Role of Plasma *S*-Nitrosothiols in Regulation of Blood Pressure in Anesthetized Rabbits with Special References to Hypotensive Effects of Acetylcholine and Nitrovasodilators

Takaharu ISHIBASHI,^{*,a,b} Tomoko MIWA,^b Naoki NISHIZAWA,^b Ikumi SHINKAWA,^b Junko YOSHIDA,^b Tomie KAWADA,^c and Matomo NISHIO^b

^a Pharmacology, School of Nursing, Kanazawa Medical University; ^b Department of Pharmacology, School of Medicine, Kanazawa Medical University; Uchinada, Ishikawa 920-0293, Japan; and ^c Department of Clinical Pharmacology, Faculty of Pharmacy, Musashino University, Nishi-Tokyo, Tokyo 202-0293, Japan.

Received March 27, 2011; accepted April 27, 2011; published online May 13, 2011

The regulatory role of plasma nitrosothiols (R-SNOs) under steady-state conditions and their possible contribution to pharmacological vasodilation were systematically examined in anesthetized rabbits. Nitrosocystein (Cys-NO), *S*-nitrosogluthathione (G-SNO), and *S*-nitrosoalbumin (Alb-SNO) were determined by HPLC-Saville's method with respective sensitivities of 1, 1, and 5 nM. These R-SNOs were not detected under steady-state conditions even in the presence of *N*-ethylmaleimide, a thiol protective agent used to prevent transnitrosation of R-SNOs. Development of plasma Alb-SNO below 300 nM was observed after intravenous injection (i.v.) of nitric oxide (NO) solution (0.1 to 3 ml/g), NOC7 (an NO releasing agent, above 1 µg/kg), and a low dose of Alb-SNO (10 nmol/kg). However, blood pressure was not significantly reduced by NO solution or Alb-SNO. Intravenous injection of a high dose of Alb-SNO (300 nmol/kg) significantly reduced blood pressure with the appearance not only Alb-SNO in micromolar level in plasma, but also G-SNO in lesser degree. Conversely, the hypotensive effect of Cys-NO (300 nmol/kg, i.v.) and G-SNO (300 nmol/kg, i.v.) accompanied development of Alb-SNO (micromolar level), but not Cys-NO or G-SNO in plasma. R-SNOs were not found in plasma during profound hypotension induced by acetylcholine (10 and 30 µg/kg/min, continuous i.v.), glyceryl trinitrate (100 µg/kg, i.v.), sodium nitroprusside (100 µg/kg, i.v.), and isosorbide dinitrate (300 µg/kg, i.v.). These results indicate that R-SNOs do not play an important role under unstimulated condition. In addition, plasma R-SNOs may not be involved in pharmacological vasodilation where contributions of NO or R-SNOs are suggested.

Key words nitrosothiol; rabbit; acetylcholine; glyceryl trinitrate; sodium nitroprusside; isosorbide dinitrate

Nitric oxide (NO) from endothelial cells is now widely accepted as a key substance in the regulation of vascular tone.¹⁾ However, due to its short half-life and susceptibility to many substances *in vivo*, the important roles of stable carriers or a reservoir of NO bioactivity in circulating blood are receiving a great deal of attention.^{2–4)} One of these is nitrite (NO₂⁻), which is catalyzed by the nitrite reductase activity of deoxygenated hemoglobin to form NO.^{2,5)} Then, it will cause vasodilation to increase blood flow, leading to improve oxygen supply at the region of oxygen deficiency. Accumulating evidence^{2,5)} under steady-state conditions and pathophysiological conditions support this idea. *S*-Nitrosothiols (R-SNOs) in plasma are also considered carriers of NO bioactivity and are expected to transport the activity downstream.^{3,6–8)} However, plasma levels of R-SNOs, even under unstimulated conditions, vary widely by species, institute, and methods, ranging from non-detectable to 9200 nM.^{3,9)} As the assessment for R-SNOs is problematic and not free from artifacts,^{9–11)} these methodological difficulties are likely responsible for divergent data and for the fragile basis of a regulatory role for plasma R-SNOs. Therefore, the presence and quantity of R-SNOs under steady-state conditions have been subject to re-evaluation with reliable methods.^{9,12–15)} Another concern is the involvement of R-SNO in pharmacological modifications of vascular tone (blood pressure *in vivo*). Endothelial stimulation by acetylcholine will cause NO formation,¹⁾ and therefore, R-SNOs may be involved in the vasodilatory effect. However, as far as we surveyed, no such report was found. Nitrovasodilators such as glyceryl trinitrate (GTN), isosorbide dinitrate (ISDN) and sodium nitroprusside (SNP) re-

quire respective unique metabolic activation before developing NO bioactivity,¹⁶⁾ and the possible involvement of R-SNOs as intermediates is indicated.^{17,18)} However, no report has systematically compared all of these NO-related vasodilators in relation to plasma R-SNOs in one species. Therefore, we systematically examined the possible involvement of R-SNOs in the regulation of vascular tone under steady-state conditions and pharmacological stimulations by acetylcholine and nitrovasodilators. For this aim, we employed quantitative devices for R-SNOs with high sensitivity avoiding artifacts as is described elsewhere.¹⁰⁾

MATERIALS AND METHODS

Animal Experiments Japanese white rabbits weighing 2.3–3.5 kg (17–23 weeks old) were anesthetized with intravenous sodium pentobarbital (30 mg/kg). Cannulae were inserted into the jugular vein (for drug administration), the carotid artery (to monitor blood pressure), and the femoral vein and artery (for blood sampling). A tachometer to measure pulse rate was triggered by pulse waves of arterial pressure. Experimental procedures were performed after a stabilization period of 20–60 min. When required, several minutes were allowed to elapse for the recovery of hemodynamic parameters after the initial control blood sampling. The Ethics Committee of Kanazawa Medical University approved experimental procedures, and the animals were treated under conditions set forth in the “Guiding Principles for the Care and Use of Laboratory Animals” of the Japanese Pharmacological Society.

* To whom correspondence should be addressed. e-mail: ishitaka@kanazawa-med.ac.jp

Blood Sampling and Treatment Rabbit arterial and venous blood was collected into vacuum blood sampling tubes containing 3 mg of ethylenediamine-*N,N,N',N'*-tetraacetic acid (EDTA)2Na (VP-NA052K, Terumo, Tokyo, Japan), supplemented with 60 μ l of 200 mM NEM (*N*-ethylmaleimide, Sigma-Aldrich, St. Louis, MO, U.S.A.). The final concentrations were expected to be 5 mM for EDTA and 8 mM for NEM when 1.5 ml of blood was mixed, enough to prevent artificial nitrosation, as well as thiolate- and ascorbate-mediated degradation of R-SNOs, by blocking SH-groups (NEM) and by inhibiting metal-catalyzed transnitrosation reactions (EDTA).^{19,20} After brief, gentle rolling, the sample was centrifuged (1500 $\times g$ for 5 min at 4 °C) to obtain supernatant, which was subsequently subjected to filtration (0.2 μ m, Millex L-G, Millipore, Bedford, MA, U.S.A.) before Alb-SNO was measured.

To quantify nitrosocystein (Cys-NO) and nitrosogluthathione (G-SNO), each blood sample mixed with NEM and EDTA was filtrated by Centriscart I (Sartorius, Göttingen, Germany), and supernatant was used for measurement.¹⁰

Preparation of NO Solution Nitric oxide gas (purity >99%) was purchased from Japan Fine Products (Tochigi, Japan). An NO solution (50 mM Tris-HCl buffer, pH 7.4) was prepared immediately before use according to Shikano *et al.*²¹ with elongation of exposure time to NO. The concentration of NO in the resultant saturated solution was estimated to be 3.3 mM.²¹ This NO solution was kept on ice and was used without any dilution.

Preparation of Nitrosothiols (R-SNOs) Cys-NO and G-SNO: These low molecular weight nitrosothiols (LMWR-SNOs) were prepared by incubating glutathione and cysteine in 10 mM HCl with equimolar NaNO₂ solution (1:4 in volume) at room temperature for 60 min.²² Excess NO₂⁻ was removed with ammonium sulfamate (50 mM in final concentration) for 1 h.

S-Nitrosoalbumin (Alb-SNO): This high molecular weight nitrosothiol was prepared according to preceding reports^{23,24} with some modifications as described below: Rabbit albumin (fraction V, Sigma) was dissolved in acetate buffer (10 mM, pH 6.0) to achieve 0.3 mM concentration (20 mg/ml, regarding a mean molecular weight of albumin as 65000). The Cys-34 thiol group was reduced by incubation with dithiothreitol (DTT: final concentration 2 mM) for 30 min at 37 °C. This reduction medium was replaced five times with acetate buffer using a centrifugal filter device equipped with 30 kDa cut-off filter (Amicon Ultra, Millipore) and was finally concentrated to a volume of 2–3 ml. This solution was mixed with 50 ml of 0.5 N HCl and 75 μ l of 300 mM NaNO₂ (final concentration approximately 0.45 mM) and incubated for 1 h at room temperature in the dark to form Alb-SNO. Excess NO₂⁻ was removed with ammonium sulfamate (50 mM in final concentration) for 1 h, and the medium was exchanged five times with acetate buffer containing NaCl (150 mM) and EDTA (0.05 g/l).

Concentrations of prepared G-SNO, Cys-NO and Alb-SNO were determined by Saville's method²⁵ and were stored at -80 °C. The R-SNO yields were around 60–70%.

Quantification of Cys-NO and G-SNO These LMWR-SNOs were separated and determined by an HPLC-flow reactor system described elsewhere.¹⁰ The detection limit was 1 nM (0.1 pmol/100 μ l) and discrimination sensitivity was 1

Fig. 1. Detection of Alb-SNO

(A) Diagram of HPLC-Flow reactor system. Mobile phase: 10 mM acetate buffer (pH 6.0) including NaCl (150 mM) and EDTA-2Na (50 mg/l) at a flow rate of 0.55 ml/min. HgCl₂: 2 mM dissolved in 10 mM acetate buffer (pH 6.0) at a flow rate of 0.2 ml/min. Griess reagent: 5% sulfanilamide and 0.25% naphthylethylenediamine, dissolved in 1.25% of HCl at a flow rate of 0.25 ml/min. (B) A typical chromatogram of a standard solution (200 μ l) containing 50 nM Alb-SNO. Peaks of Alb-SNO (retention time 14–18 min) were clearly separated from a peak (at 25 min) consisting of NO₂⁻ and unknown origin. A certain variation of molecular weight of albumin (mean value of 65000) makes a wide polyphasic peak of the substance. (C) Standard curves for Alb-SNO were constructed using different concentrations of standard solution in a volume of 200 μ l on the basis of area under curve (AUC) of their spectra ($n=3$ at each concentration). Data are mean $\pm$ S.E.M. Although the results of the statistical analysis were omitted to simplify the graph, 5 nM of Alb-SNO was significantly detectable and differences of 10 nM were differentiated at all concentrations.

and 2 nM (0.1 and 0.2 pmol/100 μ l) for G-SNO and Cys-NO, respectively.

Quantification of Alb-SNO Alb-SNO was determined by HPLC-Saville's method²⁶ as was originally described by Akaike *et al.*²⁷ with some modifications including an additional column to avoid interference by plasma proteins and composition of mobile phase (Fig. 1A and its legend). Alb-SNO was separated by a separation column (Gel filtration column CFC-200, Eicom, Kyoto, Japan or Shodex Protein KW-802.5, Showa Denko, Kawasaki, Japan). Hg⁺-sensitive NO was separated from albumin to form NO₂⁻ in the first flow reactor coil, and the albumin was absorbed by a following deproteination column (CA-ODS, Eicom, Kyoto, Japan) to avoid non-specific interference by plasma proteins. Next, NO₂⁻ was mixed with Griess reagent in the second reactor coil and absorption was read at 540 nm by the detector. Data (Fig. 1B) were stored and analyzed by a computer attached to the device (PowerChrom, AD Instruments, Tokyo, Japan). The detection limit was 5 nM and discrimination sensitivity was 10 nM (loading volume of 200 μ l; Fig. 1C).

Pure water (resistance above 18.3 megaohm and almost free of NO_x, sum of NO₂⁻ and NO₃⁻, contamination) through a reagent water system (Milli-Q SP, Millipore) was used for every preparation. To minimize NO_x contamination, all laboratory equipment except a part of ultrafiltration unit was washed five times with the pure water before use.²⁸

Chemicals All chemicals were analytical grade. Injection solutions of acetylcholine (Obisort; Daiichi Co., Ltd., Tokyo, Japan), glyceryl trinitrate (Millisrol; Nihon Kayaku Co., Ltd., Tokyo, Japan), and isosorbide dinitrate (ISDN, Nitrol; Daiichi Co., Ltd., Tokyo, Japan) were used. These solutions were diluted to desired concentrations with saline. NOC7 was purchased from Dojindo (Kumamoto, Japan) and was dissolved and diluted with 0.1 N NaOH. Sodium nitroprusside (SNP; Nacalai Tesque Inc., Kyoto, Japan) was first dissolved in distilled water and then diluted with saline.

Statistical Analysis All data are expressed as mean $\pm$ S.E.M. Within-group comparisons were made using the paired Student's *t*-test, and between-group comparisons were performed with the unpaired Student's *t*-test. For repeated measures, or for more than three groups, comparisons were made by one- or two-way analysis of variance (ANOVA) followed by Scheffé's method. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Failure to Detect R-SNOs Under Steady-State Conditions Blood samples were obtained from anesthetized rabbits under steady-state conditions (80 ± 12 mmHg in mean blood pressure and 331 ± 9 beats/min in heart rate, $n=4$) to examine the existence of plasma R-SNOs (Alb-SNO, G-SNO and Cys-NO). However, no R-SNOs were observed in either arterial or venous plasma. In another set of experiments (mean blood pressure 80 ± 14 mmHg and heart rate 331 ± 9 beats/min at control, $n=5$), NEM (200 mM solution, 0.8 ml/kg, estimated to be 2 mM in whole blood) was injected intravenously intending to protect -SH from transnitrosation, which leads to the degradation of R-SNOs *in vivo*. Three minutes after the injection, mean blood pressure decreased significantly (33 ± 4 mmHg, $p < 0.05$ by paired *t*-test), and heart rate tended to decrease (256 ± 56 beats/min, not statistically significant). Both before and 3 min after the injection, no significant amount of R-SNOs was detectable in arterial or venous plasma.

Effect of NO Solution Although bolus injection of 0.1 ml/kg NO saturated solution (estimated concentration of 3.3 mM at maximum) did not affect blood pressure or heart rate, a significant amount of Alb-SNO was detected in both arterial and venous plasma after 3 min of the infusion and increased further in a dose-dependent manner (Fig. 2). Injection of 1 and 3 ml/kg of the solution caused an initial transient decrease in mean blood pressure (5–15 s) followed by rapid recovery to reach a sustained level. Although the maximum decreases, $14.6 \pm 4.4\%$ of the control ($p < 0.05$) at 1 ml/kg and $23.2 \pm 2.3\%$ of the control ($p < 0.05$) at 3 ml/kg were statistically significant, the subsequent sustained phase exhibited no statistical significance (Fig. 2). No Cys-NO or G-SNO were observed at any dose of NO solution. Heart rate (260 ± 13 beats/min of 15 rabbits at control condition) was not significantly affected by NO solution at any dose.

Effect of NOC7 In rabbits at the control condition, the mean blood pressure and heart rate was 101 ± 3 mmHg and 276 ± 9 beats/min, respectively. Intravenous injection of NOC7, an NO releasing agent, significantly decreased the mean blood pressure dose-dependently; the maximum decreases ($22.3 \pm 6.9\%$ of the control with $0.03 \mu\text{g/kg}$, $37.0 \pm$

Fig. 2. Effect of NO Solution on Blood Pressure and Plasma Alb-SNO

Bolus injection of NO saturated solution into anesthetized rabbits caused a transient fall in blood pressure (see text in detail), followed by rapid recovery to reach sustained phase. Values of this sustained phase (3 min after administration) were shown as blood samples were taken 3 min after injection. Plasma Alb-SNO was detected in arterial and venous blood after NO solution, but LMWR-SNOs were not. Thirteen rabbits were used and only one dose was applied to each rabbit. The number of experiments (rabbits) was 5 at 0.1 and 1 ml/kg, and 3 at 3 ml/kg. Estimated NO concentrations in circulating blood by injections of 0.1, 1 and 3 ml/kg of saturated NO solution are about 4, 40 and 120 μM , respectively.

Fig. 3. Effect of NOC7 on Blood Pressure and Plasma Alb-SNO

Bolus injection of NOC7 into anesthetized rabbits caused a transient fall in blood pressure, followed by a sustained decrease. Values of sustained phase (3 min after injection) were shown as arterial and venous blood samples were taken at the time. Although the decreases in mean blood pressure from respective control values at all doses were statistically significant ($p < 0.05$ by paired *t*-test), marks denoting the significance were not indicated to simplify the figure. Arterial plasma Alb-SNO concentration by $10 \mu\text{g/kg}$ of NOC7 was significantly ($p < 0.05$) higher than those by 1 and $3 \mu\text{g/kg}$ of NOC7 and venous plasma Alb-SNO concentration by $10 \mu\text{g/kg}$ of NOC7 was significantly ($p < 0.05$) higher than that by $1 \mu\text{g/kg}$ of NOC7 (by two-way ANOVA followed by Scheffé's method). Eighteen rabbits were used and each point was constructed from injections in 3 rabbits.

5.0% with $0.1 \mu\text{g/kg}$, $49.7 \pm 11.6\%$ with $0.3 \mu\text{g/kg}$, $67.7 \pm 1.0\%$ with $1 \mu\text{g/kg}$, $68.6 \pm 2.5\%$ with $3 \mu\text{g/kg}$ and $71.5 \pm 0.6\%$ with $10 \mu\text{g/kg}$) were observed after 0.5–2 min and were followed by gradual recoveries. Changes in the mean blood pressure after 3 min (at blood sampling) are depicted in Fig. 3. Heart rate showed an initial increase in response to a de-

Fig. 4. Effect of Alb-SNO Injection on Blood Pressure and Plasma R-SNOs

In the control group, vehicle (100 nmol/kg of non-nitrosoated albumin in acetate buffer) was infused. Upper: changes in mean blood pressure in the vehicle control (open circle), low dose (10 nmol/kg; closed circle), and high dose (300 nmol/kg; closed inverse triangle) groups are presented. *Significantly ($p < 0.05$) different from other groups (by two-way ANOVA followed by Scheffe's method). $n = 5$ in each group. Lower: appearance of R-SNOs after Alb-SNO infusion. G-SNO and Alb-SNO detected in arterial and venous plasma 3 min after Alb-SNO injection are shown. No R-SNOs were detected after vehicle injection. Cys-NO was not observed under any conditions. Alb-SNO, nitrosoalbumin; G-SNO, nitrosogluthation; n.d., not detected. Open column, arterial plasma; closed column, venous plasma; $n = 5$ in each column.

crease in mean blood pressure, followed by decrease reaching near the control level without statistical significance.

Alb-SNO was not observed until 1 $\mu\text{g/kg}$ of NOC7 were used (the vasodilatory effect was submaximum), and increased further in a dose-dependent manner (Fig. 3). Cys-NO was found in 1 of 3 rabbits at 3 $\mu\text{g/kg}$ (11 nM in arterial plasma and 20 nM in venous plasma) and in 2 of 3 rabbits at 10 $\mu\text{g/kg}$ (2 and 31 nM in arterial blood and 1 and 21 nM in venous blood). A significant amount of G-SNO was not detectable at any dose.

Effect of Exogenous Alb-SNO Although 10 nmol/kg of Alb-SNO (intravenous injection (i.v.)) did not cause any change in blood pressure compared with the vehicle control group (Fig. 4, upper panel), approximately 150 nM of Alb-SNO was detected in both arterial and venous plasma without arterio-venous difference 3 min after the injection (Fig. 4, lower panel). The yield was estimated at approximately 68% (220 nM in plasma was expected on the assumption that circulating blood volume is 8% of body weight and mean hematocrit is 43% in our experience). However, no Cys-NO or G-SNO were detectable under this condition. When 300 nmol/kg of Alb-SNO was intravenously injected, a significant and sustained reduction of blood pressure was observed (Fig. 4, upper panel). Three minutes after the injection, Alb-SNO was detectable approximately 5400 nM in both arterial and venous plasma, and a low concentration of G-SNO (below 10 nM) was detectable as well; Cys-NO, was not detectable. There was no arterio-venous difference in Alb-SNO concentration and the yield of Alb-SNO was estimated at 82%.

Heart rate at the control condition was 260 ± 10 beats/min in the control (vehicle) group, 274 ± 7 beats/min in the low dose (10 nmol/kg) group and 282 ± 9 beats/min in the high

Fig. 5. Effects of Injection of Cys-NO and G-SNO on Blood Pressure and Plasma Alb-SNO

Cys-NO (300 nmol/kg) or G-SNO (300 nmol/kg) was intravenously administered. Upper: changes in mean blood pressure (mBP) for 3 min are shown. Open circle, Cys-NO ($n = 4$); closed circle, G-SNO ($n = 4$). * Significantly ($p < 0.05$) different from respective control blood pressure (time 0) by paired t -test. Lower: plasma Alb-SNO detected 3 min after injection is shown. Open column, arterial plasma; closed column, venous plasma; $n = 3$ at each column.

dose (300 nmol/kg) group. Even at a high dose, no significant change in heart rate was observed during the initial 3 min after the injection.

Effect of Exogenous Cys-NO and G-SNO Four rabbits (101 ± 4 mmHg in mean blood pressure and 283 ± 26 beats/min in heart rate at the control condition) received Cys-NO (300 nmol/kg) and 4 other rabbits (112 ± 3 mmHg in mean blood pressure and 304 ± 18 beats/min in heart rate at the control condition) received G-SNO (300 nmol/kg). Intravenous injection of these R-SNOs decreased blood pressure as is shown in Fig. 5. Significant concentrations of Alb-SNO were measured in both arterial and venous plasma after 3 min of both Cys-NO and G-SNO injections without statistical significance between the two exogenous R-SNOs. Arterio-venous difference in Alb-SNO concentration was not recognized by either R-SNO. The presence of Cys-NO and G-SNO in plasma 3 min after the injections was not confirmed in either arterial or venous plasma.

Effect of Acetylcholine Three rabbits were used in this experiment (105 ± 14 mmHg in mean blood pressure and 308 ± 28 beats/min in heart rate, just before acetylcholine injection). As stepwise increase of acetylcholine (1–30 $\mu\text{g/kg/min}$, continuous infusion) decreased mean blood pressure and heart rate in a dose-dependent manner; the maximum decrease in mean blood pressure was $39.0 \pm 6.6\%$ of the control (65 ± 8 mmHg) at 10 $\mu\text{g/kg/min}$. Further reduction was not observed at 30 $\mu\text{g/kg/min}$ (67 ± 15 mmHg). Heart rate was 264 ± 27 beats/min at 10 $\mu\text{g/kg/min}$ and 259 ± 55 beats/min at 30 $\mu\text{g/kg/min}$.

Three minutes after the injection at every dose, the existence of R-SNOs was examined in both arterial and venous blood samples. However, no signal above the detection limits was recognized in either arterial or venous plasma at any dose.

Effect of Nitrovasodilators Intravenous bolus injection of 100 $\mu\text{g/kg}$ of GTN (112 ± 2 mmHg in mean blood pressure

Fig. 6. Hemodynamic Changes by Nitrovasodilators

Changes in heart rate (HR: upper) and in mean blood pressure (mBP: lower) after intravenous administration of GTN (glyceryltrinitrate 100 μ g/kg, closed circle), ISDN (isosorbide dinitrate 300 μ g/kg, open circle), or SNP (sodium nitroprusside 100 μ g/kg, closed inverse triangle) are shown. Changes in mBP after all nitrovasodilators and changes in HR after 0.5 min of GTN and SNP were statistically significant ($p < 0.05$ by paired t -test). However, marks denoting significance are omitted to avoid confusion. $n = 4$ at each agent. * Significantly ($p < 0.05$) different from other groups (by two-way ANOVA followed by Scheffé's method).

and 285 ± 18 beats/min in heart rate at the control; $n = 4$), 300 μ g/kg of ISDN (113 ± 4 mmHg in mean blood pressure and 289 ± 23 beats/min in heart rate, at the control; $n = 4$) and 100 μ g/kg of SNP (111 ± 9 mmHg in mean blood pressure and 274 ± 24 beats/min in heart rate, at the control; $n = 4$) significantly decreased blood pressure as is shown in Fig. 6. However, no trace amount of Alb-SNO, Cys-NO, or G-SNO was found in either arterial or venous plasma 3 min after the injections.

DISCUSSION

Low molecular weight nitrosothiols such as Cys-NO and G-SNO have been proposed as candidates for circulating carriers of NO bioactivity because of their vasodilatory potencies,²⁹⁾ which are reconfirmed in the present study, and their abilities to form dinitrosyl iron complexes with NO³⁰⁾ that effectively protect NO against oxidation.³¹⁾

A larger molecule that is dominant in plasma and contains a primary cysteine residue, albumin, is significantly more stable when nitrosylated than LMWR-SNOs, although the reason for this stability is not yet fully understood.³²⁾ Circulating Alb-SNO can undergo transnitrosation reactions with low molecular weight thiols in plasma to form less stable intermediates such as Cys-NO and G-SNO, which in turn can undergo reductive or transition metal-dependent decomposition to release NO.^{33–35)}

In this study, we attempted to elucidate systematically the presence of Alb-SNO, Cys-NO, and G-SNO under steady-state conditions and their contributions to pharmacological vasodilation in one species *in vivo* with highly sensitive facilities, avoiding artifacts. The major findings of this study are: 1) failure to detect Cys-NO, G-SNO, or Alb-SNO in circulating blood under steady-state conditions even after prevention of transnitrosation by NEM; 2) *S*-nitrosoalbumin was found after exogenous NO, but only a high serum concentration of Alb-SNO could contribute to vasodilation; and 3) *S*-nitrosothiols did not appear during the hypotensive effect

caused by acetylcholine and nitrovasodilators such as GTN, ISDN and SNP. We will consider these results in order.

***S*-Nitrosothiols Are Not Detectable under Steady-State Conditions** Early trials to quantify plasma R-SNOs under steady-state have reported total R-SNO levels (all classes of molecular weights) of 20 nM to 7 μ M,^{36–39)} LMWR-SNO levels of *ca.* 220 nM^{38,40)} (consisting of mostly Cys-NO and G-SNO) and high molecular weight nitrosothiols levels of *ca.* 6.92 μ M.^{40,41)} The development of a technique to separate R-SNOs revealed the presence of 30–900 nM of Cys-NO^{33,42)} and 120–180 nM of G-SNO⁴²⁾ in plasma. However, the absence (under the detection limit) of these LMWR-SNOs has been reported by various methods with high sensitivities, such as 25, 20, or even 5 nM.^{22,38,41)}

Concentration of Alb-SNO in circulating blood under steady-state conditions has been reported at 38 to 250 nM in healthy humans^{19,43,44)} and 9.4 nM in normal pregnant women.⁴⁵⁾ In addition, an arterio-venous difference (45 nM in arterial blood and 63 nM in venous blood) in high molecular weight R-SNOs (estimated to be mostly Alb-SNO) has been reported in humans.⁴¹⁾ However, many non-detectable results have also been reported including in cats⁴⁶⁾ and in pregnant women,⁴⁷⁾ although the sensitivity of these reports was 50 nM.

In this study, no Cys-NO, G-SNO, or Alb-SNO were found in circulating blood under steady-state conditions as we have previously reported.²⁶⁾ In addition, prevention of transnitrosation leading to the degradation of R-SNOs by NEM^{19,20)} failed to detect not only Alb-SNO, but also LMWR-SNOs. Although the expected concentration of NEM in circulating blood (2 mM in whole blood) is one quarter of that used *ex vivo* (8 mM in the sampling tube; see Materials and Methods), this dose is practically the maximum *in vivo*, because higher doses of NEM caused death in our preliminary study. Therefore, at the least it would be tenable to conclude that Cys-NO and G-SNO above 1 nM, and Alb-SNO above 5 nM are not present in circulating blood under steady-state conditions. The possibility of much lower R-SNO concentrations in circulating blood remains; however, we set rather lower ceilings for R-SNOs than other reports.

Appearance of Alb-SNO after Exogenous NO-Bioactivity After the first report that Alb-SNO could be a circulating NO carrier,⁴⁰⁾ much scientific work has been devoted to investigating the possible role of Alb-SNO in mediating the effects of NO in the cardiovascular system. The importance of Alb-SNO in the hypotensive effect of NO was supported by an observation that the duration of the hypotension caused by an NO releaser, NOC7, was significantly attenuated in albuminemic rats.⁴⁸⁾ Our present study revealed the development of Alb-SNO in circulating blood by administering NO solution, NOC7, Cys-NO, G-SNO, and Alb-SNO itself. However, the appearance of Alb-SNO (<300 nM, in Fig. 2), as a result of injecting NO solution, and by low dose Alb-SNO administration (10 nmol/kg, in Fig. 4), which resulted in about 100 nM of Alb-SNO in circulating blood, did not necessarily reduce blood pressure. A similar result has been reported that approximately 400 nM of Alb-SNO by inhalation of NO or Alb-SNO injection of 17.5 nmol/kg did not cause any hemodynamic effect in cats.⁴⁶⁾ In addition, we could not recognize any arterio-venous difference (after NO inhalation, mean levels of 382 nM in arterial blood and 207 nM in venous blood have been reported⁴⁶⁾) indicating

consumption of NO bioactivity during peripheral circulation. On the contrary, Alb-SNO did not appear in circulating plasma until the hypotensive effect of NOC7 reached sub-maximal (Fig. 3, at 1 $\mu\text{g/kg}$), where an expected NO release from NOC7 for initial 5 min is approximately 6 nmol/kg (around 80 nM in whole blood). These differences may arise from different rates of NO release and/or accessibility to vascular smooth muscle. An abrupt and localized increase in NO (by NO solution or inhalation) in the blood will lead to the formation of R-SNOs in preference to vasodilation. However, a gradual release of NO from NOC7 ($t_{1/2}=5$ min at 37°C, pH 7.4) in the vicinity of vascular smooth muscle (as a result of uniform distribution in the blood) may afford to vasodilation and other portion within blood stream (a compartment apart from vessel wall) will be metabolized to form NO_2^- or R-SNOs. This will evoke the formation Alb-SNO, as well as Cys-NO in levels that are detectable after a high dose of NOC7. Notably, when a large dose of Alb-SNO (300 nmol/kg) was injected, only G-SNO, but not Cys-NO, appeared in plasma. Another interesting result is that although exogenous Cys-NO or G-SNO developed plasma Alb-SNO with significant hypotensive effect, neither Cys-NO nor G-SNO was detected in the same plasma (Fig. 5). It would be very difficult to interpret these phenomena in an integrative manner, even when marked difference in stabilities between Cys-NO and G-SNO (Cys-NO $\ll$ G-SNO, about 9 times⁴⁹⁾) are taken into consideration. NO exchange between plasma protein thiol-bound NO and low molecular weight thiol pools (transnitrosation) is known to occur *in vivo*.³³⁾ When it begins from NO (NO solution or NOC7), the first reaction may be nitrosylation of cysteine, which provides NO to albumin. On the contrary, when it starts from LMWR-SNOs (Cys-NO and G-SNO), the -NO moiety may be transferred to a more stable carrier, such as albumin, to form Alb-SNO, and LMWR-SNOs may disappear within a short period because of their short half-lives in blood. Alb-SNO levels (<400 nM) detected after NO solution or NOC7 (even at the highest dose) are not considered to contribute to vasodilation directly. However, Alb-SNO may contribute to the hypotensive effect of exogenous G-SNO and Cys-NO, because Alb-SNO levels after the injection of these agents are similar to that after a high dose of exogenous Alb-SNO (Figs. 4, 5).

Absence of R-SNOs in NO-Related Vasodilations Intravenous acetylcholine stimulates endothelial cells and activates endothelial NO synthase to form NO, which is easily diffusible to every directions. NO that reaches vascular smooth muscle will evoke vasodilation and that spills in the luminal direction will be oxidized to form NO_2^- , trapped by hemoglobin or directly or indirectly incorporated into R-SNOs.²⁾ However, we could not detect any R-SNOs after acetylcholine infusion, even at doses evoking tremendous hypotensive effect. Deducing from a report by Vallance *et al.*,⁵⁰⁾ approximately 400–500 nM of NO would be expected by our maximum dose (10 $\mu\text{g/kg/min}$), but oxyhemoglobin (the rate constant to form methemoglobin and NO_2^- is 0.3–0.4 $\mu\text{mol/M}\cdot\text{s}$), oxygen, and other substances may prevent NO (released by endothelial cells in the vasculature periphery) from gaining access to thiols uniformly distributed within the blood stream.⁴⁾ As a result, only a small or negligible amount of NO may be available for the nitrosylation of thiols.

NO formation by GTN and ISDN has been indicated not only in blood vessels, but also in other tissues.⁵¹⁾ In addition, an important role of Cys-NO (an intermediate) in the intracellular activation of GTN and SNP has been suggested,^{16,52)} although SNP is also considered to be a direct NO donor.⁵³⁾ Because Cys-NO can be membrane permeable,³⁾ some leakage of Cys-NO to plasma and successive transnitrosated R-SNOs in plasma were expected. However, these nitrovasodilators did not accompany any R-SNO appearance in plasma in the present study, in accordance with an early observation that GTN and SNP (each 100 μM) failed completely to *S*-nitrosate albumin.⁴³⁾ A possible reason why putative NO from these nitrovasodilators failed to develop R-SNOs might be similar to that of acetylcholine as described above. Another noteworthy report described a lack of GTN- and ISDN-derived NO in blood vessels with their pharmacological concentrations.⁵⁴⁾ In addition, red blood cells, which contain hemoglobin, have been shown to be the predominant site of nitrosylation by GTN, while plasma has been demonstrated to be the quantitatively least significant biological compartment.⁵⁵⁾

In summary, we could not detect Cys-NO or G-SNO higher than 1 nM or Alb-SNO than 5 nM in plasma under steady-state conditions in anesthetized rabbits, even after preventing *in vivo* decay by NEM. The presence of a certain concentration of Alb-SNO, a stable R-SNO that might be an origin of transnitrosation to LMWR-SNO leading to the development of NO bioactivity, did not reduce blood pressure. These results indicate that plasma R-SNOs do not play an important role in the regulation of vascular tone under steady-state conditions. In addition, the contributions of plasma R-SNOs are not indicated to pharmacological hypotension *via* putative NO release by acetylcholine or nitrovasodilators such as GTN, ISDN, and SNP, because their hypotensive effects were not accompanied by any R-SNO formation.

Technical Note In general, detection limits of R-SNOs by colorimetric and fluorometric methods have been reported to be 0.5 μM and 50 nM, respectively.⁵⁶⁾ More sensitive method has been reported to be a use of chemiluminescence providing a sensitivity of 5 nM.⁵⁷⁾ Even higher sensitivities of R-SNOs (1 nM for G-SNO, 1 nM for Cys-NO and 5 nM for Alb-SNO) in our devices (colorimetric method with HPLC) have been achieved by both technical support by Eicom (Kyoto, Japan) and our accumulated practices to minimize artifacts during sample processing and quantitative procedures.^{10,28)} These improvements enabled us to examine fine insight of R-SNOs in plasma.

REFERENCES

- 1) Marín J., Rodríguez-Martínez M. A., *Pharmacol. Ther.*, **75**, 111–134 (1997).
- 2) Dejam A., Hunter C. J., Schechter A. N., Gladwin M. T., *Blood Cells Mol. Dis.*, **32**, 423–429 (2004).
- 3) Giustarini D., Milzani A., Colombo R., Dalle-Donne I., Rossi R., *Clin. Chim. Acta*, **330**, 85–98 (2003).
- 4) Rassaf T., Feelisch M., Kelm M., *Free Radic. Biol. Med.*, **36**, 413–422 (2004).
- 5) Bryan N. S., *Free Radic. Biol. Med.*, **41**, 691–701 (2006).
- 6) Gow A. J., Farkouh C. R., Munson D. A., Posencheg M. A., Ischiropoulos H., *Am. J. Physiol.*, **287**, L262–L268 (2004).
- 7) Ng E. S., Kubes P., *Can. J. Physiol. Pharmacol.*, **81**, 759–764 (2003).

- 8) Hogg N., *Annu. Rev. Pharmacol. Toxicol.*, **42**, 585—600 (2002).
- 9) Giustarini D., Milzani A., Colombo R., Dalle-Donne I., Rossi R., *Trends Pharmacol. Sci.*, **25**, 311—316 (2004).
- 10) Tashimo O., Ishibashi T., Yoshida J., Tsuchida H., Nishio M., *Nitric Oxide*, **9**, 148—152 (2003).
- 11) Tsikas D., *Nitric Oxide*, **9**, 53—55 (2003).
- 12) Tsikas D., Denker K., Frölich J. C., *J. Chromatogr. A*, **915**, 107—116 (2001).
- 13) Tsikas D., *Circ. Res.*, **94**, e106 (2004).
- 14) Tsikas D., Schwedhelm E., Frölich J. C., *Circ. Res.*, **90**, e70 (2002).
- 15) Tsikas D., Frölich J. C., *Circ. Res.*, **90**, e39 (2002).
- 16) Thatcher G. R., Nicolescu A. C., Bennett B. M., Toader V., *Free Radic. Biol. Med.*, **37**, 1122—1143 (2004).
- 17) Miller M. R., Megson I. L., *Br. J. Pharmacol.*, **151**, 305—321 (2007).
- 18) Münzel T., Daiber A., Mülsch A., *Circ. Res.*, **97**, 618—628 (2005).
- 19) Rassaf T., Kleinbongard P., Preik M., Dejam A., Gharini P., Lauer T., Erckenbrecht J., Duschin A., Schulz R., Heusch G., Feelisch M., Kelm M., *Circ. Res.*, **91**, 470—477 (2002).
- 20) Rassaf T., Bryan N. S., Kelm M., Feelisch M., *Free Radic. Biol. Med.*, **33**, 1590—1596 (2002).
- 21) Shikano K., Long C. J., Ohlstein E. H., Berkowitz B. A., *J. Pharmacol. Exp. Ther.*, **247**, 873—881 (1988).
- 22) Jourdain D., Hallén K., Feelisch M., Grisham M. B., *Free Radic. Biol. Med.*, **28**, 409—417 (2000).
- 23) Stamler J. S., Simon D. I., Osborne J. A., Mullins M. E., Jaraki O., Michel T., Singel D. J., Loscalzo J., *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 444—448 (1992).
- 24) Park J. K., Kostka P., *Anal. Biochem.*, **249**, 61—66 (1997).
- 25) Saville B., *Analyst*, **83**, 670—672 (1958).
- 26) Ishibashi T., Nishizawa N., Nakamoto-Nomura M., Abe F., Liu H., Yoshida J., Kawada T., Nishio M., *Biol. Pharm. Bull.*, **34**, 528—537 (2011).
- 27) Akaike T., Inoue K., Okamoto T., Nishino H., Otagiri M., Fujii S., Maeda H., *J. Biochem.*, **122**, 459—466 (1997).
- 28) Ishibashi T., Himeno M., Imaizumi N., Maejima K., Nakano S., Uchida K., Yoshida J., Nishio M., *Nitric Oxide*, **4**, 516—525 (2000).
- 29) Myers P. R., Minor R. L. Jr., Guerra R. Jr., Bates J. N., Harrison D. G., *Nature* (London), **345**, 161—163 (1990).
- 30) Vanin A. F., *FEBS Lett.*, **289**, 1—3 (1991).
- 31) Vedernikov Y. P., Mordvintsev P. I., Malenkova I. V., Vanin A. F., *Eur. J. Pharmacol.*, **211**, 313—317 (1992).
- 32) Al-Sa'doni H., Ferro A., *Clin. Sci.*, **98**, 507—520 (2000).
- 33) Scharfstein J. S., Keaney Jr. F., Slivka A., Welch G. N., Vita J. A., Stamler J. S., Loscalzo J., *J. Clin. Invest.*, **94**, 1432—1439 (1994).
- 34) Singh R. J., Hogg N., Joseph J., Kalyanaraman B., *J. Biol. Chem.*, **271**, 18596—18603 (1996).
- 35) Tsikas D., Sandmann J., Rossa S., Gutzki F. M., Frölich J. C., *Anal. Biochem.*, **270**, 231—241 (1999).
- 36) Goldman R. K., Vlessis A. A., Trunkey D. D., *Anal. Biochem.*, **259**, 98—103 (1998).
- 37) Jourdain D., Gray L., Grisham M. B., *Biochem. Biophys. Res. Commun.*, **273**, 22—26 (2000).
- 38) Marley R., Feelisch M., Holt S., Moore K., *Free Radic. Res.*, **32**, 1—9 (2000).
- 39) Minamiyama Y., Takemura S., Inoue M., *Arch. Biochem. Biophys.*, **341**, 186—192 (1997).
- 40) Stamler J. S., Jaraki O., Osborne J., Simon D. I., Keaney J., Vita J., Singel D., Valeri C. R., Loscalzo J., *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 7674—7677 (1992).
- 41) Gladwin M. T., Shelhamer J. H., Schechter A. N., PeaseFye M. E., Wacławski M. A., Panza J. A., Ognibene F. P., Cannon R. O., *Proc. Natl. Acad. Sci. U.S.A.*, **97**, 11482—11487 (2000).
- 42) Butler A. R., Rhodes P., *Anal. Biochem.*, **249**, 1—9 (1997).
- 43) Tsikas D., Sandmann J., Luessen P., Savva A., Rossa S., Stichtenoth D. O., Frölich J. C., *Biochim. Biophys. Acta*, **1546**, 422—434 (2001).
- 44) Tsikas D., Sandmann J., Frölich J. C., *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.*, **772**, 335—346 (2002).
- 45) Tyurin V. A., Liu S. X., Tyurina Y. Y., Sussman N. B., Hubel C. A., Roberts J. M., Taylor R. N., Kagan V. E., *Circ. Res.*, **88**, 1210—1215 (2001).
- 46) Ng E. S., Jourdain D., McCord J. M., Hernandez D., Yasui M., Knight D., Kubes P., *Circ. Res.*, **94**, 559—565 (2004).
- 47) Moodley J., Gwababa S. S., *J. Obstet. Gynaecol.*, **24**, 756—759 (2004).
- 48) Minamiyama Y., Takemura S., Inoue M., *Biochem. Biophys. Res. Commun.*, **225**, 112—115 (1996).
- 49) Tullett J. M., Rees D. D., Shuker D. E., Gescher A., *Biochem. Pharmacol.*, **62**, 1239—1247 (2001).
- 50) Vallance P., Patton S., Bhagat K., MacAllister R., Radomski M., Moncada S., Malinski T., *Lancet*, **346**, 153—154 (1995).
- 51) Mülsch A., Bara A., Mordvintsev P., Vanin A., Busse R., *Br. J. Pharmacol.*, **116**, 2743—2749 (1995).
- 52) Ignarro L. J., Lippton H., Edwards J. C., Baricos W. H., Hyman A. L., Kadowitz P. J., Gruetter C. A., *J. Pharmacol. Exp. Ther.*, **218**, 739—749 (1981).
- 53) Singh R. J., Hogg N., Neese F., Joseph J., Kalyanaraman B., *Photochem. Photobiol.*, **61**, 325—330 (1995).
- 54) Kleschyov A. L., Oelze M., Daiber A., Huang Y., Mollnau H., Schulz E., Sydow K., Fichtlscherer B., Mülsch A., Münzel T., *Circ. Res.*, **93**, e104—e112 (2003).
- 55) Janero D. R., Bryan N. S., Saijo F., Dhawan V., Schwalb D. J., Warren M. C., Feelisch M., *Proc. Natl. Acad. Sci. U.S.A.*, **101**, 16958—16963 (2004).
- 56) Bryan N. S., Grisham M. B., *Free Radic. Biol. Med.*, **43**, 645—657 (2007).
- 57) Feelisch M., Rassaf T., Mnaimneh S., Singh N., Bryan N. S., Jourdain D., Kelm M., *FASEB J.*, **16**, 1775—1785 (2002).