reacts with sulfhydryl groups of cysteine residues and formed a protein-S-cGMP adduct, *via* a novel post-translational modification, namely, protein S-guanylation [12]. More recently, we investigated intracellular 8-nitro-cGMP levels and identified partly the mechanisms underlying the formation of 8-nitro-cGMP and S-guanylation [13]. Specifically, we precisely quantified NO-dependent formation of 8-nitro-cGMP in C6 glioma cells *via* liquid chromatography-tandem mass spectrometry (LC-MS/MS). Markedly high levels (>10 μM) of 8-nitro-cGMP were also evident in C6 cells that had been stimulated to express inducible NO synthase (iNOS) with excessive NO production [13]. More importantly, 8-nitro-cGMP caused S-guanylation of Kelch-like ECH-associated protein 1 (Keap1) in cells, which led to Nrf2 activation and subsequent induction of antioxidant enzymes; thus, 8-nitro-cGMP protected cells against the cytotoxic effects of oxidative stress [13]. The report was the first substantial corroboration of the biological significance of cellular 8-nitro-cGMP formation and potential roles of 8-nitro-cGMP in the Nrf2-dependent antioxidant response [13].

Thus, the biological significance of nitrated guanine derivatives, especially 8-nitro-cGMP, become evident, and therefore it is important to determine the presence and relative abundance of 8-nitro-cGMP conveniently without special analytical instruments. 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 quantitative analysis using LC-MS/MS. We previously produced monoclonal antibodies specific for 8-nitroguanine (clone NO2-52) and 8-nitro-cGMP (clone 1G6) [12]. In the present study, we performed immunocytochemistry with monoclonal antibodies NO2-52 and 1G6 in rat C6 glioma cells and rat primary cultured astrocytes. The results were compared with chemical identification and quantification by LC-MS/MS analysis.

Materials and methods

Materials

Mouse monoclonal antibodies specific for 8-nitro-cGMP (clone 1G6) and 8-nitroguanine (clone NO2-52) were generated as described previously [12]. 8-Nitro-cGMP was synthesized as described previously [12]. N^{ω}-monomethyl-L-arginine (L-NMMA), lipopolysaccharide (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-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). Interferon-γ (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 Tesque (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.

Cell culture

Rat glioma C6 cells were cultured at 37 °C in DMEM supplemented with 10% FBS and 1% penicillin–streptomycin. Primary astrocytes were prepared from Wistar rats using a modification of a technique described previously [14]. 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 polyethylene-imine-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 treated with 10 μg/ml LPS, 100 U/ml IFN-γ, 100 U/ml TNFα, and 10 ng/ml IL-1β. 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.

Western blot analysis and nitrite production

Proteins in cell lysates were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred onto a nitrocellulose membrane. Blots were incubated with Blocking One containing peroxidase-conjugated iNOS specific antibody i2G4 [14] (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 anti-rabbit 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).

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 [15] 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 [16].

Immunocytochemical analysis

Immunocytochemical analysis was carried out by the previously described method [12]. In brief, C6 cells and primary astrocyte were fixed with Zamboni solution (4% paraformaldehyde and 10 mM picric acid in 0.1 M phosphate buffer, pH 7.4) at 4 °C for 7 h. After permeabilization with 0.5% Triton X-100 at room temperature for 15 min, cells were incubated with BlockAce overnight at 4 °C to block nonspecific antigenic sites. Cells were then incubated overnight at 4 °C with anti-8-nitro-cGMP monoclonal antibody 1G6 (1 μg/ml) in Can Get Signal Immunoreaction Enhancer Solution. After three rinses with phosphate-buffered saline (PBS), cells were incubated with Cy3-labeled goat anti-mouse IgG antibody. 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).

Quantitative analysis of 8-nitro-cGMP by LC-MS/MS

Intracellular levels of 8-nitro-cGMP was measured by LC-MS/MS [13]. In brief, after treatment with LPS and cytokines, cells were washed twice with ice-cold PBS containing 5 mM NEM and were collected by using a cell scraper in 5 ml of ice-cold PBS containing 5 mM NEM, followed by centrifugation. The cell pellet thus obtained was homogenized in 5 ml of methanol containing 5 mM NEM and 40 nM ^{15}N-labeled authentic 8-$^{15}NO_2$-cGMP. After the homogenate samples were centrifuged at 5000 *g* at 4 °C, their resultant supernatant was dried *in vacuo* and then redissolved in distilled water. LC-MS/MS was performed with a Varian 1200L triple-quadrupole (Q) mass spectrometer (Varian, Inc., Palo Alto, CA, USA), after reverse-phase 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 -8 V for both analytes was applied. The signal of endogenous 8-$^{14}NO_2$-cGMP was identified simultaneously with respective ^{15}N-derivative identification.

Statistical analysis

All experiments were performed at least three times. Mean values for individual experiments are presented as means ± S.E. Statistical significance was determined by a two-tailed unpaired Student's *t* test.

Results and discussion

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 [9,12,13,17,18]. The nitration reaction to form 8-nitroguanine derivatives depends on production of NO by iNOS [9,12,13,17]. 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 [13]. Therefore, we confirmed the expression of iNOS and sGC in C6 cells and primary astrocytes after stimulation with LPS and cytokines. As shown in Fig. 2A, 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 and 24 h in C6 cells and astrocytes, respectively. NO production was also induced by the LPS/cytokines treatment in a time-dependent manner in both cells (Fig. 2B). sGC was expressed in non-treated cells, and the expression was not changed by LPS/cytokines treatment. sGC expression in astrocytes was approximately 50% of that in C6 cells.

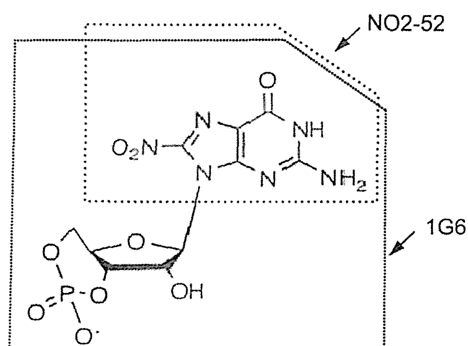

Fig. 1. The epitope structures of 8-nitroguanine derivatives that each antibody can recognize. The monoclonal antibody NO2-52 recognizes the 8-nitroguanine. On the other hand, the monoclonal antibody 1G6 was specific for 8-nitro-cGMP.

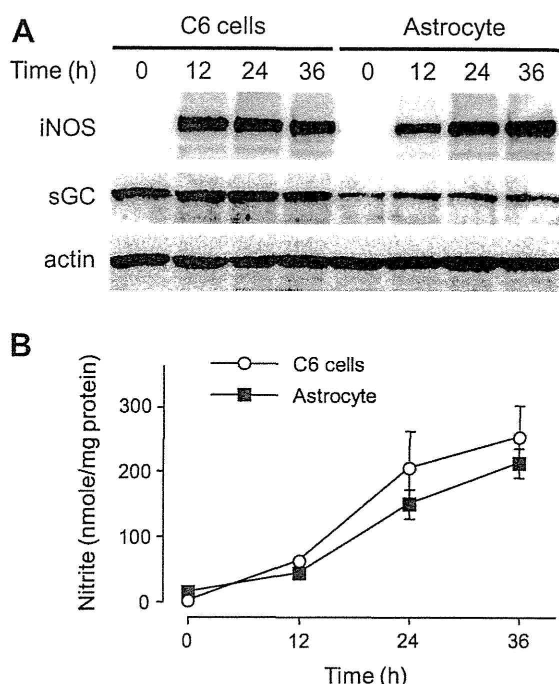

Fig. 2. Western blot analysis and nitrite production in C6 cells and primary astrocytes. (A) Expression of iNOS and sGC in C6 cells and primary astrocytes after stimulation with LPS and cytokines. Cells were stimulated with a mixture of 10 μg/ml LPS, 100 U/ml IFN-γ, 100 U/ml TNFα, and 10 ng/ml IL-1β for the indicated time periods. Cell lysates (10 μg of protein) were analyzed *via* Western blot with anti-iNOS, anti-sGC and anti-β-actin antibodies. (B) Nitrite amounts in culture supernatants of C6 cells and primary astrocytes. Cells were treated with the same mixture of LPS and 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.

Next, we examined the formation of 8-nitro-cGMP in stimulated C6 cells by means of LC-MS/MS analysis (Fig. 3). The amount of 8-nitro-cGMP endogenously formed in cells was precisely quantified using a stable-isotope dilution method [13]. Endogenous 8-$^{14}NO_2$-cGMP was quantified by comparing with the area of spiked 8-$^{15}NO_2$-cGMP. As shown in Fig. 3B, levels of 8-nitro-cGMP in C6 cells increased dramatically at late stage after stimulation (36 h). Kinetics of 8-nitro-cGMP formation is markedly delayed when compared with induction of iNOS and/or potential activation of sGC as shown in Fig. 2. Because NO itself is not sufficient to induce nitration of guanine moiety, NO would be converted to reactive nitrogen oxide species such as peroxynitrite and nitrogen dioxide radical, by reacting with reactive oxygen species (ROS) for effective guanine nitration [19]. C6 cells are known to express NADPH oxidase 2, which may be activated by LPS plus cytokine stimulation, to produce ROS [20]. In addition, our recent study showed that mitochondrial superoxide production was significantly enhanced in C6 cells upon treatment with NO donor, and kinetics of 8-nitro-cGMP formation in the cells showed good correlation to the mitochondrial superoxide production [13]. Thus, in addition to NO generation, ROS, possibly derived from NADPH oxidase as well as mitochondria, may critically involve in the production of 8-nitro-cGMP in C6 cells stimulated with LPS and cytokines.

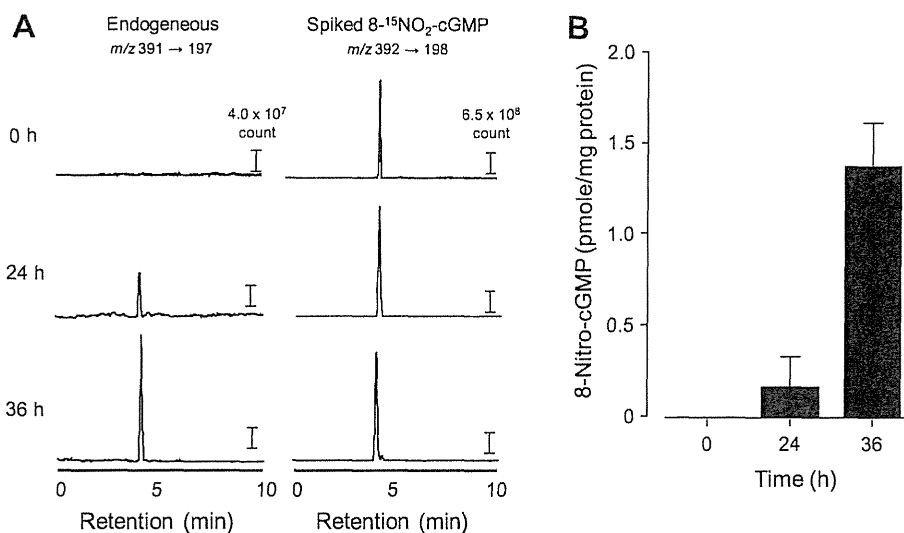

Fig. 3. Quantitative LC-MS/MS analysis for measurement of 8-nitro-cGMP formed in C6 cells. Cells were stimulated with a mixture of 10 μg/ml LPS, 100 U/ml IFN-γ, 100 U/ml TNFα, and 10 ng/ml IL-1β for the indicated time periods, and cell extracts were prepared as described under "Materials and methods".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

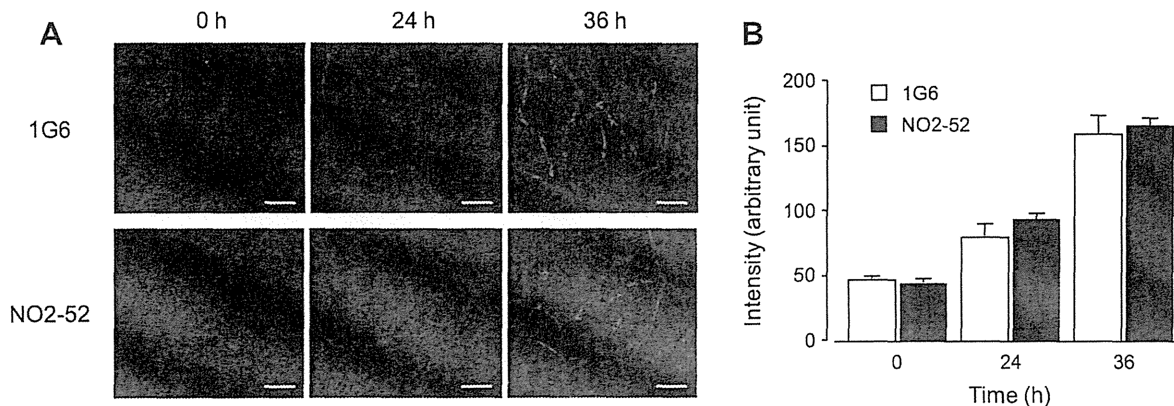

Fig. 4. Immunocytochemistry with antibodies specific for 8-nitroguanine (NO2-52) and 8-nitro-cGMP (1G6) in C6 cells. Cells were stimulated with a mixture of 10 μg/ml LPS, 100 U/ml IFN-γ, 100 U/ml TNFα, and 10 ng/ml IL-1β for the indicated time periods. (A) Immunofluorescence image of 8-nitro-cGMP and 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.

Regarding to biological effects of 8-nitro-cGMP formation at this late stage of stimulation, its marked production might have role in regulation of stress response in the cells. In fact, we clearly demonstrated that formation of 8-nitro-cGMP in cells is closely associated with the induction of adaptive response to protect the cells from oxidative stress [13,17]. Biochemical analyses revealed that 8-nitro-cGMP could activate Nrf2 signaling and subsequent induction of antioxidant enzymes, by interacting with Keap1, a negative regulator of Nrf2 *via* unique post-translational modification of Keap1 cysteine residues [13]. Cytoprotective effect of 8-nitro-cGMP was evident in cultured cells where apoptosis induced by hydrogen peroxide treatment [13] and/or bacterial infection [17] could be effectively suppressed by 8-nitro-cGMP treatments.

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. 1) [12]. 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 [12]. 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. 4), 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. 4).

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. 5). Quantitative determination of 8-nitro-cGMP by LC-MS/MS analysis demonstrated that both L-NMMA and NS 2028

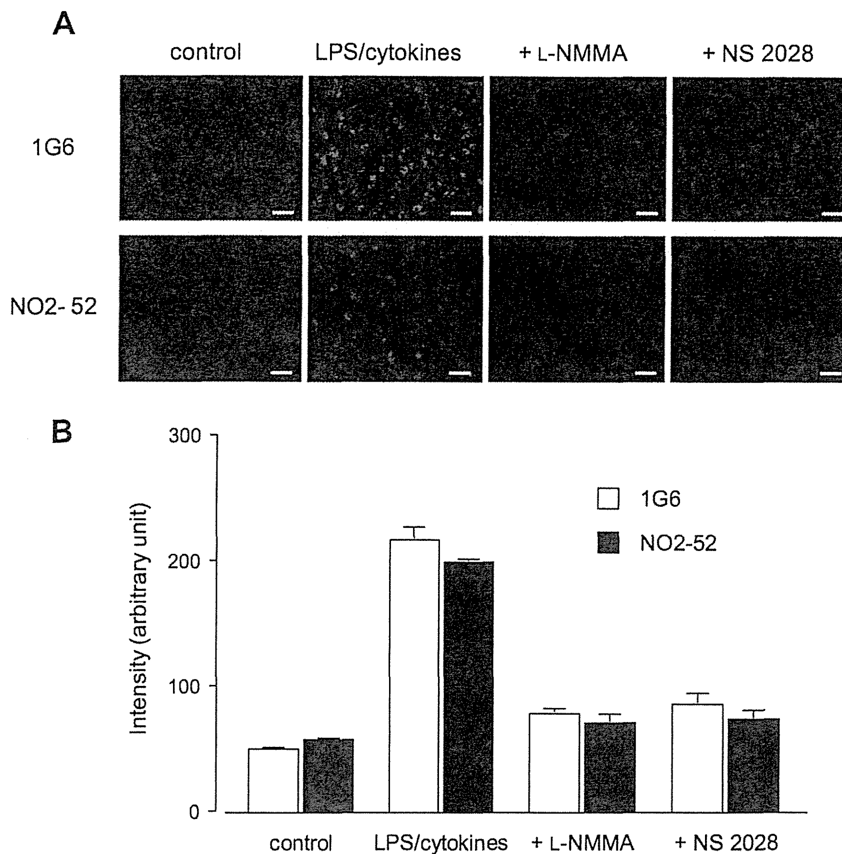

Fig. 5. Effects of inhibitors on immunocytochemistry with antibodies (NO2-52 and 1G6) in C6 cells. Cells were stimulated with a mixture of of 10 μg/ml LPS, 100 U/ml IFN-γ, 100 U/ml TNFα, and 10 ng/ml IL-1β for 36 h in the presence or absence of various inhibitors. Cells were treated with 10 mM L-NMMA or 10 μM NS 2028, beginning 1 h before the addition of LPS plus 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.

completely suppressed 8-nitro-cGMP production in C6 cells stimulated by LPS/cytokines (Fig. 6); 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. 5); 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. 5). 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 observations indicate that 8-nitro-cGMP is the most predominant derivative of 8-nitroguanine in cells.

Previously, we confirmed the chemical identity of 8-nitro-cGMP and other nitrated purines by means of high-performance liquid chromatography (HPLC) coupled with electrochemical detection (ECD) [12]. We applied the lysate of RAW 264.7 cells, a murine macrophage cell line, which had been stimulated with LPS and cytokines for the expression of iNOS, to the immunoaffinity column containing anti-8-nitroguanine and anti-8-nitro-cGMP antibodies in order to separate and enrich the 8-nitroguanine derivatives, and subsequently performed HPLC-ECD analysis. Of the derivatives, 8-nitro-cGMP was the major product of RAW 264.7 cells; 8-nitroguanine and 8-nitroxanthine were minor nitrated products, and no appreciable peak of 8-nitroguanosine was observed with the cell lysate [12]. These observations are consistent with our present NO2-52 immunochemistry results.

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. We analyzed guanine nitration in stimulated astrocytes *via* 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. 2). As shown in Fig. 7, strong immunostaining of activated astrocytes is apparent with both types of antibodies. Treatment with 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 thus 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. In our preliminary experiments, we identified the cellular formation of 8-nitro-cGMP

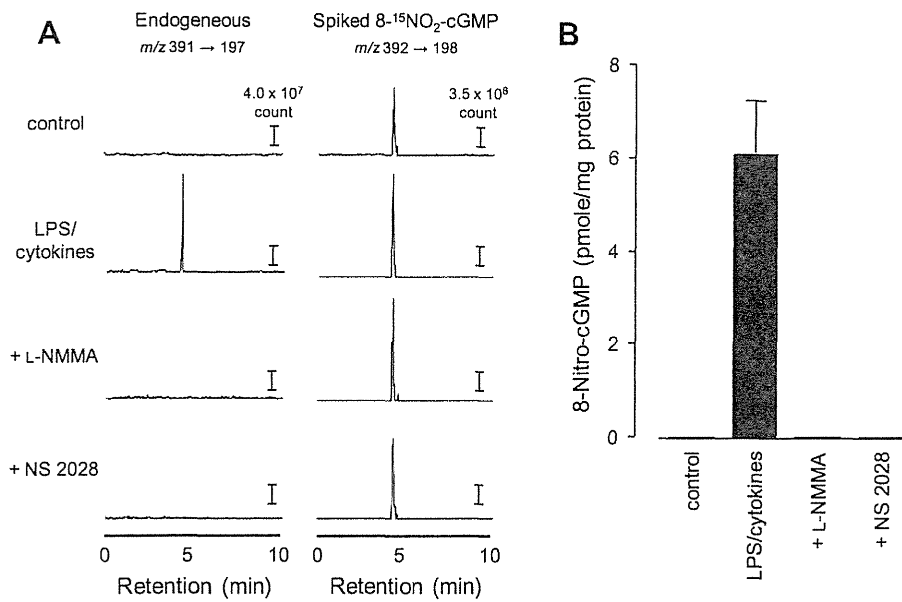

Fig. 6. Effects of inhibitors on 8-nitro-cGMP formation in C6 cells; LC-MS/MS analyses. Cells were stimulated with a mixture of of 10 μg/ml LPS, 100 U/ml IFN-γ, 100 U/ml TNFα, and 10 ng/ml IL-1β for 36 h in the presence or absence of various inhibitors. Cells were treated with 10 mM L-NMMA or 10 μM NS 2028, beginning 1 h before the addition of LPS plus 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. ($n = 3$).

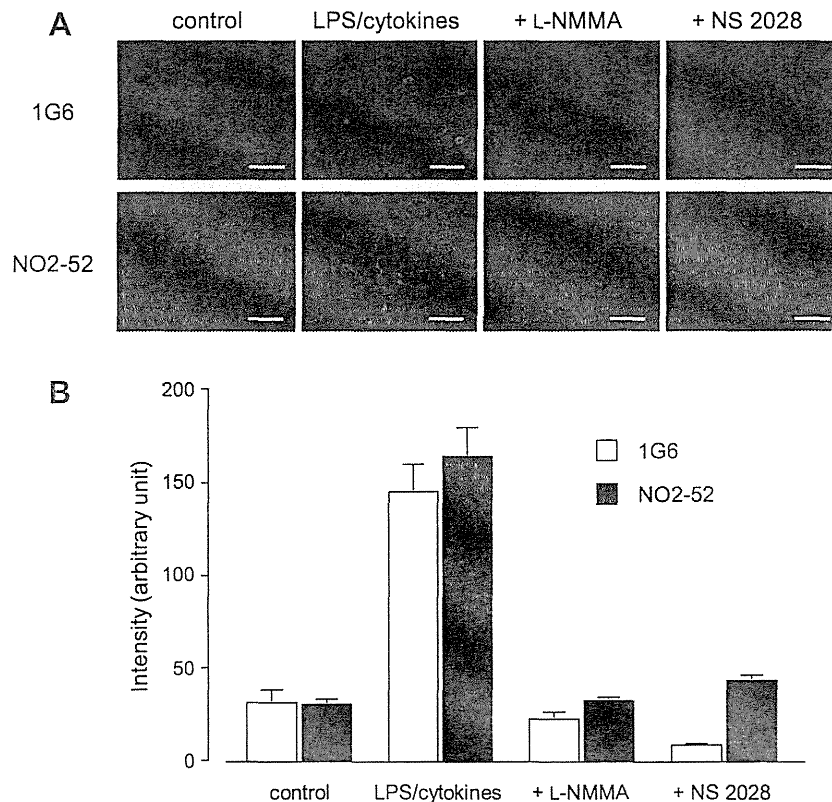

Fig. 7. Effects of inhibitors on immunocytochemistry with antibodies (NO2-52 and 1G6) in primary astrocytes. Cells were stimulated with a mixture of of 10 μg/ml LPS, 100 U/ml IFN-γ, 100 U/ml TNFα, and 10 ng/ml IL-1β for 36 h in the presence or absence of various inhibitors. Cells were treated with 10 mM L-NMMA or 10 μM NS 2028, beginning 1 h before the addition of LPS plus 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.

in rat primary astrocytes by means of LC-MS/MS. Consistent with immunocytochemical data (Fig. 7), the levels of 8-nitro-cGMP were significantly increased upon stimulation (data not shown). However, to perform multiple analyses to examine the association of 8-nitro-cGMP formation with iNOS or sGC levels by means of LC-MS/MS, it requires more than 10^7 cells. Because we could obtain

only ~10^6 primary astrocytes from 5 to 6 rat embryos (see Materials and Methods section), we have to use more than 60 embryos to complete the experiments by means of LC-MS/MS. As demonstrated in the present study, our immunocytochemical analysis requires remarkably smaller numbers of cells (~10^5 cells) than that with LC-MS/MS (usually more than 10^6 cells). Thus, we can reduce the number of cells used, and hence, number of animals sacrificed. Therefore, there is a great benefit to use immunocytochemistry particularly when only limited amount of samples are available (e.g., primary astrocytes).

Conclusion

The present immunochemical analysis in C6 glioma cells stimulated with LPS/cytokines using the anti-8-nitro-cGMP (1G6) antibody demonstrated that the intensities of immunostaining are in good agreement with the results of chemical quantification of 8-nitro-cGMP by LC-MS/MS. 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. Similar results were obtained in the primary astrocytes stimulated with LPS/cytokines. 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 [9,12,13,17,18]. Immunocytochemistry is a conventional and fairly straightforward method for determining the presence and relative abundance of an antigen of interest in cultured cells. 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.

Acknowledgments

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Regulation of Redox Signaling Involving Chemical Conjugation of Protein Thiols by Nitric Oxide and Electrophiles

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Nitric oxide (NO), a gaseous free radical that is biologically synthesized by nitric oxide synthases, participates in a critical fashion in the regulation of diverse physiological functions including vascular and neuronal signal transduction, host defense, and cell death regulation. This article reviews the chemical and biochemical mechanisms of protein thiol modifications caused by NO and by electrophiles derived from NO metabolism. The classical NO signaling pathway involves formation of the second messenger guanosine 3′,5′-cyclic monophosphate (cGMP). Post-translational modifications of redox-sensitive protein thiols have also been shown to be important in this signaling pathway. For instance, redox-sensitive thiols are targets for NO conjugation and formation of *S*-nitrosothiols. Electrophiles generated by reactions of NO or reactive nitrogen oxide species and biomolecules (i.e., fatty acids) effect thiol conjugations through S-alkylation. Among this class of reactions, S-guanylation is particularly emphasized. S-Guanylation is a novel type of S-alkylation with nitrated cGMP that contributes to the cytoprotective effects of NO. Post-translational modifications of thiols affect protein structures and functions: allosteric effects may alter protein structure, modification of active centers of enzymes may suppress enzyme actions, and modifications may modulate protein–protein interactions. Better understanding of protein modification by NO-derived electrophiles and the molecular basis of NO signaling would be useful in the development of new diagnostic methods and treatment of diseases related to NO metabolism.

INTRODUCTION

Nitric oxide (NO) is a gaseous free radical that is synthesized by nitric oxide synthases (NOSs[1]). NOSs catalyze the oxidation of L-arginine to form NO and L-citrulline (*1*). In spite of its simple structure, NO participates in a critical fashion in the regulation of diverse physiological phenomena such as vascular and neuronal signal transduction, host defense, and cell death regulation (*2–5*). Signal transduction of NO primarily involves activation of soluble guanylate cyclase to form the second messenger molecule guanosine 3′,5′-cyclic monophosphate (cGMP) (*6*). cGMP thus formed binds to allosteric regulatory domains of target proteins, including protein kinases, ion channels, and phosphodiesterases (PDEs), with a variety of downstream biological consequences that allow cells to adapt to changes in environmental conditions and metabolic demands (*6*).

Post-translational modification (PTM) is the process that attaches functional groups such as acetate and phosphate to amino acid side chains of proteins. PTM plays major roles in regulating diverse biological processes through a wide variety of mechanisms that include changes in protein activities, interactions, and subcellular localizations (*7–9*). Accumulating evidence has suggested that PTM of redox-sensitive protein thiols contributes to the regulation of NO signaling via a cGMP-independent mechanism (*10*). A well-characterized example of PTM by NO is S-nitrosylation, i.e., the conjugation of an NO moiety to a reactive cysteine thiol to form an *S*-nitrosothiol (*11–13*). A number of proteins have been identified as targets for S-nitrosylation, and the functional impact of the PTM has been extensively studied (*13–15*). Recent studies have suggested the importance of electrophilic metabolites of NO in PTMs other than S-nitrosylation. Biomolecules such as fatty acids and nucleotides reacts with NO or other reactive nitrogen oxide species (RNOS) to form electrophilic metabolites, and these electrophiles modify protein thiols to regulate NO signaling. Thus, better understanding of the modification by NO-derived metabolites would contribute to the development of new diagnostic methods and treatments of NO-related diseases. In this review article, we discuss the chemical and biochemical mechanisms of protein thiol conjugation mediated by NO and electrophiles and the impact of such modification on redox signaling. We place particular emphasis on S-guanylation, a novel type of S-alkylation, which is involved in cytoprotective signaling mediated by NO (*16–19*).

TYPES OF PROTEIN THIOL MODIFICATIONS BY NITRIC OXIDE AND RELATED ELECTROPHILES

S-Nitrosylation. Although nitrosothiol is formed on exposure to NO in solution under aerobic conditions, possibly through the formation of the potent nitrosating agent N_2O_3 (*20*), its efficacy is drastically enhanced in the presence of Cu^{2+}-containing proteins such as ceruloplasmin (*11, 21*). Ceruloplas-

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[1]Abbreviations: ASK1, apoptotic signal-regulating kinase-1; cGMP, 3′,5′-cyclic monophosphate; GSH, glutathione; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; HO-1, heme oxygenase-1; HIF-1, hypoxia-inducible factor 1; iNOS, inducible isoform of nitric oxide synthase; Keap1, Kelch-like ECH-associating protein 1; MTs, metallothioneins; Nrf2, NF-E2-related factor 2; NOSs, nitric oxide synthases; 8-nitro-cGMP, 8-nitroguanosine 3′,5′-cyclic monophosphate; ODD, oxygen-dependent degradation; PDE, phosphodiesterase; PTM, post-translational modification; PTP1B, protein tyrosine phosphatase 1B; RNOS, reactive nitrogen oxide species; ROS, reactive oxygen species; RSOH, sulfenic acid; Trx-1, thioredoxin-1; vHL, von Hipple-Lindau.

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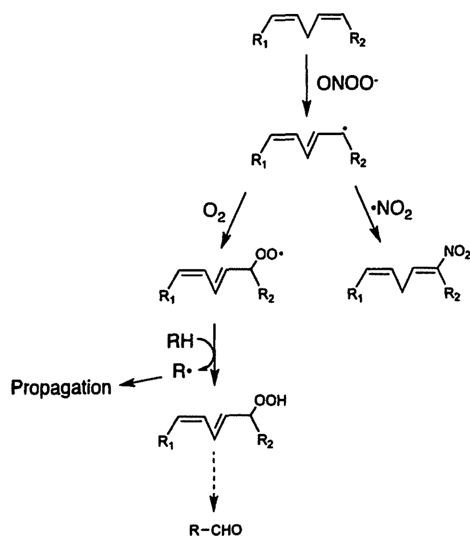

Figure 1. Proposed mechanisms for the peroxynitrite-induced formation of nitrated and oxidized fatty acids.

min catalyzes one-electron oxidation of NO to form the nitrosonium ion equivalent that favors electrophilic attack of thiols to form nitrosothiol.

Because deprotonation of thiols makes them more reactive toward NO, RNOS, and electrophiles (*22, 23*), the pK_a value of the specific thiol defines the reactivity of a thiol-containing protein under physiological pH. The pK_a value of the thiol of cysteine is 8.33, and pK_a values of protein-associated cysteine thiols usually stay between 8.2 and 8.5 (*23*). The environment that surrounds cysteine thiols can modulate the pK_a: the pK_a value of the cysteine thiol is decreased when proximal to (a) basic amino acids such as histidine, lysine, and arginine; (b) aromatic amino acids such as tyrosine and tryptophan; or (c) metal centers such as heme–metal complexes (*13, 23*). Allosteric regulators can also modulate the reactivity of cysteine thiols by affecting the accessibility of thiols and/or reactivity of cysteine thiols (*13, 24*). In this context, we recently found that S-nitrosylation of human serum albumin by NO donors and low-molecular-weight nitrosothiols can be greatly enhanced by binding of endogenous ligands such as fatty acids and bilirubin to the protein (*25*). Thus, protein allostery may be a significant factor in the regulation of thiol modifications and warrants further investigation.

Oxidation and Disulfide Formation. Reaction of a thiol with an RNOS such as peroxynitrite, a coupling product of NO and superoxide (*26*), results in oxidation of the thiol to form sulfenic acid (RSOH) (*27*). RSOH can react with proximal thiol groups to form intra- or intermolecular disulfide bonds (*28*). Thus, RSOH may play an important role in glutathionylation, the covalent attachment of GSH to protein thiols via a mixed disulfide bond. Intra- or intermolecular disulfide bond formation can also be facilitated through formation of nitrosothiols (*28*).

S-Alkylation. In addition to direct modifications of protein thiols by RNOS, thiol S-alkylation caused by electrophilic secondary metabolites may also play an important role in the regulation of redox signaling. Examples of such electrophiles include nitrated fatty acids and nitrated guanine nucleotides.

Mechanisms of fatty acid nitration and oxidation by RNOS have been extensively studied by Freeman and colleagues (*29*). Carbon-centered radicals formed from hydrogen abstraction by oxidant react with nitrogen dioxide to form nitrated fatty acids (Figure 1). At low RNOS environment, however, lipid peroxidation proceeds predominantly to form peroxidation products including several electrophiles such as lipid aldehyde (e.g., 4-hydroxynonenal) rather than forming nitrated electrophiles (Figure 1).

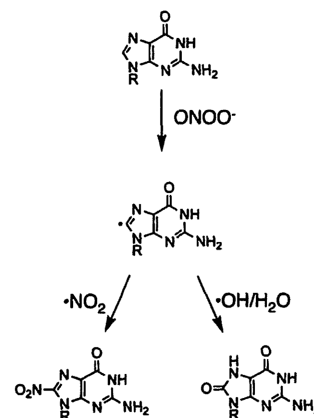

Figure 2. Proposed mechanisms for the peroxynitrite-induced formation of nitrated and oxidized guanine derivatives.

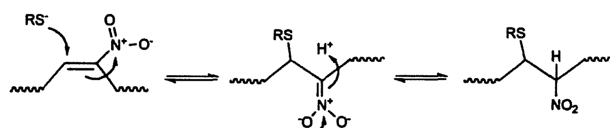

Figure 3. Michael addition reaction of nitroalkene derivatives with protein thiols (thiolates, RS-).

Peroxynitrite represents a biologically relevant oxidizing and nitrating agent. In physiological pH, peroxynitrite is rapidly converted to form nitrogen dioxide and hydroxyl radical; the latter can abstract hydrogen atom from guanine moiety to form guanine radical (Figure 2) (*30, 31*). Guanine radical thus formed reacts with nitrogen dioxide or hydroxyl radical to form nitroguanine and hydroxyguanine, respectively (Figure 2). Physiologically relevant levels of carbon dioxide can facilitate peroxynitrite-mediated nitration of guanine derivatives (*32*), possibly through formation of nitrosoperoxycarbonate ($ONOOCO_2^-$) (*33*).

Biological formation of nitrated fatty acids has been reviewed recently (*29*). Other reports support the endogenous generation and presence of nitrated fatty acids (*34, 35*). The first such nitrated fatty acid was observed in bovine papillary muscles: vicinal nitrohydroxyeicosatrienoic acid (*36*). Nitrolinoleate has been detected in human blood plasma, and cholesteryl nitrolinoleate has been found in human plasma and lipoproteins (*37, 38*), with hyperlipidemic and postprandial conditions elevating plasma levels of nitrated fatty acids.

The nitrated fatty acids involve conjugated nitroalkene functions, and the electron-withdrawing nature of the nitro groups facilitates the Michael addition of thiols to the alkenes. The reaction of cysteine thiols (or corresponding thiolates) with nitrated fatty acids has been shown in vivo (S-nitroalkylation) (*39*) (Figure 3). The rate constants of those reactions exceed those with most fatty acid-derived electrophiles, with second-order rate constants of up to 355 M^{-1} s^{-1} at pH 7.4 and 37 °C (*40*). Those constants are orders of magnitude greater than those for the glutathione (GSH) reaction with H_2O_2 and electrophilic lipids such as 8-*iso*-prostaglandin A_2 and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2. It is noteworthy that S-nitroalkylation is reversible, and liberated nitroalkenes can react with thiols of other proteins or peptides (trans-alkylation) (*39*). Protein adducts of nitrated fatty acids have been detected in clinical environments, which is evidence of a metabolic and redox-sensitive mechanism for regulating protein distribution and function. Readers may refer to a review article by Freeman and colleagues for details of the chemistry and biology of nitrated fatty acid signaling (*29*).

Nitration products of guanine derivatives have been identified in various in vitro and in vivo settings, most of which were

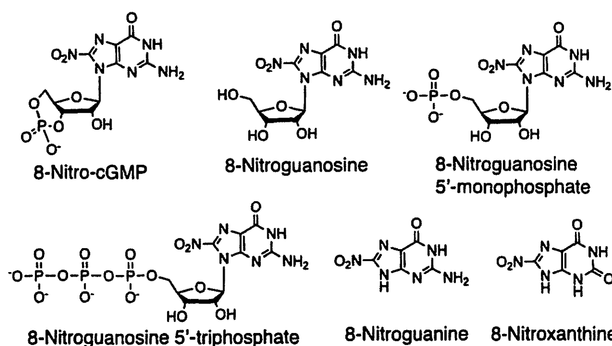

Figure 4. Chemical structures of 8-nitro-cGMP and related compounds.

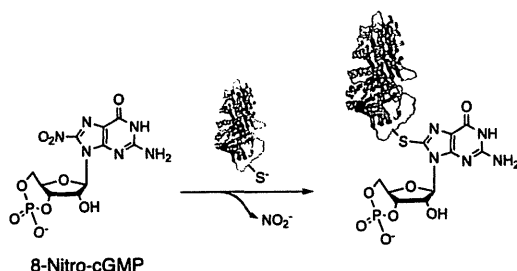

Figure 5. Schematic representation of protein S-guanylation. Nucleophilic protein thiolates attack the C8 carbon of 8-nitro-cGMP, which results in adduction of cGMP moieties to cysteine residues in proteins, with concomitant release of a nitrite anion.

associated with inflammatory conditions (*30, 41−48*). Detailed analyses of cell culture experiments revealed that nitrated cGMP (8-nitroguanosine 3′,5′-cyclic monophosphate [8-nitro-cGMP], Figure 4) was the major product formed in cells (*16*). 8-Nitro-cGMP was first identified in the mouse macrophage cell line RAW 264.7 when cells were stimulated with interferon-γ and lipopolysaccharide to produce NO via the inducible isoform of NO synthase (iNOS) (*16*). Infection with the gram-negative bacterium *Salmonella* facilitated the formation of 8-nitro-cGMP in mouse macrophages with the wild-type iNOS gene but not in iNOS-deficient macrophages. As another example, human hepatoma HepG2 cells treated with an NO donor under glucose starvation showed enhanced 8-nitro-cGMP formation (*16*).

8-Nitro-cGMP is the first endogenous cGMP derivative that forms a stable conjugate with cysteine thiols at the purine base. This conjugation reaction is called "protein S-guanylation" (*16*) and proceeds without enzymatic stimulation in vitro. The occurrence of S-guanylation in vivo was unambiguously proved by using a specific antibody and mass spectrometry. Figure 5 shows the mechanism of the reaction, in which the nitro group of 8-nitro-cGMP is replaced by a thiol group, with the release of NO_2^-. In contrast to S-nitrosylation and S-nitroalkylation (which are conjugations via addition), S-guanylation is believed to be irreversible under physiological conditions. Thus, as a signaling principle, deguanylation may be catalyzed by certain enzyme systems, which would be analogous to dephosphorylation catalyzed by phosphatases, and a search for such biological deguanylation mechanisms is warranted (*17*).

Kinetic analyses indicate that reactivity of 8-nitro-cGMP with cysteine thiols is moderate compared with that of other biological electrophiles (*16*). For instance, the reaction rate constants for GSH at pH 7.4 and 37 °C are 0.03, 0.7, 1.3, 183, and 355 $M^{-1}\ s^{-1}$ for 8-nitro-cGMP, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2, 4-hydroxynonenal, nitrooleic acid, and nitrolinoleic acid, respectively. These data suggest that only proteins bearing highly nucleophilic reactive cysteine thiols as determined, for example, by low pK_a values, may be targets for S-guanylation.

MODULATION OF PROTEIN FUNCTIONS BY THIOL MODIFICATIONS

Modifications of cysteine thiols by NO, RNOS, and electrophiles have been reported to regulate the activity of numerous metabolic enzymes, including oxidoreductases, proteases, and protein kinases and phosphatases, as well as receptors, ion channels and transporters, transcription factors, and others (Table 1).

The best-characterized examples of protein function regulation include enzyme inhibition by modifications of active center thiols, thiol modifications that affect protein function and structure (allosteric regulation), and regulation of protein−protein interactions (*13, 49*).

Active Center Thiol Modification. Some enzymes contain cysteine residues at the active site that are involved in catalysis. Mannick et al. demonstrated that procaspase-3, an enzyme that executes apoptotic signals, is constitutively S-nitrosylated in situ at its active site thiol and that it is selectively denitrosylated after apoptotic stimulation by Fas (*50*). Phosphatases provide another example of thiol modifications associated with inhibition of enzyme activity. Protein tyrosine phosphatase 1B (PTP1B) is one of the protein tyrosine phosphatases that catalyze the removal of phosphate residues and regulate the activity of phosphotyrosyl proteins involved in numerous cell signaling pathways (*51*). The pK_a value of the active site thiol of PTP1B is reportedly 5.4, which suggests that the thiol may be susceptible to modification by NO and RNOS (*23*). Li and Whorton reported inhibition of PTP1B activity by *S*-nitrosothiols through modification of the active site cysteine of PTP1B, with a concomitant increase in the amount of phosphorylated epidermal growth factor receptor (*52*). This finding supports the idea that regulation of PTP1B activity through cysteine thiol modifications may contribute to regulation of phosphorylation-dephosphorylation signaling.

Allosteric Regulation. Thiol modifications can also affect protein functions by means of an allosteric mechanism. Cysteine residues that coordinate to metal ions, especially the nonoxidizable Zn^{2+}, have reduced pK_a values and are thereby susceptible to redox-based modification. Matrix metalloproteinases are enzymes that are critically involved in disintegration and remodeling of the extracellular matrix (*53*). Okamoto et al. demonstrated that matrix metalloproteinases can be activated through modification of Zn^{2+}-coordiated cysteine in a manner dependent on peroxynitrite and low-molecular-weight thiols such as GSH (*54*). Peroxynitrite was thus found to strongly activate matrix metalloproteinases in the presence of GSH. Mass spectrometric analyses revealed that *S*-nitroglutathione forms in the reaction of peroxynitrite with GSH, after which it forms mixed disulfide at the Zn^{2+}-coordinated cysteine, which leads to enzyme activation. Activation of metalloproteinases by NO via S-nitrosylation of Zn^{2+}-coordinated thiols has been reported for tumor necrosis factor α-converting enzyme, a member of a metalloproteinase−disintegrin family (*55*). Other experiments suggest, however, that NO donors or nitrosothiols such as *S*-nitrosoglutathione failed to induce direct activation of matrix metalloproteinases in spite of their ability to nitrosylate Zn^{2+}-coordiated cysteine (*56*). This may thus suggest that S-nitrosylation of matrix metalloproteinases is insufficient to activate the enzyme. Such a discrepancy observed for the impact of S-nitrosylation in the metalloproteinase activation may be explained by release of zinc ion from the zinc−thiolate complex as reported earliear (*56*).

A similar complicated mechanism for zinc release during S-nitrosylation of zinc-coordinated thiolate is described for

Table 1. Protein Targets for Thiol Modifications by NO, RNOS, and Electrophiles

protein[a]	effect[a]	modifications (refs)
procaspase-3	inhibition of enzyme activity	nitrosylation (*50*)
PTP1B	inhibition of enzyme activity	glutathionylation (*76*), nitrosylation (*52*)
proMMPs	activation of enzyme activity	glutathionylation (*54*)
Keap1	enhancement of Nrf2 translocation into the nucleus	alkylation (*16, 77*), oxidation (*78*)
HIF-1α	stabilization of HIF-1α via inhibition of vHL	nitrosylation (*71*)
GAPDH	promotion of GAPDH binding with Siah1, which leads to nuclear translocation and cell death inhibition of enzyme activity	nitrosylation (*73*), alkylation (*39*), glutathionylation (*79*)
Trx-1	inhibition of oxidoreductase activity and release of ASK1 by Cys32 and/or Cys35 modification	alkylation (*80*), nitrosylation (*74, 75*)
NMDA receptor	inhibition of excessive NMDA current	nitrosylation (*81, 82*)
TRP channels	activation of TRP channels (Ca^{2+} entry)	nitrosylation (*83*)
PDI	inhibition of enzyme activity	alkylation (*84*), nitrosylation (*85*)
NF-κB	inhibition of DNA binding by NF-κB	nitrosylation (*86, 87*)

[a] Abbreviations: PTP1B, protein tyrosine phosphatase 1B; proMMPs, precursors of matrix metalloproteinases; Keap1, Kelch-like ECH-associating protein 1; HIF-1α, hypoxia-inducible factor 1α; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Trx-1, thioredoxin-1; NMDA receptor, *N*-methyl-D-aspartate receptor; TRP channels, transient receptor potential channels; PDI, protein disulfide-isomerase; NF-κB, nuclear factor-κB; vHL, von Hippel-Lindau; ASK1, apoptotic signal-regulating kinase-1.

metallothioneins (MTs). MTs are a family of small (6−7 kDa) metal-binding proteins that bind seven divalent metal (Zn^{2+}, Cd^{2+}) via metal−thiolate coordination (*57*). Under aerobic conditions, NO via thiolate-nitrosylation induces the release of Zn^{2+} from MTs in vitro (*58*). Unbound, free zinc ion is known to activate gene expression and, hence, regulate cellular signaling, through activation of metal-response element-binding transcription factor-1 (*59*). Recent study has demonstrated that the NO-mediated zinc release may play a role in regulation of hypoxic pulmonary vasoconstriction (*60*). It is therefore conceivable that NO-mediated zinc release via thiolate modifications may participate in zinc-mediated redox signaling (*61*).

Regulation of Protein−Protein Interactions. Thiol modifications reportedly also control protein−protein interactions. Keap1 (Kelch-like ECH-associated protein 1) is a cysteine-rich redox-sensitive regulatory protein that binds to NF-E2-related factor 2 (Nrf2) and represses its transcriptional activity (*62, 63*). Modifications of Keap1 thiols by reactive oxygen species (ROS), RNOS, and electrophiles facilitate dissociation of Keap1 from Nrf2, which results in nuclear translocation of Nrf2 and transactivation of an array of genes involving antioxidant, anti-inflammatory, drug-metabolizing, and cytoprotective enzymes (*62, 63*). Mutation studies have suggested the importance of certain cysteine residues such as Cys151, Cys273, and Cys288 for the interaction of Keap1 and Nrf2 (*64*). Holland et al. recently reported that S-glutathionylation of Cys434 and Cys368 may cause marked alterations in the structure of Keap1 at the interface of the Nrf2-binding domain (*65*). Further study is needed to clarify the functional impact of thiol modification at cysteines other than Cys151, Cys273, and Cys288 in Keap1.

Similar regulatory mechanisms of inhibition of protein−protein interactions may also operate in stabilization of hypoxia-inducible factor 1 (HIF-1), a master transcriptional factor (*66*). HIF-1 is a heterodimeric protein that consists of the constitutively expressed HIF-1β/ARNT subunit and the highly regulated HIF-1α subunit (*67*). The overall activity of HIF-1 is determined by the intracellular HIF-1α level. Under normoxic conditions, HIF-1α is hydroxylated by oxygen-activated prolylhydroxylases at proline residues 402 and 564 in the oxygen-dependent degradation (ODD) domain (*68, 69*). This hydroxylation drives HIF-1α toward ubiquitylation by E3 ubiquitin protein ligase, which is part of the von Hippel-Lindau (vHL) tumor suppressor protein complex (*70*). Ubiquitylated HIF-1α is then rapidly degraded by the proteasome pathway. Li et al. demonstrated that NO can stabilize HIF-1α protein in cells (*71*). This HIF-1α stabilization is largely mediated by S-nitrosylation of the Cys533 in the ODD domain, which leads to inhibition of binding between the ODD domain and vHL (*71*).

Alternatively, thiol modification can facilitate protein−protein interactions. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a classic glycolytic enzyme, and accumulating evidence has suggested that GAPDH is a multifunctional protein (*72*). In particular, GAPDH plays an important role in regulating cell death mediated by NO. Hara et al. demonstrated that S-nitrosylation of GAPDH facilitates binding to Siah1, an E3 ubiquitin ligase with a nuclear localization signal (*73*). The GAPDH-Siah1 protein complex, in turn, translocates to the nucleus and mediates cell death.

Many proteins contain multiple cysteine residues that can be targets for thiol modifications. This information suggests that a single protein can be controlled in different ways by site-specific modifications. One well-characterized example is thioredoxin-1 (Trx-1). Trx-1 is an oxidoreductase that is essential for regulation of the cellular redox state, and it binds and inactivates apoptotic signal-regulating kinase-1 (ASK1). Haendeler et al. showed that Trx-1 is S-nitrosylated constitutively in endothelial cells at a single allosteric cysteine residue (Cys69) that lies outside the active site (which contains Cys32 and Cys35) and that S-nitrosylation of Cys69 is necessary for its basal antiapoptotic function in endothelial cells (*74*). However, treatment with *S*-nitrosoglutathione can reportedly induce S-nitrosylation of Trx-1 in intact cells and dissociate ASK1, possibly by targeting the active site Cys32 and/or Cys35. In contrast to S-nitrosylation of Cys69, S-nitrosylation of active site cysteine residues can affect apoptosis, perhaps through the release of ASK1 (*75*). Thus, the overall effect of thiol modifications may be regulated in a manner dependent on site-specific modifications, which may be determined by the amount and types of reactive metabolites that can modify thiols.

Table 1 includes other examples of thiol modifications that are not explained here in detail, and readers may refer to articles cited in Table 1 (*76−87*).

NITRIC OXIDE SIGNALING MEDIATED BY 8-NITRO-CGMP

8-Nitro-cGMP participates in the regulation of NO signaling in two ways. This molecule retains the activity of its parental cGMP and it possesses its own unique property: activation of a cytoprotective response. The latter was studied in a murine salmonellosis model (*19*). Production of NO by iNOS reportedly played an important role in cytoprotective responses in this model (*88, 89*), but the molecular mechanism of these responses has remained elusive. As mentioned earlier, 8-nitro-cGMP forms in cells infected with *Salmonella*, so the effect of this molecule in cell culture experiments using *Salmonella*-infected mouse macrophages was examined. 8-Nitro-cGMP clearly induced such a cytoprotective response (*19*). However, 8-bromoguanosine 3′,5′-cyclic monophosphate, a stable analogue of cGMP, did not provide such cytoprotection, which suggests that 8-nitro-

cGMP induces a cytoprotective response independent of cGMP-like activity. In macrophages that were infected with *Salmonella* and/or treated with 8-nitro-cGMP, marked induction of S-guanylation of Keap1 and associated overexpression of Nrf2-regulated cytoprotective genes including heme oxygenase-1 (HO-1) were seen, which may indicate that 8-nitro-cGMP can induce a cytoprotective response by activating the Keap1/Nrf2 system through protein S-guanylation. Similar activation of a cytoprotective response that depends on the Keap1/Nrf2 system was identified in HepG2 cells under conditions of glucose starvation and exposure to hydrogen peroxide (unpublished data). NO-induced activation of Nrf2 system has been reported previously (*78, 90*). Buckley et al. recently demonstrated the occurrence of thiol oxidation on intracellular Keap1 in response to NO donors (*78*). Thus, NO and RNOS exposure may induce several types of thiol modifications in Keap1, including S-guanylation, oxidation, and presumably nitrosylation. Further studies are warranted to clarify relative contribution of those thiol modifications in Nrf2 activation, particularly identification of modification sites and their impact on Keap1 function.

Consistent with those in vitro experiments, *Salmonella* infection of mice significantly increased the formation of 8-nitro-cGMP in the liver, with colocalization of HO-1 upregulation (*19*). Pharmacological intervention with an HO-1 inhibitor (pegylated zinc protoporphyrin IX) (*91−93*) further supported the importance of HO-1 upregulation in cytoprotective signaling mediated by NO in salmonellosis.

With regard to the regulation of vascular tone, 8-nitro-cGMP has unique biphasic effects: vasoconstriction at low concentrations and vasorelaxation at high concentrations (*16*). The vasorelaxation effect is mediated by the mechanism identical to that of the parental cGMP, which involves activation of cGMP-dependent protein kinase. However, vasoconstriction is caused by ROS formation secondary to NOS uncoupling (*41, 94*) caused by 8-nitro-cGMP (*16*).

Although the metabolic fate of 8-nitro-cGMP needs elucidation, this molecule may not undergo conventional cGMP metabolism because of its resistance to PDEs. PDEs are enzymes that catalyze hydrolysis of the 3′,5′-cyclic monophosphate moiety of cyclic nucleotides including cGMP and adenosine 3′,5′-cyclic monophosphate to form 5′-monophosphates of corresponding nucleotides (*95*). PDEs constitute members of a 21-gene family that are grouped into 11 different primary isoenzymes (with a total of 48 isoforms) on the basis of substrate affinity, selectivity, and regulation mechanisms (*95*). Of these enzymes, PDE5, PDE6, and PDE9 are highly selective for cGMP; PDE1, PDE2, and PDE11 have dual substrate affinity (*95*). In vitro experiments revealed that 8-nitro-cGMP was resistant to hydrolytic activity of PDE1 and PDE5 (*16*).

The GSH-dependent detoxification pathway may function as an alternative metabolic route for 8-nitro-cGMP. GSH is the most abundant nonprotein thiol in cells (*96, 97*). GSH protects cells against endogenous and exogenous toxins including ROS and RNOS (*96, 97*). ROS and RNOS are removed via nonenzymatic reduction with GSH. Conjugation of GSH with electrophilic compounds, in some cases, mediated the glutathione-S-transferases, and subsequent excretion of those conjugates from the cells also serves to regulate the levels of those electrophilic compounds (*96*). Our previous observation suggested that cellular formation of 8-nitro-cGMP was regulated partially by GSH (*16*). For example, depletion of cellular GSH by using an inhibitor for GSH biosynthesis, L-buthionine sulfoximine, resulted in moderate increase of the cellular 8-nitro-cGMP levels (*16*). This limited effect of GSH on 8-nitro-cGMP levels may be due to the moderate reactivity of 8-nitro-cGMP with GSH as mentioned above. In addition, our preliminary data shows that 8-nitro-cGMP is insensitive to glutathione-S-

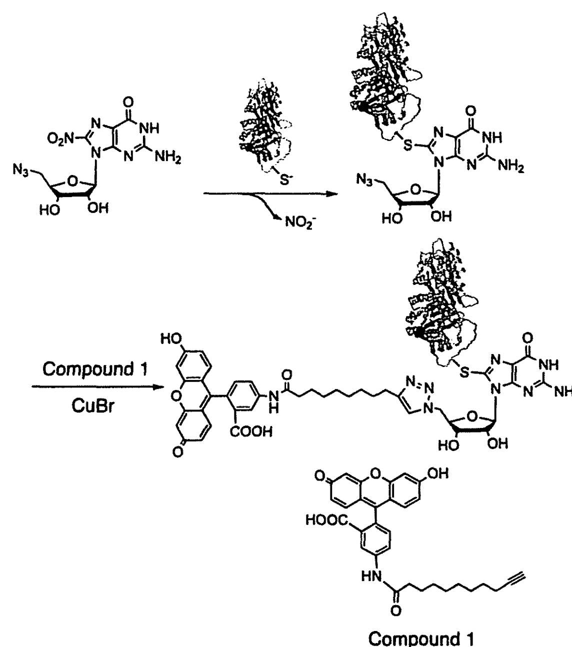

Figure 6. Click chemistry-based fluorescent labeling at sites of protein S-guanylation. 5′-Azido derivatives of 8-nitroguanosine undergo S-guanylation to introduce the azide group into proteins, with subsequent conjugation to fluorophores via the Huisgen reaction.

transferase-mediated glutathione conjugation (unpublished observation). These results are consistent with the finding that 8-nitro-cGMP gradually accumulates in cells during a long time period (~11 h) after exposure to NO from endogenous and exogenous sources (*16*). Thus, with respect to temporal regulation, NO signaling mediated by 8-nitro-cGMP and NO signaling mediated by cGMP may differ. All these data together indicate that nitration of cGMP brings unique chemical and biological features to 8-nitro-cGMP including electrophilicity, redox activity, and PDE resistance, which establishes this molecule as a new cGMP messenger.

METHODOLOGIES TO DETECT PROTEIN S-GUANYLATION

Elucidation of temporal and spatial regulation of the formation of 8-nitro-cGMP and associated protein S-guanylation is important for understanding how protein S-guanylation contributes to the regulation of NO signaling. Anti-8-nitroguanine monoclonal antibodies were developed and used to localize the cellular formation of 8-nitroguanine derivatives. Among these antibodies, clone 1G6 exhibits the highest specificity against 8-nitro-cGMP compared with other 8-nitroguanine derivatives, with immunoreactivity decreasing in the order 8-nitro-cGMP > 8-nitroguanosine, 8-nitroguanosine 5′-monophosphate > nitrated RNA > 8-nitroguanosine 5′-triphosphate; no reactivity against 8-nitroguanine and 8-nitroxanthine was observed (*16*). Confocal laser microscopic analysis with the use of clone 1G6 and organelle-specific markers revealed immunostaining for 8-nitro-cGMP (1G6) mainly in the cytosol, with colocalization partly with mitochondria rather than endoplasmic reticulum (*16*). Mitochondrial localization of this nitrated derivative may implicate 8-nitro-cGMP in regulation of mitochondrial functions such as energy metabolism and cell death regulation, and further study related to this issue is ongoing.

Anti-8-thioalkoxy-cGMP antibodies that recognize the S-guanylation moiety have also been developed to detect protein S-guanylation by means of immunohistochemistry, Western blotting, and immunoaffinity purification coupled with chromatography (*16*). Western blotting of cell lysates obtained from

macrophages infected with *Salmonella* revealed multiple reactive bands except for S-guanylated Keap1 (*16*). Further characterization, via mass spectrometry, of the S-guanylation proteome is currently underway.

Appropriately labeled chemical probes are powerful tools that complement specific antibodies. Most important, the low molecular weight of the probes results in excellent membrane permeation that allows imaging of protein S-guanylation in living cells (*18*). We developed an azido derivative of 8-nitroguanosine for use in click chemistry-based fluorescent labeling of S-guanylated proteins (*18*) (Figure 6). This 5′-azido analogue efficiently upregulated HO-1 in a manner similar to that of 8-nitro-cGMP, which suggests that the azido analogue induces Keap1 S-guanylation and subsequently activates Nrf2 signaling. Proteins that are S-guanylated by this azido analogue can be labeled with fluorophore by means of click chemistry and can thus readily be detected by fluorescence scanning of electrophoresed gels. Prelabeling with a series of fluorophores is also possible with the azido derivatives, and the labeled 8-nitroguanosines have been shown to be incorporated into cells (unpublished data). This technique may allow analysis of the formation, localization, and metabolism of S-guanylated proteins in living cells with the use of organelle-specific markers and confocal microscopy.

CONCLUSIONS

Redox-sensitive protein thiols can become targets for chemical modifications caused by NO, RNOS, and electrophiles derived from reactions between RNOS and biomolecules, the result being formation of various protein thiol conjugates. Although those covalent modifications were previously regarded as nonspecific injurious changes to protein functions associated with oxidative stress, those modifications were recently recognized as involved in a critical fashion in regulation of diverse biological signaling processes (such as redox signaling). As mentioned earlier, chemical modification of redox-sensitive thiols affects protein functions through various mechanisms including modification of active center thiols, alteration of protein structure by allosteric effects, and modulation of protein−protein interactions, which in turn contribute to regulation of NO signaling. The total number of cysteine residues encoded in the human genome is estimated at approximately 214 000 (*49*). Among them, 21 000−42 000 residues (10−20% of the total) may be redox-sensitive (*49*). Thus, a number of proteins that possess redox-sensitive thiols remain to be discovered as redox-sensing targets in regulation of redox signaling.

The redox microenvironment that affects thiol reactivity may differ in each cell compartment. Hansen et al. reported that the relative redox states of these different compartments, which can be determined via the ratio of free thiols to thiols forming disulfide, from most reducing to most oxidizing are mitochondria > nuclei > cytoplasm > endoplasmic reticulum > extracellular space (*98*). In this context, mitochondrial proteins are assumed to be important targets of redox-dependent signaling through thiol modifications. Recent development of mass spectrometry-based proteomics may provide a powerful tool for identification of novel redox-sensitive protein targets and regulation of thiol modifications in each organelle. In fact, dozens of proteins that undergo S-nitrosylation and S-glutathionylation have been discovered (*14, 99*). Further analysis is now warranted to achieve comprehensive understanding of target proteins for S-alkylation reactions including S-guanylation and S-nitroalkylation.

Protein thiol modification is critically important in the regulation of physiological signaling. Impaired regulation of this modification, which results mainly from overproduction of reactive substances such as RNOS and ROS that are associated with oxidative stress, may contribute to development of diseases via disruption of well-organized signaling networks involving protein thiol modification (*100, 101*). Better understanding of the molecular mechanisms by which redox-sensitive thiol modifications regulate cell signaling will provide the basis for novel therapeutic approaches to restore normal signaling and control and thereby prevent pathological and toxicological consequences of oxidative stress.

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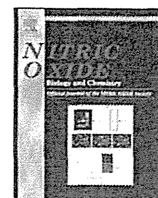

One-step preparation of S-nitrosated human serum albumin with high biological activities

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ABSTRACT

S-Nitrosated human serum albumin (SNO-HSA) is a large molecular weight nitric oxide carrier in human plasma, and because of its many beneficial effects in different tests, it is currently under investigation as a cytoprotective agent. However, making SNO-HSA preparations is a complicated and time-consuming process. We found that binding of caprylic acid (CA) and N-acetyl-L-tryptophan (N-AcTrp) to defatted mercaptalbumin increased S-nitrosation by S-nitrosoglutathione (GS-NO) by making Cys-34 of HSA more accessible and by protecting it against oxidation, respectively. Fortunately, HSA solutions for clinical use contain high concentrations of CA and N-AcTrp as stabilizers. By making use of that fact it was possible to work-out a fast and simple procedure for producing SNO-HSA: incubation of a commercial HSA formulation with GS-NO for only 1 min results in S-nitrosation of HSA. The biological usefulness of such a preparation was tested in a rat ischemia–reperfusion liver injury model. Although our procedure for making SNO-HSA is fast and straightforward, the cytoprotective effect of the preparation was similar to, or better than, that of a preparation made in a more traditional way. The clinical development of SNO-HSA as a strong cytoprotective agent is under way using this method in collaboration with clinicians and industrial developers.

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Introduction

Post-translational modifications are essential in their functional regulation. Among these, changes of the redox state of cysteine residues are of great importance. The sulfhydryl moiety can interact with nitric oxide (NO) and thereby form S-nitrosothiols [1–3]. S-Nitrosothiols may function as NO reservoirs and preserve the antioxidant and other activities of NO [4,5]. For example, it has been reported that S-nitrosated human serum albumin (SNO-HSA) may serve *in vivo* as a circulating reservoir for NO produced by the endothelial cells [6]. The reservoir function was also reported to be operative when application of SNO-HSA to animals suffering from ischemia–reperfusion injury minimized the extent of tissue damage associated with reperfusion [7–9]. Therefore, SNO-HSA is under investigation as a therapeutic agent in humans. However, efficient SNO-HSA preparations are difficult to produce.

HSA is a single, non-glycosylated polypeptide that organizes to form a heart-shaped protein with approximately 67% α-helix but no β-sheet [10]. Except for Cys-34, all of the 35 cysteine residues are involved in the formation of stabilizing disulfide bonds. Cys-34 represents the largest fraction of free thiols in the circulation (mercaptalbumin). The residue is located in "a crevice" on the surface in the N-terminal part of the protein, and such a location can impede the access of S-nitrosating agents to the sulfhydryl group. In addition, normally about half of the Cys-34 residues are oxidized or involved in ligand binding (non-mercaptalbumin), and that kind of hindrance could also make an efficient S-nitrosation difficult.

In previous investigations we found that binding of N-acetyl-L-tryptophanate (N-AcTrp) protects the sulfhydryl group of HSA against oxidation [11], and that binding of oleic acid increases S-nitrosation of HSA [12]. In the present work we found that

Abbreviations: HSA, human serum albumin; SNO-HSA, S-nitrosated HSA; GS-NO, S-nitrosoglutathione; N-AcTrp, N-acetyl-L-tryptophanate; CA, caprylic acid; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HO-1, heme oxygenase-1.

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simultaneous binding of N-AcTrp and caprylic acid (CA) greatly increased *S*-nitrosation of HSA by *S*-nitrosoglutathione (GS-NO) by protecting the sulfhydryl group against oxidation and by making it more accessible to GS-NO, respectively. HSA solutions for clinical use contain high concentrations of just N-AcTrp and CA, and we took advantage of that fact to work-out a simple, one-step method to make a biological active and stable SNO-HSA preparation which most probable also can be of clinical use, see the illustrative overview in Fig. 1.

Experimental procedures

Materials

HSA solutions (20%) were donated by the Chemo-Sera-Therapeutic Research Institute (Kumamoto, Japan). As stabilizers the formulation contained N-AcTrp (6.2 mol/mol HSA) and CA (5.3 mol/mol HSA). Part of the albumin was defatted by treatment with charcoal as described by Chen [13], dialyzed against deionized water, freeze-dried, and then stored at −20 °C until used. According to density analysis of Coomassie Brilliant Blue (CBB)-stained protein bands on 12.5% SDS–PAGE, the purity of albumin in the original solutions and of the defatted samples was more than 97%. N-AcTrp, CA and 1,4-dithiothreitol (DTT) were purchased from Sigma–Aldrich (St. Louis, MO). Sulfanilamide, naphthylethylenediamine-hydrochloride, $HgCl_2$, $NaNO_2$ and $NaNO_3$ were obtained from Nakalai Tesque (Kyoto, Japan). GS-NO, 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) and diethylenetriaminepentaacetic acid (DTPA) were obtained from Dojindo Laboratories (Kumamoto, Japan). Other chemicals were of the best grades commercially available, and all solutions were made in deionized water. Sephadex G-25 desalting column (ϕ 1.6 × 2.5 cm) was from Amersham Pharmacia Biotech (Tokyo, Japan).

Preparation of HSA-ligand solutions

First, Cys-34 of defatted HSA was reduced by treatment with DTT as follows. HSA (300 μM) was incubated with DTT (molar ratio, protein:DTT = 1:10) for 5 min at 37 °C. After incubation, DTT was quickly removed by Sephadex G-25 gel filtration using 10 mM phosphate-buffered saline (pH 7.4) (PBS; Ca^{2+}, Mg^{2+} free). We checked that the final SH/HSA ratio was less than 1 by using the DTNB assay described below. Stock-solutions of 20 mM CA and 20 mM N-AcTrp were made in methanol–H_2O (1:1, v/v) and H_2O, respectively. Later, these stock-solutions were diluted with PBS. In all cases, the resulting solutions were mixed with PBS containing HSA. The final methanol concentrations in the CA-containing solutions were 0.75% or less. The protein content of all protein preparations used in this study was determined by the Bicinchoninic acid protein assay.

Accessibility of Cys-34 in the presence and absence of ligands

DTT-treated HSA (100 μM) was incubated with 0.5 mM Ellman's reagent, DTNB, for 60 min at room temperature. During incubation, the absorbance of the samples was monitored at 405 nm. The accessibility was evaluated as A_5/A_{60}, where A_5 and

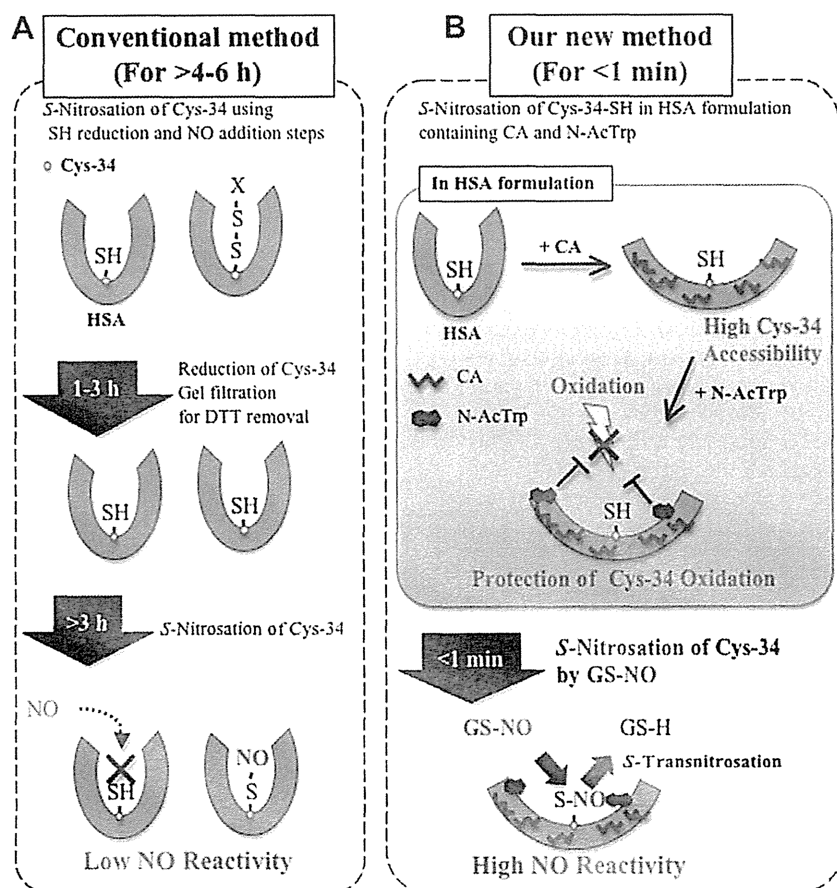


Fig. 1. Mechanism of a novel SNO-HSA preparation strategy using HSA formulation and GS-NO. The conventional method (A) contains two time-consuming steps (SH reduction and NO addition), which take more than 4–6 h to complete. On the other hand, our new method (B) contains only one step (GS-NO incubation), which takes less than 1 min. In case of our method, CA and N-AcTrp contained in HSA formulations play the roles of not only stabilizers but also as a Cys-34 accessible inducer and a Cys-34 oxidation inhibitor, respectively. The presence of these two ligands makes it possible to *S*-nitrosate HSA within 1 min.

A_{60} are the sample absorbance after 5 and 60 min (maximal absorbance), respectively, of incubation with DTNB [14].

SH content of Cys-34 in the presence and absence of ligands

The amount of free SH-groups was also estimated with Ellman's reagent, DTNB. Briefly, HSA (100 μM) was incubated with 0.5 mM DTNB for 60 min at room temperature, and afterwards the absorbance at 405 nm was measured. The thiol concentration was calculated from a standard curve prepared with cysteine.

Oxidation of Cys-34 and protein polymerization by heat treatment in the presence and absence of ligands

DTT-treated HSA (100 μM) without or with ligand (5 mol/mol HSA) was incubated in PBS (pH 7.4) for 15 or 60 min at 60 °C. The mercaptalbumin fraction was determined from a standard curve prepared with cysteine using the DTNB assay. Any changes in molecular weight and aggregation of heated HSA samples were visualized by non-reduced 10% SDS–PAGE with 3 μg of protein in each lane.

S-Nitrosation of HSA in the presence and absence of ligands

SNO-HSA was prepared with protection against light and according to previous reports [15,16]. Briefly, DTT-treated HSA (100 μM) without or with ligand (5 mol/mol HSA) or the original solution from Chemo-Sera-Therapeutic Research Institute was incubated with GS-NO as NO donor (molar ratio, protein:NO donor = 1:5) in PBS for 1, 15 or 60 min at 37 °C. To remove the NO donor, the *S*-nitrosated products were applied to a Sephadex G-25 column, eluted with PBS containing 0.5 mM DTPA, and concentrated by ultrafiltration. These samples were stored at −80 °C until analyzed. For the *in vivo* ischemia–reperfusion studies, the SNO-HSA samples with and without ligand were adjusted to the same *S*-nitrosation efficiency by means of different incubation times with GS-NO. Actually, the HSA samples with or without ligand were incubated with GS-NO for 1 or 60 min, respectively.

Determination of S-nitrosation efficiency

The amounts of the *S*-nitroso moiety of SNO-HSA were quantified by HPLC coupled with a flow-reactor system, as previously reported [15,17]. The HPLC column was a gel filtration column for *S*-nitrosated proteins (ϕ 8 × 300 mm), Diol-120, YMC, Kyoto, Japan. Briefly, the eluate from the HPLC column was mixed with a $HgCl_2$ solution to decompose SNO compounds to yield NO_2^- (via NO^+). The NO_2^- generated was then detected after reaction with Griess reagent in the flow-reactor system.

CD spectroscopy

CD spectra were measured at 25 °C using a J-720-type spectropolarimeter (JASCO, Tokyo, Japan). Far-UV and near-UV spectra were recorded at protein concentrations of 5 and 15 μM, respectively, in 20 mM sodium phosphate buffer (pH 7.4). For calculation of mean residue ellipticity, [θ], the molecular weight of the albumins was taken as 66,500.

Cytoprotective effect of SNO-HSA in vivo

A rat ischemia–reperfusion liver injury model served for investigation of the cytoprotective effect of SNO-HSA, according to a previous report [16]. Male Wistar rats weighing between 200 and 230 g (Kyudo, Inc., Kumamoto, Japan) were used. The animals were fasted for 9 h before surgery but were allowed access to water. The rats were anesthetized with ether during the operation. After the abdomen was shaved and disinfected with 70% ethanol, a complete midline incision was made. The portal vein and hepatic artery were exposed and cross-clamped for 30 min with a noncrushing microvascular clip. Saline, as the vehicle control, GS-NO or DTT-treated HSA without or with *S*-nitrosation or the original formulation without or with *S*-nitrosation (0.1 μmol protein (0.03 μmol SNO)/rat) were given via the portal vein immediately after reperfusion was initiated. Then the abdomen was closed in two layers with 2–0 silk. The rats were kept under warming lamps until they awakened and became active. Because blood loss caused by frequent blood sampling could affect liver functions, the animals were sacrificed by taking whole circulating blood via abdominal aorta under anesthesia at various time points after reperfusion was initiated. Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were measured by using a sequential multiple AutoAnalyzer system from Wako Chemicals, with activities expressed in international units per liter. All animal experiments were carried out according to the guidelines of the Laboratory Protocol of Animal Handling, Graduate School of Medical Sciences, Kumamoto University.

Purification of intracellular proteins and Western blot analysis

Frozen liver samples, originally taken 6 h after hepatic ischemia–reperfusion, were homogenized in a lysis buffer (50 mM HEPES, 5 mM EDTA, and 50 mM NaCl; pH 7.5) containing protease inhibitors (10 mg/mL aprotinin, 1 mM PMSF, and 10 mg/mL leupeptin). Supernatant was taken after centrifugation at 15,000*g*. Equal amounts of protein from the supernatant were loaded onto 12.5% SDS–PAGE gels and blotted onto polyvinylidine difluoride membranes (Millipore). Polyclonal antibody against rat heme oxygenase-1 (HO-1; StressGen) and horseradish conjugated secondary antibody (Jackson ImmunoResearch) were used at 1:2000 and 1:5000 dilutions, respectively. The ECL Western blotting system (Amersham Life Sciences) was used for detection. Bands were visualized and quantified using a lumino-analyzer (LAS-1000, Fuji photo Film). Density of the bands for HO-1 and β-actin was quantitatively analyzed using the NIH Image J Software.

Statistical analysis

The statistical significance of collected data was evaluated using the ANOVA analysis followed by Newman–Keuls method for more than 2 means. Differences between groups were evaluated by the Student's *t* test. $P < 0.05$ was regarded as statistically significant.

Results

Effect of ligand binding on the SH-accessibility of Cys-34

The effect of CA and/or N-AcTrp binding on the SH-accessibility of defatted, DTT-treated HSA was evaluated by the DTNB assay. Binding of 1–5 mol CA/mol HSA increased significantly and in a dose-dependent manner the accessibility of the SH-group (Fig. 2A). By contrast, addition of 5 mol of N-AcTrp per mol of HSA had no significant effect on SH-accessibility (Fig. 2B). Furthermore, addition of N-AcTrp had no influence on the improving effect of CA (Fig. 2B).

Protective effect of ligands on oxidation and aggregation of mercaptalbumin exposed to heat treatment

Defatted, DTT-treated HSA without and with ligands (N-AcTrp and/or CA) was incubated at 60 °C. Heat incubation was used, be-

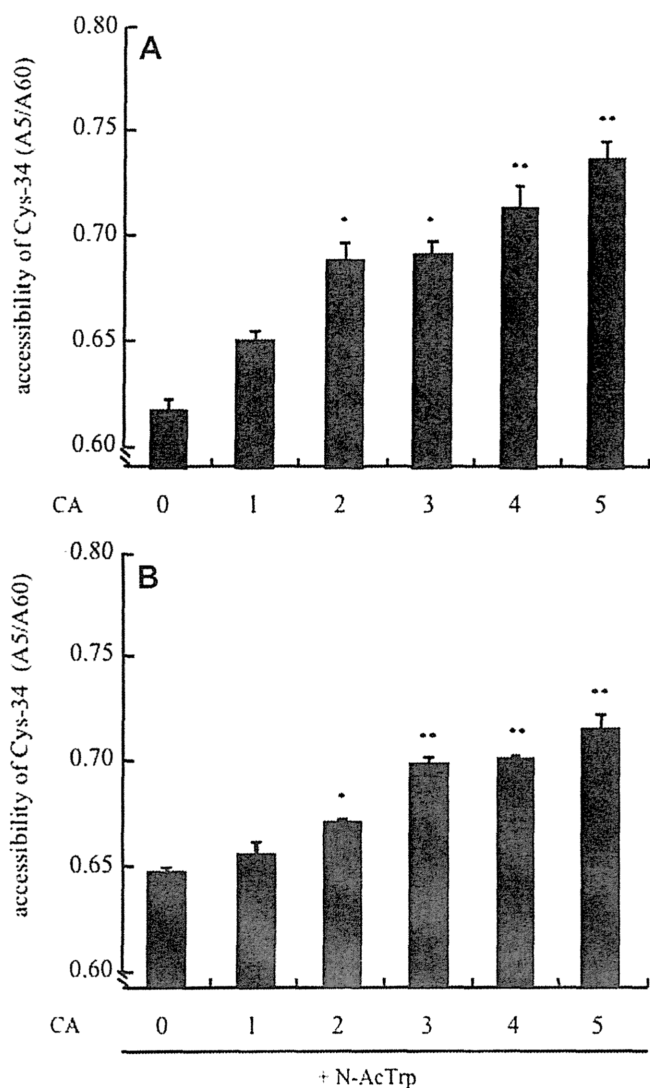


Fig. 2. Effect of ligand binding on the SH-accessibility of Cys-34. The effect of CA (CA/HSA = 0, 1, 2, 3, 4, 5) on the SH-accessibility of defatted, DTT-treated HSA was studied in the absence (A) and presence of N-AcTrp (N-AcTrp/HSA = 5) (B). DTT-treated HSA (100 μM) was incubated with 0.5 mM Ellman's reagent, DTNB, for 60 min at room temperature. The accessibility was evaluated as the ratio of DTNB absorbance at 405 nm after 5 and 60 min of incubation (A_5/A_{60}). A_5 and A_{60} are the sample absorbance at 405 nm after 5 min (initial rate period) and 60 min (maximal absorbancy), respectively, of incubation with DTNB. Data are expressed as means ± SEM (n = 4). *, $p < 0.05$, **, $p < 0.01$, compared with control (CA = 0).

cause in this way we could study both thiol oxidation and protein aggregation [11,18,19]. From Table 1 it is seen that N-AcTrp is an effective protector of the sulfhydryl group against heat-facilitated oxidation; less than 10% of the groups have been oxidized after 60 min of incubation. By contrast, binding of CA increases oxidation; after 60 min of heat treatment ca. 75% of Cys-34 have been oxidized, whereas only ca. 35% of the groups have been modified in the absence of ligands. Finally, simultaneous binding of CA diminishes somewhat the protective effect of N-AcTrp. The increasing effect of CA on oxidation is most probably due to the fact that CA binding increases the accessibility of Cys-34 (see above). Often oxidation of cysteine residues is accompanied by protein aggregation. Whether or not this has taken place in our heat-treated HSA samples was analyzed for by using non-reduced SDS–PAGE (Fig. 3). Lane 1 shows no clear presence of light or heavy aggregated HSA in the non-heated sample. Heating for 15 min at 60 °C actually results in formation of both types of aggregations (lane 2), and heating for 60 min results in a major fraction of heavy aggregation (lane 6). Addition of N-AcTrp diminishes aggregation (lanes 3 and 7); especially the formation of heavy aggregation is strongly depressed. By contrast, binding of CA has no inhibitory effect on aggregation (lanes 4 and 8). Interestingly, apparently CA has no influence on the protective effect of N-AcTrp (lanes 5 and 9). Thus, N-AcTrp, but not CA, minimizes HSA aggregation and protects Cys-34 against oxidation.

Table 1
Mercaptalbumin ratio (% of total albumin) after oxidation by heat incubation.

Heal incubation time (mm)	0	15	60
HSA alone	82.0 ± 2.4	75.6 ± 3.1	54.3 ± 3.9
HSA with N-AcTrp	82.0 ± 2.4	77.5 ± 2.6	74.6 ± 2.6**
HSA with CA	82.0 ± 2.4	21.3 ± 5.9*	19.4 ± 8.4**
HSA with CA and N-AcTrp	82.0 ± 2.4	63.9 ± 4.0*	61.0 ± 5.0*

Results are given as means ± SEM (n = 6).
* $P < 0.05$ as compared with HSA alone.
** $P < 0.01$ as compared with HSA alone.

Improving effects of ligand binding on S-nitrosation of HSA

Now, we investigated whether an increased accessibility to Cys-34 (caused by CA binding) and/or protection of the residue against oxidation and aggregation (caused by N-AcTrp binding) can promote *S*-nitrosation of HSA. Incubation of defatted, DTT-treated HSA alone with GS-NO for 60 min resulted in the formation of 0.31 mol *S*-nitroso moieties per mol of HSA (Table 2). This value, and those determined at 1 and 15 min of incubation, is not changed by addition of N-AcTrp. By contrast, at all incubation times CA significantly increased *S*-nitrosation. Simultaneous addition of both ligands increased this effect further, and after 1 min of incubation the number of *S*-nitroso moieties (0.42 mol SNO/mol HSA) is even higher than that obtained after 60 min of HSA without ligand. Thus, co-binding of CA and N-AcTrp results in efficient and fast *S*-nitrosation of HSA. HSA formulations for clinical applications usually contain high concentrations of CA and N-AcTrp for stabilizing and protecting the protein against oxidation during the heat treatment performed for destroying potential contamination with

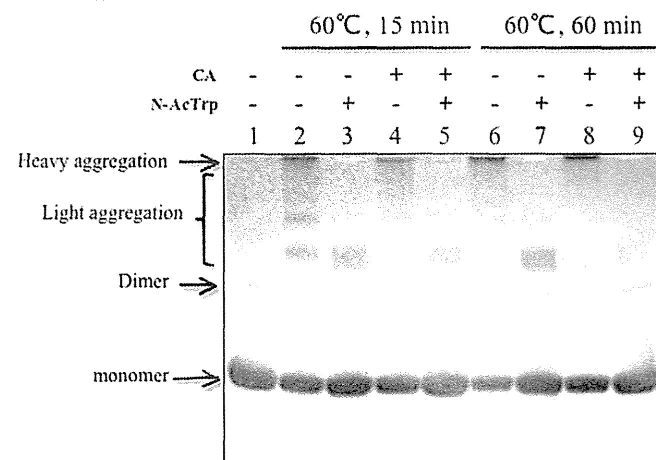


Fig. 3. Effect of ligands on aggregation of heat-treated mercaptalbumin. Defatted, DTT-treated HSA without or with ligand (5 mol/mol HSA) was incubated in PBS (pH 7.4) for 15 or 60 min at 60 °C. Afterwards, protein samples (3 μg) were analyzed by non-reduced 10% SDS–PAGE; the gel was stained by CBB. Lane 1 represents non-heated HSA. The arrows indicate heavy aggregation, light aggregation, dimer and monomer from the top.

Table 2
S-Nitroso moieties (SNO mol/mol USA) after incubation with GS-NO.

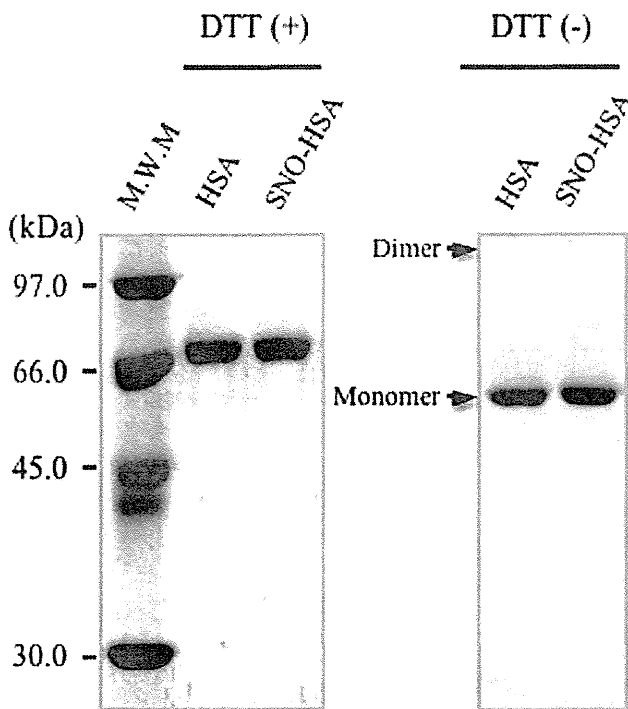
GS-NO incubation time (min)	0	1	15	60
HSA alone	0.00 ± 0.00	0.06 ± 0.03	0.12 ± 0.03	0.31 ± 0.03
HSA with N-AcTrp	0.00 ± 0.00	0.08 ± 0.02	0.13 ± 0.02	0.33 ± 0.06
HSA with CA	0.00 ± 0.00	0.29 ± 0.06**	0.31 ± 0.06**	0.41 ± 0.08**
HSA with CA and N-AcTrp	0.00 ± 0.00	0.42 ± 0.09**	0.45 ± 0.10**	0.51 ± 0.09**
HSA formulation	0.00 ± 0.00	0.35 ± 0.05**	0.41 ± 0.10**	0.45 ± 0.09**

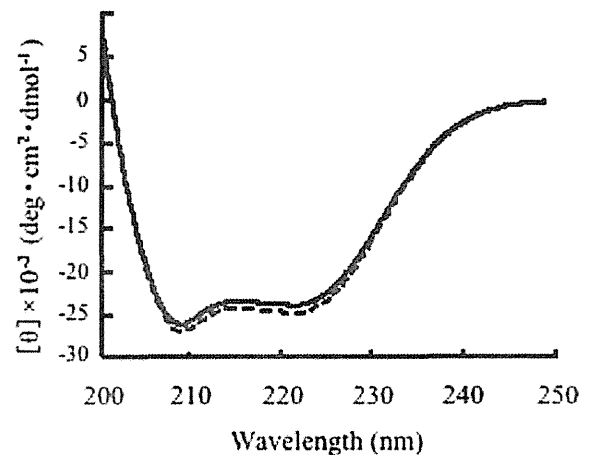
Results are given as means ± SEM (n = 3–10).
** $P < 0.01$, as compared with HSA alone.

viral or other biological agents [11]. We used the solutions from Chemo-Sera-Therapeutic Research Institute as an illustrative example, and this formulation has 5–6 mol of each of the two ligands per mol of HSA. Furthermore, it has a high percentage of mercaptalbumin, i.e. 71 ± 8% (n = 6). Therefore, we investigated whether a simple incubation of the formulation with GS-NO would result in significant amounts of SNO-HSA. As seen from Table 2, this one-step procedure also resulted in an efficient and fast S-nitrosation of HSA.

Physicochemical properties and stability of S-nitrosated HSA

According to reduced and non-reduced SDS–PAGE, the one-step S-nitrosation of formulation HSA did not result in the formation of dimer via disulfide bond formation, fragmentation or other gross conformational changes (Fig. 4A). Any minor conformational changes were examined for by using circular dichroism spectroscopy. The far-UV spectra (Fig. 4B) and the near-UV spectra (Fig. 4C) show that S-nitrosation of Cys-34 had no evident effect on the secondary or tertiary structure of HSA. Previous examinations with non-reducing SDS–PAGE revealed that S-nitrosation of defatted, DTT-treated HSA only results in, if any, changes in the tertiary structure of the protein [9]. Thus, our data propose that S-nitrosation does not induce significant conformational changes in any of our protein preparations. The stability of SNO-HSA with 5 mol of CA and N-AcTrp per mol of protein was tested in two different ways. First, the half-life of the S-nitroso moiety of SNO-HSA with the ligands was 50 days in phosphate buffer, pH 7.4, in the dark. Without the ligands the half-life was only 25 days. Second, lyophilization with CA and N-AcTrp resulted in only a slight decrease (10%) in S-nitroso content. Thus, SNO-HSA with CA and N-AcTrp is very stable.





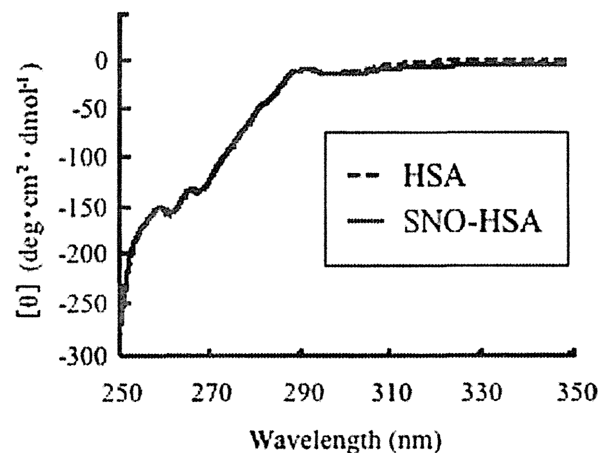


Fig. 4. Structural integrity of HSA and SNO-HSA. (A) Reduced and non-reduced SDS–PAGE of HSA and SNO-HSA. 1 μg of protein was added to each lane, and the gel was stained by CBB. Molecular mass markers are indicated at the left of the gel. B and C show far-UV and near-UV CD spectra, respectively, of HSA and SNO-HSA. The proteins were unmodified and S-nitrosated HSA formulation.