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Nanomolar Quantification and Identification of Various Nitrosothiols by High Performance Liquid Chromatography Coupled with Flow Reactors of Metals and Griess Reagent[1]

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Nitrosothiols (RS-NOs) appear to be critically involved in various signal transduction mechanisms. We describe here a specific and highly sensitive quantification method for RS-NOs by using high performance liquid chromatography (HPLC) combined with a flow reactor system. RS-NOs were applied to an HPLC system of C_{18}-reverse phase or a gel filtration column and eluted with 10 mM sodium acetate buffer (pH 5.5) plus 0.5 mM diethylenetriamine pentaacetic acid with or without either 0-7% methanol or 0.15 M NaCl. The eluate from the HPLC column was mixed with a solution containing 1.75 mM $HgCl_2$ or 1.75 mM $CuSO_4$ for RS-NO decomposition in a reaction coil *via* a three-way connector. NO_2^- generated *via* the metal-induced RS-NO decomposition was then reacted with Griess reagent, which was infused through a second three-way connector, yielding a diazo-compound detected at 540 nm. In a separate experiment, a copper particle-loaded column was used for RS-NO degradation instead of the metal-ion flow reactor. In all RS-NOs tested, *i.e.*, nitrosoglutathione (GS-NO), nitroso-L-cysteine, and nitrosoalbumin, the nitroso-group was converted to NO_2^- by the Hg^{2+}-reaction system as well as copper-loaded column, and the recovery was almost 100%. The Cu^{2+}-solution flow reaction system, however, yielded only 30% recovery of RS-NOs as NO_2^-. Also, the RS-NOs could be identified at nanomolar concentrations: detection limit, 3.0 nM in a 150-μl aliquot. These RS-NOs showed well-resolved elution profiles even in the presence of NO_2^- and NO_3^-. More importantly, biological generation of GS-NO was quantitatively demonstrated with RAW264 cells in culture incorporating free GSH in the medium. In conclusion, our novel RS-NO assay will be useful to examine the formation and functions of RS-NOs in biological systems.

Key words: HPLC-flow reactor, nanomolar quantification, nitrosothiols, nitrosoglutathione formation.

Nitric oxide (NO) exhibits a diverse array of physiological functions such as neuronal signal transduction, regulation of vascular tone, and antimicrobial effects (*1*). One of its most interesting chemical properties is that it is converted into different redox forms of nitrogen monoxide, *i.e.*, $^{\bullet}NO$, nitrosonium cation (NO^+), and nitroxyl anion (NO^-) (*2-5*). It seems that the nitrosonium cation, existing eventually as an adduct of thiol compounds (nitrosothiols or thionitrites, RS-NOs) in biological systems, plays an important role in NO-mediated signalling cascades (*6*) such as downregulation of *N*-methyl-D-asparate receptor (*7*) and regulation of transcriptional factors (*8*), and it may be involved in non-adrenergic and non-cholinergic neuronal (NANC) responses (*9*). Therefore, it is critically important to specifically identify and quantify various nitrosothiols such as nitrosoglutathione (GS-NO), nitroso-L-cysteine (Cys-NO), and nitrosated proteins, *e.g.*, *S*-nitrosoalbumin and *S*-nitrosohemoglobin, generated in biological systems.

Several RS-NO measurement procedures have been reported that use special detection systems. The most traditional and conventional method, described by Saville in 1958 (*10*), is to assay RS-NO by measuring NO_2^- generated from it by Hg^{2+} solution with Griess reagent (*11*), after RS-NO is decomposed to NO^+, followed by HNO_2 (NO_2^-) formation with OH^-, through a simple and quantitative reaction of Hg^{2+} with RS-NO. However, for RS-NO determination in biological materials, Saville's method (a batch method) has a number of limitations, not only in its sensitivity but also in the specificity to differentiate between RS-NO molecular species, because the RS-NO reacts with Hg^{2+} solution in a mixture of NO_2^-/

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Abbreviations: NO, nitric oxide; RS-NO, nitrosothiol; GS-NO, nitrosoglutathione; Cys-NO, nitroso-L-cysteine; *S*-NO-BSA, *S*-nitrosobovine serum albumin; iNOS, inducible nitric oxide synthase; DTPA, diethylenetriaminepentaacetic acid; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); PCMB, *p*-chloromercuribenzoate; SOD, superoxide dismutase; L-NMMA, N^{ω}-monomethyl-L-arginine; KRP, Krebs-Ringer-phosphate buffer.

NO_3^- and various interfering substances which are often present in biological samples.

Analytical techniques with sufficient sensitivity to allow measurement of various RS-NOs separately have recently been reported by Loscalzo's group (*12*). Each RS-NO species is initially separated by high performance liquid chromatography (HPLC), then the peak of RS-NO eluted is identified either by chemiluminescence for NO formed *via* the photolytic decomposition of RS-NO, or by use of an electrochemical (EC) detector (*12*). However, these detection systems are complicated, and their responses are not necessarily specific for RS-NOs. For instance, not only RS-NO but also nitrosyl iron complexes such as nitrosyl hemoglobin (NO-Hb) and dinitrosyl iron sulfur complex may release NO on photolysis. EC detection will also bring about similar nonspecific responses. Therefore, a convenient and truly specific method for detection of RS-NOs is yet to be established.

Here we describe a novel quantification system using HPLC combined with highly sensitive and specific determination techniques for RS-NOs. In this system, metal-catalyzed and Griess reagent flow reactors are serially connected to HPLC columns, and thus RS-NOs of various molecular sizes are converted to NO^+ or NO_2^- through the rapid and quantitative metal-catalyzed reactions with Hg^{2+} or Cu^{2+} (Cu^{1+}), followed by peak detection with Griess reagent. Moreover, each RS-NO is identifiable in the nanomolar range after separation through HPLC: the detection limit, 3 nM, is more than 10-fold lower than with the previous methods (*12*). The present analytical method will be useful to obtain better understanding of physiology of RS-NOs in a variety of biological phenomena involving NO.

MATERIALS AND METHODS

Chemicals—GS-NO, Cys-NO, diethylenetriaminepentaacetic acid (DTPA), propylamine NONOate (NOC-7), and 5,5′-dithiobis[2-nitrobenzoic acid] (DTNB) were obtained from Dojindo Laboratories, Kumamoto. *S*-Nitroso-bovine serum albumin (*S*-NO-BSA) was prepared by using isoamyl nitrite with dithiothreitol (DTT)-treated bovine serum albumin (BSA) according to the previous method (*13*). The propylamine NONOate spontaneously releases NO (2 mol of NO per mol of NONOate; $T_{1/2}$, 5 min) in neutral solution (*14*). $HgCl_2$, $CuSO_4$, $NaNO_2$, $NaNO_3$, naphthylethylenediamine-HCl, sulfanilamide, and BSA (crystal), were purchased from Nacalai Tesque, Osaka. Reduced form of GSH, *p*-chloromercuribenzoate (PCMB) and Cu,Zn-superoxide dismutase (SOD) from bovine erythrocytes, were obtained from Sigma Chemicals, St. Louis, MO, USA. BSA was further purified by gel filtration column chromatography using Sephadex G-100 (Pharmacia, Uppsalla, Sweden), before nitrosation with isoamyl nitrite or NO. N^{ω}-Monomethyl-L-arginine (L-NMMA) was a product of Calbiochem., La Jolla, CA, USA. Murine interferon-γ (IFN-γ) was purchased from Genzyme, Cambridge, MA, USA, and lipopolysaccharide (LPS: *Escherichia coli* 026B) was a product of Difco Laboratories, Detroit, MI, USA. Highly pure copper metal was produced by electroplating with Cu^{2+} ion ($CuSO_4$) solution and used for preparation of the copper-loaded column. The purities of GS-NO, Cys-NO, and *S*-NO-BSA were determined by Saville's method (*10*). Briefly, the amount of NO_2^- formed by the reaction of various RS-NOs with $HgCl_2$ was quantified by an NO_2^-/NO_3^- analyzer (Eicom, Kyoto). The protein and thiol contents in each protein preparation were determined by Lowry's method (*15*) and a colorimetric analysis for free thiols with DTNB (*16*), respectively. Other chemicals were of the highest purity commercially available.

HPLC Analysis and RS-NO Detection—RS-NO measurement was performed by using an HPLC column combined with a flow-reactor system for specific detection of RS-NOs with Griess reagent as shown in Fig. 1. An aliquot (150 μl) of RS-NOs was applied to the appropriate HPLC column, C_{18}-reverse phase (4.6×250 mm; TSKgel ODS-80Ts; Tosoh, Tokyo) for the low-molecular weight RS-NOs or a gel filtration column (8×300 mm; Diol-120; YMC, Kyoto) for the *S*-nitrosoproteins. As shown in a flow diagram in Fig. 1A, the HPLC column was eluted to separate each RS-NO with 10 mM sodium acetate buffer (pH 5.5) containing 0.5 mM DTPA with or without either 0–7% methanol (C_{18} column) or 0.15 M NaCl (gel filtration column) at a flow rate of 0.55 ml/min (pump 1). The eluate from the HPLC column was then mixed with a flow solution (flow rate: 0.2 ml/min; pump 2) containing metal ions (Hg^{2+}, Cu^{2+}) for RS-NO decomposition, which was infused into the system *via* the three-way connector. In this first

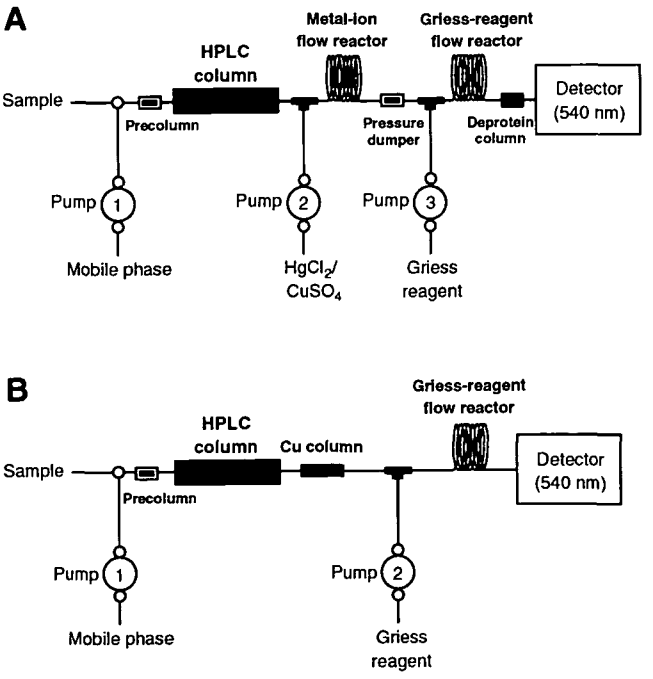

Fig. 1. **Flow diagrams of the HPLC-flow reactor systems for measurement of various RS-NOs using either a Hg^{2+}/Cu^{2+}-flow reactor (A) or a copper metal-loaded column (B) for RS-NOs decomposition.** In both systems, the Griess reagent flow reactor was employed for the colorimetric detection (540 nm) of NO_2^-; the RS-NO eluted from the HPLC column was detected as NO_2^- after metal-induced decomposition. In system (A), a C_{18}-reverse phase HPLC column (TSK gel ODS-80Ts) and a gel filtration column (YMC Diol-120) were used for low-molecular weight and high-molecular weight RS-NOs, respectively. A deproteinization column was included in the system for the *S*-NO-protein measurement before access to the detector. See text for details.

reaction coil, 1.75 mM $HgCl_2$ or 1.75 mM $CuSO_4$ solution decomposed RS-NOs to form NO^+ (HNO_2) or NO, respectively (*17, 18*), followed by conversion to NO_2^-. NO_2^- thus generated was reacted with Griess reagent in a flow reactor (*11*), which is fed through a second three-way connector at a flow rate of 0.24 ml/min (pump 3), and the diazo-compound yielded was detected at 540 nm by using a visible detector (Eicom, Kyoto) and an integrator (System Instruments, Tokyo). Accordingly, RS-NOs initially separated on the HPLC column could be identified as NO_2^- produced after decomposition with Hg^{2+} or Cu^{2+}.

In a separate experiment, a copper particle-packed column (5×8 mm) was used for RS-NOs degradation instead of the metal-ion flow reactor (Fig. 1B). The eluate from the HPLC column (mobile phase, same as Hg^{2+}-flow reactor; flow rate: 0.33 ml/min; pump 1) was passed through the copper column, which was connected to the flow reactor to mix with Griess reagent (flow rate: 0.13 ml/min) (pump 2). The Griess reagent used in our experiment consisted of 0.1% naphthylethylenediamine, 1.0% sulfanilamide, and 2.0% phosphoric acid in H_2O with 0-7% methanol.

Quantitative Conversion of RS-NOs to NO_2^-—To examine whether RS-NOs were converted to NO_2^- in a stoichiometric manner by the flow-reactor system or the copper-loaded column, the peak area of each RS-NO recorded with the HPLC/Hg^{2+} or Cu^{2+}-flow reactor system was compared with that of NO_2^- generated by the treatment of RS-NOs with Hg^{2+} before injection into the C_{18}-reverse phase HPLC/flow reactor system. Specifically, each RS-NO (1 μM) was incubated with a 10-fold molar excess of Hg^{2+} in 10 mM sodium acetate buffer (pH 5.5) at 37°C for 30 min, then 150 μl of each reaction mixture was applied to the HPLC-flow reactor analysis. Simultaneously, 150 μl of RS-NO (1 μM) solution without Hg^{2+} treatment was injected into the HPLC-flow reactor. The quantitative conversion was assessed by comparing the peak area of RS-NOs with that of NO_2^- produced by Hg^{2+} treatment of RS-NOs.

Treatment of GSH and Albumin with NO—To examine RS-NO formation from the thiol compounds and NO, GSH, and BSA were reacted with the propylamine NONOate under aerobic conditions. Specifically, various concentrations of GSH were incubated with 10 μM NONOate in the presence or absence of 1,000 U/ml of Cu,Zn-SOD in 200 μl of Krebs-Ringer-phosphate buffer (KRP, pH 7.4) at 37°C. For the reaction of BSA with NO, it was treated with equimolar DTT to albumin in 10 mM sodium phosphate-buffered 0.15 M NaCl (PBS, pH 7.4) with 1.0 mM DTPA at 37°C for 1 h, followed by Sephadex G-100 column chromatography to obtain a reduced form of BSA. The content of the free cysteine of BSA was almost 1 mol per mol of the protein by the DTNB method. The reduced BSA at a final concentration of 10 μM was then reacted with various concentrations of the NONOate in a same manner as GSH. After incubation for 45 min, 200 μl of 2-fold concentrated elution buffer (acetate buffer, pH 5.5) containing 1 mM DTPA was added to the reaction mixture, so that metal-catalyzed degradation of RS-NOs was inhibited by DTPA. Aliquots (150 μl) were then applied to the HPLC analysis with Hg^{2+}/Griess reagent reactor system as just described.

In some experiments, the free thiol of BSA was blocked by treatment of the protein with PCMB as reported earlier (*19*). The PCMB-treated BSA was then reacted with the NONOate as just described, after removal of PCMB from the reaction mixture by Sephadex G-100 column chromatography.

Quantification of GS-NO Generated from RAW264 Cells—GS-NO produced extracellularly in RAW264 cells (a murine macrophage cell line) was investigated by using the HPLC-flow reactor system with Hg^{2+} and Griess reagent. RAW264 cells were cultured in 24-well plates (Falcon, Lincoln Park, NJ, USA) with Dulbecco's minimal essential medium (DMEM) (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS) and nonessential amino acids (Gibco) as reported (*20*), and cells at saturation density (1×10^6 cells/well) were stimulated with 100 U/ml IFN-γ and 10 μg/ml LPS for 12 h at 37°C in a CO_2 incubator (5% CO_2/95% air, v/v). The culture medium was removed and the cells were washed three times with KRP (pH 7.4). They were further incubated with 200 μl of KRP containing 1 mM L-arginine with or without various concentrations of GSH or 1,000 U/ml of Cu,Zn-SOD at 37°C in the CO_2 incubator. After incubation for 45 min, the reaction medium was harvested and mixed with the same volume of pure water containing 10% methanol and 2 mM DTPA (pH 7.4), followed by centrifugation at 10,000×*g* for 10 min at 4°C. The resultant supernatant (150 μl) was then applied to the HPLC-flow reactor system with Hg^{2+}.

Simultaneously, NO released from the cells was monitored by using liposome PTIO (phosphatidylcholine liposome-encapsulated 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide) with electron spin resonance spectroscopy (ESR) as described recently (*20, 21*). Briefly, liposome PTIO was added to the cells in culture after stimulation with LPS and IFN-γ as just described, and ESR measurement was performed for the assay of NO generated extracellularly by the cells. In addition, expression of inducible NO synthase (iNOS) mRNA in the RAW264 cells was confirmed by Northern blot analysis as described previously (*22, 23*).

Statistical Analysis—Unless otherwise specified, data are expressed as means±SD of three different experiments.

RESULTS

Specific Identification of RS-NOs by HPLC-Flow Reactor Systems—The elution profiles of GS-NO, Cys-NO, and NO_2^- on the HPLC system are shown in Fig. 2. Our RS-NO detection is based on the production of a red azo-dye formed in the reaction of Griess reagent for NO_2^- generated by metal-induced decomposition of RS-NOs. Thus, to specifically identify the RS-NO, it is essential that each RS-NO is clearly separated from NO_2^- with the HPLC system before access to the flow reactor. On the reverse-phase HPLC (TSKgel ODS-80Ts) combined with Hg^{2+} and Griess reagent flow reactors, peaks of both Cys-NO and GS-NO were clearly separated from that of NO_2^- (Fig. 2A). When NO_3^- solution was injected into the HPLC-flow reactors, no appreciable response was observed. Similar results were obtained for Cys-NO and GS-NO by using the HPLC/Cu-column system (data not shown). On the basis of the elution pattern, the use of two different elution buffers with or without methanol is preferable to detect Cys-NO or

GS-NO, respectively.

In addition, a high-molecular weight RS-NO, *i.e.*, *S*-NO-BSA, was examined by the HPLC system with a gel filtration column (YMC Diol-120). As is shown in Fig. 2B, the elution profile of *S*-NO-BSA, but not native BSA, was clearly identified by the HPLC/Hg^{2+}-flow reactor system. But when the copper column was used for *S*-NO-BSA with the HPLC analysis, the catalytic activity of copper was diminished, possibly due to nonspecific adsorption of the protein to the copper metal. Thus, the copper column is not applicable for the detection of nitrosated proteins or in the presence of large amount of protein.

Furthermore, the efficacy of conversion of RS-NOs to NO_2^- was tested either in the Hg^{2+}/Cu^{2+}-flow reactor system or in the copper column RS-NO decomposition system. Specifically, RS-NOs treated or untreated with $HgCl_2$ solution were injected to the HPLC/flow reactor systems, and the peak area of the RS-NO without $HgCl_2$ treatment was compared with that of NO_2^- generated by $HgCl_2$ treatment of RS-NOs. The results in Fig. 3 showed that the peak area of GS-NO was consistent with that of NO_2^- for GS-NO treated with $HgCl_2$ before injection into the HPLC system. This indicates that almost 100% of GS-NO was converted to NO_2^- by the Hg^{2+} and Griess reagent flow reactors. Similar results were obtained for Cys-NO as well as *S*-NO-BSA, and by using the copper particle column. However, only 30% of NO_2^- conversion was observed with use of the Cu^{2+} (solution)-flow reactor with all of RS-NOs tested (data not shown).

Sensitivity of RS-NOs with HPLC-Flow Reactor Systems—The detection limit of RS-NO measurement was tested with the HPLC analysis coupled with the RS-NO decomposition system of the Hg^{2+}-flow reactor or copper column. GS-NO at the concentrations varying from 3.3 nM to 6.6 μM was applied to the HPLC-flow reactor analyses, and the peak area recorded with each concentration of GS-NO was plotted against its concentration. The HPLC analysis with Hg^{2+}-flow reactor revealed that the peak area of GS-NO correlated well with GS-NO concentration over a wide range (3.3 nM to 6.6 μM, $r^2=1.000$) (Fig. 4A). The detection limit for GS-NO with this method was more than 3 nM; a similar range of sensitivity was found for Cys-NO. Also, by using Cu^{2+}-flow reactor, a linear correlation was observed between the peak area and the GS-NO concentration ($r^2=1.000$) (Fig. 4B). The peak area obtained with the copper column system was about two times larger than that with Hg^{2+}-flow reactor. This is due to the difference in the elution conditions employed: the flow rate of the mobile

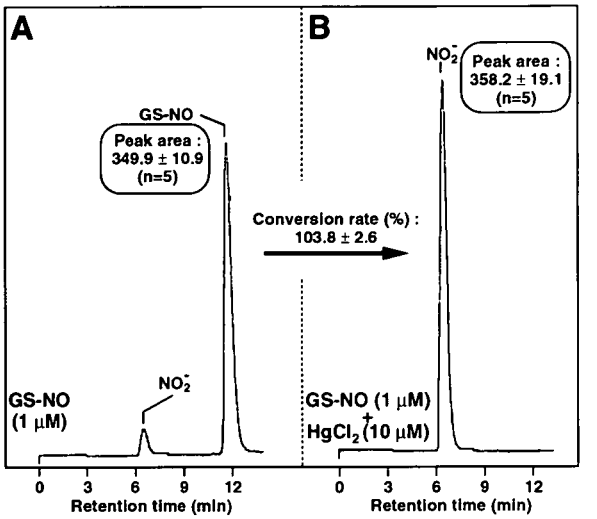

Fig. 3. **The efficacy of NO_2^- generation from GS-NO in the HPLC/flow reactor systems.** GS-NO (1 μM) treated (B) or untreated (A) with 10 μM $HgCl_2$ was applied to the HPLC/Hg^{2+}-flow reactor system using the C_{18}-reverse phase HPLC eluted with the same buffer as in Fig. 2 containing 7% methanol. Typical elution profiles obtained are shown in the figures. See text for details.

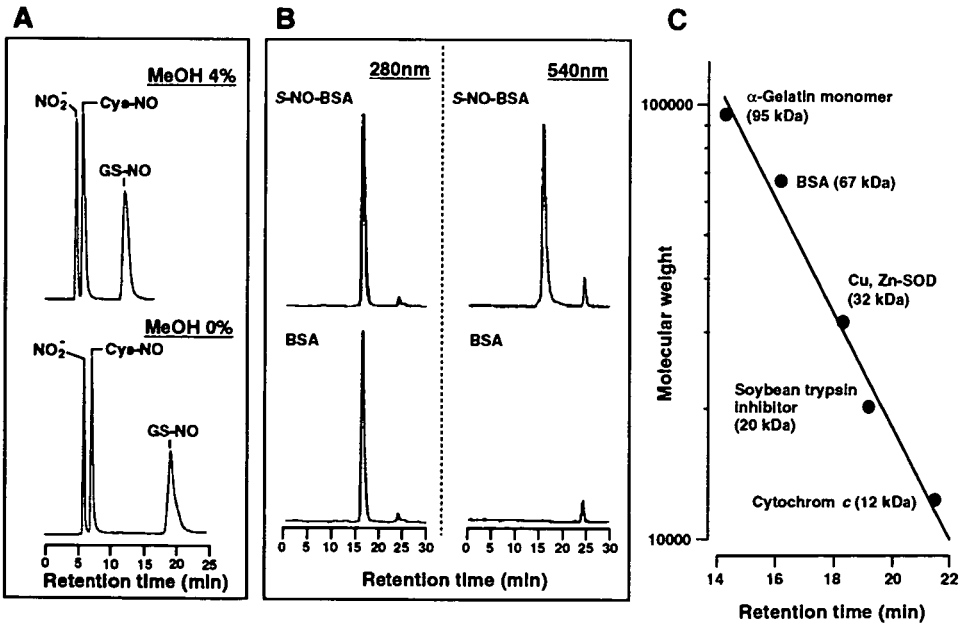

Fig. 2. **Elution profiles of various RS-NOs on the HPLC/Hg^{2+}-flow reactor systems.** A: A mixture (150 μl) of 2 μM each of NO_2^-, Cys-NO, and GS-NO was applied to the C_{18}-reverse phase HPLC combined with Hg^{2+}-flow reactor, in which 10 mM sodium acetate buffer (pH 5.5) containing 0.5 mM DTPA with or without 4% methanol (MeOH) was used for the reverse phase HPLC. B: A 150-μl aliquot of 4 μM *S*-NO-BSA or native (non-nitrosylated) BSA was injected to the HPLC/Hg^{2+}-flow reactor in a same manner as in (A), except that a gel filtration column was used for the HPLC analysis, and the elution peak on the column was monitored by using the Griess-reagent detection (540 nm) as well as UV spectrophotometry (280 nm) without use of Hg^{2+}/Griess reagent flow reactor. Note that a good linear correlation between molecular mass (10-100 kDa) of the various proteins and their retention times was obtained with the gel filtration HPLC (YMC Diol 120) (C). See text for details.

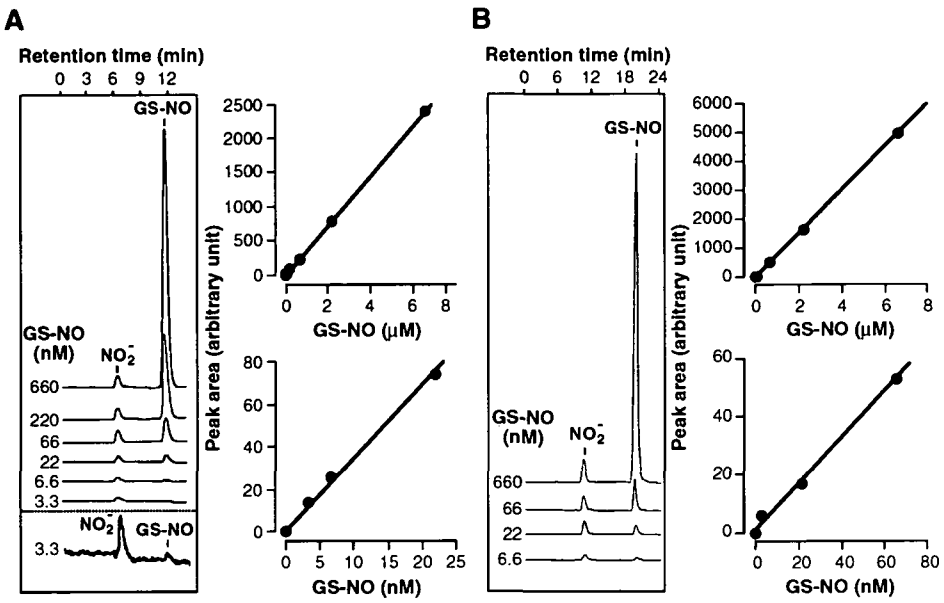

Fig. 4. **Quantitation of GS-NO using HPLC-flow reactor systems.** GS-NO ranging from 3.3 nM to 6.6 μM was applied to the HPLC analyses combined with either Hg^{2+}-flow reactor system (A) or Cu column (B). The HPLC-flow reactor analyses were performed in a same manner as in Fig. 3, and the peak area of GS-NO calculated by the integrator was correlated with each concentration of GS-NO. Data are expressed as means of three different experiments. See text for details.

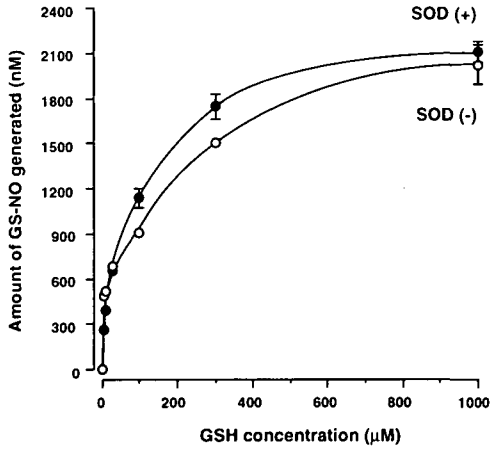

Fig. 5. **Generation of GS-NO in the reaction system of propylamine NONOate and GSH.** GSH was incubated with the NONOate in the presence or absence of 1,000 U/ml of Cu,Zn-SOD in KRP (pH 7.4) for 45 min at 37°C under the aerobic condition, then the amount of GS-NO generated was quantified by the HPLC/Hg^{2+}-flow reactor analysis as described in Fig. 4. See text for details.

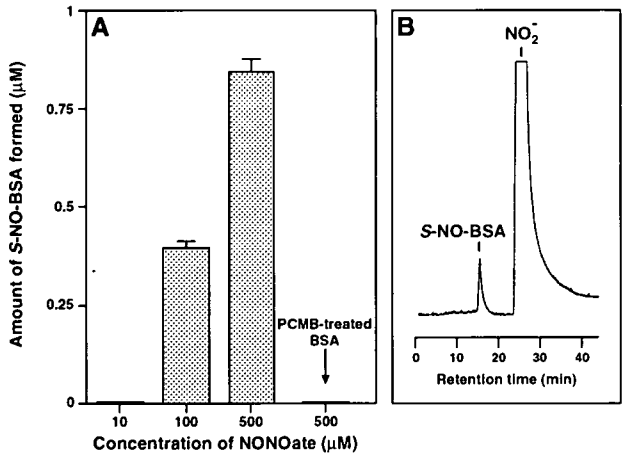

Fig. 6. **Generation of *S*-NO-BSA in the reaction of NONOate with BSA.** BSA (10 μM) that had been treated with either DTT or PCMB was reacted with various concentrations of propylamine NONOate in the same manner as in Fig. 5. The reaction mixture was then applied to the HPLC-flow reactor system to measure the amount of *S*-NO-BSA formed in the reaction. Data shown in (A) are means ± SD ($n=3$). A typical elution pattern of the reaction mixture of 10 μM BSA with 500 μM NONOate is shown in (B). See text for details.

phase in the HPLC/Hg^{2+}-flow reactor was almost twice that with the copper-column system. In any event, the linear correlation of the peak yield and RS-NO concentration was highly reproducible in both flow reactor systems.

Formation of RS-NOs in the Reaction of Thiol Compounds with NONOate—GSH or BSA was reacted with the propylamine NONOate in KRP (pH 7.4) at 37°C for 45 min under aerobic conditions, then the amount of GS-NO or *S*-NO-BSA formed with NO released from the NONOate was quantified by the HPLC/Hg^{2+}-flow reactor system. As shown in Fig. 5, GS-NO was generated in a dose-dependent manner with GSH supplied to the reaction system. GSH at a concentration of 3 μM provided 500 nM GS-NO formation in the NO reaction system (10 μM NONOate), and GS-NO formation reached a plateau at above 200 μM GSH. GS-NO formation in the GSH+NONOate system was not affected appreciably by SOD supplied to the reaction mixture, which is in clear contrast to the GS-NO formation in the RAW264 cells as described below. Similar but less efficient *S*-NO-BSA production was observed in the reaction system of BSA with the NONOate (Fig. 6). Albumin that had been treated with PCMB did not show any appreciable generation of *S*-NO-BSA, indicating that the nitrosothiol moiety is formed through a specific reaction of NO or its oxidized derivatives (NO_x; N_2O_3) with the free cysteine in the protein.

Generation of GS-NO in the Cells in Culture—Production of GS-NO from the cells was further investigated with

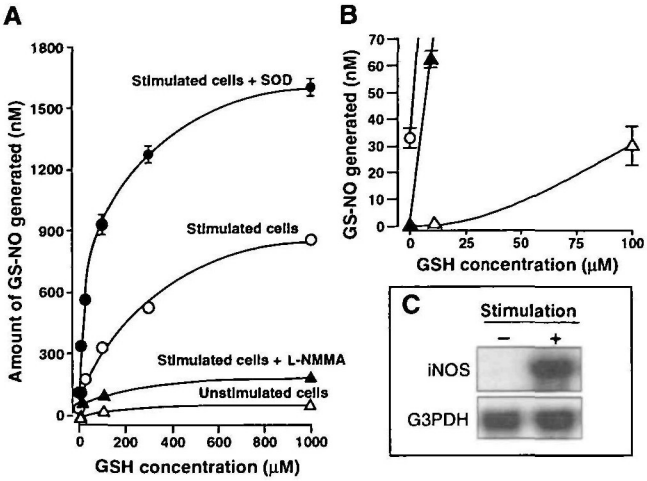

Fig. 7. **Formation of GS-NO in the RAW264 cells stimulated with both LPS and IFN-γ.** RAW264 cells at a density of 1×10^6 cells/well were stimulated with 10 μg/ml LPS and 100 U/ml IFN-γ in DMEM supplemented with 10% FBS and nonessential amino acids at 37°C for 12 h, then incubated with 200 μl KRP containing 1 mM L-arginine with or without various concentrations of GSH or 1,000 U/ml of Cu,Zn-SOD at 37°C for 45 min. The supernatant of the reaction mixture was then subjected to the HPLC/Hg^{2+}-flow reactor analysis (A, B). A part of the vertical axis in (A) is enlarged for clear demonstration of the low level of GS-NO production (B); each symbol is given in a same manner as in (A). C: Northern blot analysis for iNOS mRNA expression in the cells. G3PDH, glyceraldehyde-3-phosphate dehydrogenase. See text for details.

a murine macrophage cell line, RAW264 cells in culture. Briefly, RAW264 cells stimulated with LPS/IFN-γ or unstimulated were incubated with or without various concentrations of GSH in KRP (pH 7.4) for 45 min. The amount of GS-NO released or produced extracellularly was measured by the HPLC-flow reactor system as described above. Dose-dependent increase in GS-NO production was observed with GSH added to the cell culture, which was strongly, though not completely, inhibited by addition of L-NMMA (Fig. 7A). It should be noted that a small amount of GS-NO (33.0 ± 3.6 nM) was released from the cells stimulated with LPS/IFN-γ in the absence of GSH supplied exogenously to the cultured cells (Fig. 7B). Similarly, a low level of GS-NO generation was demonstrated in the cells without LPS/IFN-γ stimulation in the presence of more than 100 μM GSH (Fig. 7B).

The expression of iNOS mRNA was most evident in the LPS/IFN-γ-stimulated cells but not in unstimulated cells, by Northern blot analyses (Fig. 7C). The amount of NO produced by the stimulated cells, determined by ESR spectroscopy with liposome PTIO, was 18.0 ± 2.5 μM in 200 μl of the cell culture for 45 min at 37°C.

In a separate experiment, we found that L-NMMA treatment did not completely abrogate the NO production from RAW264 cells stimulated with LPS/IFN-γ. Similarly, a low but appreciable level of NO generation was observed without LPS/IFN-γ stimulation in the RAW264 cell line used. Therefore, these low levels of NO production in the cells may contribute to formation of the small amount of GS-NO in the L-NMMA-treated RAW264 cells or the cells unstimulated with LPS/IFN-γ.

DISCUSSION

We have described a novel RS-NO detection method using HPLC coupled with flow reactor systems. Three different flow reactor systems were constructed for the effective decomposition of various RS-NOs to NO_2^- with use of heavy metals such as mercury and copper, which subsequently can be detected by using a Griess reagent flow reactor and visible spectroscopy (integrator). The HPLC analysis combined with Hg^{2+}/Griess reagent flow reactor was found to be most useful and applicable to a wide range of RS-NOs including low-molecular and high-molecular weight nitroso-compounds.

Our detection system is based on the colorimetric assay using Griess reagent, which forms a diazo dye having a strong absorbance at 540 nm at acidic pH: the extinction coefficient at 540 nm is 53,000 $M^{-1}\cdot cm^{-1}$ (*24*). It is reported that NO_2^- itself can be measured at nM levels by using HPLC analysis coupled with a Griess reagent flow reactor (*25*). Therefore, if RS-NOs are decomposed to NO_2^- in a stoichiometric manner, they could be quantified in a same range of concentration (low nanomolar concentration) as the HPLC assay for NO_2^-. For this purpose, each RS-NO and NO_2^- must be separated before the RS-NO decomposition to NO_2^-, which becomes feasible with use of appropriate HPLC techniques as demonstrated in the present report. In fact, our results provided evidence that any type of RS-NO can be successfully identified and quantified by using HPLC/Hg^{2+} and the Griess reagent flow reactor system at nanomolar range (minimum detection limit: >3 nM), and a linear correlation was obtained between the peak area recorded and RS-NO concentration over a wide range.

The present HPLC-flow reactor analysis system for RS-NOs is based on the stoichiometric and precisely quantitative conversion of RS-NOs to NO_2^-. This is carried out by using Hg^{2+} or copper. A number of reports indicated recently that RS-NOs are decayed by heavy metals such as iron and copper ions (*17, 18*). For instance, Cu^{2+} ion reacted readily with RS-NOs to form NO according the following equation as proposed by Williams (*18*).

$$RS^- + Cu^{2+} \longrightarrow RS^\bullet + Cu^+ \quad (1)$$

$$RSNO + Cu^+ \longrightarrow RS^- + Cu^{2+} + NO \quad (2)$$

$$RSNO + RS^\bullet \longrightarrow RSSR + NO \quad (3)$$

However, Cu^+/Cu^{2+} is known to undergo oxygen radical generation in the presence of molecular oxygen, and thus these reactive oxygen species produced *via* copper-catalyzed autooxidation may interfere with the NO formation from RS-NOs. In fact, effective conversion of RS-NOs to NO_2^- was not obtained by using our HPLC analysis combined with Cu^{2+}-flow reactor system.

Meanwhile, another heavy metal that has been used for RS-NO assay is mercury ions (Hg^{2+}:$HgCl_2$). Hg^{2+} reacts with RS-NOs according to the following equation.

$$RSNO + Hg^{2+} + H_2O \longrightarrow RSHg^+ + HNO_2 + H^+ \quad (4)$$

This assay was originally described by Saville (*10*). As described earlier, NO^+ or HNO_2 (NO_2^-) is released from RS-NOs by Hg^{2+} stiochiometrically rather than catalytically and the reaction proceeds much faster than the Cu^{2+}

reaction regardless of the structure of R-group in RS-NO molecules (*18*). Thus, use of Hg^{2+} seems most beneficial and reliable, and it will be widely applicable for the specific and sensitive RS-NO quantitation by the HPLC-flow reactor system.

There is an interesting report by Wink's group that RS-NOs can be measured conveniently based on the Hg^{2+}/Cu^{2+}-induced decomposition of RS-NOs (*26*). They used 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) and 2,3-diaminonaphthalene (DAN), instead of Griess reagent, for colorimetric and fluorometric measurement of RS-NOs, respectively, as coupled with Hg^{2+}/Cu^{2+}-dependent decomposition of RS-NOs. In particular, nanomolar quantitation could be successfully achieved by fluorometric detection with DAN. The assay, however, might be susceptible to interference by various biological reducing substances including GSH as suggested in the report (*26*). In contrast, our flow reactor systems is combined with HPLC analysis to separate such interfering substances. Therefore, not only Griess reagent but also a fluorogenic reagent with high sensitivity such as DAN might be applicable to our present HPLC system.

HPLC analysis with ozone-chemiluminescence detection of RS-NOs *via* the photolytic decomposition was also reported previously (*12*). Similarly, EC detector can be applied for the HPLC analysis of RS-NOs as described by Loscarzo's group (*12*). Complicated and expensive instrumental techniques, however, are required for the photolysis-chemiluminescence detection, and yet photolytic reaction may not be specific for RS-NOs: NO-Hb as well as NO-iron complexes may be susceptible to the photolysis. Because EC detection is based on the redox potential of RS-NOs, biological compounds with the same redox potential as RS-NOs could give nonspecific responses.

It is now accepted that EDRF is nitric oxide *per se*, and its identity as an RS-NO is not widely recognized, although many lines of evidence support this notion. RS-NOs, however, possess very unique chemical properties in that they may act as carriers which transfer and even store of NO in the biological system (*2, 13, 18*). In this context, of particular importance is our finding that release or generation of GS-NO from a macrophage cell line was clearly demonstrated by the HPLC/Hg^{2+} and Griess reagent flow reactor assay, and that addition of SOD to the cell culture resulted in significant enhancement of GS-NO formation with the cells particularly expressing iNOS *via* stimulation with LPS/IFN-γ (Fig. 7). In contrast, we found recently that peroxynitrite ($ONOO^-$), a product of the reaction of O_2^- and NO, showed very little potential to form GS-NO from GSH (data not shown). These results suggest that GS-NO is formed effectively by the NO-producing cells and that GS-NO generation was regulated by O_2^- released simultaneously by the cells.

Another interesting point about our present data is the clear demonstration of *S*-NO-BSA formation in the reaction of NO with bovine albumin under aerobic conditions. This indicates that not only low-molecular weight RS-NOs but also high-molecular weight compounds can be analyzed in specific and quantitative manners at nanomolar levels by using the HPLC/Hg^{2+}-flow reactor system.

In conclusion, our novel analytical technique with the HPLC-flow reactor will be useful to explore the unique biochemical and physiological aspects of NO *via* formation of RS-NOs in diverse biological phenomena.

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