on guanine nucleotide nitration, we used the NO-releasing agent P-NONOate, which spontaneously decomposes to release NO [19]. 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 [18]: 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).

Hypochlorous acid/nitrite system

HOCl (hypochlorous acid) is a product of the MPO-catalysed oxidation of chlorine ion (Cl^-) in the presence of H_2O_2. HOCl reportedly reacts with nitrite to form NO_2Cl [20]. 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).

Effect of ROS scavengers

To determine the roles of ROS on guanine nucleotide nitration, we used three different ROS scavengers: SOD and tiron for scavenging superoxide and catalase for scavenging H_2O_2. For the experiments described below, PEGylated SOD and catalase were used for effective delivery of those enzymes into cells [12].

Cell treatment

Rat C6 glioma cells were cultured at 37 °C in DMEM (Dulbecco's modified Eagle's medium; Wako Pure Chemical Industries) supplemented with 10% FBS (fetal bovine serum) 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-ESI (electrospray ionization)-MS/MS, and at a density of 1×10^5 cells per chamber in BD Falcon Culture Slides (BD Biosciences) for immunocytochemistry. To study 8-nitro-cGMP formation, cells were stimulated for 36 h with a mixture of 10 μg/ml LPS (from *Escherichia coli*; Sigma–Aldrich, catalogue number L8274) and 200 units/ml IFN-γ (interferon-γ), 500 units/ml TNF-α (tumour necrosis factor-α) and 10 ng/ml IL-1β (interleukin-1β) (all cytokines from R&D Systems). 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 certain experiments, cells were treated with an LPS/cytokine mixture for 0, 3, 12, 24 and 36 h followed by detection of mitochondrial ROS by MitoSOX™ Red, as described below. 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 NADPH oxidase $p47^{phox}$-specific siRNA before stimulation as described below.

Immunocytochemistry

Formation of 8-nitro-cGMP in C6 cells was analysed by means of immunocytochemistry with an anti-(8-nitro-cGMP) mouse monoclonal antibody and Cy3 (indocarbocyanine)-labelled goat anti-mouse IgG antibody (10 μg/ml; Amersham Biosciences, catalogue number PA43002), as described previously [3,5]. Fluorescence intensity values from three different experiments were obtained, and the average relative fluorescence intensity (as the percentage fluorescence intensity) was determined for LPS/cytokine-stimulated cells. We confirmed that LPS/cytokines and PEG-derivatized proteins had no significant effects on quenching or augmentation of fluorescence by using non-immune antiserum (results not shown).

LC-ESI-MS/MS analysis for intracellular formation of 8-nitro-cGMP

Intracellular levels of 8-nitro-cGMP were quantified by means of LC-ESI-MS/MS as described previously [5]. Amounts of endogenous cGMP and 8-nitro-cGMP were determined via the stable isotope dilution method based on the recovery efficiency of stable isotope-labelled derivatives (c[$^{15}N_5$]GMP and 8-$^{15}NO_2$-cGMP) spiked with the cell extract as described previously [5].

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, we used MitoSOX™ Red [21] and DHE [22] for detection of mitochondrial superoxide production and DCDHF-DA [23] for detection of cellular oxidants. C6 cells were washed with Hank's buffer [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 $MgSO_4$ and 4.2 mM $NaHCO_3$ (pH 7.4)], and were then stained with 2.5 μM MitoSOX™ Red or with 100 nM DHE 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 and 543 nm for DHE (the red photomultiplier channel of the confocal microscope was used for image acquisition); for DCDHF-DA, excitation was at 488 nm (the green photomultiplier channel of the confocal microscope was used for image acquisition). To minimize run-to-run variations, the laser intensity and photomultiplier tube voltage were kept constant, and microscopic observations were performed on all sample sets at same time. Images were captured and processed by means of Nikon EZ-C1 software. Further image processing and quantification were performed using Adobe Photoshop Elements version 2.0 (Adobe Systems). Fluorescence intensity values from three different experiments were obtained, and the average relative fluorescence intensity (as the percentage fluorescence intensity) was determined for LPS/cytokine-stimulated cells. In other experiments, the percentage relative fluorescence intensity was determined for PBS-treated cells.

Transfection of $p47^{phox}$ siRNA

A 25-nucleotide $p47^{phox}$ siRNA (manufactured by Invitrogen, catalogue number 1320003, Oligo ID, MSS206956 and MSS275934) was used for transfection using Lipofectamine™ RNAiMAX transfection reagent (Invitrogen). 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) 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

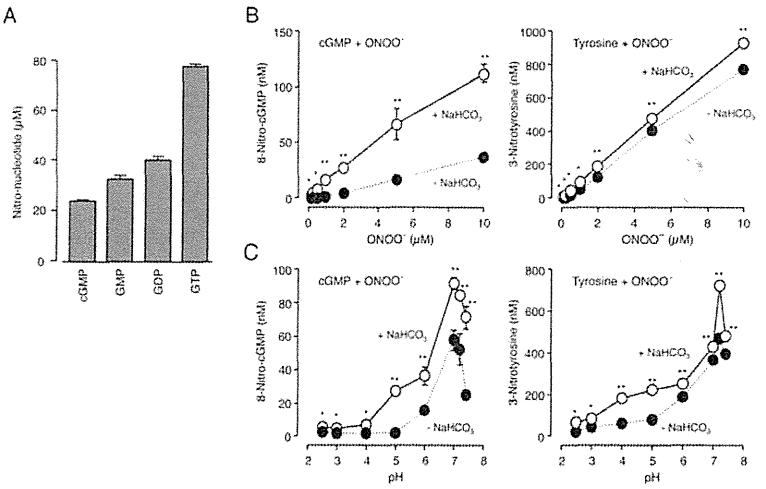

Figure 1 ONOO⁻-dependent nitration of guanine nucleotides and tyrosine

(**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-hand panel) and tyrosine (right-hand 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.M. ($n = 3$). $^*P < 0.05$ and $^{**}P < 0.01$, compared with the group in the absence of $NaHCO_3$.

(RNA interference) negative control (high GC; Invitrogen) was used as a negative control siRNA as described above. In other experiments, cells were treated with 10 μM rotenone for 15 min before stimulation with LPS/cytokines for 36 h.

Western blot analysis

Proteins were separated using SDS/PAGE and transferred on to a PVDF membrane. The blot was blocked with 5 % non-fat skimmed milk followed by 1 h of incubation with an anti-$p47^{phox}$ antibody (Millipore) and a goat anti-rabbit HRP-conjugated IgG secondary antibody. The immunoreactive bands were detected using chemiluminescence reagent (Millipore) with a luminescent image analyser (LAS-1000, Fujifilm).

Statistical analysis

All cell culture results were obtained from at least three separate wells or three separate dishes, and the data are represented as means ± S.E.M. Statistical analyses were performed using Student's *t* test.

RESULTS

Nitration of guanine nucleotides by various RNOS: *in vitro* chemical analyses

We investigated the effects of various RNOS on nitration of guanine nucleotides *in vitro*. We first examined nitration of guanine nucleotides in the reaction with $ONOO^-$, a potent nitrating and oxidizing species formed from the reaction of NO and superoxide. As shown in Figure 1(A), $ONOO^-$ nitrated all guanine nucleotides. However, the efficacy of nitration varied depending on the structure of the nucleotides; GTP showed 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 shown in Figure 1(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) (Figure 1C). To exclude the possibility that nitrite that may be contaminating the $ONOO^-$ solution could affect the induction of cGMP nitration, we examined cGMP nitration in a reaction with decomposed $ONOO^-$ solution that would presumably contain the same amount of contaminating nitrite as an intact $ONOO^-$ solution. Results indicated no 8-nitro-cGMP formation in the reaction of cGMP with decomposed $ONOO^-$ (results not shown).

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 superoxide, caused both cGMP and tyrosine nitration. This result suggests that $ONOO^-$ formed from NO and superoxide 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 (Figure 2). 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 a SOD mimic and has been used as a superoxide scavenger [24]. In the present study, however, tiron

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Review

Cell signaling mediated by nitrated cyclic guanine nucleotide

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ABSTRACT

We recently clarified the physiological formation of 8-nitroguanosine 3′,5′-cyclic monophosphate (8-nitro-cGMP) and its critical roles in nitric oxide (NO) signal transductions. This discovery of 8-nitro-cGMP is the first demonstration of a nitrated cyclic nucleotide functioning as a new second messenger in mammals since the identification of cGMP more than 40 years ago. By means of chemical analyses, e.g., liquid chromatography–tandem mass spectrometry, we unequivocally identified 8-nitro-cGMP formation, which depended on NO production, in several types of cultured cells, including macrophages and glial cells. Most important, we previously showed that 8-nitro-cGMP as an electrophile reacted with particular sulfhydryls of proteins to generate a unique post-translational modification that we called protein S-guanylation. In fact, certain specific intracellular proteins, such as the redox-sensor protein Keap1, readily underwent S-guanylation induced by 8-nitro-cGMP. 8-Nitro-cGMP activated the Nrf2 signaling pathway by triggering dissociation of Keap1, via S-guanylation of its highly nucleophilic cysteine sulfhydryls. We also determined that S-guanylation of Keap1 was involved in cytoprotective actions of NO and 8-nitro-cGMP by inducing oxidative stress response genes such as heme oxygenase-1. Such unique chemical properties of 8-nitro-cGMP shed light on new areas of NO and cGMP signal transduction. Protein S-guanylation induced by 8-nitro-cGMP may thus have important implications in NO-related physiology and pathology, pharmaceutical chemistry, and development of therapeutics for many diseases.

Contents

Introduction

Reactive oxygen species (ROS) and reactive nitrogen oxide species (RNOS) formed during infection and inflammation or by chemical means cause oxidative stress and contribute to the development of various diseases [1–3]. Diseases involving oxidative stress-related pathogenesis include inflammation, cancer, ischemia–reperfusion injuries such as cerebral and cardiac infarctions, diabetes, and atherosclerosis among others [4–7]. Organisms may, however, respond successfully to oxidative stress by inducing antioxidant and detoxifying enzymes, cellular reactions known as antioxidant and adaptive responses to oxidative stress. For some time, we have been investigating the mechanism of such cellular responses, with a focus on guanine nitration induced by RNOS produced from nitric oxide (NO) and ROS, which is being recognized as contributing to an important cytoprotective signaling mechanism against oxidative stress (Fig. 1) [8–12]. In 2003, for example, we first reported the *in vivo* formation of 8-nitroguanosine and 8-nitroguanine, which

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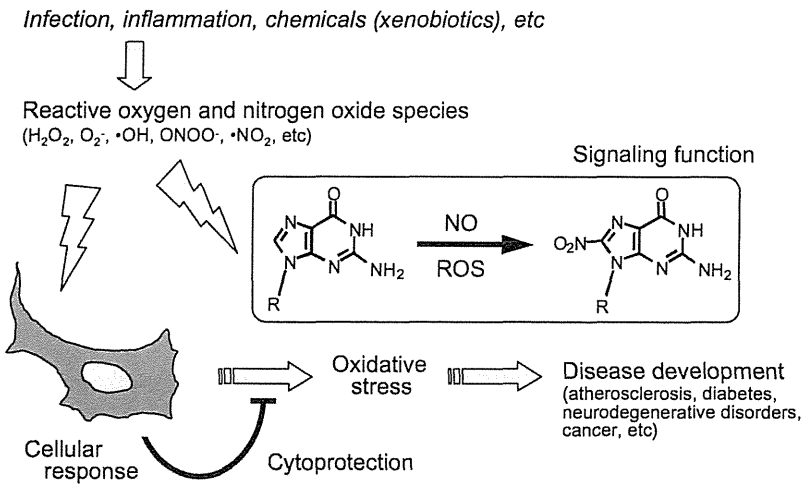

Fig. 1. A schematic drawing of potential involvement of nitrated nucleotide signaling molecules during the antioxidant adaptive response of cells to oxidative stress caused by ROS and RNOS.

depended on NO generated from inducible NO synthase (iNOS), in airway epithelial cells in mice infected with influenza virus [13].

NO was initially discovered as a signaling molecule regulating vascular tone and neuronal systems [14,15]. These functions are mainly mediated through a cGMP-dependent mechanism, but other pathways that are not directly related to cGMP appear to be responsible for many aspects of NO signaling [16–19]. Such a cGMP-independent pathway may be driven by nitrosylation and nitration of amino acids, proteins, lipids, and nucleotides, these processes being induced by RNOS (which are derived from NO and ROS), such as peroxynitrite ($ONOO^-$) and nitrogen dioxide (NO_2) [3,4,8,18–20].

In fact, nitration of nucleic acids is known to be caused by RNOS. For several years, the presence of nitrated guanine derivatives such as 8-nitroguanine and 8-nitroguanosine 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 [13,21–24]. Of particular importance is the redox activity of 8-nitroguanosine, which suggests that guanine nitration may have a biological effect [13,25]. In this regard, our recent discovery of a novel nitrated cyclic nucleotide, 8-nitroguanosine 3,5-cyclic monophosphate (8-nitro-cGMP), has many implications in that 8-nitro-cGMP, while remaining as a potential activator of cGMP-dependent protein kinase (protein kinase G [PKG]), shows the strongest redox-active property among nitrated guanine derivatives [8,11]. Related to this redox activity is the characteristic of 8-nitro-cGMP acting as an electrophile so that it can react with particularly reactive sulfhydryl groups of cysteine residues to form a protein-(8-*S*-Cys)-cGMP adduct, which is a novel post-translational modification (PTM) of proteins that we called protein *S*-guanylation [8].

In this review article, we describe cutting edge concepts of ROS/RNOS signaling arising from the unique chemical and physiological properties of the nitrated cyclic nucleotide 8-nitro-cGMP, specifically with regard to its potent signaling functions via protein *S*-guanylation that contribute to the adaptive cellular responses to oxidative stress (Fig. 1).

Formation of nitrated nucleotides in cells

To analyze the biological formation of nitroguanine and its derivatives, we first synthesized various derivatives, including 8-nitroxanthine and 8-nitroguanine, 8-nitroguanosine, and the monophosphate, triphosphate, and cyclic monophosphate of 8-nitroguanosine, as standard authentic compounds to be used for the chemical and immunochemical determination of guanine nitration occurring *in vivo* (Fig. 2) [8]. We expected that guanine nucleotides

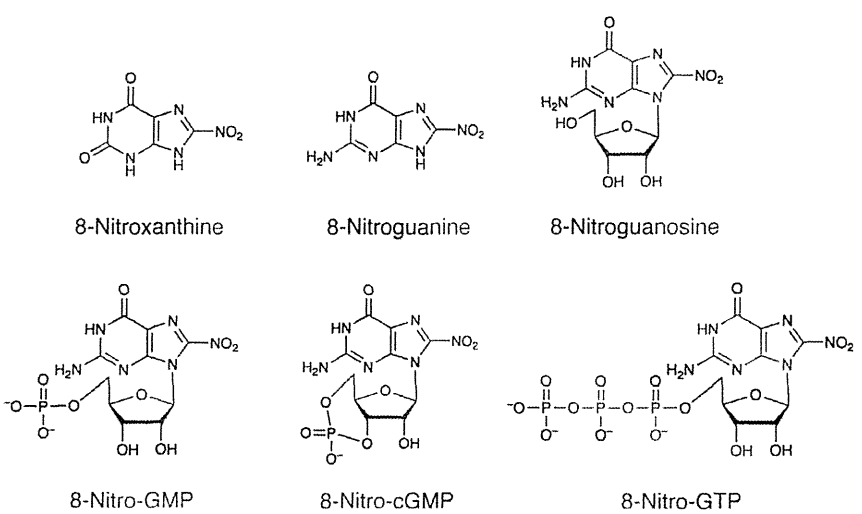

Fig. 2. Chemical structures of 8-nitroxanthine and 8-nitroguanine derivatives including several nucleotides such as 8-nitro-cGMP.

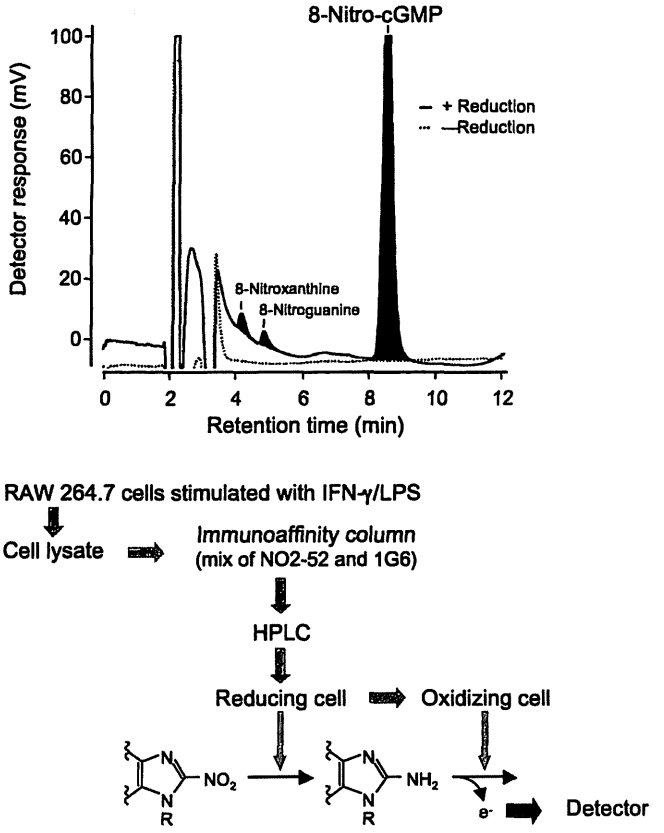

Fig. 3. Chemical identification of 8-nitro-cGMP formed in RAW 264.7 cells. Cells were stimulated with a mixture of lipopolysaccharide (LPS) (10 μg/ml) and interferon-γ (IFN-γ) (100 U/ml) for 11 h. 8-Nitro-cGMP formed in cells was detected by means of HPLC–ECD analysis (upper panel), whose principle is illustrated by the diagram (lower panel). Modified from Sawa et al. [8].

stored in cells could become targets of nitration by RNOS. We then prepared a series of polyclonal and monoclonal antibodies for these nitrated guanine nucleotides. For various analyses, we mainly used, from the series of successfully obtained antibodies, a monoclonal antibody, clone 1G6, that reacted strongly with 8-nitro-cGMP plus another clone, NO2-52, that exclusively recognized 8-nitropurine-containing structures. These antibodies were indeed useful not only for immunochemical analyses but also for chemical identification of 8-nitroguanine-related compounds that were to be separated from crude cell and tissue extracts and even biological samples such as urine. We first utilized the antibodies for chemical identification of nitrated purines generated in cells, as reported earlier [8]. For example, we confirmed the chemical identity of 8-nitro-cGMP and other nitrated purines by means of high performance liquid chromatography (HPLC) with electrochemical detection (ECD) (Fig. 3). We applied the lysate of RAW 264.7 cells, a murine macrophage cell line, which had been stimulated with lipopolysaccharide (LPS) and cytokines to express iNOS, to an immunoaffinity column containing the anti-nitroguanine antibodies (clones 1G6 and NO2-52) to separate and enrich the 8-nitroguanine derivatives, followed by HPLC–ECD analysis. The elution profile in Fig. 3 clearly contains a large peak for 8-nitro-cGMP in addition to small peaks for 8-nitroxanthine and 8-nitroguanine. Liquid chromatography–tandem mass spectrometry (LC–MS/MS) confirmed that the large peak was 8-nitro-cGMP, which indicated that 8-nitro-cGMP is the major nitration product among various nitrated guanine derivatives, as we documented earlier [8].

It is also critically important to provide proof of the biological relevance of endogenous 8-nitro-cGMP formation. We therefore performed extensive LC–MS/MS analyses utilizing the stable isotope-dilution technique with C6 cells, a rat glial cell line expressing iNOS, so that we could quantify the exact amount of 8-nitro-cGMP produced in cells [12]. We thus paid particular attention to cell production of not only 8-nitro-cGMP but also cGMP, because clarification of distinctive production kinetics (quantity and time-dependent profile), and thus differentiation between these two compounds, may lead to better understanding of their physiological and pathological significance. In fact, assessment of the exact quantity of endogenous 8-nitro-cGMP became possible via our stable isotope-dilution technique and LC–MS/MS analysis applied in a spike-and-recovery study with control nucleotides synthesized with stable ^{15}N isotopes. A surprising finding was a greatly improved recovery of 8-nitro-cGMP by means of methanol cell extraction: recovery efficiency increased up to 100-fold after spiking with exogenous ^{15}N-labeled 8-nitro-cGMP (i.e., 8-$^{15}NO_2$-cGMP and 8-$^{14}NO_2$-c[$^{15}N_5$]GMP), whereas recovery of cGMP was not appreciably affected by the same c[$^{15}N_5$]GMP spiking (Fig. 4A). This finding may indicate that a large part of 8-nitro-cGMP formed in the cells remained as a cellular component during methanol cell extraction, but that it could be efficiently removed and stabilized for recovery in the cell extract by adding the same authentic ^{15}N-labeled compound, which would permit effective quantification of 8-nitro-cGMP via LC–MS/MS analysis.

Fig. 4B illustrates that unexpectedly high levels of 8-nitro-cGMP were formed in the cells, a much greater amount than that of cGMP detected simultaneously in the same cells. Such a large amount of 8-nitro-cGMP leads us to suggest that GTP rather than cGMP may first be nitrated and thereby converted to 8-nitro-GTP, which would then become a substrate for soluble guanylate cyclase (sGC), as we proposed in our recent publication [12].

Moreover, because we now know that an appreciable fraction of 8-nitro-cGMP that formed in cells remained there even after methanol cell extraction, a measurable amount of 8-nitro-cGMP could conceivably be identified by means of immunocytochemical analysis (albeit not strictly quantitative), even after various fixative treatments of cells and tissues. Immunostaining is a conventional tool that allows analysis of clear and apparently dynamic expression profiles of various biological molecules produced in cells, as long as they remain intracellular. One successful immunocytochemical analysis with the anti-8-nitro-cGMP antibody 1G6 revealed strong immunostaining for 8-nitro-cGMP in the murine macrophage cell line RAW 264.7 and rat glial C6 cells that had been stimulated with interferon-γ (IFN-γ) and LPS to produce NO via iNOS [8,10,12]. As an intriguing result, intracellular 8-nitro-cGMP immunostaining colocalized with mitochondria rather than endoplasmic reticulum [8,24]. This intracellular localization may have certain implications for the mechanism and physiological effects of 8-nitro-cGMP formation. Specifically, guanine nitration may occur in mitochondria or their related compartments, possibly by means of RNOS generated from NO and ROS in the vicinity of this particular cellular organelle, as evidenced by our recent preliminary data [12]. Localized 8-nitro-cGMP production is further supported by our earlier observation of guanine nitration in human lung tissues [24].

These robust experimental data now provide evidence of a new cyclic nucleotide entity, i.e., 8-nitro-cGMP, that is physiologically generated in cells in a manner depending on NO and possibly ROS, as they both form RNOS.

Protein cysteine modification by 8-nitro-cGMP

That 8-nitro-cGMP as an electrophile has unique chemical reactivity for protein cysteine residues was also revealed. In other words, 8-nitro-cGMP undergoes nucleophilic substitution with sulfhydryls to release the nitro group and form a cysteine-cGMP

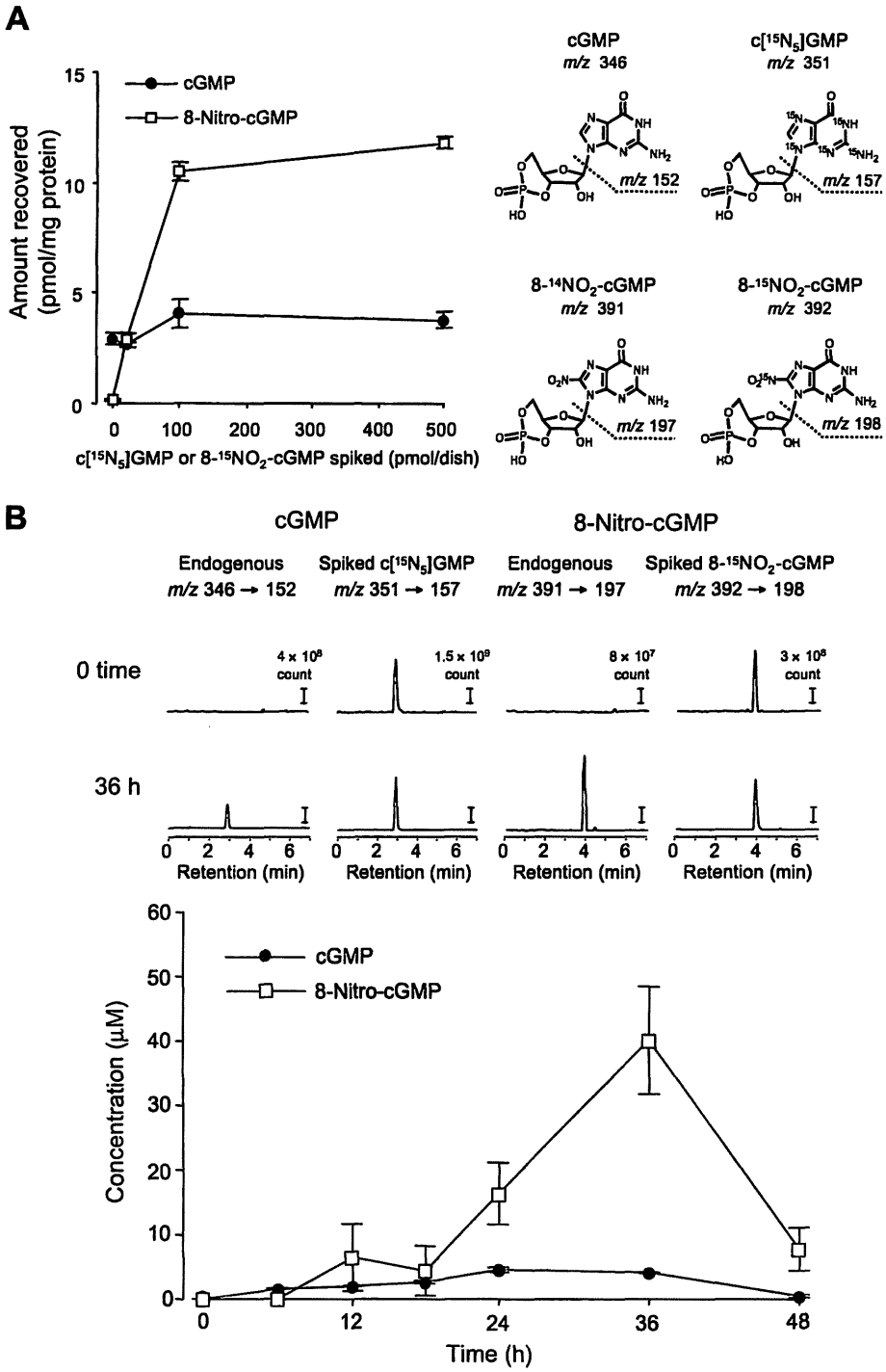

Fig. 4. Quantitative LC–MS/MS analysis for measurement of cGMP and 8-nitro-cGMP formed in cells stimulated with LPS plus cytokines. (A) Recovery efficiency of cGMP and 8-nitro-cGMP as determined in the spike-and-recovery study [12]. Recovery was evaluated by using rat glial C6 cells stimulated with a mixture of LPS (10 μg/ml), IFN-γ (200 U/ml), tumor necrosis factor α (500 U/ml), and interleukin-1β (10 ng/ml) for 27 h, and by using stable isotope-labeled nucleotides (right panel). Although the amount of endogenous cGMP recovered from cells was not affected by different amounts of exogenous c[$^{15}N_5$]GMP, recovery of endogenous 8-nitro-cGMP improved greatly with increasing concentrations of 8-$^{15}NO_2$-cGMP exogenously spiked to the cell extract (left panel). (B) C6 cells were stimulated in the same manner as in (A). The amounts of endogenous cGMP (c[^{14}N]GMP) and 8-nitro-cGMP (8-$^{14}NO_2$-cGMP) were quantified by the stable isotope-dilution-LC–MS/MS technique [12]. Upper panel, representative LC–MS/MS chromatograms of cGMP and 8-nitro-cGMP. Lower panel, time profiles of intracellular 8-nitro-cGMP and cGMP concentrations as determined with LC–MS/MS. Data represent means ± SEM ($n = 3$). Modified from Fujii et al. [12].

adduct. We called this new PTM protein *S*-guanylation (Fig. 5). Indeed, endogenous protein *S*-guanylation occurring in cells is currently a major focus of our studies, which may stimulate a new era of NO and ROS research.

One of the most important target proteins for *S*-guanylation is Keap1, which is being increasingly recognized as a potent redox-sensing protein (Fig. 5). Keap1 is a negative regulator of Nrf2, a transcription factor regulating phase 2-detoxifying and antioxidant enzymes for electrophiles and ROSs [26,27]. Binding of Keap1 to Nrf2 maintains cytosolic localization of Nrf2 and mediates rapid degradation of Nrf2 by proteasomes (Fig. 5). Because Keap1 has highly reactive Cys residues, chemical modification of the sulfhy-

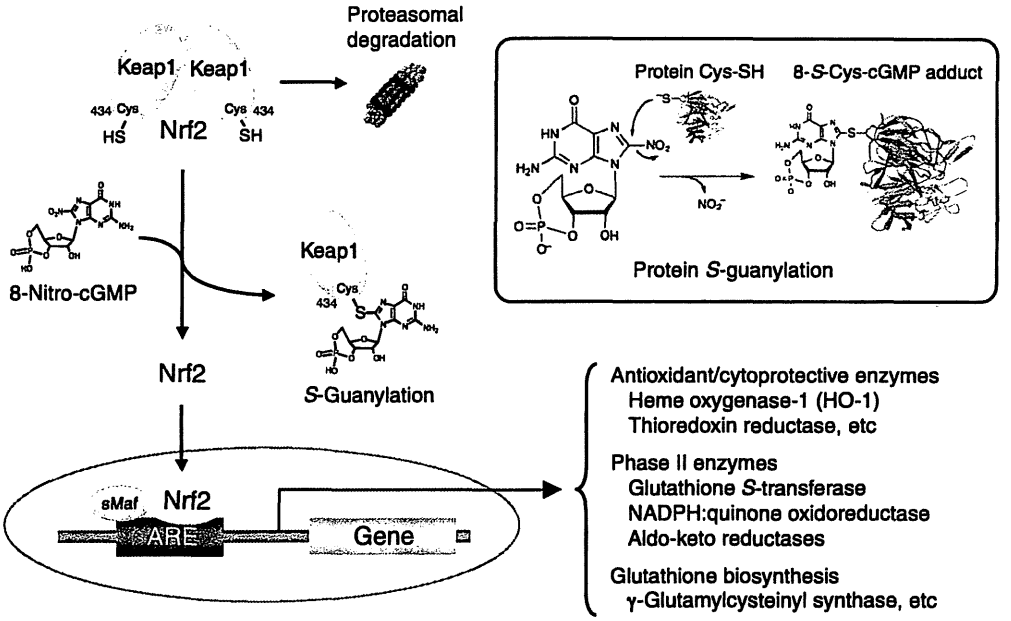

Fig. 5. A scheme illustrating the mechanism of Nrf2–Keap1 transcriptional regulation via *S*-guanylation of the Cys sulfhydryls of Keap1 and activation of subsequent antioxidant enzymes induced by ROS and electrophiles. The model of activation of Nrf2-dependent downstream antioxidant signaling pathways, resulting from Keap1 modifications caused by ROS and various electrophiles, was originally proposed by Dr. Masayuki Yamamoto's group [28,29]. The upper right panel shows a reaction scheme for protein *S*-guanylation caused by 8-nitro-cGMP. Note that the nitro moiety of 8-nitro-cGMP is lost as nitrite that is released during nucleophilic substitution of the Cys sulfhydryl of the protein by 8-nitro-cGMP. ARE, antioxidant responsive element. Modified from Sawa et al. [8] and Fujii et al. [12].

dryl group of Cys residues by electrophiles and ROSs has been proposed to trigger dissociation of Nrf2, which leads to its stabilization and nuclear translocation. Activated Nrf2 then binds to the antioxidant responsive element (ARE) to induce expression of various cytoprotective enzymes, which contribute to the adaptive response to oxidative stress [28,29].

The exact physiological function and structural characterization of Keap1 *S*-guanylation remained to be explored, however. We thus examined whether Keap1 could indeed be modified by NO and oxidative stress in cells. In one study, cultured murine macrophages were infected with a pathogenic bacterium, *Salmonella*, and Keap1 protein was analyzed for its *S*-guanylation by means of Western blotting using anti-*S*-guanylation antibody after Keap1 isolation via immunoprecipitation [10]. With wild-type macrophages after *Salmonella* infection, when 8-nitro-cGMP formation was clearly observed, strong *S*-guanylation of Keap1 occurred, as evidenced by Western blotting. Similar Keap1 *S*-guanylation was clearly illustrated with C6 cells in culture via the same immunoprecipitation technique used with Western blotting [12]. Indeed, the general proteomic profiling of *S*-guanylated proteins, which we recently clarified, indicated that Keap1 is the major target that is *S*-guanylated after NO exposure, and its *S*-guanylated structure derives solely from RS-cGMP adducts [12] (Fig. 5). Other chemical modifications, such as sulfhydryl oxidation and *S*-nitrosylation, that are often caused by NO and ROS [19,30,31] were not observed in Keap1 Cys residues in cells treated with the NO donor *S*-nitroso-*N*-acetylpenicillamine (SNAP) [12]. That Keap1 may play a predominant role in the NO signaling pathway that depends on 8-nitro-cGMP produced in cells is therefore highly plausible. The structural basis of Keap1 modification leading to Nrf2 activation is discussed in greater detail below.

Characterization of 8-nitro-cGMP electrophilicity and its biological significance

Because several electrophiles are known to form endogenously, it is important to compare their chemical properties, e.g., reactivity with sulfhydryls, reversibility of electrophilic adduction (*S*-alkylation), compartmentalization, and biological stability. In general, electrophiles react readily with various sulfhydryl-containing peptides and proteins because of their high reaction rate constants with sulfhydryls. Most highly reactive electrophiles appear to undergo reversible electrophilic sulfhydryl adduction, so the stability of such an adduction produced in cells may be greatly affected by glutathione (GSH), which is a major cellular low-molecular-weight sulfhydryl. Because of the redox-recycling potential of abundant GSH present in cells, the C–S bonds of *S*-alkylation protein adducts will be effectively dissociated, and released electrophiles are again bound covalently with GSH to form low-molecular-weight *S*-alkylation adducts (Fig. 6).

Specific intracellular factors may therefore be required for electrophiles to have notable biologically relevant effects on particular cellular proteins, enzymes, and signaling molecules. For example,

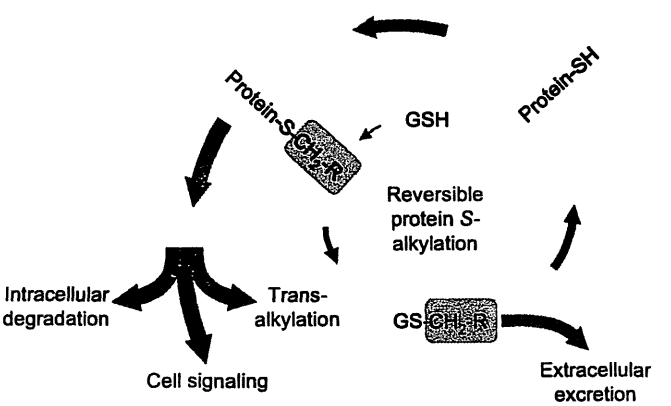

Fig. 6. A schematic illustration showing transalkylation of electrophilic residues of *S*-alkylation adducts among various Cys sulfhydryls. Transalkylation confers reversibility of protein *S*-alkylation induced by reactive endogenous electrophiles.

even reactive electrophiles readily undergoing reversible protein *S*-alkylation may participate in intracellular signaling, if it occurs in a molecular compartment in which low-molecular-weight sulfhydryl compounds such as GSH are scarce. In this situation, the electrophiles could react specifically with particular protein sulfhydryl residues that would serve as biologically functioning acceptors to form stable *S*-alkylation Cys adducts involved in cell signaling [11,32].

In contrast, irreversible *S*-alkyl adductions with electrophiles may cause permanent loss or activation of protein functions and thus be regarded as pathological rather than physiological reactions. An exceptional case of apparently irreversible protein *S*-alkylation occurs endogenously, however, for which such a simplistic explanation cannot apply. This situation is illustrated by 8-nitro-cGMP-induced protein *S*-guanylation, which is a unique, irreversible PTM of protein Cys residues [11]. The best characterized of the chemical and pharmacological features of 8-nitro-cGMP is its relative stability in cells, so that it can maintain its electrophilicity and PKG-activating potential. The electrophilicity of 8-nitro-cGMP is generally lower than that of other endogenous electrophiles, as shown by the second-order rate constant for the reaction of the sulfhydryl of GSH: 0.03 M^{-1} s^{-1} at pH 7.4, 37 °C [8]. 8-Nitro-cGMP is thought to be much less reactive compared with several other electrophiles such as α,β-unsaturated aldehydes, ω−6 and ω−3 unsaturated fatty acids, and nitroalkene fatty acids, which include, e.g., 4-hydroxynonenal, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2, and nitrolinoleic and nitrooleic acids. The reaction rate constants with GSH are 1.3 M^{-1} s^{-1} (4-hydroxynonenal), 0.7 M^{-1} s^{-1} (15-deoxy-$\Delta^{12,14}$-prostaglandin J_2), 355 M^{-1} s^{-1} (nitrolinoleic acid), and 183 M^{-1} s^{-1} (nitrooleic acid) at pH 7.4, 37 °C [11,33,34]. Therefore, these compounds seem to react with the sulfhydryls of GSH more than 20–10,000 times more rapidly than does 8-nitro-cGMP [11].

Such stability of 8-nitro-cGMP as an electrophile may support the considerable biological relevance of 8-nitro-cGMP formed in cells. As discussed above, we recently used an isotope-dilution-LC–MS/MS analysis to extensively evaluate and precisely determine the quantity of 8-nitro-cGMP formed. The fact that a large amount of 8-nitro-cGMP remained in cells may not be consistent with its electrophilic nature, because electrophilic compounds are generally thought to react readily with sulfhydryls and thus be degraded. 8-Nitro-cGMP undergoes, during its reaction with sulfhydryls, denitration to release its nitro moiety, so that it in turn loses its unique electrophilicity. However, in view of the reaction rate constants for sulfhydryls just noted, we now know that 8-nitro-cGMP, compared with other biological electrophiles, is inert in terms of electrophilicity. For example, the reaction rate constant for 8-nitro-cGMP and GSH is orders of magnitude lower than the rate constants of most fatty acid-derived electrophiles, as just mentioned. This inert electrophilic property appears to agree well with our recent finding that 8-nitro-cGMP generation was not greatly affected after GSH was eliminated by administration of buthionine sulfoximine to SNAP-treated and LPS-cytokine-treated cells [12].

Furthermore, for highly reactive electrophiles, nucleophilic amino acids other than Cys, especially histidine and lysine, also become targets during their electrophilic reactions. Because of the very high electron-withdrawing potential of these electrophiles, they undergo unstable, reversible *S*-alkylation, which eventually can be transferred to other sulfhydryls of Cys of different proteins, a process known as transalkylation (Fig. 6). This fact indicates that many endogenous electrophiles, except 8-nitro-cGMP, may not necessarily produce site-specific PTMs, which can transduce signaling (Fig. 6). These electrophiles may need, therefore, specific reaction conditions or compartmentalization in which they can serve as signaling molecules and have significant effects on sulfhydryls of acceptor proteins. For example, certain unique structural characteristics, although yet not identified, may be prerequisites for much of the stable covalent binding of electrophilic protein *S*-alkylation maintained near PTM sites.

It is also important to emphasize that 8-nitro-cGMP can produce a unique electrophilic PTM. The major distinction between *S*-guanylation and other *S*-alkylations is that *S*-guanylation is apparently a quite stable and irreversible sulfhydryl modification,

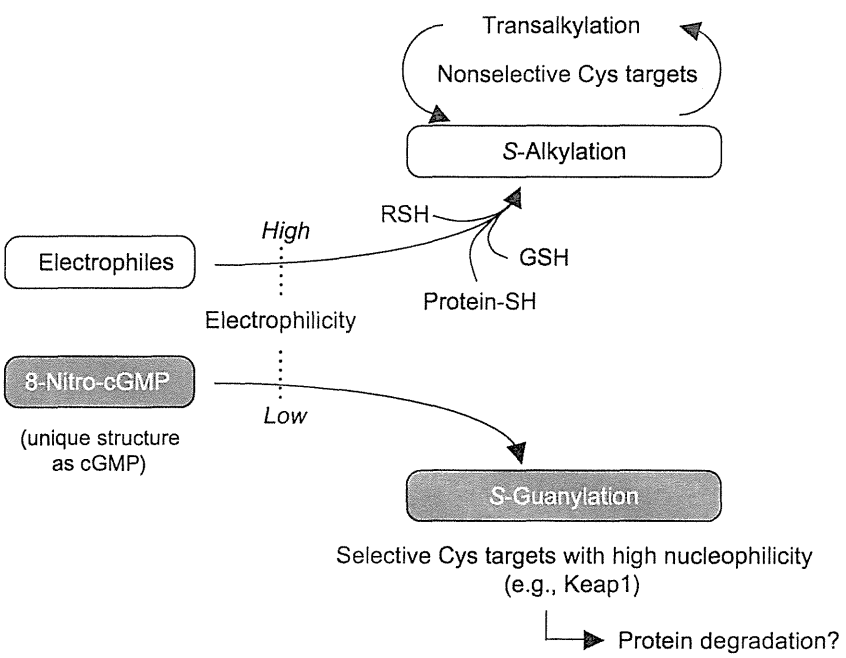

Fig. 7. A hypothetical scheme for protein *S*-guanylation as an irreversible but selective PTM contributing to precisely regulated electrophilic signaling mediated by 8-nitro-cGMP formed in cells. Although highly reactive electrophiles produce reversible protein *S*-alkylation, which readily undergoes nonselective transalkylation with protein Cys sulfhydryls, a much less reactive 8-nitro-cGMP may affect only Cys residues with high nucleophilicity. The fate of *S*-guanylated proteins remains unclear, but they may be processed via a type of proteolytic degradation in cells.

because the nucleophilic nitro moiety of its purine structure is lost during adduct formation with protein Cys residues. Another important property of 8-nitro-cGMP is that, because of its relatively inert chemical reactivity as an electrophile compared with other electrophiles as just discussed, *S*-guanylation occurs almost exclusively with sulfhydryls possessing high nucleophilicity, as indicated by the pK_a of Cys moiety sulfhydryls (Fig. 7). This finding is strongly supported by the finding that Keap1 Cys is highly susceptible to 8-nitro-cGMP-induced *S*-guanylation [8–12].

Another interesting characteristic of 8-nitro-cGMP is its possible molecular interaction with cyclic nucleotide (cGMP) receptor proteins such as PKG and other cGMP-binding proteins. The function of *S*-guanylated proteins may therefore be affected by the structural characteristics of parental cGMP. Also, of great importance is that cGMP-protein adducts acquire resistance to phosphodiesterases, which may confirm the strong, steady stimulation conferred during signal transduction via *S*-guanylation [8]. All these unique characteristics of *S*-guanylation together indicate that despite the irreversibility of protein *S*-guanylation, which is quite different from the situation for most electrophilic PTMs among various sulfhydryls, 8-nitro-cGMP may serve as an effective electrophilic regulator of intracellular signaling by virtue of its elegant chemistry via ingenious electrophilic reactions with protein sulfhydryls and *S*-guanylation structures thereby generated in cells.

Signal transduction via protein *S*-guanylation

Involvement of *S*-guanylation in signal transduction caused by NO and ROS was clearly verified in terms of Nrf2-related gene expression, which led to activation of downstream enzymes including heme oxygenase-1 (HO-1), a well-known antioxidant and cytoprotective enzyme [35]. As discussed above, we determined that the redox-sensor protein Keap1 is a major target of *S*-guanylation. Keap1 is now widely accepted as a negative regulator of Nrf2, as described earlier. Activated Nrf2 then binds to the ARE to induce expression of various cytoprotective enzymes, thereby contributing to the phase 2-detoxifying and adaptive response to toxic electrophiles and ROS-induced oxidative stress [26,27].

In this context, considerable evidence from our recent work shows that Keap1 *S*-guanylation and subsequent Nrf2 activation are mediated by 8-nitro-cGMP formed via NO derived from NO donors or generated by iNOS [8–12]. It is intriguing that proteomic analysis for endogenously modified Keap1 with matrix-assisted laser desorption/ionization time-of-flight-MS/MS analysis revealed that 8-nitro-cGMP *S*-guanylated the Cys434 residue of Keap1 in a site-specific manner [12]. In recombinant Keap1 reacted with 8-nitro-cGMP in an *in vitro* cell-free system, however, 18 of 25 total cysteine residues were *S*-guanylated, so that Cys434 *S*-guanylation was not dominant. Therefore, Cys434 of Keap1 may have structural or chemical properties that are related to its specific *S*-guanylation and that are distinct from those of other cysteine residues of Keap1. A number of studies have examined Keap1-electrophile adducts generated *in vitro* in the presence of high concentrations of electrophilic reagents [28,30,36–39]. Yamamoto's group reported the *in vivo* significance of Cys151, Cys273, and Cys288 for Keap1 functions in a transgenic complementation rescue experiment in mice [40]. Although verification of the *in vivo* requirement for Cys434 is yet to come, a recent study demonstrated that Cys434 in Keap1 is one of the cysteine residues that are most sensitive to *S*-glutathionylation and that disulfide adduct formation of Cys434 with GSH causes marked structural changes in the Nrf2-binding surface of the Keap1 molecule [41]. Thus, the specific *S*-guanylation of Cys434 by 8-nitro-cGMP may play an important role in a molecular sensing system operated by Keap1 for RNOS and/or ROS [12].

Yamamoto's group also determined that the Keap1–Nrf2 complex consists of one molecule of Nrf2 and two molecules of Keap1, possibly as a result of high- and low-affinity binding between Keap1 and Nrf2 [42–45]. The BTB domains at the N-terminal regions of two molecules of Keap1 bind each other directly to maintain a homodimer, and the DC domains of Keap1 located at the C-terminal region form a spherical shape at both ends of the whole dimer complex by virtue of their tertiary structure [45]. A co-crystallization study showed that two motifs of Nrf2, i.e., DLG and ETGE, interact with the β-propeller structure at the bottom of the sphere of the DC domain [43,44]. Structural alterations in the integrity of the Keap1–Nrf2 complex may disrupt the low-affinity binding between the ETGE/DLG motifs and the DC domains, which

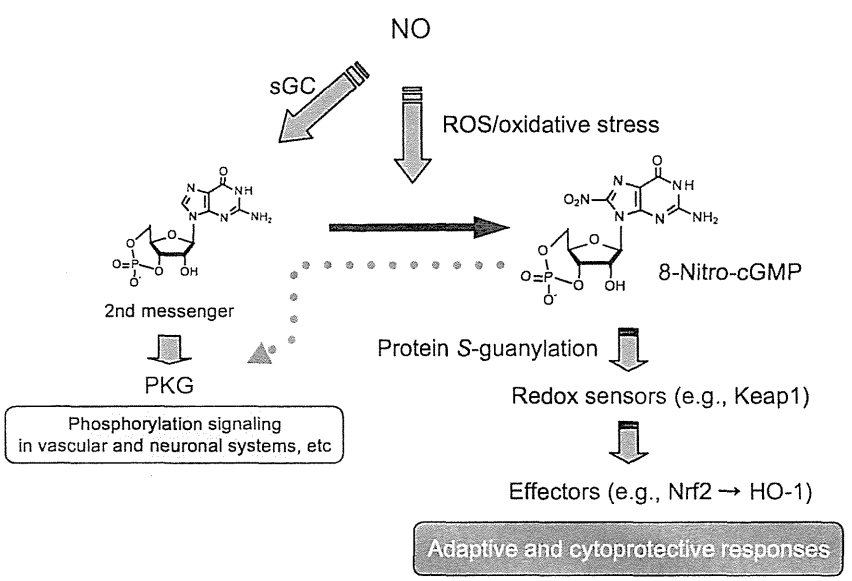

Fig. 8. Overview of unique cell signaling regulated by a nitrated cyclic nucleotide (8-nitro-cGMP) generated through a classical NO-soluble guanylate cyclase (sGC) pathway coupled with a nitration reaction dependent on NO or ROS (possibly RNOS). Various nucleotides, which are abundant in cells, may serve as redox (ROS/NO) sensors and evoke many downstream signaling pathways.

would then distort or dissociate this tertiary Keap1–Nrf2 complex, stabilize Nrf2, and trigger translocation of Nrf2 to nuclei for transcriptional activation. X-ray crystallographic analysis showed that Cys434 is located at blade 3 and is exposed to the outer surface of the β-propeller structure of the Keap1 DC domain [44,46]. *S*-guanylation of Keap1 Cys434 may therefore cause Nrf2 activation, via two possible mechanisms: *S*-guanylation of Cys434 may weaken Keap1 binding to the ETGE and DLG motifs of Nrf2, because Cys434 lies close to the Nrf2-binding region of the DC domain; or the Cys434 modification may affect the integrity of the entire Keap1–Nrf2 complex.

In fact, 8-nitro-cGMP can mediate the cytoprotective response through *S*-guanylation of Keap1, as evidenced by our finding that treatment of C6 cells with 8-nitro-cGMP reduced cell death induced by oxidative stress related to H_2O_2 exposure [12]. We also found that 8-nitro-cGMP increased the nuclear accumulation of Nrf2 and expression of HO-1 in C6 cells [12]. NO-induced expression of HO-1 reportedly contributes to cell survival in solid tumor models and during bacterial infection [10,47–49]. Therefore, the cytoprotection against oxidative stress that 8-nitro-cGMP confers is associated, at least in part, with increased HO-1 expression. That 8-nitro-cGMP participates in the major NO signaling pathway for cytoprotection or adaptive responses to ROS and oxidative stress is thus conceivable (Fig. 8).

Concluding remarks

In summary, we have clarified the NO-dependent formation of 8-nitro-cGMP and simultaneous *S*-guanylation of Keap1 induced by 8-nitro-cGMP in cells in culture. This NO- and 8-nitro-cGMP-mediated signaling pathway leads to Nrf2 activation and cytoprotective gene expression, including functioning of HO-1, which seems to be involved in the general adaptive response to oxidative stress. Most important, chemical identification of specific Keap1 *S*-guanylation unequivocally indicated a remarkable structural alteration of Keap1, which can maintain activation of Nrf2 for its ARE transcriptional regulation. These findings therefore suggest that protein *S*-guanylation induced by 8-nitro-cGMP is a unique pathway for modulating various cellular functions mediated by NO and ROS signaling. Present evidence of protein *S*-guanylation induced by 8-nitro-cGMP may thus warrant further extensive study that would reveal new aspects of NO-related physiology and pathology, pharmaceutical chemistry, and development of therapeutics for diseases.

Acknowledgments

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

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8-Nitro-cGMP (8-nitroguanosine 3′,5′-cyclic monophosphate) is a nitrated derivative of cGMP, which can function as a unique electrophilic second messenger involved in regulation of an antioxidant adaptive response in cells. In the present study, we investigated chemical and biochemical regulatory mechanisms involved in 8-nitro-cGMP formation, with particular focus on the roles of ROS (reactive oxygen species). Chemical analyses demonstrated that peroxynitrite-dependent oxidation and myeloperoxidase-dependent oxidation of nitrite in the presence of H_2O_2 were two major pathways for guanine nucleotide nitration. Among the guanine nucleotides examined, GTP was the most sensitive to peroxynitrite-mediated nitration. Immunocytochemical and tandem mass spectrometric analyses revealed that formation of 8-nitro-cGMP in rat C6 glioma cells stimulated with lipopolysaccharide plus pro-inflammatory cytokines depended on production of both superoxide and H_2O_2. Using the mitochondria-targeted chemical probe MitoSOX™ Red, we found that mitochondria-derived superoxide can act as a direct determinant of 8-nitro-cGMP formation. Furthermore, we demonstrated that Nox2 (NADPH oxidase 2)-generated H_2O_2 regulated mitochondria-derived superoxide production, which suggests the importance of cross-talk between Nox2-dependent H_2O_2 production and mitochondrial superoxide production. The results of the present study suggest that 8-nitro-cGMP can serve as a unique second messenger that may be implicated in regulating ROS signalling in the presence of NO.

Key words: mitochondrion, nitric oxide, oxidative stress, peroxynitrite, reactive oxygen species (ROS), reactive oxygen species (ROS) signalling.

INTRODUCTION

cGMP is a cyclic nucleotide formed from GTP by the catalytic action of the enzymes called guanylyl cyclases [1,2]. In vertebrates, two guanylyl cyclase isoforms have been identified, membrane-bound pGC (particulate-type guanylyl cyclase) and sGC (soluble-type guanylyl cyclase), that are expressed in almost all cell types [2]. These enzymes are activated in response to specific signals, such as NO for sGC and peptide ligands for pGC, to produce cGMP. Subsequently, cGMP functions as a second messenger for these signals and regulates a wide variety of cell physiological functions such as vascular smooth muscle motility, host defence, intestinal fluid and electrolyte homoeostasis, and retinal phototransduction [2]. Such biological actions of cGMP may be primarily mediated by activation of downstream effector molecules such as cGMP-dependent protein kinase, ion channels and phosphodiesterases [2].

A nitrated derivative of cGMP, 8-nitro-cGMP (8-nitroguanosine 3′,5′-cyclic monophosphate), has been identified in mammalian cells [3–6]. 8-Nitro-cGMP possesses unique biochemical properties, e.g. it behaves as an electrophile and reacts with protein sulfhydryls, which results in cGMP adduction to protein sulfhydryls [3,5,7,8]. This post-translational modification by 8-nitro-cGMP via cGMP adduction is named protein S-guanylation [3,5,7,8]. Furthermore, 8-nitro-cGMP can induce an antioxidant adaptive response in cells via S-guanylation of the redox sensor protein Keap1 (Kelch-like ECH-associated protein 1), which results in transcriptional activation of Nrf2 with concomitant expression of a battery of genes that encode an array of phase II detoxifying or antioxidant enzymes, as well as other cytoprotective proteins [5]. Thus 8-nitro-cGMP may function as a potent electrophilic second messenger involved in regulation of redox signalling [7–9].

To explore how and when 8-nitro-cGMP is involved in regulating cell physiology via its unique electrophilic properties, 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 (reactive nitrogen oxide species), formed from the reaction of NO and ROS (reactive oxygen species), can nitrate guanine derivatives under biologically relevant conditions [10,11]. An example of RNOS include peroxynitrite ($ONOO^-$), which is a potent oxidizing and nitrating agent formed from the reaction of NO and superoxide (O_2^-). In the present study, we investigated the role 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 H_2O_2 and MPO (myeloperoxidase) may

Abbreviations used: DCDHF-DA, 2′,7′-dichlorodihydrofluorescein diacetate; DHE, dihydroethidium; DTPA, diethylenetriamine penta-acetic acid; ECD, electrochemical detection; ESI, electrospray ionization; HRP, horseradish peroxidase; IFN-γ, interferon-γ; IL-1β, interleukin-1β; Keap1, Kelch-like ECH-associated protein 1; LC, liquid chromatography; LPS, lipopolysaccharide; mETC, mitochondrial electron transport chain; MPO, myeloperoxidase; MS/MS, tandem MS; 8-nitro-cGMP, 8-nitroguanosine 3′,5′-cyclic monophosphate; Nox2, NADPH oxidase 2; PDA, photodiode array; PEG, poly(ethylene glycol); pGC, particulate-type guanylyl cyclase; PI3K, phosphoinositide 3-kinase; 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; sGC, soluble-type guanylyl cyclase; SIN-1, 3-morpholinosydnonimine; siRNA, small interfering RNA; SOD, superoxide dismutase; TNF-α, tumour necrosis factor-α.

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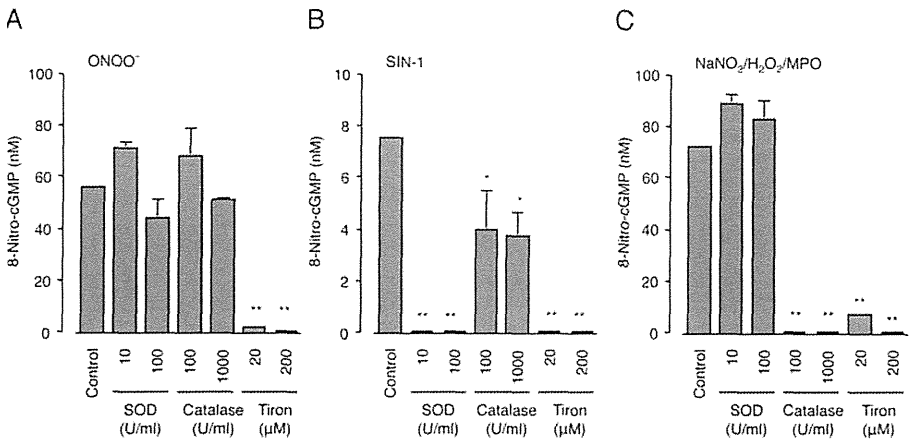

Figure 2 Effect of ROS scavengers on the formation of 8-nitro-cGMP induced by various RNOS systems

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 units/ml), catalase (100 and 1000 units/ml) or tiron (20 and 200 μM). Data are expressed as means $\pm$ S.E.M. ($n = 3$). $^*P < 0.05$ and $^{**}P < 0.01$ compared with the control.

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

ND, not detected.

Conditions	Product formed (nM)	
	8-Nitro-cGMP	3-Nitrotyrosine
NONOate (100 μM), 4h†	ND	16.7 $\pm$ 1.1*
$NaNO_2$ (100 μM), at pH 3.0, 1 h‡	ND	95.8 $\pm$ 1.8*
$NaNO_2$ (100 μM), at pH 4.0, 1 h‡	ND	30.3 $\pm$ 3.0*
SIN-1 (50 μM), 2 h§	7.7 $\pm$ 0.3*	267.3 $\pm$ 5.6*
SIN-1 (100 μM), 2 h§	14.5 $\pm$ 0.7*	402.9 $\pm$ 8.3*
$NaNO_2$ (100 μM), H_2O_2 (100 μM), MPO (10 nM), 4 h‖	67.3 $\pm$ 1.5*	9307.0 $\pm$ 11.1*
$NaNO_2$ (100 μM), MPO (10 nM), 4 h‖	ND	ND
H_2O_2 (100 μM), MPO (10 nM), 4 h‖	ND	ND
$NaNO_2$ (100 μM), H_2O_2 (100 μM), 4 h‖	ND	ND
$NaNO_2$ (100 μM), H_2O_2 (100 μM), HRP (23.8 μM), 4 h‖	ND	803.5 $\pm$ 1.6*
HOCl (100 μM), $NaNO_2$ (100 μM), 4 h¶	ND	48.3 $\pm$ 1.9*

*$P < 0.01$, compared with the control (no RNOS treatment).
†In 0.1 M sodium phosphate buffer (pH 7.4) and 0.1 mM DTPA at 37 °C.
‡In 0.1 M sodium citrate buffer (pH 2.5–4.5) and 100 μM $NaNO_2$ at 37 °C.
§In 0.1 M sodium phosphate buffer (pH 7.4), 0.1 mM DTPA and 25 mM $NaHCO_3$ at 37 °C.
‖In 0.1 M sodium phosphate buffer (pH 7.4) at 37 °C.
¶In 0.1 M citric acid buffer (pH 4.5) at 37 °C.

effectively suppressed cGMP nitration mediated by all RNOS systems examined. Similarly, tiron effectively suppressed tyrosine nitration caused by authentic $ONOO^-$ (Supplementary Figure S1 at http://www.BiochemJ.org/bj/441/bj4410719add.htm). It was also found that tiron effectively suppressed tyrosine nitration by P-NONOate, suggesting that tiron can inhibit nitration by either $ONOO^-$ or aerobic NO (NO_2), possibly via scavenging NO_2, independent of its superoxide scavenging activity.

Formation of 8-nitro-cGMP in rat C6 glioma cells: involvement of cellular ROS production

Our chemical analyses clearly demonstrated that NO itself is not sufficient to cause nitration of guanine nucleotides, but requires ROS, including superoxide and H_2O_2, for that reaction to occur. To study the roles of ROS in 8-nitro-cGMP formation in cells, we used C6 cells in culture as a model system.

Immunocytochemical analyses provided the baseline formation of 8-nitro-cGMP in non-stimulated C6 cells (Figure 3A). Formation of 8-nitro-cGMP was markedly enhanced in C6 cells when cells were stimulated with LPS/cytokines (Figure 3A). Treatment with PEG–SOD, which is reportedly a membrane-permeant SOD derivative [12], reduced the immunostaining in a manner dependent on PEG–SOD concentration (Figure 3B). This result suggests the essential role of superoxide for cell formation of 8-nitro-cGMP in stimulated C6 cells. Similar to PEG–SOD, PEG–catalase, a membrane-permeant catalase derivative, suppressed 8-nitro-cGMP formation in C6 cells stimulated with LPS/cytokines (Figure 3C). LC-ESI-MS/MS analyses verified 8-nitro-cGMP formation in C6 cells and its modulation by PEG–SOD and PEG–catalase. In agreement with immunocytochemical data, these analyses detected a certain level of 8-nitro-cGMP in non-stimulated C6 cells (Figure 4). As Figure 4 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 approximately 5.6-fold higher than that of cGMP under the experimental conditions used in the present study. 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 was almost completely nullified by treatment with both PEG–SOD and PEG–catalase, a finding that agrees with results obtained by immunocytochemistry. Thus these data suggest that formation of 8-nitro-cGMP depends greatly on cell production of both superoxide and H_2O_2. Under these condition, PEG–SOD and PEG–catalase treatments did not affect NO production in C6 cells (Supplementary Figure S2 at http://www.BiochemJ.org/bj/441/bj4410719add.htm).

Cellular production of ROS and related oxidants was analysed by using chemical probes that become fluorescent in response to ROS and oxidants. With MitoSOX™ Red, a mitochondria-targeted superoxide-sensitive fluorigenic probe [21], we found that stimulation of C6 cells with LPS/cytokines significantly induced production of mitochondrial superoxide (Figure 5A). A time-course study showed that mitochondrial superoxide production gradually increased and reached a plateau at 24 h after LPS/cytokine stimulation (Supplementary Figure S3 at http://www.BiochemJ.org/bj/441/bj4410719add.htm). On

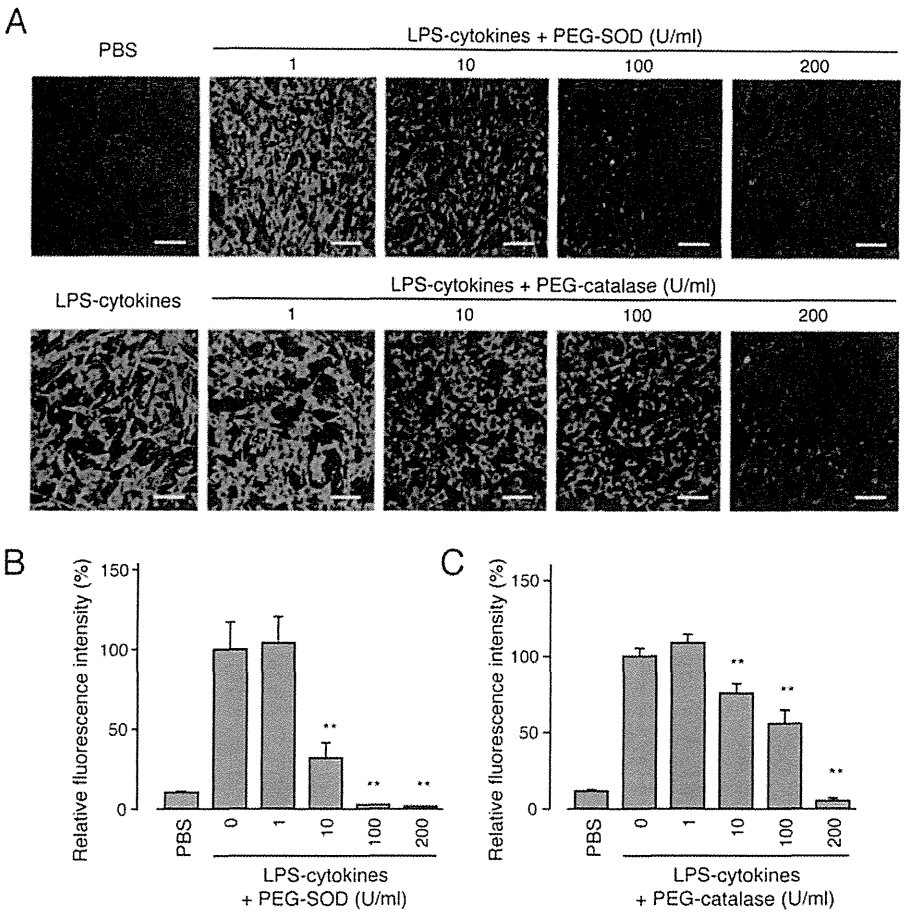

Figure 3 Immunocytochemical analysis of 8-nitro-cGMP formation in rat C6 glioma cells and modulation of its formation by ROS scavengers

Cells were stimulated with a mixture of LPS (10 μg/ml), IFN-γ (200 units/ml), TNF-α (500 units/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 in the Materials and methods section, followed by immunocytochemical detection of intracellular 8-nitro-cGMP with the use of the 1G6 monoclonal antibody against 8-nitro-cGMP. (**A**) Cells were treated with different concentrations of PEG–SOD (top panels) or PEG–catalase (bottom panels), 1 h before the addition of LPS/cytokines and during stimulation with LPS/cytokines for 36 h, and the immunocytochemical detection of 8-nitro-cGMP was then performed. Scale bars = 50 μm. (**B**) Concentration-dependent decrease in relative fluorescence intensity of 8-nitro-cGMP in C6 cells after the addition of the cell-permeant superoxide scavenger PEG–SOD (1–200 units/ml) during stimulation with LPS/cytokines. (**C**) Concentration-dependent decrease in relative fluorescence intensity of 8-nitro-cGMP in C6 cells after the addition of the cell-permeant H_2O_2 scavenger PEG–catalase (1–200 units/ml) during stimulation with LPS/cytokines. Data are expressed as means ± S.E.M. ($n = 3$). $^{**}P < 0.01$, compared with the LPS/cytokine-treated group.

the basis of this result, analyses of ROS production were carried out at 36 h after stimulation. We also used double staining of cells with MitoTracker® Green and MitoSOX™ Red to investigate whether the MitoSOX™ Red signal was derived from mitochondria. As shown in Supplementary Figure S4 (at http://www.BiochemJ.org/bj/441/bj4410719add.htm), MitoSOX™ Red staining co-localized well with MitoTracker® Green staining, which suggests that the superoxide detected by MitoSOX™ Red was primarily of mitochondrial origin.

Treatment with PEG–SOD significantly reduced the fluorescence intensity originating with MitoSOX™ Red (Figure 5B). PEG–catalase also suppressed production of superoxide, as shown by reduced MitoSOX™ Red-derived fluorescence. These results suggest that mitochondrial superoxide production may be regulated by H_2O_2 production. We then studied production of H_2O_2 using DCDHF-DA, a cell-permeant and oxidation-sensitive fluorescent probe. Microscopic observation of DCDHF-DA-derived fluorescence clearly showed oxidant production in C6 cells after stimulation with LPS/cytokines (Figure 5A). A significant inhibitory effect of PEG–catalase (Figure 5B) suggests that H_2O_2 is produced in stimulated C6 cells and acts as a major oxidant involved in induction of DCDHF-derived fluorescence. The specificity of superoxide detection was confirmed using DHE, and the results were consistent with those obtained by MitoSOX™ Red analysis (Figure 5).

As mentioned above, tiron can act not only as a superoxide scavenger, but also as an antioxidant to inhibit guanine nucleotide nitration caused by $ONOO^-$ and $NaNO_2/H_2O_2$/MPO (Figure 2). Immunocytochemical analyses revealed that tiron effectively suppressed formation of 8-nitro-cGMP in C6 cells stimulated with LPS/cytokines (Figure 6). Similarly, tiron treatment significantly reduced fluorescence derived from both MitoSOX™ Red and DCDHF-DA (Figure 6). Under these conditions, tiron did not cause any detectable cytotoxic effects (Supplementary Figure S5 at http://www.BiochemJ.org/bj/441/bj4410719add.htm).

mETC (mitochondrial electron transport chain) complexes, particularly complexes I and III, are the main source of ROS produced from mitochondria [25]. The mETC inhibitor rotenone reportedly accelerates or inhibits mitochondrial ROS production, depending on the cell type studied [26,27]. We thus studied whether modulation of mitochondrial ROS production with the mETC complex inhibitor rotenone affected formation of 8-nitro-cGMP in C6 cells. As shown in Figure 7, rotenone treatment increased 8-nitro-cGMP formation in C6 cells stimulated with

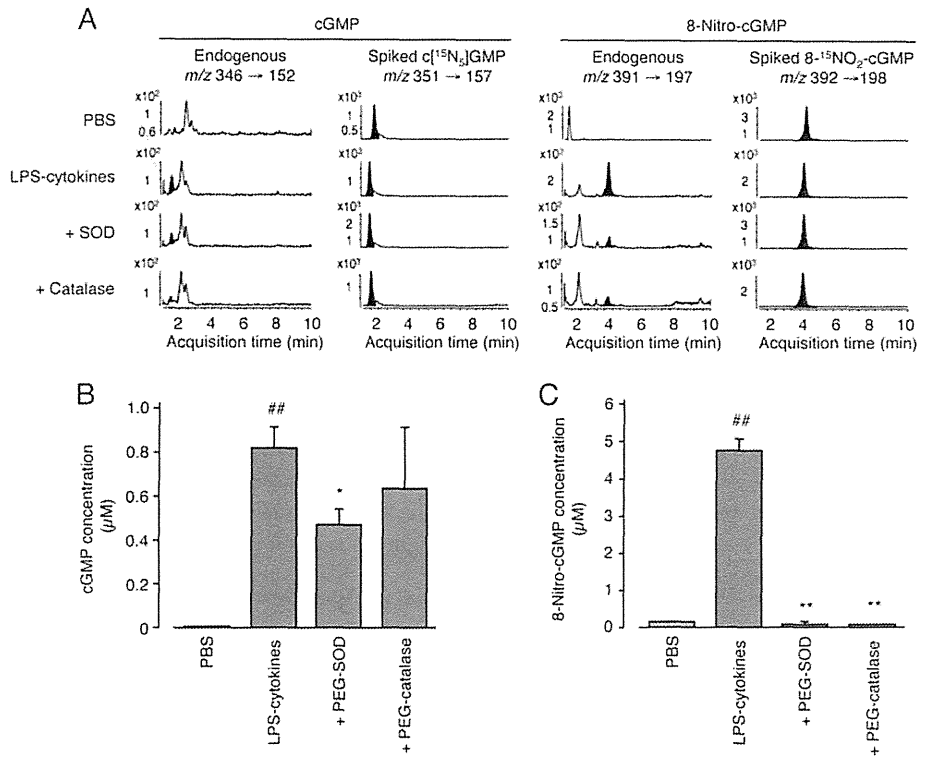

Figure 4 LC-ESI-MS/MS analysis of 8-nitro-cGMP formation in rat C6 glioma cells and modulation of its formation by ROS scavengers

Cells were stimulated with a mixture of LPS (10 μg/ml), IFN-γ (200 units/ml), TNF-α (500 units/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 in the Materials and methods section, followed by LC-ESI-MS/MS quantification of cGMP and 8-nitro-cGMP formed in cells. (**A**) LC-ESI-MS/MS chromatograms of cGMP and 8-nitro-cGMP in untreated cells and cells treated with 200 units/ml PEG–SOD or 200 units/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 units/ml) determined with LC-ESI-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 units/ml) determined with LC-ESI-MS/MS. Data are expressed as means $\pm$ S.E.M. ($n = 3$). $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the LPS/cytokine-treated group. $\#\#P < 0.01$, compared with the PBS-treated group.

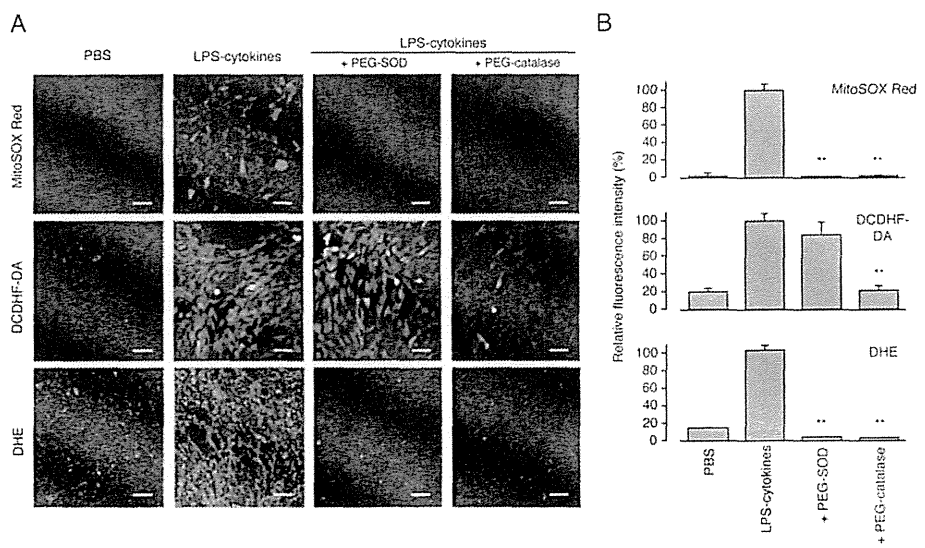

Figure 5 Fluorescence microscopic determination of ROS production in rat C6 glioma cells stimulated with LPS/cytokines

Cells were stimulated with a mixture of LPS (10 μg/ml), IFN-γ (200 units/ml), TNF-α (500 units/ml) and IL-1β (10 ng/ml) for 36 h in the absence or presence of PEG–SOD or PEG–catalase. Cells were then analysed for the presence of mitochondrial superoxide and cellular H_2O_2, as described in the Materials and methods section. (**A**) MitoSOX^{TM} Red (top panels), DCDHF-DA (middle panels) and DHE (bottom panels) staining of untreated cells and cells treated with 200 units/ml PEG–SOD or 200 units/ml PEG–catalase from 1 h before the addition of LPS/cytokine and during stimulation with LPS/cytokine for 36 h, as detected by the Nikon EZ-C1 confocal laser microscope (for MitoSOX^{TM} Red: excitation at 420 nm and red photomultiplier channel; for DCDHF-DA: excitation at 488 nm and green photomultiplier channel; and for DHE: excitation at 543 nm and red photomultiplier channel). Scale bars = 50 μm. (**B**) Relative fluorescence intensity for MitoSOX^{TM} Red staining (top panel), DCDHF-DA staining (middle panel) and DHE staining (bottom panel) of untreated C6 cells or cells treated with 200 units/ml PEG–SOD or 200 units/ml PEG–catalase from 1 h before the addition of LPS/cytokine and during stimulation with LPS/cytokine for 36 h. Data are expressed as means $\pm$ S.E.M. ($n = 3$). $^{**}P < 0.01$, compared with the LPS/cytokine-treated group.

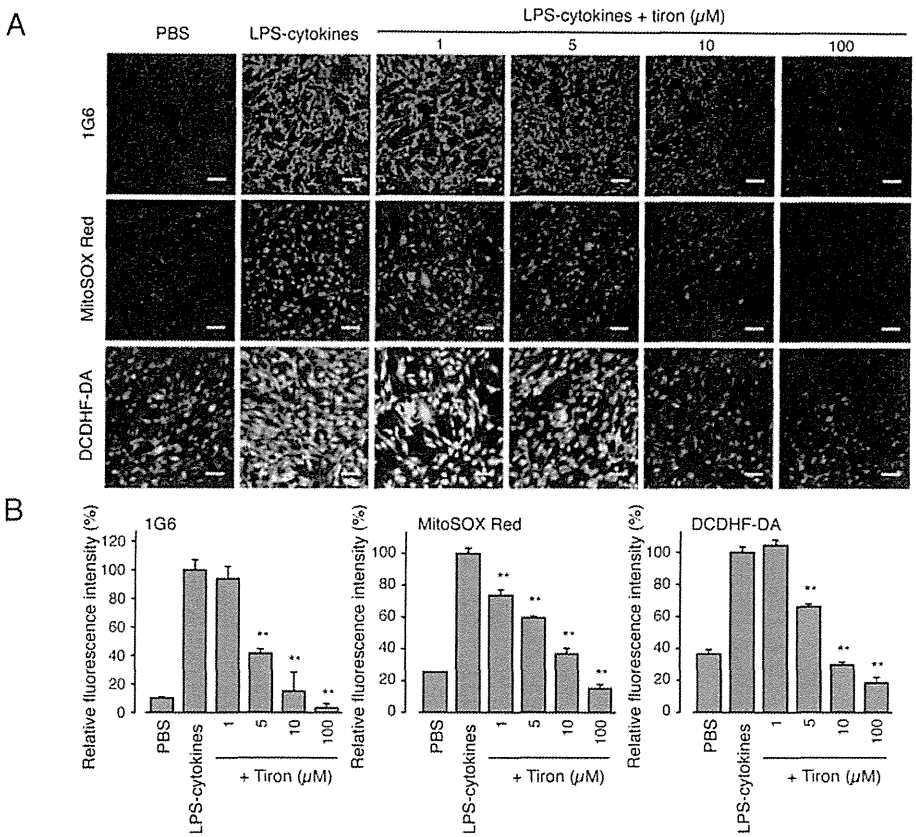

Figure 6 Effect of the SOD mimic tiron on formation of 8-nitro-cGMP and ROS production in rat C6 glioma cells

Cells were stimulated with a mixture of LPS (10 μg/ml), IFN-γ (200 units/ml), TNF-α (500 units/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 a 1G6 monoclonal antibody against 8-nitro-cGMP, or by direct staining for mitochondrial superoxide or cellular H_2O_2 as described in the Materials and methods section. Cells were untreated or treated with 1–100 μM tiron from 1 h before the addition of LPS/cytokine and during stimulation with LPS/cytokines for 36 h, followed by immunocytochemical detection of 8-nitro-cGMP (**A**, top 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**, bottom panels), via a Nikon EZ-C1 confocal laser microscope (excitation, 488 nm; green photomultiplier channel). Scale bars = 50 μm. (**B**) Relative fluorescence intensity of C6 cells, treated as described above, for 8-nitro-cGMP immunocytochemical staining (left-hand panel), MitoSOX™ Red staining (middle panel) and DCDHF-DA staining (right-hand panel). Data are expressed as means ± S.E.M. ($n = 3$). $^{**}P < 0.01$, compared with the LPS/cytokine-treated group.

LPS/cytokines, as shown by immunocytochemistry and LC-ESI-MS/MS results: both methods revealed a similar increase, by approximately 1.5-fold. Under the same experimental conditions, rotenone treatment significantly increased MitoSOX™ Red-derived fluorescence intensity without affecting DCDHF-DA-derived fluorescence (Figure 7B). 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 superoxide production. However, treatment of non-stimulated cells with rotenone alone did not significantly affect the production of 8-nitro-cGMP, and, in fact, an increased MitoSOX™ Red signal was observed (Figure 7). This result suggests that 8-nitro-cGMP production requires simultaneous production of both NO and ROS.

Nox2 is a member of the NADPH oxidase family and is an important source of ROS, particularly in immunologically stimulated cells [28]. To clarify the implications of Nox2-dependent ROS production for 8-nitro-cGMP formation, we performed knockdown of $p47^{phox}$, a critical component of Nox, by using $p47^{phox}$ siRNA. The efficacy of the $p47^{phox}$ knockdown was confirmed by Western blotting (Figure 8). In C6 cells treated with $p47^{phox}$ siRNA, immunocytochemistry revealed marked suppression of 8-nitro-cGMP formation (Figure 8). The $p47^{phox}$ knockdown also significantly suppressed production of mitochondrial superoxide and cellular oxidant as determined by fluorescence microscopy (Figure 8). As shown in Supplementary Figure S6 (at http://www.BiochemJ.org/bj/441/bj4410719add.htm), superoxide and H_2O_2 production were remarkably augmented by LPS/cytokine stimulation and suppressed by $p47^{phox}$ siRNA treatment. Levels of H_2O_2 were approximately 5-fold lower than those of superoxide. This may be due, at least in part, to the different methods used. Superoxide was captured by cytochrome *c in situ* when generated extracellularly. On the other hand, concentrations of H_2O_2 were determined after 10 min accumulation in culture supernatant. During a 10 min incubation, some of the H_2O_2 produced extracellularly would be consumed by cells so that values determined for H_2O_2 accumulation reflect the differences between the production and consumption.

We also performed investigations to see whether mitochondria could produce ROS under conditions of $p47^{phox}$ knockdown. As seen in Supplementary Figure S7 (at http://www.BiochemJ.org/bj/441/bj4410719add.htm), we found that rotenone treatment along with LPS/cytokines in Nox2-knockdown cells increased mitochondrial superoxide production, independent of the Nox2-generated ROS. This finding suggests that even though the mitochondrial respiratory chain is intact in Nox2-knockdown cells, its superoxide production is impaired under the stimulation conditions used in the present study. Taken together, these results suggest that Nox2 contributes to the formation of 8-nitro-cGMP

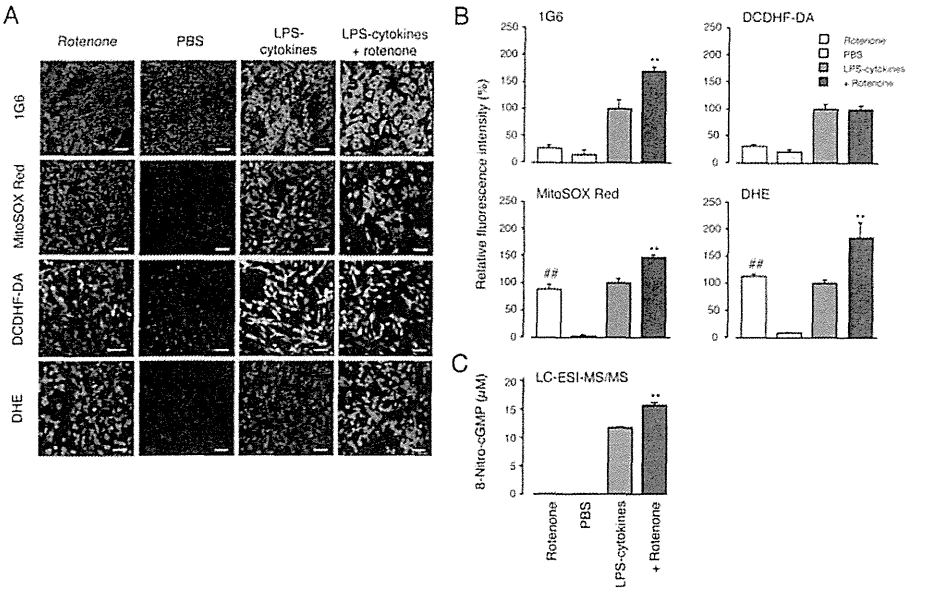

Figure 7 Modulation of 8-nitro-cGMP formation by rotenone in rat C6 glioma cells

Cells were stimulated with a mixture of LPS (10 μg/ml), IFN-γ (200 units/ml), TNF-α (500 units/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 in the Materials and methods section, followed by immunocytochemical detection of intracellular 8-nitro-cGMP with the use of the 1G6 monoclonal antibody against 8-nitro-cGMP, or by direct staining for mitochondrial superoxide or cellular H_2O_2. Cells were treated with 10 μM rotenone for 15 min before the addition of LPS/cytokine (or were untreated), during stimulation with LPS/cytokines for 36 h, followed by immunocytochemical detection via a Nikon EZ-C1 confocal laser microscope of 8-nitro-cGMP (**A**, top panels); MitoSOX™ Red staining (excitation, 420 nm; red photomultiplier channel) (**A**, upper middle panels); DCDHF-DA staining (excitation, 488 nm; green photomultiplier channel) (**A**, lower middle panels) and DHE staining (excitation, 543 nm; red photomultiplier channel) (**A**, bottom panels). Scale bars = 50 μm. (**B**) Relative fluorescence intensity of C6 cells, treated as described above, for 8-nitro-cGMP immunocytochemical staining (top left-hand panel); MitoSOX™ Red staining (bottom left-hand panel); DCDHF-DA staining (top right-hand panel); and DHE staining (bottom right-hand panel). (**C**) Intracellular 8-nitro-cGMP concentrations in cells after stimulation with LPS/cytokines with or without 10 μM rotenone pretreatment, as determined with LC-ESI-MS/MS. Data are expressed as means ± S.E.M. ($n = 3$). $^{**}P < 0.01$, compared with the LPS/cytokine-treated group. $^{\#\#}P < 0.01$ compared with the PBS-treated group.

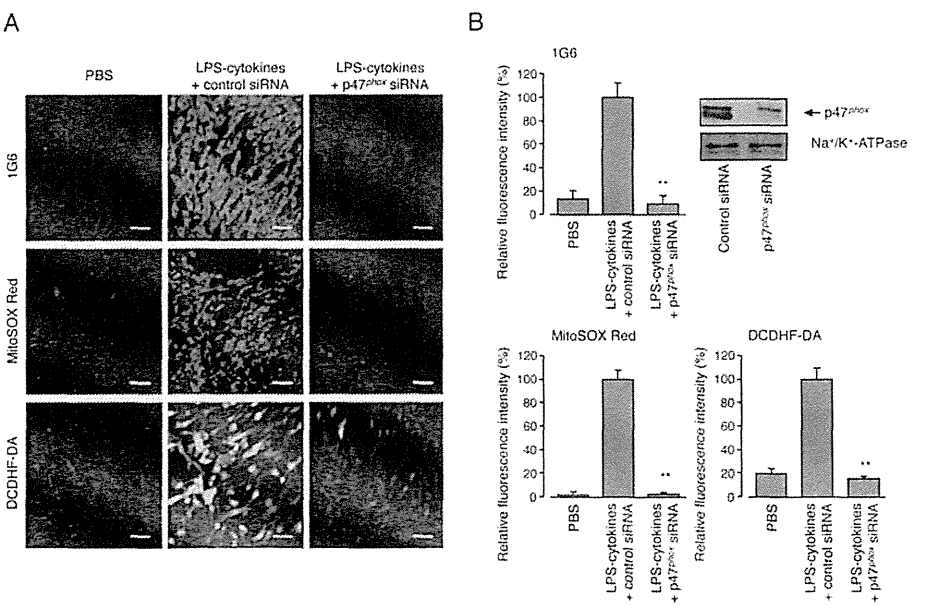

Figure 8 Effects of Nox2 gene knockdown on formation of 8-nitro-cGMP and ROS production in rat C6 glioma cells

Cells were transfected with control siRNA or p47^{phox}-specific siRNA, as described in the Materials and methods section, followed by stimulation with a mixture of LPS (10 μg/ml), IFN-γ (200 units/ml), TNF-α (500 units/ml) and IL-1β (10 ng/ml) for 36 h. Immunocytochemistry with the 1G6 monoclonal antibody against 8-nitro-cGMP was used to detect intracellular 8-nitro-cGMP, or direct staining was used for mitochondrial superoxide or cellular H_2O_2. (**A**) Immunocytochemistry for 8-nitro-cGMP (1G6; top panels); fluorescent staining of mitochondrial superoxide (MitoSOX™ Red; middle panels); and fluorescent staining of intracellular H_2O_2 (DCDHF-DA; bottom panels). Scale bars = 50 μm. (**B**) Relative fluorescence intensity of C6 cells, treated as described above, for 8-nitro-cGMP immunocytochemical staining (top left-hand panel), MitoSOX™ Red staining (bottom left-hand panel) and DCDHF-DA staining (bottom right-hand panel). The top right-hand panel shows the Western blot analysis for the p47^{phox} knockdown. Results are expressed as means ± S.E.M. ($n = 3$). $^{**}P < 0.01$, compared with the LPS/cytokine plus negative control siRNA-treated group.

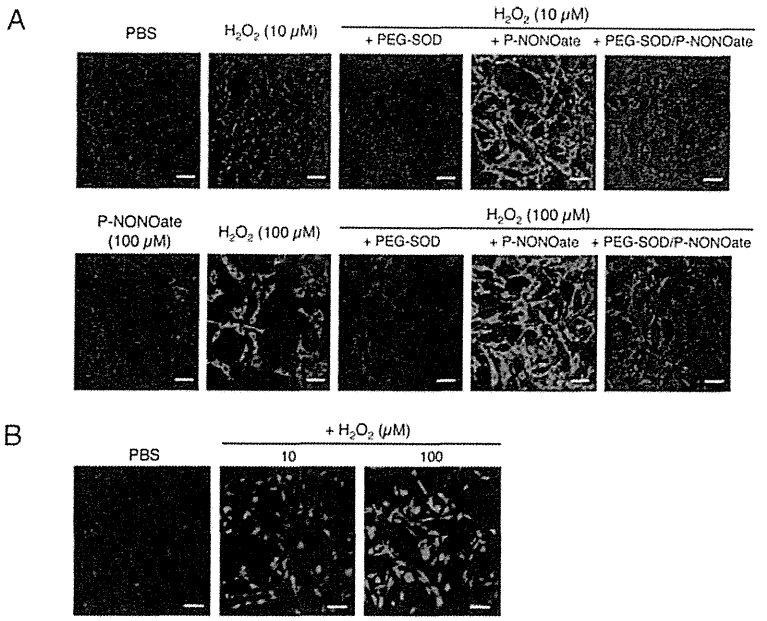

Figure 9 Increase in 8-nitro-cGMP formation and mitochondrial superoxide production in rat C6 glioma cells by H_2O_2 and NO treatment

(**A**) Cells were untreated or treated with 10 or 100 μM H_2O_2 for 36 h, plus PEG–SOD (200 units/ml), P-NONOate (100 μM) or PEG–SOD (200 units/ml) plus P-NONOate (100 μM). Immunocytochemistry for intracellular 8-nitro-cGMP using the 1G6 monoclonal antibody against 8-nitro-cGMP, as described in the Materials and methods section, was carried out. Scale bars = 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 superoxide generation by MitoSOX™ Red staining as described in the Materials and methods section (left-hand panels). Scale bars = 50 μm.

via production of H_2O_2, which in turn enhances mitochondrial superoxide production.

To study the role of H_2O_2 in formation of 8-nitro-cGMP and its association with mitochondrial superoxide production, we treated C6 cells with NO donors and H_2O_2. P-NONOate alone had a negligible effect on 8-nitro-cGMP formation (Figure 9A and Supplementary Figure S8A at http://www.BiochemJ.org/bj/441/bj4410719add.htm). Treatment with H_2O_2 slightly increased 8-nitro-cGMP formation, and PEG–SOD treatment suppressed this increase. 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 extracellular H_2O_2 can directly accelerate mitochondrial superoxide production, we examined the effect of H_2O_2 treatment on MitoSOX™ Red fluorescence. In fact, we found that H_2O_2 treatment significantly increased mitochondrial superoxide production (Figure 9B and Supplementary Figure S8B). Taken together, in stimulated C6 cells, H_2O_2 derived from Nox2 may play a role in NO-dependent formation of 8-nitro-cGMP, possibly via accelerating superoxide production in mitochondria.

DISCUSSION

In the present study, we have investigated the chemical and biochemical mechanisms of 8-nitro-cGMP formation, with particular focus on the roles of ROS. Our 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. At physiological pH, both $ONOO^-$ and its conjugated acid ONOOH ($pK_a = 6.8$) exist, and the latter decomposes via homolysis to give the hydroxy radical ($^{\bullet}OH$) and NO_2 [29]. 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 [30]. Reduction potentials have been reported for $^{\bullet}OH$ ($E^0 = 1.9$–2.1 V) [31], NO_2 ($E^0 = 1.04$ V) [32], $CO_3^{\bullet -}$ ($E^0 = 1.5$ V) [33] and guanine ($E^0 = 1.29$ V) [34]. Oxidation of guanine by $^{\bullet}OH$ or $CO_3^{\bullet -}$ is thus believed to be thermodynamically favourable and would result in formation of the guanine radical cation. This cation undergoes recombination with NO_2 to form 8-nitroguanine [11]. A similar mechanism may therefore operate for nitration of guanine nucleotides induced by $ONOO^-$ and $ONOOCO_2^-$. The $NaNO_2/H_2O_2/MPO$ system used in the present 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 [35]. This compound I may also directly oxidize guanine nucleotides because of its strong reduction potential ($E^0 = 1.35$ V) [36]. In contrast, we found no production of nitrated guanine nucleotides 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 [37]. Therefore HRP compound I could not oxidize guanine nucleotides to form the corresponding cation radical.

We determined that GTP, among the guanine nucleotides examined, 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 signalling through activation of GTP-binding proteins and production of the second messenger cGMP [38]. Electrophilic activity of nitrated guanine nucleotides varies depending on their structures (Md. M. Rahaman, T. Sawa and T. Akaike, unpublished work). Among the 8-nitroguanine derivatives examined, 8-nitro-cGMP showed the highest electrophilic activity at neutral pH, and the electrophilic activity decreasing in the following order: 8-nitro-cGMP>>8-nitroguanosine>8-nitro-GTP/8-nitro-GMP. The second-order rate constants for the

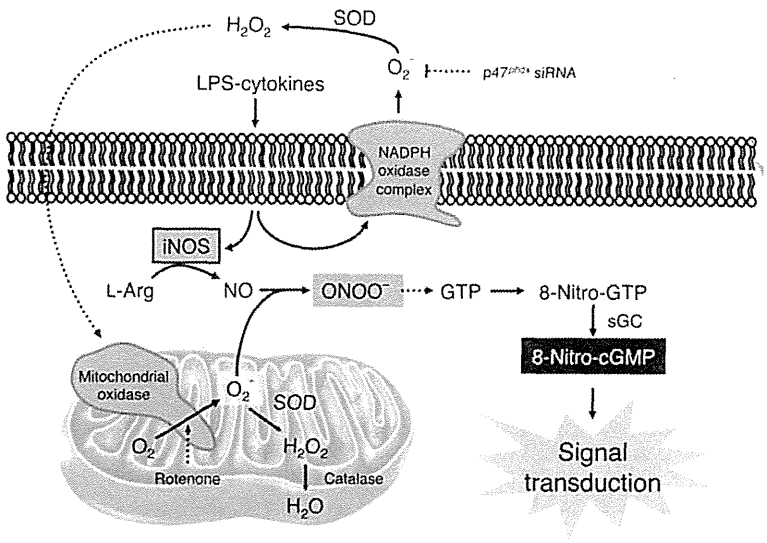

Figure 10 Schematic diagram of possible mechanisms involved in cell formation of 8-nitro-cGMP in rat C6 glioma cells stimulated with LPS/cytokines

iNOS, inducible NOS; L-Arg, L-arginine.

reaction of glutathione with those molecules were determined to be 0.05 (8-nitro-cGMP), 0.018 (8-nitroguanosine), 0.01 (8-nitro-GTP), and 0.008 (8-nitro-GMP) $M^{-1} \cdot s^{-1}$ respectively ([3] and Md. M. Rahaman, T. Sawa and T. Akaike, unpublished work). In this context, 8-nitro-GTP may stably be present in cytosol where glutathione is present abundantly. On the other hand, our previous work demonstrated that 8-nitro-GTP can act as a substrate for sGC, with 8-nitro-cGMP formed as a product [5]. These data suggest that 8-nitro-GTP may be implicated in electrophile signalling via acting as an excellent substrate for guanylyl cyclase to produce 8-nitro-cGMP, rather than inducing direct protein S-guanylation. Although ONOO$^-$ has been considered as a toxic by-product of NO and ROS formation, accumulating evidence suggests a signalling function. Kang et al. [39] reported that ONOO$^-$ formed under sulfur amino acid deprivation activates the Nrf2 signal via the PI3K (phosphoinositide 3-kinase) [39]. Our previous study revealed that the Nrf2 signal can be activated, at least in part, via Keap1 S-guanylation, as supported by the fact that reduction of 8-nitro-cGMP formation by sGC inhibitor appreciably suppressed Nrf2 nuclear translocation and haem oxygenase-1 gene expression, without affecting RNOS formation [5]. It is thus suggested that ONOO$^-$ may contribute to activation of Nrf2-dependent responses via multiple mechanisms, including increased production of electrophilic molecules such as 8-nitro-cGMP and activation of the PI3K pathway.

The present study shows 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. The levels of H_2O_2 from stimulated C6 cells were determined to be 1.63 ± 0.03 nmol/min per mg of protein (or 3.0 ± 0.2 μM in culture supernatant for 10 min incubation under the conditions used in the present study). In separate experiments, 10 μM H_2O_2 added exogenously to non-stimulated cells sufficiently induced mitochondrial superoxide production and 8-nitro-cGMP formation in the presence of NO donor (Supplementary Figure S8). These observations suggest that ~10 μM H_2O_2 is required to promote mitochondrial superoxide production in C6 cells. Recent studies have suggested that superoxide production in mitochondria is accelerated by H_2O_2 [40]. We therefore speculate that H_2O_2 derived from Nox2 contributes to the acceleration of mitochondrial superoxide generation, and hence, 8-nitro-cGMP formation as illustrated in Figure 10. In this context, a study by Zhang et al. [41] demonstrated local production of superoxide by Nox4 in sarcoplasmic reticulum, suggesting regulation of ROS signalling by different Nox enzymes in certain cell types, warranting further investigation of 8-nitro-cGMP signalling in these cells.

In summary, in the present study we have verified that ROS play a pivotal role in the formation of 8-nitro-cGMP in C6 cells stimulated with LPS/cytokines. Superoxide, most probably derived from mitochondria, is directly involved in the formation of 8-nitro-cGMP, whereas H_2O_2 generated by Nox2 also has an important role by increasing mitochondrial superoxide production. Our data thus suggest that 8-nitro-cGMP may serve as a unique second messenger for ROS signalling in the presence of NO. Greater understanding of 8-nitro-cGMP formation in relation to mitochondrial function and NADPH oxidase regulation may help us develop new diagnostic methods and treatment of diseases related to dysregulation of NO and ROS [42].

AUTHOR CONTRIBUTION

Khandaker Ahtesham Ahmed and Tomohiro Sawa designed and performed experiments, analysed the data and wrote the paper. Hideshi Ihara, Shingo Kasamatsu and Jun Yoshitake contributed to the acquisition of LC-MS/MS data. Tatsuya Okamoto, Md. Mizanur Rahaman and Shigemoto Fujii performed immunocytochemistry, cytotoxicity assays, superoxide and H_2O_2 measurements, and Western blotting experiments. Takaaki Akaike designed and supervised the project, and wrote the paper. All authors read and approved the final version of the manuscript.

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