ROS scavengers including SOD and catalase to the intracellular compartment, those enzymes were chemically modified by conjugation with PEG to obtain the pegylated superoxide dismutase (PEG-SOD) and pegylated catalase (PEG-catalase) (47). In brief, enzyme amino groups that originated from lysine or N-terminal amino acids were conjugated with an amino-reactive succinimidyl derivative of PEG according the literature (48). SOD (10 mg/ml) or catalase (10 mg/ml) was reacted with succinimidyl PEG (155 mg/ml for SOD and 120 mg/ml for catalase) in 0.5 M sodium phosphate buffer (pH 7.4) for 2 h in 4 ^{o}C under stirring. After the reaction, unreacted PEG derivatives and other impurities were removed by using an ultrafiltration device (Centricon 3K; Amicon Bioseparations, Millipore Corporation, Bedford, MA, USA), as previously reported (48). Authentic $ONOO^-$ was synthesized from acidified nitrite and H_2O_2 by a quenched-flow method according to the literature (49). Contaminating H_2O_2 was then decomposed by using manganese dioxide. Mouse monoclonal antibodies specific for 8-nitro-cGMP (clone 1G6) and 8-nitroguanine (clone NO2-52) were generated as described previously (24). N^{ω}-monomethyl-L-arginine (L-NMMA), LPS (from *Escherichia coli*; L8274), deoxyribonuclease I (DNase I), penicillin-streptomycin, trypsin and anti-β-actin antibody were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). Anti-p47-phox antibody (Cat # 07-497) was purchased from (Millipore, Temecula, CA, USA. Anti-sGC β1 subunit antibody was from Cayman Chemical (Ann Arbor, MI,

USA). Fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT, USA). Horse serum was purchased from Gibco-Invitrogen (Grand island, NY, USA). IFN-γ, tumor necrosis factor α (TNFα), and interleukin-1β (IL-1β) were obtained from R&D Systems, Inc. (Minneapolis, MN, USA). Dulbecco's modified Eagle's medium (DMEM) and NS 2028 were from Wako Pure Chemical Industries (Osaka, Japan). Blocking One and *N*-ethylmaleimide (NEM) were obtained from Nacalai Tesuque (Kyoto, Japan). BlockAce was from Dainippon Pharmaceutical (Osaka, Japan). Can Get Signal Immunoreaction Enhancer Solution was purchased from Toyobo (Osaka, Japan). Cy3-labeled goat anti-mouse IgG antibody and peroxidase-conjugated anti rabbit secondary antibody were from GE Healthcare (Piscataway, NJ, USA). All other chemicals and reagents were from common suppliers and were of the highest grade commercially available.

2.2 Synthesis of Various Guanine Nucleotides

Authentic 8-nitro-cGMP labeled with a stable isotope or unlabeled (8-$^{15}NO_2$-cGMP or 8-$^{14}NO_2$-cGMP, respectively) was prepared according to the method we reported previously (24). ^{15}N-Labeled cGMP, i.e., [U-$^{15}N_5$, 98%] c[$^{15}N_5$]GMP, was synthesized from [U-$^{15}N_5$, 98%] ([$^{15}N_5$]GTP; Cambridge Isotope Laboratories, Inc., Andover, MA) via an enzymatic reaction by utilizing purified sGC. Specifically, 1 mM [$^{15}N_5$]GTP was incubated with sGC purified from bovine lung as described previously (50) in 50 mM sodium phosphate buffer (pH 7.4) at 37 °C for 12 h, and a

product of the sGC reaction—c[$^{15}N_5$]GMP—was then obtained via high-performance liquid chromatography (HPLC) as reported earlier (25). All these stable isotope-labeled guanine nucleotides were used as internal standards in the stable isotope dilution technique with LC-MS/MS analysis, as described below. Authentic 8-nitro-GMP, 8-nitroguanosine 5′-diphosphate (8-nitro-GDP), and 8-nitro-GTP were prepared by reacting 1 mM GMP, GDP, and GTP, respectively, with 2 mM $ONOO^-$ in 0.1 M sodium phosphate buffer (pH 7.4) containing 25 mM $NaHCO_3$ and 0.1 mM DTPA.

2.3 Chemical Analysis of Guanine Nucleotide Nitration In Vitro

Guanine nucleotides were reacted with various RNOS systems in vitro to determine the nitrating potential of each RNOS system. Formation of nitrated derivatives was analyzed by means of reverse-phase (RP) high performance liquid chromatography-photodiode array detection (HPLC-PDA) or with HPLC-ECD (24). HPLC-ECD was used for analysis of cGMP nitration, whereas HPLC-PDA was used for analysis of nitration of other guanine nucleotides. For HPLC-ECD, HPLC separation was carried out with a RP column (150 mm long, 3.0 mm inner diameter; Eicom Pak SC-5 ODS; Eicom, Kyoto, Japan) eluted with 0.4 ml/min of 200 mM sodium phosphate buffer (pH 5.0) plus 5.5% acetonitrile. 8-Nitro-cGMP was detected electrochemically by means of an online reductive activation method with electrode settings of -500 mV (first cell for reduction) and +250 mV (second cell for oxidation) (HTEC-500 and

PEC-510; Eicom) (24). Tyrosine nitration by RNOS was analyzed for comparison. 3-Nitrotyrosine was detected via HPLC-ECD under the following conditions: +100 mV, -600 mV, 0.4 ml/min, 0.2 M sodium phosphate buffer (pH 3.5), with 2% CH_3CN plus 5 μg/ml EDTA (24). For HPLC-PDA, HPLC plus PDA detection was performed with a UV detector using an MCM C-18 column (150 mm long, 4.6 mm inner diameter; MC Medical, Inc., Tokyo, Japan). Samples were eluted with 0-40% CH_3CN in 1.0% dibutylammonium acetate buffer with a 0.7 ml/min flow rate; detection was by HPLC-PDA, with a UV detector (SPD-M10A VP; Shimadzu, Kyoto, Japan).

2.3.1 Authentic $ONOO^-$ System

The guanine nucleotides cGMP, GMP, GDP, and GTP (each at 1 mM) were reacted under vortex mixing with authentic $ONOO^-$ (2 mM) in 0.1 M sodium phosphate buffer (pH 7.4) containing 25 mM $NaHCO_3$ and 0.1 mM DTPA. Nitration of cGMP by $ONOO^-$ was further analyzed as a function of $ONOO^-$ concentration and buffer pH. That is, cGMP (50 μM) was reacted under vortex mixing with $ONOO^-$ (up to 10 μM) in 0.1 M sodium phosphate buffer (pH 7.4) containing 0.1 mM DTPA with or without 25 mM $NaHCO_3$. The effect of pH was examined for the pH range of 2.5-7.4. Buffers used included 0.1 M citric acid buffer (pH 2.5-5.0) and 0.1 M sodium phosphate buffer (pH 5.5-7.4), containing 0.1 mM DTPA with or without 25 mM $NaHCO_3$.

2.3.2 SIN-1 System

SIN-1 was used to study the effect of simultaneous production of NO and O_2^- (51) on guanine nucleotide nitration. cGMP (50 μM) or tyrosine (50 μM) was reacted

with SIN-1 (0-100 μM) in 0.1 M sodium phosphate buffer (pH 7.4) in the presence of 0.1 mM DTPA and 25 mM $NaHCO_3$.

2.3.3 Nitrite/H_2O_2/Heme Peroxidases System

Heme peroxidases catalyze oxidation of nitrite in the presence of H_2O_2 to form the potent nitrating agent NO_2 (52-54). In my study here, two heme peroxidases—MPO and HRP—were used as catalysts. cGMP (50 μM) or tyrosine (50 μM) was reacted at 37 °C for 3 h with either MPO (10 nM) or HRP (23.8 nM) in 0.1 M sodium phosphate buffer (pH 7.4) containing 100 μM $NaNO_2$ and 100 μM H_2O_2.

2.3.4 Aerobic NO Production System

NO is oxidized to form NO_2, an oxidizing and nitrating agent, under aerobic conditions (55). To study the effect of NO and NO_2 on guanine nucleotide nitration, I used the NO-releasing agent P-NONOate, which spontaneously decomposes to release NO (56, 57). cGMP (50 μM) and tyrosine (50 μM) were reacted with P-NONOate (0-100 μM), an NO donor in 0.1 M sodium phosphate buffer (pH 7.4), in the presence of 0.1 mM DTPA and 25 mM $NaHCO_3$, followed by measurement of the nitrated derivatives of cGMP and tyrosine. Nitration of cGMP and tyrosine was also examined in an acidified nitrite system (55, 58): cGMP (50 μM) and tyrosine (50 μM) were reacted for 1 h at 37 °C with $NaNO_2$ (100 μM) in 0.1 M sodium citrate buffer (pH 2.5-4.5)

2.3.5 Hypochlorous Acid/Nitrite System

Hypochlorous acid (HOCl) is a product of the MPO-catalyzed oxidation of

chlorine ion (Cl^-) in the presence of H_2O_2. HOCl reportedly reacts with nitrite to form NO_2Cl (59). To determine the effect of NO_2Cl on guanine nucleotide nitration, cGMP (50 μM) and tyrosine (50 μM) were reacted for 4 h at 37 °C with HOCl (100 μM) plus $NaNO_2$ (100 μM) in 0.1 M sodium phosphate buffer (pH 7.4).

2.4 Nitration of Guanine Nucleotide in Presence of ROS Scavengers

To determine the roles of ROS on guanine nucleotide nitration, I used three different ROS scavengers: SOD and tiron for scavenging O_2^- and catalase for scavenging H_2O_2. For cellular experiments as described below, PEG-SOD and PEG-catalase were used to effectively delivered those enzymes into cells (47).

2.5 Cell Treatment

Rat C6 glioma cells were cultured at 37 °C in DMEM (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% FBS and 1% penicillin-streptomycin. Cells were plated at a density of 1.5×10^6 cells per 60-mm dish to prepare cell extracts for LC-MS/MS and at a density of 1×10^5 cells per chamber in BD Falcon Culture Slides (BD Biosciences, San Jose, CA) for immunocytochemistry. Primary astrocytes were prepared from Wistar rats using a modification of a technique described previously (60). Briefly, brain cortices from 20-days-old rat embryos were cleaned of their meninges, cut into blocks, and dissociated with 0.25% trypsin. An equal volume of horse serum supplemented with 0.1 mg/ml of DNase I was added to the medium to

inactivate the trypsin, and the tissues were centrifuged at 300×*g* for 5 min. The tissue sediments were resuspended in DMEM containing 10% FBS, 1% penicillin-streptomycin. The cells were plated on 100-mm diameter polyethyleneimine-coated plastic dishes at a density of 0.8–1.3 × 10^5 cells/cm^2. Cultures were maintained at 37 °C in 5% CO_2 and 95% air, and the medium was changed every 3 days. After 1 week, astrocytes were replated to remove the neurons using a standard trypsin treatment technique.

To study 8-nitro-cGMP formation, cells were stimulated for 36 h with a mixture of 10 μg/ml LPS (from *Escherichia coli*; L8274; Sigma-Aldrich Corporation, St. Louis, MO) and 200 U/ml IFN-γ, 500 U/ml TNFα, and 10 ng/ml IL-1β (all cytokines from R&D Systems, Inc., Minneapolis, MN). In some experiments, to investigate the mechanism of ROS-dependent 8-nitro-cGMP production, cells were stimulated in the presence of ROS scavengers, including PEG-SOD, tiron, and PEG-catalase, followed by analyses for 8-nitro-cGMP formation and ROS generation. In other experiments to investigate involvement of mitochondrial ROS generation in 8-nitro-cGMP formation, cells were pretreated for 15 min with 10 μM rotenone before LPS-cytokine stimulation. To study the role of Nox2 in cellular ROS production, cells were transfected with Nox2 $p47^{phox}$-specific siRNA before stimulation as described bellow.

To investigate the mechanism of 8-nitro-cGMP production, cells were stimulated in the presence of an NOS inhibitor (L-NMMA) and the sGC inhibitor (NS 2028), followed by a series of various analyses for 8-nitro-cGMP formation.

2.6 Immunocytochemistry

Formation of 8-nitro-cGMP in C6 cells was analyzed by means of immunocytochemistry with anti-8-nitro-cGMP monoclonal antibody, as described previously (24, 25). In brief, after LPS-cytokine stimulation, cells were washed with phosphate-buffered saline (PBS, pH 7.4) and were fixed with Zamboni fixative (4% paraformaldehyde and 10 mM picric acid 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 again with PBS. To block nonspecific antigenic sites, cells were incubated at 4 °C overnight with BlockAce (Snow Brand Milk Products Co. Ltd, Tokyo, Japan). Cells were then incubated overnight at 4 °C with the monoclonal antibody (10 μg/ml) in PBS with 10% goat serum (Invitrogen, Carlsbad, CA), 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; Amersham Biosciences Corporation, Piscataway, NJ, USA) in PBS with 10% goat serum. Cells were washed with PBS, covered with SlowFade Light Antifade Kit (S-7461; Molecular Probes, Inc., Eugene, OR), and examined with a Nikon-ECLIPSE TE 2000-E (Nikon EZ-C1) confocal laser microscope (Nikon Corporation, Tokyo, Japan), with excitation at 488 nm. The red photomultiplier channel of the confocal microscope was used for image acquisition. Images were captured and processed by means of Nikon EZ-C1 software. Further image processing and quantification were

performed by using Adobe Photoshop Elements v. 2.0 (Adobe Systems, Waltham, MA).

In other experiment cells were stained and then examined with a fluorescence microscope (ECLIPSE Ti; Nikon, Tokyo, Japan) equipped with an ORCA-R2 camera (Hamamatsu Photonics, Hamamatsu**,** Japan). Images were processed to reduce noise by using a deconvolution software (AutoDeblur, Lexi, Tokyo, Japan). Further image processing and quantification were performed by using Adobe Photoshop v. 7.0 (Adobe Systems, Waltham, MA, USA). Fluorescence intensity values from three different experiments were obtained, and the average relative fluorescence intensity (as percent fluorescence intensity) was determined for LPS-cytokine-stimulated cells.

2.7 LC-MS/MS Analysis for the Intracellular Formation of 8-Nitro-cGMP

Intracellular levels of 8-nitro-cGMP were quantified by means of LC-MS/MS as described previously (25). In brief, after C6 cells were plated on 60-mm dishes and were stimulated as described above, they were washed twice with ice-cold PBS and collected with a cell scraper (BD Biosciences) in 5 ml of ice-cold PBS, followed by centrifugation. The cell pellet thus obtained was homogenized in 5 ml of methanol containing 5 mM NEM (Sigma-Aldrich Corporation, St. Louis, MO), and 20 nM c[^{15}N]GMP and 100 nM 8-$^{15}NO_2$-cGMP were used for recovery standardization in the stable isotope dilution technique. After the homogenate samples were centrifuged at 5000 × *g* at 4 °C, their supernatants were dried in vacuo and then redissolved in distilled water. The samples were mixed with hexane to obtain an aqueous phase. This

fraction was dried in vacuo and then reconstituted with 50 μl of water, followed by LC-MS/MS coupled with the stable isotope dilution method.

LC-MS/MS was performed with an Agilent 6430 Triple Quadrupole LC/MS (Agilent Technologies, Santa Clara, CA, USA), after reverse phase high performance liquid chromatography (RP-HPLC) on a Mightysil RP-18 column (50 × 2.0 mm inner diameter; Kanto Chemical, Tokyo, Japan), with a linear 0-100% CH_3CN gradient for 5 min in 0.1% formic acid at 40 °C. The total flow rate was 0.15 ml/min, and the injection volume was 20 μl. Ionization was achieved by using electrospray in the positive mode with the spray voltage set at 3500 V. Nitrogen was used as the nebulizer gas and nebulizer pressure was set at 50 psi. The dissolving gas (nitrogen) was heated to 350 °C and was delivered at a flow rate of 10 L/min. For collision-induced dissociation, high-purity nitrogen was used as the collision gas at a pressure of 0.05 MPa. Quantification was performed by using the multiple-reaction monitoring mode at *m/z* 391 → 197 for 8-NO_2-cGMP, *m/z* 392 → 198 for 8-$^{15}NO_2$-cGMP, *m/z* 346 → 152 for cGMP, and *m/z* 351 → 157 for c[$^{15}N_5$]GMP. The fragment energies of MS1 for all materials were set at 80 V. The optimized collision energies of 10 eV and 20 eV were used for nitro-cGMP and cGMP, respectively.

In other experiment LC-MS/MS was performed with a Varian 1200L triple-quadrupole (Q) mass spectrometer (Varian, Inc., Palo Alto, CA, USA), after RP-HPLC. The observed parent ion masses were *m/z* 391 and *m/z* 392 and the product

ion masses were *m/z* 197 and *m/z* 198 for endogenous 8-$^{14}NO_2$-cGMP and spiked 8-$^{15}NO_2$-cGMP, respectively. Collision energy of -8V for both analytes was applied. The signal of endogenous 8-$^{14}NO_2$-cGMP was identified simultaneously with respective ^{15}N-derivative identification.

Amounts of endogenous cGMP and 8-nitro-cGMP were determined via the stable isotope dilution method based on the recovery efficiency of stable isotope-labeled derivatives (c[$^{15}N_5$]GMP and 8-$^{15}NO_2$-cGMP) spiked with the cell extract as described above. For each sample measurement, signals of endogenous ^{14}N-guanine nucleotides were identified simultaneously with those of the respective ^{15}N-derivatives, so that the exact values of endogenous nucleotide concentrations were obtained after correction by the amount of derivatives finally recovered by MS analyses. Our earlier study demonstrated the validity of this present spike-and-recovery study (25).

2.8 Determination of Cellular ROS Production

Cellular ROS production was determined by means of fluorescence microspectrometry with chemical probes that become fluorescent on reaction with ROS. Specifically, I used MitoSOX™ Red (61) and DCDHF-DA (62) for detection of mitochondrial O_2^- production and cellular oxidants, respectively. C6 cells were washed with Hank's buffer pH 7.4 (0.137 M NaCl, 5.4 mM KCl, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$, 4.2 mM $NaHCO_3$) and were then stained with 2.5 µM MitoSOX™ Red dissolved in Hank's buffer, or with 5 µM

DCDHF-DA dissolved in PBS, for 15 min at 37 °C in the dark. Cells were then washed carefully with Hank's buffer, mounted with mounting buffer, covered with coverslips, and examined with a Nikon EZ-C1 confocal laser microscope. Excitation was at 420 nm for MitoSOX™ Red (the red photomultiplier channel of the confocal microscope was used for image acquisition), and excitation was at 488 nm for DCDHF-DA (the green photomultiplier channel of the confocal microscope was used for image acquisition). Images were captured and processed by means of Nikon EZ-C1 software. Further image processing and quantification were performed by using Adobe Photoshop Elements v. 2.0 (Adobe Systems). Fluorescence intensity values from three different experiments were obtained, and the average relative fluorescence intensity (as percent fluorescence intensity) was determined for LPS-cytokine-stimulated cells.

2.9 Transfection of p47 ^{phox} siRNA

A 25-nucleotide p47^{phox} siRNA (Catalog no. 1320003, Oligo ID, MSS206955 andMSS275934; manufactured by Invitrogen, Carlsbad, CA) was used for transfection using the Lipofectamine RNAiMAX transfection reagent (Invitrogen, Carlsbad, CA). Briefly, C6 cells were seeded in 12-well plates at a density of 2×10^5 cells/well. Cells were transfected with p47^{phox} siRNA (60 pmol/well) by using Lipofectamine RNAiMAX transfection reagent. At 72 h after transfection, cells were harvested, but just before the harvest they were treated with LPS-cytokines for 36 h. Stealth RNAi

negative control (high GC; Invitrogen, Carlsbad, CA, USA) was used as a negative control siRNA according to the user's manual.

2.10 Reverse Transcriptase PCR

The efficiency of transfection and gene silencing was confirmed by isolating the total RNA followed by analyzing Nox2 $p47^{phox}$ mRNA expression. Total RNA was isolated from C6 cells by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. First-strand cDNAs were synthesized by using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA). For RT-PCR analysis of Nox2 $p47^{phox}$, the forward and reverse primers used were 5′-AGGACTGCTGTTACGTTCAAGGTCGGCCTG-3′ and 5′-ACAGCTGGATCTTATGGTCGCATTTTC-3′, respectively. Expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was determined as an internal control. The forward and reverse primers used were 5′-AAACCCATCACCATCTTCCA-3′ and 5′-CAGGGGTTTCTTACTCCTTG-3′, respectively. PCR was performed by using a multiple cycler (Astec, Yokohama, Japan). The template for the 50-µl PCR reaction was 1 µl of the first-strand product. The cycling parameters consisted of one cycle at 95 °C for 3 min and then 35 cycles at 95 °C for 0.5 min, 55 °C for 0.5 min, 72 °C for 1 min, followed by a single 5-min cycle at 72 °C for extension. RT-PCR products were electrophoresed on a 2% agarose gel.

2.11 Western Blot Analysis

Proteins in cell lysates were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred onto PVDF membrane. The blot was blocked with skim milk followed by 1h incubation with anti-p47-phox antibody (Millipore, Temecula, CA, USA) and a goat anti-rabbit HRP conjugated IgG secondary antibody. In other experiment the proteins were transferred onto a nitrocellulose membrane. Blots were incubated with Blocking One containing peroxidase-conjugated iNOS specific antibody i2G4 (60) (1 μg/ml), anti-sGC β1 antibody (1 μg/ml), and anti-β-actin antibody (0.5 μg/ml) overnight at 4 °C. The membrane that reacted with the anti-sGC and anti-β-actin antibodies was further incubated for 1 h with peroxidase-labeled antirabbit IgG secondary antibody (0.1 μg/ml). The immunoreactive bands were detected by using a chemiluminescence reagent (Millipore, Bedford, MA, USA) with a luminescent image analyzer (LAS-1000; Fujifilm, Tokyo, Japan).

2.12 Nitrite Production and Protein Measurement

NO synthesis was determined by assaying nitrite, a stable reaction product of NO with molecular oxygen, in the culture supernatants. In brief, 100 μl of culture supernatant was allowed to react with 100 μl of Griess reagent (63) and was incubated at room temperature for 15 min. The optical density of the assay samples was measured spectrophotometrically at 540 nm. Fresh culture media served as the blank

in all experiments. Nitrite concentrations were calculated from a standard curve derived from the reaction of $NaNO_2$ in the assay.

Protein concentrations were determined via the Bradford method with bovine serum albumin (BSA) as the standard (64).

2.13 Statistical Analysis

All data are shown as means ± S.E. Data for each experimental condition were acquired from at least three independent experiments. Statistical analyses were performed by using Student's *t* test.

RESULTS

PART A

REGULATION BY MITOCHONDRIAL SUPEROXIDE AND NADPH OXIDASE OF CELL FORMATION OF NITRATED CYCLIC GMP: POTENTIAL IMPLICATIONS FOR ROS SIGNALING

3.1 Nitration of Guanine Nucleotides by Various RNOS: In V itro Chemical Analyses

The effect of various RNOS on nitration of guanine nucleotides was investigated in vitro. I first examined nitration of guanine nucleotides in the reaction with $ONOO^-$, a potent nitrating and oxidizing species formed from the reaction of NO and O_2^-. As Fig. 5*A* shows, $ONOO^-$ nitrated all guanine nucleotides. However, the efficacy of nitration varied depending on the structure of the nucleotides; GTP evidenced the highest production of nitrated derivative, with nitration efficiency then decreasing in the following order: GDP > GMP > cGMP.

Nitration of guanine nucleotides by $ONOO^-$ was further examined as a function of $ONOO^-$ concentration and pH of the reaction mixtures, with cGMP used as a model substrate. As illustrated in Fig. 5*B*, formation of 8-nitro-cGMP depended on the concentration of $ONOO^-$. 8-Nitro-cGMP formation was markedly enhanced in the presence of $NaHCO_3$. This enhanced effect of $NaHCO_3$ on $ONOO^-$-mediated nitration was more obvious for cGMP than for tyrosine. The efficacies of $ONOO^-$-mediated nitration of both cGMP and tyrosine were maximum at neutral pH (pH 7-7.2) (Fig. 5*C*).

Table 1 summarizes the effects of various RNOS systems on nitration of cGMP and tyrosine. The NO donor P-NONOate did not cause detectable nitration of cGMP even under aerobic conditions. An acidic nitrite system did cause tyrosine nitration but no detectable formation of 8-nitro-cGMP. SIN-1, which simultaneously generates NO and O_2^-, caused both cGMP and tyrosine nitration.

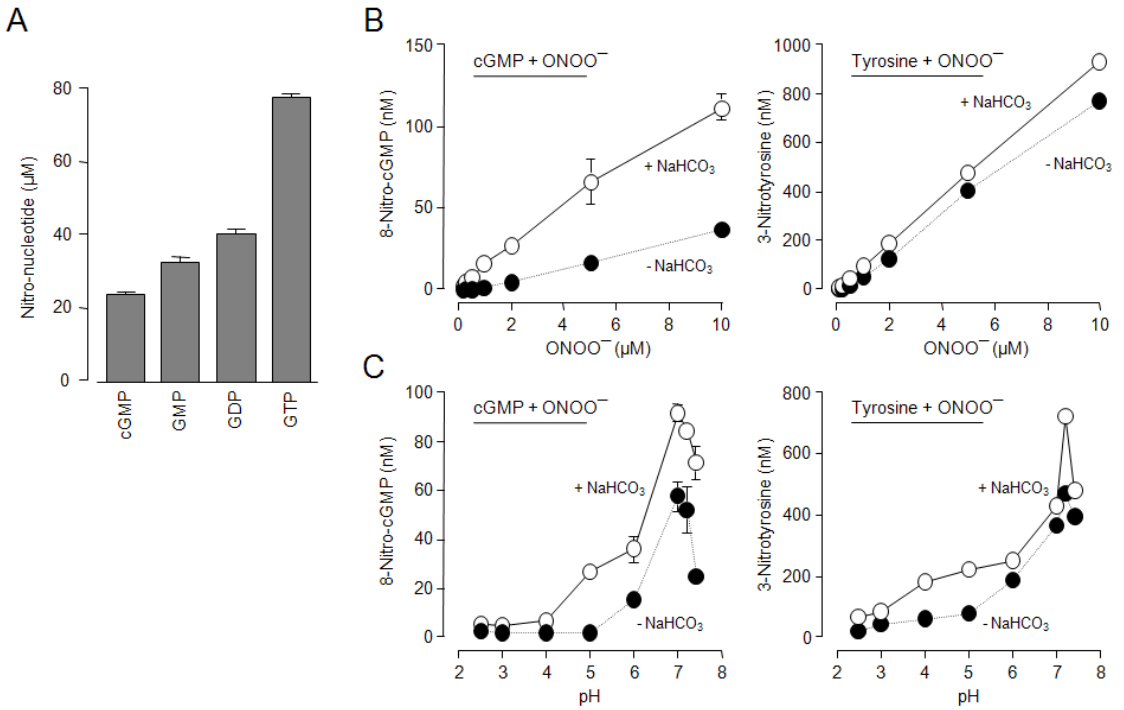

Fig. 5 ONOO$^-$-dependent nitration of guanine nucleotides and tyr osine. *A*, nitration of various guanine nucleotides by ONOO$^-$. The guanine nucleotides cGMP, GMP, GDP, and GTP (1 mM each) were reacted with 2 mM ONOO$^-$ in 0.1 M sodium phosphate buffer (pH 7.4) in the presence of 25 mM $NaHCO_3$. *B*, nitration of cGMP (left panel) and tyrosine (right panel) by ONOO$^-$ as a function of ONOO$^-$ concentration. *C*, pH dependence of ONOO$^-$-mediated nitration of cGMP and tyrosine. In *B* and *C*, cGMP (50 μM) or tyrosine (50 μM) was reacted with the indicated concentration of ONOO$^-$ and different pH range respectively. Data are expressed as means ± S.E. (n = 3).

Table 1. Nitration of cGMP (50 µM) and tyrosine (50 µM) by various RNOS system

Conditions	Product formed (nM)	
	8-Nitro-cGMP	3-Nitrotyrosine
NONOate (100 µM), 4 h[a]	ND[b]	16.7 ± 1.1
$NaNO_2$ (100 µM), at pH 3.0, 1 h[c]	ND	95.8 ± 1.8
$NaNO_2$ (100 µM), at pH 4.0, 1 h[c]	ND	30.3 ± 3.0
SIN-1 (50 µM), 2 h[d]	7.7 ± 0.3	267.3 ± 5.6
SIN-1 (100 µM), 2 h[d]	14.5 ± 0.7	402.9 ± 8.3
$NaNO_2$ (100 µM), H_2O_2 (100 µM), MPO (10 nM), 4 h[e]	67.3 ± 1.5	8307.0 ± 11.1
$NaNO_2$ (100 µM), MPO (10 nM), 4 h[e]	ND	ND
H_2O_2 (100 µM), MPO (10 nM), 4 h[e]	ND	ND
$NaNO_2$ (100 µM), H_2O_2 (100 µM), 4 h[e]	ND	ND
$NaNO_2$ (100 µM), H_2O_2 (100 µM), HRP (23.8 µM), 4 h[e]	ND	803.5 ± 1.6
HOCl (100 µM), $NaNO_2$ (100 µM), 4 h[f]	ND	48.3 ± 1.9

[a]In 0.1 M sodium phosphate buffer (pH 7.4), 0.1 mM DTPA, at 37 °C.
[b]ND, not detected.
[c]In 0.1 M sodium citrate buffer (pH 2.5-4.5) , 100 µM $NaNO_2$, at 37 °C.
[d]In 0.1 M sodium phosphate buffer (pH 7.4), 0.1 mM DTPA, 25 mM $NaHCO_3$, at 37 °C.
[e]In 0.1 M sodium phosphate buffer (pH 7.4), at 37 °C.
[f]In 0.1 M citric acid buffer (pH 4.5), at 37 °C.

This result suggests that $ONOO^-$ formed from NO and O_2^- is an effective nitrating agent for guanine nucleotides. In addition to SIN-1, the complete $NaNO_2/H_2O_2$/MPO system led to marked nitration of cGMP. Omission of just one component from this complete system resulted in no detectable level of 8-nitro-cGMP. HOCl is a strong oxidant produced by MPO. $NaNO_2$ in the presence of HOCl effectively nitrated tyrosine but not cGMP. No 8-nitro-cGMP formed after replacement of MPO by HRP in the complete system, which suggests that nitration of guanine nucleotides depends on the type of peroxidase even when both nitrite anion and H_2O_2 are available simultaneously.

Nitration of cGMP by RNOS was inhibited by specific inhibitors and scavengers of ROS (Fig. 6). SOD completely suppressed SIN-1-mediated cGMP nitration, whereas it failed to suppress nitration of cGMP mediated by authentic $ONOO^-$ or $NaNO_2/H_2O_2$/MPO. Catalase, however, was an effective inhibitor of only $NaNO_2/H_2O_2$/MPO-mediated nitration of cGMP. Tiron is a small molecule that is an SOD mimic and has been used as a O_2^- scavenger (65). In my study here, however, tiron effectively suppressed cGMP nitration mediated by all RNOS systems examined.

3.2 Formation of 8-Nitro-cGMP in Rat C6 Glioma Cells: Involvement of Cellular ROS Production

The chemical analyses clearly demonstrated that NO itself is not sufficient to cause nitration of guanine nucleotides but requires ROS including O_2^- and H_2O_2 for that

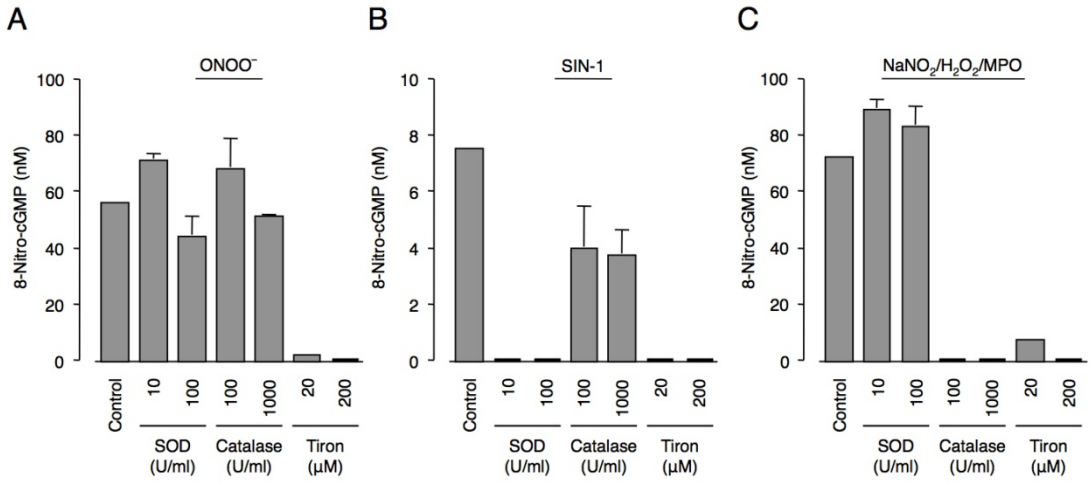

Fig. 6 Effects of ROS scavengers on fo rmation of 8-nitro-cGMP induced by various RNOS systems. cGMP (50 μM) was reacted with authentic $ONOO^-$ (5 μM) (*A*), with SIN-1 (100 μM) (*B*), or with $NaNO_2$ (100 μM)/H_2O_2 (100 μM)/MPO (10 nM) (*C*) in the absence or presence of SOD (10 and 100 U/ml), catalase (100 and 1000 U/ml), or tiron (20 and 200 μM). Data are expressed as means ± S.E. (n = 3).

reaction to occur. To study the roles of ROS in 8-nitro-cGMP formation in cells, I used C6 cells in culture as a model system.

Immunocytochemical analyses provided the baseline formation of 8-nitro-cGMP in nonstimulated C6 cells (Fig. 7*A*). Formation of 8-nitro-cGMP was markedly enhanced in C6 cells when cells were stimulated with LPS-cytokines (Fig. 7*A*). Treatment with PEG-SOD, which is reportedly a membrane-permeable SOD derivative (48), reduced the immunostaining in a manner dependent on PEG-SOD concentration (Fig. 7*B*). This result suggests the essential role of O_2^- for cell formation of 8-nitro-cGMP in stimulated C6 cells. Similar to PEG-SOD, PEG-catalase, a membrane-permeable catalase derivative, suppressed 8-nitro-cGMP formation in C6 cells stimulated with LPS-cytokines (Fig. 7*C*).

Formation of 8-nitro-cGMP in C6 cells and its modulation by PEG-SOD and PEG-catalase was verified by means of LC-MS/MS analyses. In agreement with immunocytochemical data, these analyses detected a certain level of 8-nitro-cGMP in nonstimulated C6 cells (Fig. 8). As Fig. 8 shows, stimulation by LPS-cytokines significantly promoted formation of both cGMP and 8-nitro-cGMP in C6 cells. The concentration of 8-nitro-cGMP was about 5.6 times higher than that of cGMP under current experimental conditions. PEG-SOD treatment moderately reduced the level of cGMP. A similar trend was observed with PEG-catalase, although it was not statistically significant. Formation of 8-nitro-cGMP, however, was almost completely nullified by treatment with both PEG-SOD and PEG-catalase, a finding that agrees with

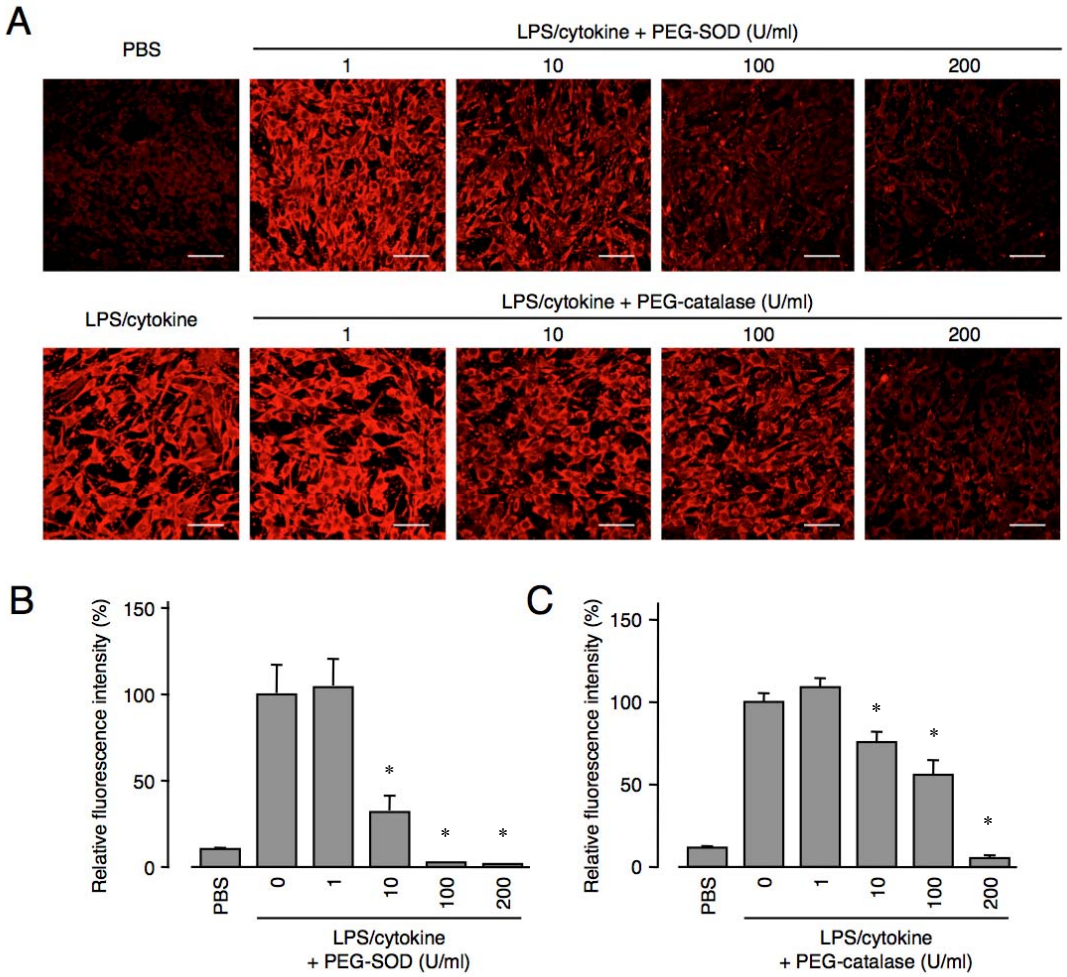

Fig. 7 Immunocytochemical analysis of 8- nitro-cGMP formation in rat C6 glioma cells and modulation of its formation by ROS sc avengers. Cells were stimulated with a mixture of LPS (10 μg/ml), IFN-γ (200 U/ml), TNFα (500 U/ml), and IL-1β (10 ng/ml) for 36 h in the absence or presence of the indicated concentrations of PEG-SOD or PEG-catalase. Cells were then fixed with Zamboni fixative as described under "Materials and Methods," followed by immunocytochemical detection of intracellular 8-nitro-cGMP with the use of 1G6 monoclonal antibody against 8-nitro-cGMP. *A*, cells were treated with different concentrations of PEG-SOD (upper panels) or PEG-catalase (lower panels) (from 1 h before addition of LPS-cytokines), during stimulation with LPS-cytokines for 36 h, followed by immunocytochemical detection of 8-nitro-cGMP. Scale bars indicate 50 μm. *B,* concentration-dependent decrease in relative fluorescence intensity of 8-nitro-cGMP in C6 cells after addition of the cell-permeable O_2^- scavenger PEG-SOD (1-200 U/ml) during stimulation with LPS-cytokines. *C,* concentration-dependent decrease in relative fluorescence intensity of 8-nitro-cGMP in C6 cells after addition of the cell-permeable H_2O_2 scavenger PEG-catalase (1-200 U/ml) during stimulation with LPS-cytokines. Data are expressed as means ± S.E. ($n = 3$). *, $p < 0.01$, compared with the LPS-cytokine-treated group.

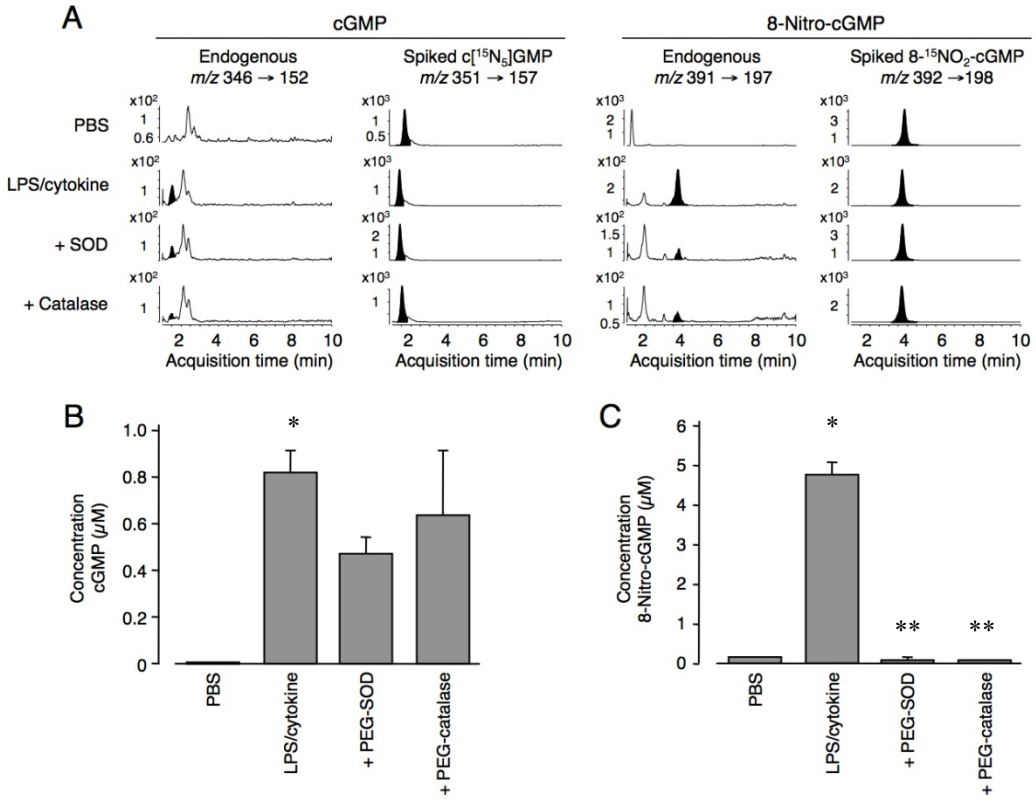

Fig. 8 LC-MS/MS analysis of 8-nitro-cGMP formation in rat C6 glioma cells and modulation of its format ion by ROS scavengers. Cells were stimulated with a mixture of LPS (10 μg/ml), IFN-γ (200 U/ml), TNFα (500 U/ml), and IL-1β (10 ng/ml) for 36 h in the absence or presence of PEG-SOD or PEG-catalase, and cell extracts were prepared as described under "Materials and Methods," followed by LC-MS/MS quantification of cGMP and 8-nitro-cGMP formed in cells. *A*, LC-MS/MS chromatograms of cGMP and 8-nitro-cGMP in untreated cells and cells treated with 200 U/ml PEG-SOD or 200 U/ml PEG-catalase (from 1 h before LPS-cytokine addition), during stimulation with LPS-cytokines for 36 h. *B*, intracellular cGMP concentrations in cells after stimulation with LPS-cytokines in the presence or absence of PEG-SOD or PEG-catalase (each at 200 U/ml) determined with LC-MS/MS. *C*, intracellular 8-nitro-cGMP concentrations in cells after stimulation with LPS-cytokines in the presence or absence of PEG-SOD or PEG-catalase (each at 200 U/ml) determined with LC-MS/MS. Data are expressed as means ± S.E. (n = 3). *, $p < 0.01$, compared with the PBS-treated control group. **, $p < 0.01$, compared with the LPS-cytokine-treated group.

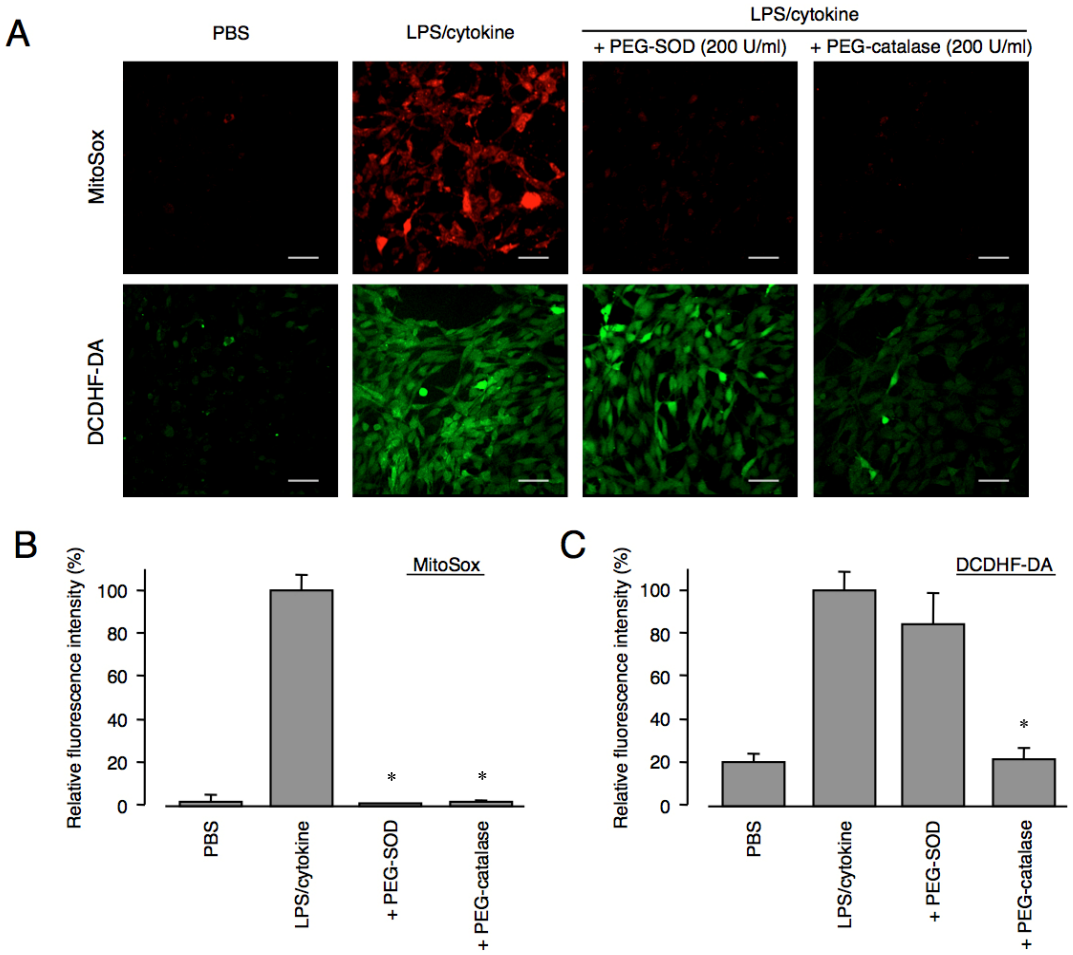

Fig. 9 Fluorescence microscopic determination of ROS pr oduction in rat C6 glioma cells stimulated with LPS plus cytokines. Cells were stimulated with a mixture of LPS (10 μg/ml), IFN-γ (200 U/ml), TNFα (500 U/ml), and IL-1β (10 ng/ml) for 36 h in the absence or presence of PEG-SOD or PEG-catalase. Cells were then analyzed for the presence of mitochondrial O_2^- and cellular H_2O_2, as described under "Materials and Methods." *A*, MitoSOX Red (upper panels) and DCDHF-DA (lower panels) staining of untreated cells and cells treated with 200 U/ml PEG-SOD or 200 U/ml PEG-catalase (from 1 h before LPS-cytokine addition), during stimulation with LPS-cytokines for 36 h, as detected by the Nikon EZ-C1 confocal laser microscope (excitation at 420 nm and red photomultiplier channel for MitoSOX Red; excitation at 488 nm and green photomultiplier channel for DCDHF-DA). Scale bars indicate 50 μm. Relative fluorescence intensity for MitoSOX Red staining (*B*) and DCDHF-DA staining (*C*) of untreated C6 cells or cells treated with 200 U/ml PEG-SOD or 200 U/ml PEG-catalase (from 1 h before LPS-cytokine addition), during stimulation with LPS-cytokines for 36 h. Data are expressed as means ± S.E. ($n = 3$). *, $p < 0.01$, compared with the LPS-cytokine-treated group.

results obtained by immunocytochemistry. Thus, these data suggest that formation of 8-nitro-cGMP depends greatly on cell production of both O_2^- and H_2O_2.

Cell production of ROS and related oxidants was analyzed by using chemical probes that become fluorescent in response to ROS and oxidants. With MitoSOX^{TM} Red, a mitochondria-targeted O_2^--sensitive fluorogenic probe (61), I found that stimulation of C6 cells with LPS-cytokines significantly induced production of mitochondrial O_2^- (Fig. 9*A*). Treatment with PEG-SOD significantly reduced the fluorescence intensity originating with MitoSOX^{TM} Red (Fig. 9*B*). PEG-catalase also suppressed production of O_2^-, as shown by reduced MitoSOX^{TM} Red-derived fluorescence. These results suggest that mitochondrial O_2^- production may be regulated by H_2O_2 production. I then studied production of H_2O_2 by using DCDHF-DA, a cell-permeable and oxidation-sensitive fluorescent probe. Microscopic observation of DCDHF-DA-derived fluorescence clearly showed oxidant production in C6 cells after stimulation with LPS-cytokines (Fig. 9*A*). A significant inhibitory effect of PEG-catalase (Fig. 9*C*) suggests that H_2O_2 is produced in stimulated C6 cells and acts as a major oxidant involved in induction of DCDHF-derived fluorescence.

As mentioned above, tiron can act not only as a O_2^- scavenger but also as an antioxidant to inhibit guanine nucleotide nitration caused by $ONOO^-$ and $NaNO_2/H_2O_2$/MPO (Fig. 6). Immunocytochemical analyses revealed that tiron effectively suppressed formation of 8-nitro-cGMP in C6 cells stimulated with LPS-cytokines (Fig. 10). Similarly, tiron treatment significantly reduced fluorescence

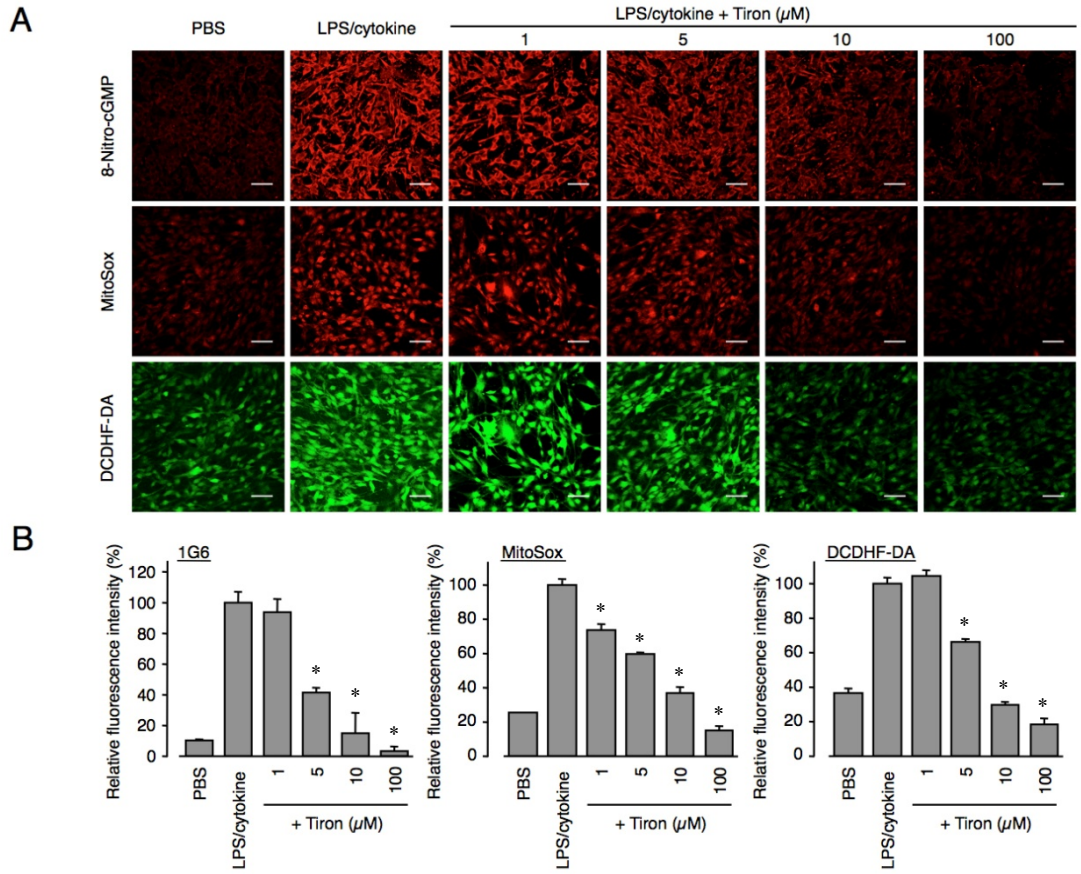

Fig. 10 Effect of the SOD mimic tiron on formation of 8-nitro-cGMP and ROS production in rat C6 glioma cells. Cells were stimulated with a mixture of LPS (10 μg/ml), IFN-γ (200 U/ml), TNFα (500 U/ml), and IL-1β (10 ng/ml) for 36 h in the absence or presence of tiron (1-100 μM), followed by immunocytochemical detection of intracellular 8-nitro-cGMP with the use of 1G6 monoclonal antibody against 8-nitro-cGMP, or by direct staining for mitochondrial O_2^- or cellular H_2O_2 as described under "Materials and Methods." Cells were untreated or treated with 1-100 μM tiron (from 1 h before LPS-cytokine addition), during stimulation with LPS-cytokines for 36 h, followed by immunocytochemical detection of 8-nitro-cGMP (*A*, upper panels); detection of MitoSOX Red staining (*A*, middle panels), via a Nikon EZ-C1 confocal laser microscope (excitation, 420 nm; red photomultiplier channel); and detection of DCDHF-DA staining (*A*, lower panels), via a Nikon EZ-C1 confocal laser microscope (excitation, 488 nm; green photomultiplier channel). Scale bars indicate 50 μm. *B*, relative fluorescence intensity of C6 cells, treated as described above, for 8-nitro-cGMP immunocytochemical staining (left panel), MitoSOX Red staining (middle panel), and DCDHF-DA staining (right panel). Data are expressed as means ± S.E. (n = 3). *, $p <$ 0.01, compared with the LPS-cytokine-treated group.

derived from both MitoSOX^{TM} Red and DCDHF (Fig. 10).

Mitochondrial electron-transport chain (mETC) complexes, particularly complexes I and III, are the main source of ROS produced from mitochondria (66, 67). The mETC inhibitor rotenone reportedly increased mitochondrial ROS production (68). I thus studied whether modulation of mitochondrial ROS production with the mETC complex inhibitor rotenone affected formation of 8-nitro-cGMP in C6 cells. As Fig. 11 shows, rotenone treatment increased 8-nitro-cGMP formation in C6 cells stimulated with LPS-cytokines, as evidenced by immunocytochemistry and LC-MS/MS (Fig. 11*B*, upper right panel) results: both methods revealed a similar increase, by approximately 1.5 fold. Under the same experimental conditions, rotenone treatment significantly increased MitoSOX^{TM} Red-derived fluorescence intensity without affecting DCDHF-derived fluorescence (Fig. 11*B*). This finding suggests that the effect of rotenone was specific to mitochondrial ROS production and hence that 8-nitro-cGMP formation in C6 cells is closely related to mitochondrial O_2^- production.

Nox2 is a member of the NADPH oxidase family and is an important source of ROS, particularly in immunologically stimulated cells (69, 70). To clarify the implications of Nox2-dependent ROS production for 8-nitro-cGMP formation, I performed knockdown of p47^{phox}, a critical component of Nox2, by using p47^{phox} siRNA. The efficacy of the p47^{phox} knockdown was confirmed by RT-PCR (Fig. 12). In C6 cells treated with p47^{phox} siRNA, immunocytochemistry revealed marked suppression of 8-nitro-cGMP formation (Fig. 12). The p47^{phox} knockdown also significantly

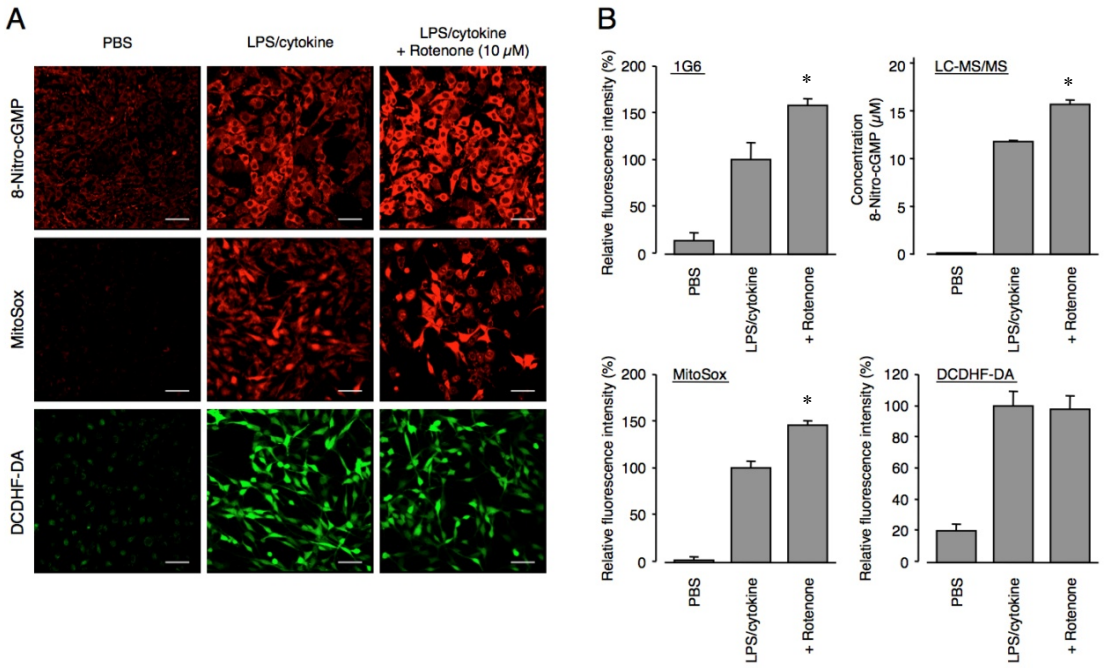

Fig. 11 Modulation of 8-nitro-cGMP formation by rotenone in rat C6 glioma cells. Cells were stimulated with a mixture of LPS (10 μg/ml), IFN-γ (200 U/ml), TNFα (500 U/ml), and IL-1β (10 ng/ml) for 36 h in the absence or presence of rotenone (10 μM). Cells were then fixed with Zamboni fixative as described under "Materials and Methods," followed by immunocytochemical detection of intracellular 8-nitro-cGMP with the use of 1G6 monoclonal antibody against 8-nitro-cGMP, or by direct staining for mitochondrial O_2^- or cellular H_2O_2. Cells were pretreated with 10 μM rotenone (from 15 min before LPS-cytokine addition) or were untreated, during stimulation with LPS-cytokines for 36 h, followed by immunocytochemical detection of 8-nitro-cGMP (*A*, upper panels); detection of MitoSOX Red staining (*A*, middle panels), via a Nikon EZ-C1 confocal laser microscope (excitation, 420 nm; red photomultiplier channel); and detection of DCDHF-DA staining (*A*, lower panels), via a Nikon EZ-C1 confocal laser microscope (excitation, 488 nm; green photomultiplier channel). Scale bars indicate 50 μm. *B*, relative fluorescence intensity of C6 cells, treated as described above, for 8-nitro-cGMP immunocytochemical staining (upper left panel); MitoSOX Red staining (lower left panel); and DCDHF-DA staining (lower right panel). The upper right panel shows intracellular 8-nitro-cGMP concentrations in the cells after stimulation with LPS-cytokines with or without 10 μM rotenone pretreatment, as determined with LC-MS/MS. Data are expressed as means ± S.E. ($n = 3$). *, $p < 0.01$, compared with the LPS-cytokine-treated group.

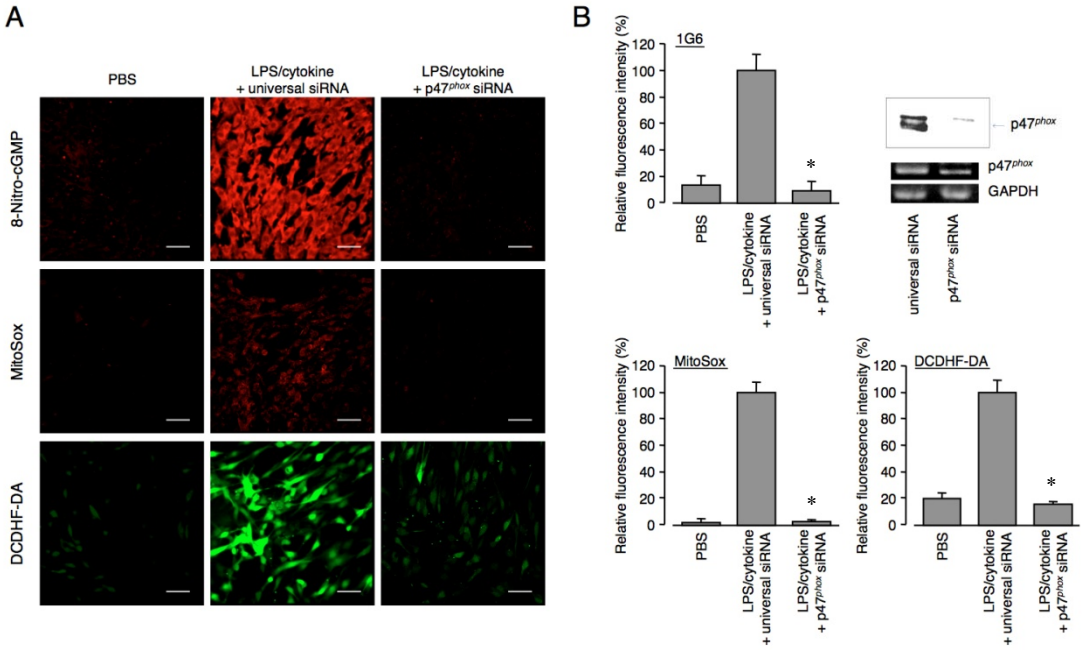

Fig. 12 Effects of Nox2 gene knockdow n on formation of 8-nitro-cGMP and ROS production in rat C6 glioma cells. Cells were transfected with negative control siRNA or p47^{phox}-specific siRNA as described under "Materials and Methods," followed by stimulation with a mixture of LPS (10 μg/ml), IFN-γ (200 U/ml), TNFα (500 U/ml), and IL-1β (10 ng/ml) for 36 h. Immunocytochemistry with 1G6 monoclonal antibody against 8-nitro-cGMP was used to detect intracellular 8-nitro-cGMP, or direct staining was used for mitochondrial O_2^- or cellular H_2O_2. *A*, immunocytochemistry for 8-nitro-cGMP (upper panels); fluorescent staining of mitochondrial O_2^- (middle panels); and fluorescent staining of intracellular H_2O_2 (lower panels). Scale bars indicate 50 μm. *B*, relative fluorescence intensity of C6 cells, treated as described above, for 8-nitro-cGMP immunocytochemical staining (upper left panel); MitoSOX Red staining (lower left panel); and DCDHF-DA staining (lower right panel). The upper right panel shows western blot and RT-PCR for the p47^{phox} knockdown. Data are expressed as means ± S.E. (n = 3). *, p < 0.01, compared with the LPS-cytokine plus negative control siRNA-treated group.

suppressed production of mitochondrial O_2^- and cellular oxidant as determined by fluorescence microscopy (Fig. 12). These data suggest that Nox2 contributes to the formation of 8-nitro-cGMP via production of H_2O_2, which in turn enhances mitochondrial O_2^- production.

To study the role of H_2O_2 in formation of 8-nitro-cGMP and it's association with mitochondrial O_2^- production, I treated C6 cells with NO donors and H_2O_2. P-NONOate alone had a negligible effect on 8-nitro-cGMP formation (Fig. 13*A*). Treatment with H_2O_2 slightly increased 8-nitro-cGMP formation, and PEG-SOD treatment suppressed this increment. 8-Nitro-cGMP formation significantly increased in C6 cells that were simultaneously treated with both P-NONOate and H_2O_2. Furthermore, PEG-SOD treatment markedly suppressed 8-nitro-cGMP formation induced by P-NONOate plus H_2O_2. To clarify whether H_2O_2 can directly affect mitochondrial O_2^- production, I examined the effect of H_2O_2 treatment on MitoSOX^{TM} Red fluorescence. In fact, I found that H_2O_2 treatment significantly increased mitochondrial O_2^- production, as evidenced by increased fluorescence intensity derived from MitoSOX^{TM} Red (Fig. 13*B*). Taken together, these findings suggest the possibility that mitochondrial O_2^- production, which can be enhanced by H_2O_2, may play a role in NO-dependent formation of 8-nitro-cGMP in cells.

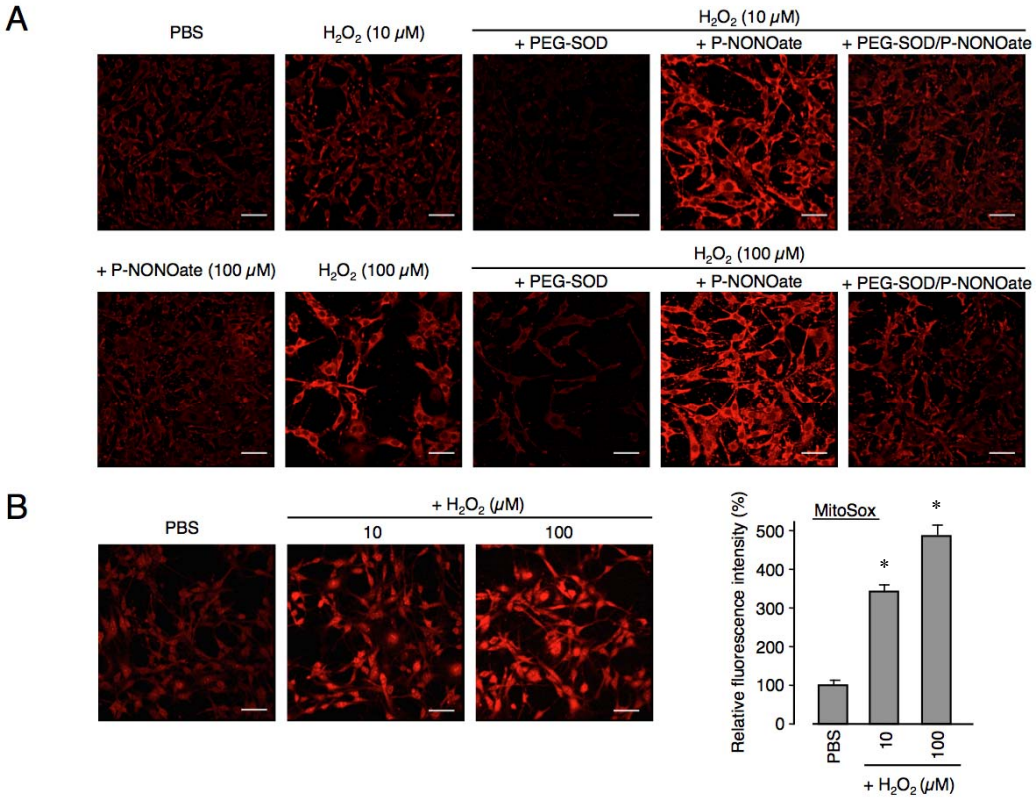

Fig. 13 Increase in 8-nitro-cGMP formation and mitochondrial O_2^- production in rat C6 glioma cells by H_2O_2 and NO treatment. *A*, cells were untreated or treated with 10 or 100 μM H_2O_2 for 36 h, plus PEG-SOD (200 U/ml), P-NONOate (100 μM), or PEG-SOD (200 U/ml) plus P-NONOate (100 μM). Immunocytochemistry for intracellular 8-nitro-cGMP with the use of 1G6 monoclonal antibody against 8-nitro-cGMP, or direct staining for mitochondrial O_2^-, as described under "Materials and Methods," followed. Scale bars indicate 50 μm. *B*, in other experiments, cells were untreated or treated only with 10 or 100 μM H_2O_2, followed by detection of mitochondrial O_2^- generation by MitoSOX Red staining (left panels). Scale bars indicate 50 μm. The right panel presents the relative fluorescence intensity for MitoSOX Red staining compared with 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.

PART B

METHODOLOGICAL PROOF OF IMMUNOCHEMISTRY FOR SPECIFIC IDENTIFICATION OF 8-NITROGUANOSINE 3′,5′-CYCLIC MONOPHOSPHATE FORMED IN GLIA CELLS

3.3 Expression of iNOS and sGC, and 8-Nitro-cGMP Formation

The presence of nitrated guanine derivatives such as 8-nitroguanine, 8-nitroguanosine, and 8-nitro-cGMP and their formation were observed in various cultured cells and specimens of tissues with viral pneumonia and human lung disease, and even in human urine (14, 17, 24, 25, 28). The nitration reaction to form 8-nitroguanine derivatives depends on production of NO by iNOS (14, 24, 25, 28). sGC, which is an enzyme catalyzing the formation of cGMP from GTP, is also involved in the formation of the novel nitrated cyclic nucleotide 8-nitro-cGMP (25). Therefore, I confirmed the expression of iNOS and sGC in C6 cells and primary astrocytes after stimulation with LPS-cytokines. As shown in Fig. 14A, iNOS protein expression was induced by LPS-cytokine treatment in both C6 cells and primary astrocytes. iNOS expression was induced faster in C6 cells than in astrocytes; iNOS expression reached a plateau at 12 h and 24 h in C6 cells and astrocytes, respectively. NO production was also induced by the LPS-cytokine treatment in a time-dependent manner in both cells (Fig. 14B). sGC was expressed in non-treated cells, and the expression was not changed by LPS-cytokine treatment. sGC expression in astrocytes was approximately 50% of that in C6 cells.

I then examined the formation of 8-nitro-cGMP in stimulated C6 cells by means of LC-MS/MS analysis (Fig. 15). The amount of 8-nitro-cGMP endogenously formed in cells was precisely quantified using a stable-isotope dilution method (25). Endogenous 8-$^{14}NO_2$-cGMP was quantified by comparing with the area of spiked

8-$^{15}NO_2$-cGMP. As shown in Fig. 15B, levels of 8-nitro-cGMP in C6 cells increased dramatically at late stage after stimulation (36 h). Kinetics of 8-NO_2-cGMP formation is markedly delayed when compared with induction of iNOS and/or potential activation of sGC as shown in Fig. 14. Because NO itself is not sufficient to induce nitration of guanine moiety, NO would be converted to RNOS such as $ONOO^-$ and NO_2 radical, by reacting with ROS for effective guanine nitration (16). As described in the earlier part of the study (Part A), C6 cells produce ROS in response to the stimulation (LPS-cytokines), originated from Nox2 and mitochondria. Furthermore, ROS produced play a critical role on the formation of 8-nitro-cGMP. These observations suggests that 8-nitro-cGMP formation is activated under the conditions when both ROS and NO are available.

3.4 Immunocytochemistry with Antibodies Specific for 8-Nitroguanine (NO2-52) and 8-Nitro-cGMP (1G6) in Activated C6 Cells

As previously reported, the monoclonal antibody NO2-52 recognizes the 8-nitro moiety of the guanine base, which reacted with 8-nitroguanine, 8-nitroxanthine, 8-nitroguanosine, 8-nitro-cGMP, 8-nitro-GMP, and 8-nitro-GTP (Fig. 16) (24). On the other hand, the monoclonal antibody 1G6 was specific for 8-nitro-cGMP and had no cross-reactivity with nitrated purines including 8-nitroxanthine and 8-nitroguanine (24). Immunocytochemical analysis using these two different clones of antibodies is expected to provide useful information about the formation of nitrated guanine derivatives in

cells.

Immunocytochemistry utilizing the 1G6 monoclonal antibody indicated that the 1G6 immunostaining increased markedly in C6 cells expressing increased amounts of iNOS after treatment with LPS-cytokines (Fig. 17), consistent with the above LC-MS/MS analyses. Similarly, the intensity of 8-nitroguanine immunostaining using NO2-52 monoclonal antibody markedly increased in C6 cells after stimulation with LPS-cytokines (Fig. 17).

Furthermore, to examine whether iNOS and sGC are involved in 1G6 and NO2-52 immunostaining, immunocytochemistry using 1G6 and NO2-52 was performed with C6 cells that were treated or not treated with inhibitors for NOS (L-NMMA) and sGC (NS 2028) (Fig. 18). Quantitative determination of 8-nitro-cGMP by LC-MS/MS analysis demonstrated that both L-NMMA and NS 2028 completely suppressed 8-nitro-cGMP production in C6 cells stimulated by LPS-cytokines (Fig 19); this result indicate that 8-nitro-cGMP was the downstream signal of NO/cGMP. The results of immunocytochemistry performed using 1G6 showed that treatment of cells with L-NMMA and NS 2028 almost completely nullified the elevated immunoreactivity (Fig. 18); this result is consistent with that of the above mentioned LC-MS/MS. The immunocytochemistry performed using NO2-52 also showed that treatment of cells with L-NMMA and NS 2028 almost completely nullified the elevated immunoreactivity (Fig. 18). The effect produced by NS 2028 is notable because immunoreactivity of NO2-52 is not only dependent on iNOS activity but also on sGC activity. These

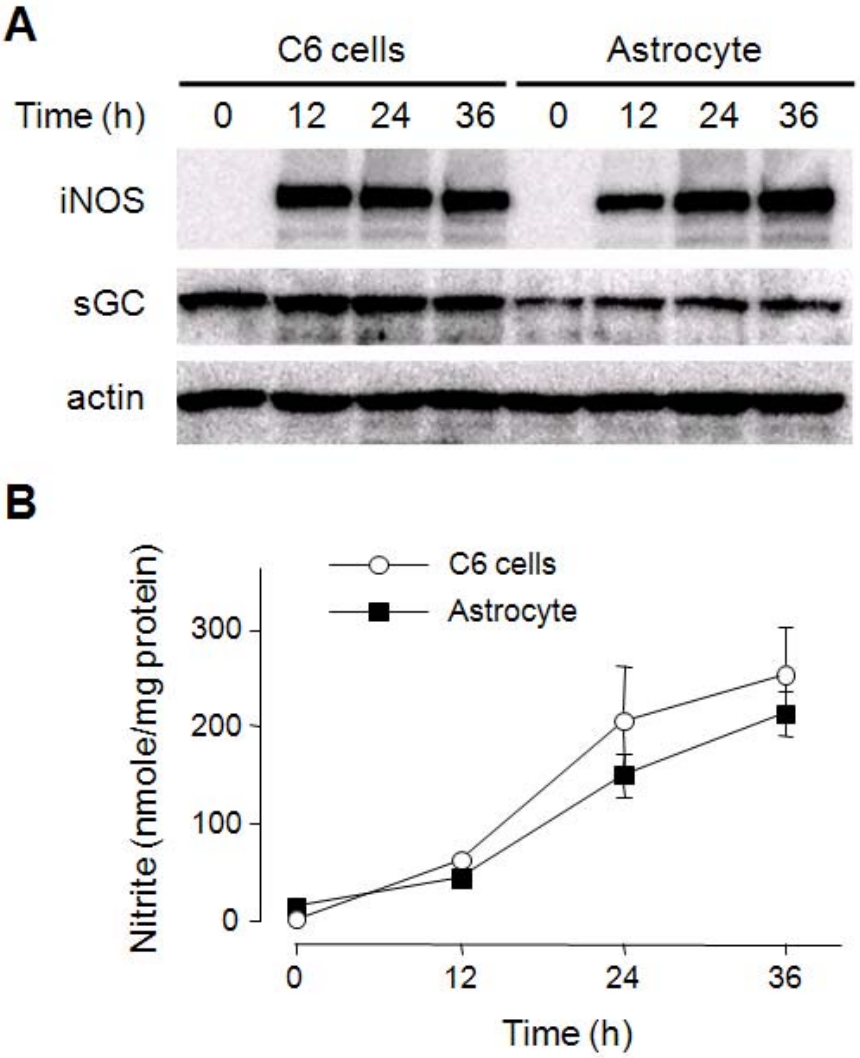

Fig. 14 Western blot analysis and nitrit e production in C6 cells and primary astrocytes. (A) Expression of iNOS and sGC in C6 cells and primary astrocytes after stimulation with LPS-cytokines. Cells were stimulated with a mixture of 10 μg/ml LPS, 100 U/ml IFN-γ, 100 U/ml TNFα, and 10 ng/ml IL-1β for the indicated time periods. Cell lysates (10 μg of protein) were analyzed via Western blot with anti-iNOS, anti sGC and anti-actin antibodies. (B) Nitrite amounts in culture supernatants of C6 cells and primary astrocytes. Cells were treated with the same mixture of LPS-cytokines as in (A). Nitrite amounts were normalized by protein amounts of cell lysates. Data represent means ± S.E. (n = 3). No significant difference was found between nitrite amounts in both culture supernatants

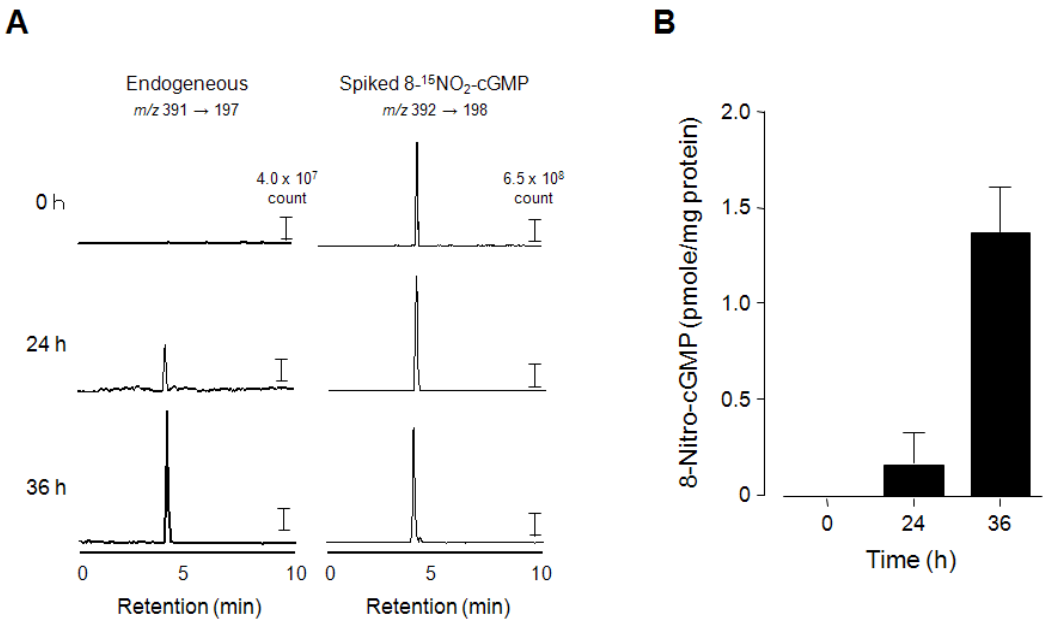

Fig. 15 **Quantitative LC-MS/MS analysis for measurement of 8-nitr o-cGMP fromed in C6 cells.** Cells were stimulated with a mixture of 10 μg/ml LPS, 100 U/ml IFN-γ, 100 U/ml TNFα, and 10 ng/ml IL-1β for the indicated time periods, and cell extracts were prepared as described under "Materials and Methods." (A) Representative LC-MS/MS chromatograms of 8-nitro-cGMP. (B) Amounts of 8-nitro-cGMP in C6 cells after LPS-cytokine treatment. Data represent means ± S.E. (n = 3).

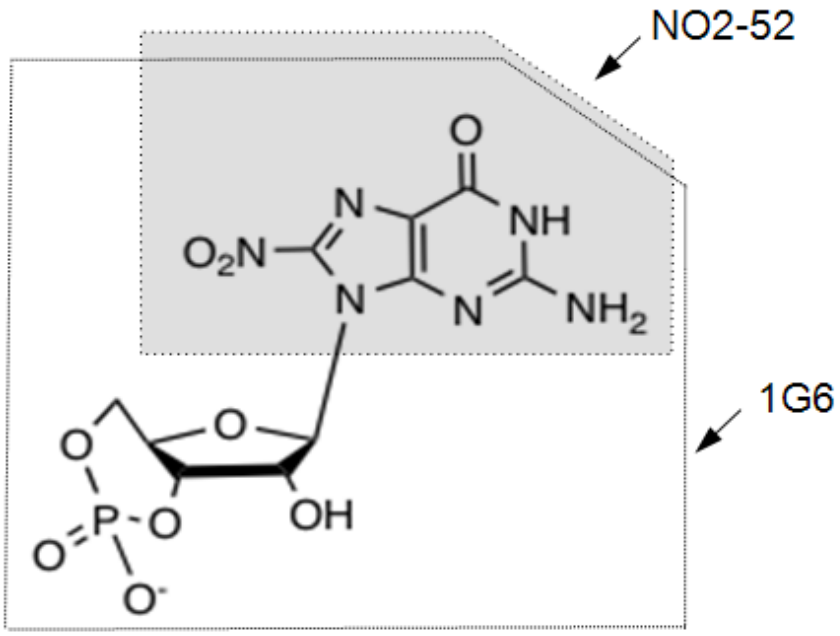

Fig. 16 The epitope structures of 8-nitroguanine derivatives that each antibody can recognize. The monoclonal antibody NO2-52 recognizes the 8-nitroguanine. On the other hand, the monoclonal antibody 1G6 was specific for 8-nitro-cGMP.

observations indicate that 8-nitro-cGMP is the most predominant derivative of 8-nitroguanie in cells.

3.5 Immunocytochemistry with Antibodies Specific for 8-Nitroguanine (NO2-52) and 8-Nitro-cGMP (1G6) in Activated Astrocytes

C6 glioma cells express glial fibrillary acidic protein, which is an astrocyte marker protein, are easily maintained and consistently available. Therefore, they are widely used as a model for studying the metabolism and function of astrocytes. To date, there have been no reports on 8-nitro-cGMP formation in primary cultured astrocytes, however. Guanine nitration in stimulated astrocytes was examined by means of immunocytochemistry with 1G6 and NO2-52. Primary astrocytes expressed moderate levels of sGC, and these astrocytes as well as C6 cells were easily activated by LPS-cytokines to express iNOS (Fig. 14). As shown in Fig. 20, strong immunostaining of activated astrocytes is apparent with both types of antibodies (1G6 and NO2-52). Treatment with an NOS inhibitor (L-NMMA) completely eliminated this positive immunostaining result. The same cells without stimulation showed only marginal immunoreactivity. These results indicate that 8-nitroguanine-related compounds (e.g., 8-nitro-cGMP) are produced via NO derived from iNOS, which also produced in primary astrocytes.

Furthermore, treatment with an sGC inhibitor (NS 2028) eliminated positive immunostaining not only with 1G6 but also with NO2-52 in astrocytes. These data

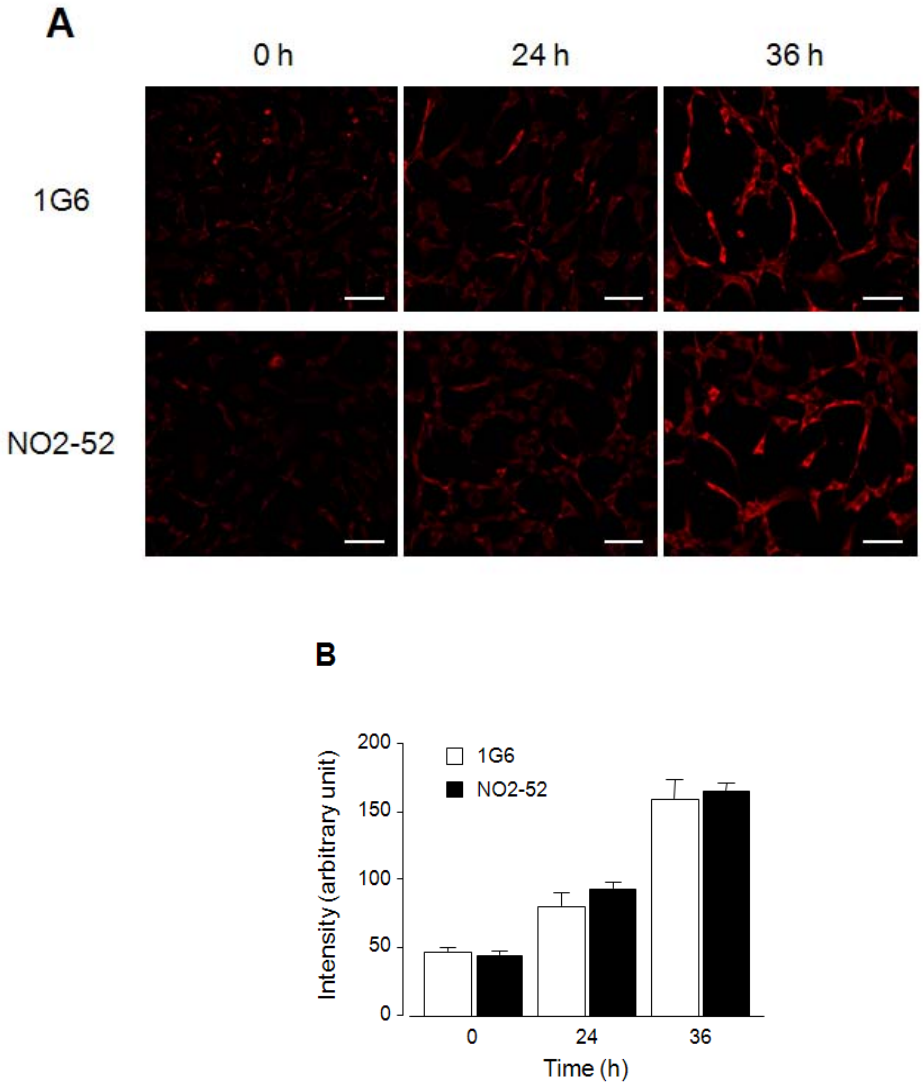

Fig. 17 Immunocytochemistry with antibodies specific for 8-nitroguanine (NO2-52) and 8-nitro-cGMP (1G6) in C6 cells. Cells were stimulated with a mixture of 10 μg/ml LPS, 100 U/ml IFN-γ, 100 U/ml TNFα, and 10 ng/ml IL-1β for the indicated time periods. (A) Immunofluorescence image of 8-nitro-cGMP and 8-nitroguanine in C6 cells. Cells were stained with anti-8-nitro-cGMP (1G6; 10 μg/ml) and anti-8-nitroguanine (NO2-52; 10 μg/ml) antibodies after LPS-cytokine treatment. Scale bars indicate 50 μm. (B) Morphometric determination for the immunofluorescence image (A) showing the time-dependent increase in relative fluorescence intensity of 8-nitro-cGMP and 8-nitroguanine in C6 cells.

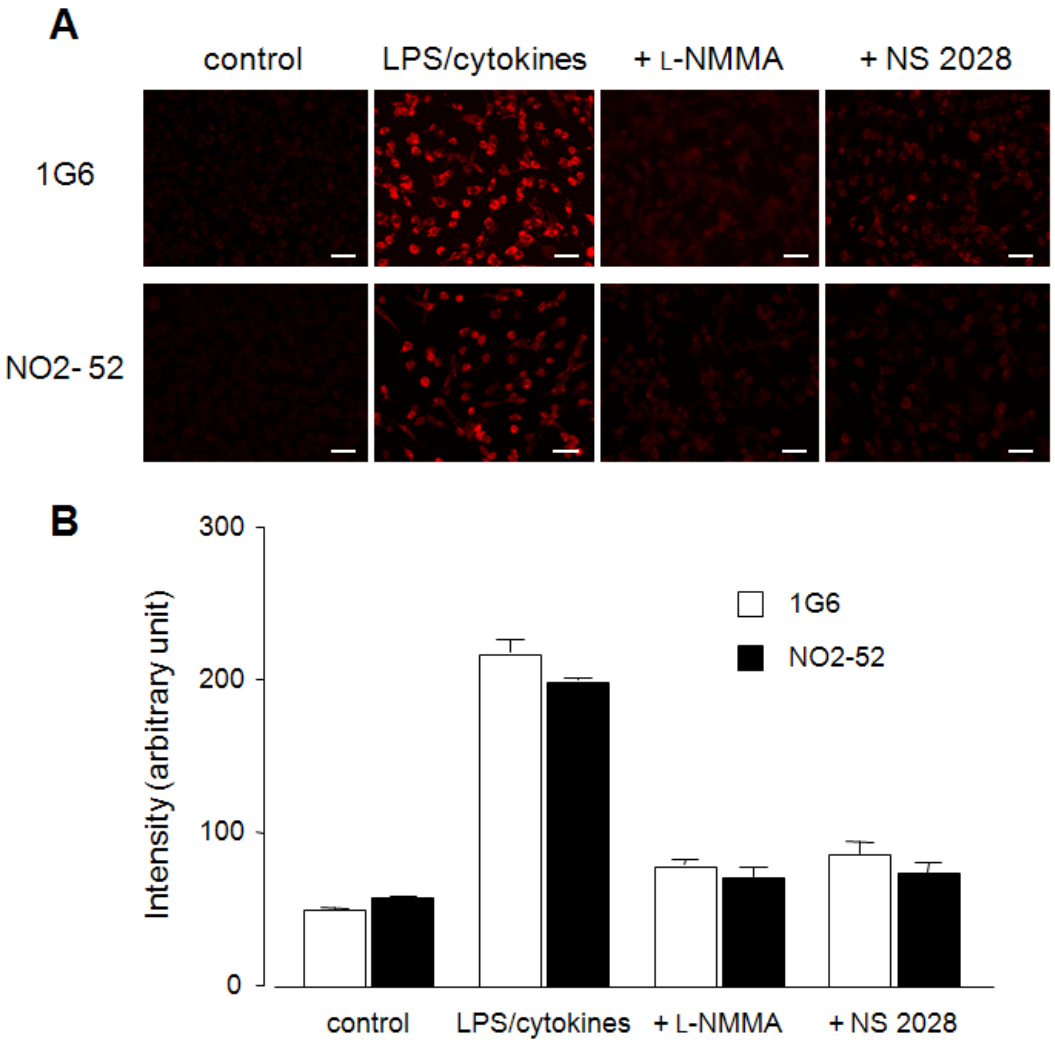

Fig. 18 Effects of inhibitors o n immunocytochemistry with antibodies (NO2-52 and 1G6) in C6 cells. Cells were stimulated with a mixture of of 10 μg/ml LPS, 100 U/ml IFN-γ, 100 U/ml TNFα, and 10 ng/ml IL-1β for 36 h in the presence or absence of various inhibitors. Cells were treated with 10 mM L-NMMA or 10 μM NS 2028, beginning 1 h before the addition of LPS-cytokines. (A) Immunofluorescence image of 8-nitro-cGMP and 8-nitroguanine in C6 cells. After stimulation with LPS-cytokines, cells were stained with anti-8-nitro-cGMP (1G6; 10 μg/ml) and anti-8-nitroguanine (NO2-52; 10 μg/ml) antibodies. Scale bars indicate 50 μm. (B) Morphometric determination for the immunofluorescence image (A) showing the effects of inhibitors on fluorescence intensity of 8-nitro-cGMP and 8-nitroguanine in C6 cells.

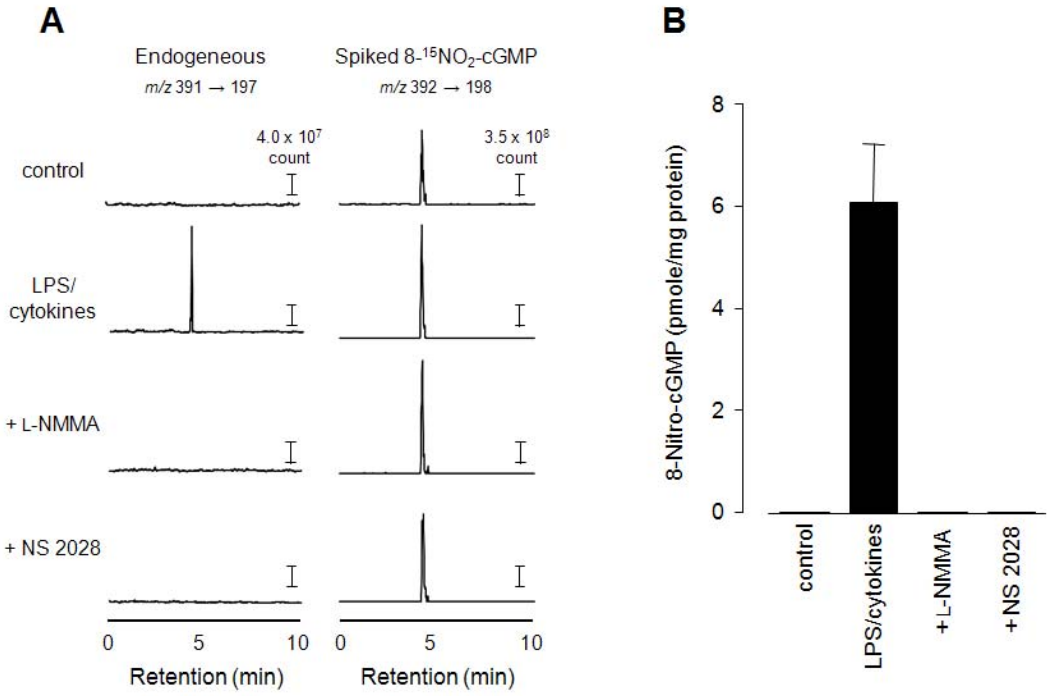

Fig. 19 Effects of inhibitors on 8-nitro-cGMP formation in C6 cells (LC-MS/MS analyses). Cells were stimulated with a mixture of of 10 μg/ml LPS, 100 U/ml IFN-γ, 100 U/ml TNFα, and 10 ng/ml IL-1β for 36 h in the presence or absence of various inhibitors. Cells were treated with 10 mM L-NMMA or 10 μM NS 2028, beginning 1 h before the addition of LPS-cytokines. After stimulation, cell extracts were prepared as described under "Materials and Methods." (A) Representative LC-MS/MS chromatograms of 8-nitro-cGMP. (B) Amounts of 8-nitro-cGMP in C6 cells after LPS-cytokines treatment. Data represent means ± S.E. (n = 3).

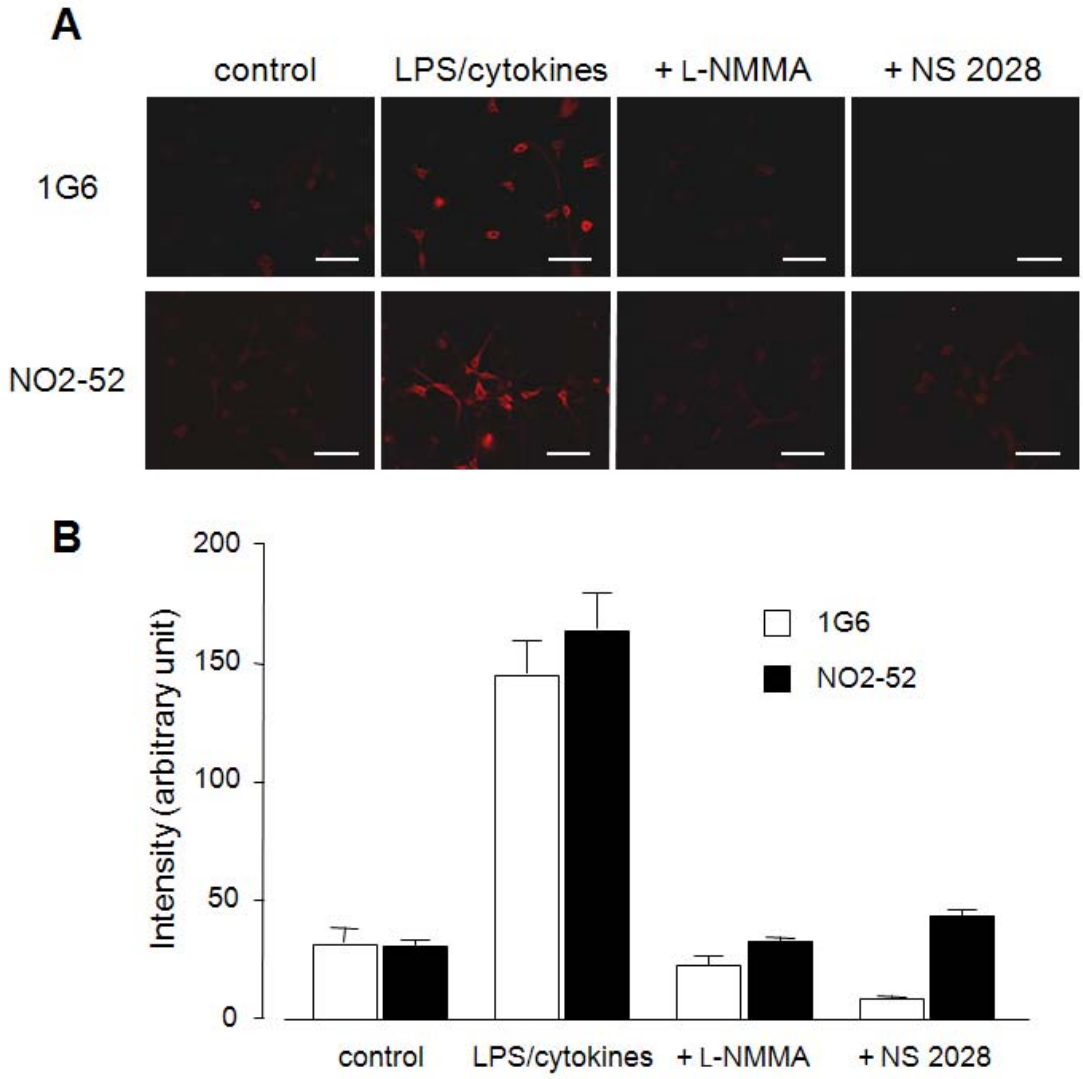

Fig. 20 Effects of inhibitors o n immunocytochemistry with antibodies (NO2-52 and 1G6) in primary astr ocytes. Cells were stimulated with a mixture of of 10 μg/ml LPS, 100 U/ml IFN-γ, 100 U/ml TNFα, and 10 ng/ml IL-1β for 36 h in the presence or absence of various inhibitors. Cells were treated with 10 mM L-NMMA or 10 μM NS 2028, beginning 1 h before the addition of LPS-cytokines. (A) Immunofluorescence image of 8-nitro-cGMP and 8-nitroguanine in C6 cells. After stimulation with LPS-cytokines, cells were stained with anti-8-nitro-cGMP (1G6; 10 μg/ml) and anti-8-nitroguanine (NO2-52; 10 μg/ml) antibodies. Scale bars indicate 50 μm. (B) Morphometric determination for the immunofluorescence image (A) showing the effects of inhibitors on fluorescence intensity of 8-nitro-cGMP and 8-nitroguanine in primary astrocytes.

suggest that most of 8-nitroguanine-related compounds in primary astrocytes stimulated with LPS-cytokines are also 8-nitro-cGMP. Thus, 8-nitro-cGMP is generated in not only C6 glioma cells but also in primary astrocytes; indicating possible involvement of 8-nitro-cGMP in physiological brain function.

DISCUSSION

In this study, I investigated the chemical and biochemical mechanisms of 8-nitro-cGMP formation, with particular focus on the roles of ROS. Chemical analyses showed that NO itself is not sufficient to nitrate guanine nucleotides in vitro. Two reaction systems, $ONOO^-$ and $NaNO_2/H_2O_2/MPO$, effectively produced nitrated guanine nucleotides. $ONOO^-$ is an unstable intermediate formed by the diffusion-controlled reaction between NO and O_2^- (71, 72). At physiological pH, both $ONOO^-$ and its conjugated acid ONOOH (pK_a = 6.8) exist, and the latter decomposes via homolysis to give the hydroxyl radical ($^{\bullet}OH$) and NO_2 (71, 72). In the presence of CO_2, $ONOO^-$ reacts with CO_2 to form nitrosoperoxycarbonate anion ($ONOOCO_2^-$), which undergoes homolysis to give $CO_3^{\bullet-}$ and NO_2 (73). Reduction potentials have been reported for $^{\bullet}OH$ (E^0 = 1.9-2.1 V) (74), NO_2 (E^0 = 1.04 V) (75), $CO_3^{\bullet-}$ (E^0 = 1.5 V) (76), and guanine (E^0 = 1.29 V) (77). Oxidation of guanine by $^{\bullet}OH$ or $CO_3^{\bullet-}$ is thus believed to be thermodynamically favorable and would result in formation of the guanine radical cation. This cation undergoes recombination with NO_2 to form 8-nitroguanine (46). A similar mechanism may therefore operate for nitration of guanine nucleotides induced by $ONOO^-$ and $ONOOCO_2^-$. However, although NO under aerobic conditions is oxidized to form NO_2, guanine oxidation by NO_2 is not sufficient to oxidize the guanine base and initiate guanine modification. The $NaNO_2/H_2O_2/MPO$ system used in this study was another potent mechanism for nitration of guanine nucleotides. MPO reacts with H_2O_2 to form MPO compound I, which can oxidize nitrite and produce NO_2 (78). This compound I may also directly

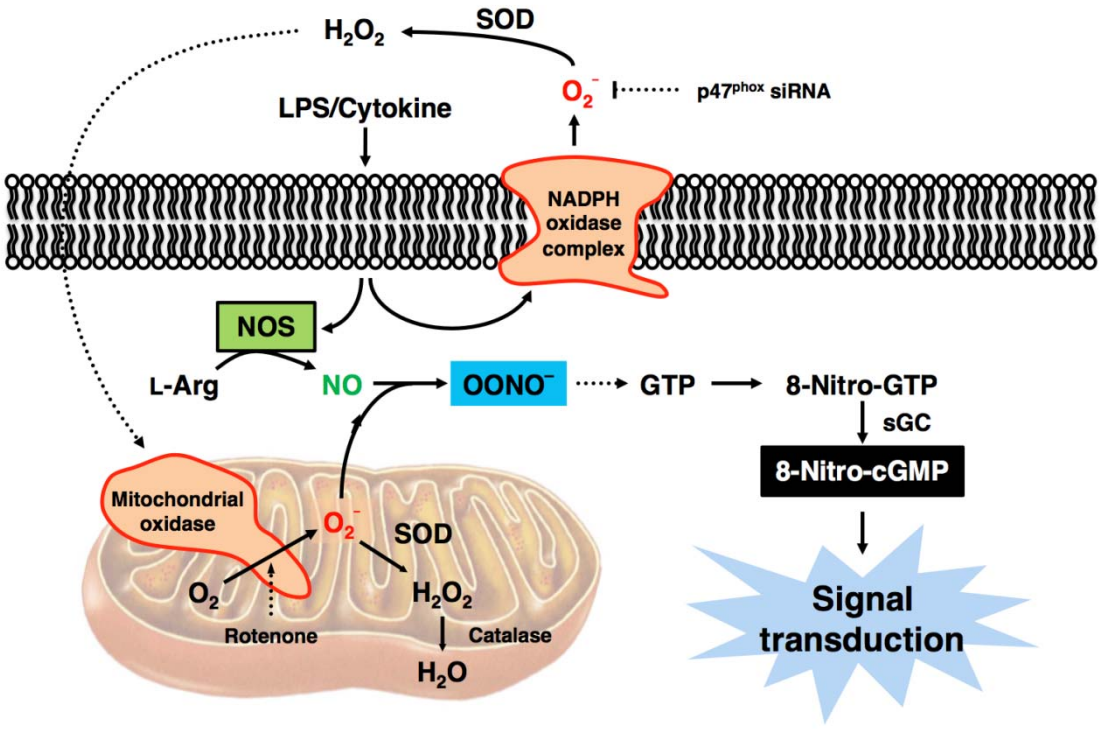

Fig. 21 **Schematic drawing of possible mechanisms involved in cell formation of 8-nitro-cGMP in rat C6 glioma cells stimulated with LPS plus cytokines.**

oxidize guanine nucleotides because of its strong reduction potential (E^0 = 1.35 V) (79). Subsequently, NO_2 and radical cations of guanine nucleotides react with each other to form 8-nitroguanine nucleotides. However, there was no production of nitrated guanine nucleotides found in the reaction of guanine nucleotides with $NaNO_2/H_2O_2$/HRP. HRP compound I is reportedly a much weaker one-electron oxidant compared with MPO compound I (80). Therefore, HRP compound I could not oxidize guanine nucleotides to form the corresponding cation radical, which is a key step for guanine nucleotide nitration.

Among the guanine nucleotides examined, GTP was the most susceptible to nitration induced by $ONOO^-$. GTP makes up nearly 25% of the total intracellular nucleotide triphosphate pool, and it acts as a versatile nucleotide, as it participates in many critical physiological functions including RNA synthesis, cell signaling through activation of GTP-binding proteins, and production of the second messenger cGMP (81). Because of its abundance in the intracellular nucleotide pool, GTP has been reported to be a significant target for oxidative modification induced by ROS (82). Oxidized GTP can be mutagenic when incorporated into DNA or RNA (83). Our recent work suggested that 8-nitro-GTP can act as a substrate for sGC, with 8-nitro-cGMP formed as a product (25). All these findings together suggest that cell formation of 8-nitro-cGMP may depend largely on the formation of 8-nitro-GTP, which can be converted into 8-nitro-cGMP by sGC, rather than on direct nitration of cGMP.

The present study showed that Nox2 and mitochondria are two important sources of ROS in rat C6 glioma cells stimulated by LPS-cytokines and are critically involved in regulation of 8-nitro-cGMP formation. It was found that mitochondrial O_2^- production depended greatly on Nox2 activity, because siRNA knockdown of Nox2

almost completely abolished mitochondrial O_2^- production, to the basal level determined for unstimulated cells (Fig. 12). The effects of ROS on mitochondrial O_2^- production have been reported, with the phenomenon being named ROS-induced ROS release (RIRR) (84, 85). Two modes of RIRR were reported: mitochondrial inner membrane anion channel (IMAC)-dependent and mitochondrial permeability transition pore (MPTP)-dependent mechanisms (86). These two anion channels, along with the voltage-dependent anion channel (87), are thought to be the main pathways for cytosolic release of O_2^- generated by the electron-transport chain. Thus, either IMAC- or MPTP-dependent mechanisms may function, in a manner dependent on Nox2 activation, to increase mitochondrial O_2^- generation in C6 cells.

In view of the finding that PEG-SOD treatment almost completely nullified 8-nitro-cGMP formation without affecting H_2O_2-like oxidant production in stimulated C6 cells, I propose that O_2^-, possibly derived from mitochondria, may have direct effects on nitration of guanine nucleotides, as Fig. 21 illustrates. In stimulated cells, as NO is also actively generated by iNOS, $ONOO^-$-mediated nitration of guanine nucleotides may occur. Data showing that 8-nitro-cGMP formation in C6 cells treated with chemical reagents including P-NONOate and H_2O_2 can be effectively suppressed by PEG-SOD further support the idea that O_2^- functions as a downstream molecular species involved in effective guanine nitration.

Mitochondrial O_2^- production is an important source of ROS in most mammalian cells (66, 67). The main sites of O_2^- production in the respiratory chain are Complex I and III (88, 89), but other mitochondrial enzymes, such as Complex II, glycerol-3-phosphate dehydrogenase, and 2-oxoglutarate dehydrogenase, are also involved in mitochondrial production of O_2^- (90). Increased mitochondrial O_2^-

production reportedly occurred under various conditions such as anticancer drug treatment (91), Alzheimer disease (92), apoptosis (68), and aging (66, 93, 94). Pathological effects of such increased mitochondrial O_2^- production have been studied, particularly with respect to oxidative damage to DNA, proteins, and lipids caused by ROS (93, 94). However, accumulating evidence has also suggested that ROS play an important role as signaling molecules in regulation of diverse physiological phenomena (95-97). The data demonstrate that mitochondrial O_2^- production in the presence of NO is a potential mechanism for generating the unique electrophilic signaling molecule 8-nitro-cGMP. Thus, formation and signaling functions of 8-nitro-cGMP merit study as related to physiological and pathological conditions associated with increased mitochondrial O_2^- production.

In the second part of this study, I examined the reliability of immunological approaches for determining 8-nitro-cGMP formation in cells. Immunocytochemistry is a conventional and fairly straightforward method for determining the presence and relative abundance of an antigen of interest in cultured cells. As demonstrated in the present study, immunocytochemical analysis requires remarkably smaller numbers of cells (~10^5 cells) than that with LC-MS/MS (usually more than 10^6 cells). Thus, I can reduce the number of cells used which is economically viable. Therefore, there is a great benefit to use immunocytochemistry particularly when only limited amount of samples are available (e.g., primary astrocytes). This suggests that immunocytochemistry itself could be used as a fundamental detection tool for nitration of guanine nucleotide in cells particularly cGMP. Additionally, it could be widely usable method due to its easy handling methodology as well as economic feasibility.

It has become clear that nitrated guanine nucleotides are generated by reactive

nitrogen oxides in cultured cells and tissues, and play important roles in biological functions (14, 17, 24, 25, 28). The present immunocytochemical data further proved that treatment with an NOS inhibitor (L-NMMA) and sGC inhibitor (NS 2028) eliminated positive immunostaining not only with 1G6 but also with NO2-52 in both C6 and astrocytes. These finding supports that most of 8-nitroguanine-related compounds in C6 cells and primary astrocytes stimulated with LPS-cytokines are solely 8-nitro-cGMP indicating possible involvement of 8-nitro-cGMP in physiological brain function. Thus the anti-8-nitroguanine (NO2-52) and anti-8-nitro-cGMP (1G6) antibodies could be powerful tools for analyzing nitrated guanine nucleotides by means of immunocytochemistry. Furthermore, comparison with results of immunocytochemistry using anti-8-nitroguanine antibody (NO2-52) indicated that 8-nitro-cGMP is a major component of 8-nitroguanine derivatives produced in cells. Thus use of this conventional, economically viable and easy handling immunocytochemical method will promote the progress of research involving ROS signaling and 8-nitro-cGMP.

In summary, I herein verified that ROS play a pivotal role in the formation of 8-nitro-cGMP in C6 cells stimulated with LPS-cytokines. O_2^-, most likely derived from mitochondria, is directly involved in formation of 8-nitro-cGMP, whereas H_2O_2 generated by Nox2 also has an important role by increasing mitochondrial O_2^- production. The above data thus suggest that 8-nitro-cGMP may serve as a unique second messenger for ROS signaling in the presence of NO. Greater understanding of 8-nitro-cGMP formation in relation to mitochondrial functions and Nox2 regulation may help us develop new diagnostic methods and treatment of diseases related to dysregulation of NO and ROS (98).

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