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Ascorbic Acid and Reducing Agents Regulate the Fates and Functions of *S*-Nitrosothiols

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Although *S*-nitrosoglutathione (GS-NO) and other *S*-nitrosothiols (RS-NO) exhibit activity attributable to nitric oxide (NO), the dynamic aspects of their metabolism remain to be elucidated. To determine the fates and functions of RS-NO, the stability of GS-NO was analyzed in plasma, and various fractions of liver and kidney. GS-NO was fairly stable under physiological conditions in plasma and buffer solutions. However, GS-NO was rapidly decomposed in the presence of either homogenates of rat liver and kidney or their supernatant fractions. The ability of the supernatants to decompose GS-NO remained unchanged after the removal of proteins and large molecular weight compounds. Physiological levels of reducing agents, such as reduced glutathione (GSH), ascorbic acid (AsA), and cysteine, also enhanced the decomposition of RS-NO; the order of their potency was AsA > cysteine > GSH. Considering their intra-cellular concentrations and potency, AsA might principally be responsible for the enhanced decomposition of GS-NO. NO, GS-NO, and related RS-NO inhibited the respiration of Ehrlich ascites tumor cells. The inhibitory effect of GS-NO was enhanced by the reducing agents (cysteine > AsA > GSH). Intravenously administered GS-NO exhibited a depressor action through some ascorbic acid-enhancable mechanism. Thus, the metabolism and biological function of GS-NO and related RS-NO might be affected by AsA and other reducing agents.

Key words: ascorbic acid, cysteine, glutathione, nitric oxide, *S*-nitrosothiol.

Nitric oxide (NO) and/or its metabolite(s) rapidly react with various molecules, such as heme and nonheme irons (*1*), molecular oxygen (*2, 3*), the superoxide anion (*4*), and GSH, cysteine, and cysteinyl residues in proteins (*5*). Although the lifetime of NO is believed to be short, *S*-nitrosothiols (RS-NO) are fairly stable under physiological conditions (*6*); the half-lives of *S*-nitrosoglutathione (GS-NO) and *S*-nitrosoalbumin are longer than 10 h. In fact, bronchial lavage fluid and plasma from healthy human were found to contain about 0.3 and 7 μM GS-NO and other RS-NO, respectively (*7, 8*). Because GS-NO, *S*-nitrosocysteine (Cys-NO), and *S*-nitrosoalbumin exhibit the ability to induce vasorelaxation, similar to that of NO (*9-12*), the formation and degradation of RS-NO have been postulated to play important roles in the regulation of the circulatory status (*12, 13*).

Based on the observations that platelet lysates and vascular smooth muscle cells enhanced the release of NO from GS-NO and *S*-nitroso-*N*-acetylpenicillamine, some cellular component(s) have been postulated to affect the metabolism of RS-NO (*14, 15*). In fact, fairly high concentrations of copper ions have been shown to enhance the decomposition of RS-NO (*16, 17*). However, the physiological concentrations of free copper ions in plasma and tissues are extremely low. Although reducing agents, such as cysteine, glutathione (GSH), and ascorbic acid (AsA), also enhance the decomposition of RS-NO (*14, 18-20*), their concentrations differ significantly from one cell type to another. Thus, the biological significance of these compounds in the metabolism of RS-NO *in vivo* remains to be elucidated.

NO has been shown to inhibit the respiration of mitochondria and ascites tumor cells (*21-25*). Because NO rapidly reacts with molecular oxygen (*2, 3*), the inhibitory effect was stronger under low oxygen tension than with high concentrations of it (*21-23*). Thus, the *in vivo* fates and functions of NO and RS-NO might also be affected by the local concentrations of oxygen, reducing agents and metals. However, only limited information is available regarding the quantitative aspects of the reactivity of RS-NO with these molecules. The present paper describes the fates of RS-NO in plasma, and homogenates of liver and kidney, and their effects on tumor cell respiration under different oxygen tension and on the circulatory status.

EXPERIMENTAL PROCEDURES

Chemicals—GSH, AsA, D-cysteine, L-cysteine, *N*-acetylcysteine (NAC), and sodium nitrite were obtained from

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Abbreviations: GS-NO, *S*-nitrosoglutathione; RS-NO, *S*-nitrosothiol; Cys-NO, *S*-nitrosocysteine; NAC-NO, *S*-nitroso-*N*-acetylcysteine; EATC, Ehrlich ascites tumor cells; TCA, trichloroacetic acid; AsA, ascorbic acid; GSH, reduced glutathione; EDTA, ethylenediaminetetraacetic acid, disodium salt; DTPA, diethylenetriamine-*N,N,N',N'',N''*-pentaacetic acid; γ-GTP, γ-glutamyltransferase.

Wako Pure Chemical Industries (Osaka). Diethylenetriamine-*N,N,N′,N″,N″*-pentaacetic acid (DTPA), bathocuproine disulfonic acid and ethylenediaminetetraacetic acid (EDTA) were obtained from Dojin Chemical (Kumamoto). Other chemicals used were of the highest grade commercially available.

Synthesis of RS-NO—RS-NO was synthesized as reported by Saville (*26*). Briefly, a reaction mixture containing either GSH, D-cysteine, L-cysteine, or NAC was incubated with an equimolar amount of sodium nitrite under acidic conditions (pH 2.0) for 15 min. To the reaction mixture was added NaOH to give a final pH of 7.5. Then, the mixture was added to 20 mM Tris-HCl buffer (pH 7.5). The amount of RS-NO formed was determined spectrophotometrically at 545 nm. The disappearance of thiols and sodium nitrite was confirmed by the methods of Ellman (*27*) and Green *et al.* (*28*), respectively. The yield of RS-NO generated from thiols was measured spectrophotometrically (*29*) (ε_{545} = 13.0 $M^{-1}\cdot cm^{-1}$), and was higher than 96%.

Preparation of Human Plasma Samples—Human blood was obtained from healthy donors. Blood samples were collected in succinate-containing tubes. After centrifugation of the blood samples at 12,000×*g* for 5 min, fresh plasma samples were obtained and used for experiments.

Preparation of Rat Liver and Kidney Fractions—Under ether anesthesia, rats were exsanguinated, and then their livers or kidneys were perfused with ice-cold saline. Each perfused liver or kidney was excised and homogenized in 5 volumes of ice-cold 0.1 M mannitol-containing 10 mM Tris-HCl buffer (pH 7.4) and 10 mM $MgCl_2$ (*30*). After centrifugation at 10,000×*g* for 30 min, the supernatant fraction was obtained. The pellet was washed twice with the mannitol solution by repeated centrifugation. The pellet was resuspended in the buffer (12 mg/ml). To eliminate macromolecules with molecular weights higher than 10,000, the supernatant fraction was subjected to ultrafiltration through a Centricut filter (Kurabou, Osaka). To eliminate proteins and large peptides from the supernatant, trichloroacetic acid (TCA) was added to the supernatant to a final concentration of 5%. After removing the denatured proteins by centrifugation at 12,000×*g* for 10 min, the supernatant fraction was neutralized with NaOH. The four fractions thus obtained were added to 20 mM EDTA and then used for experiments.

Measurement of RS-NO—The concentration of RS-NO was determined spectrophotometrically (*29*) (ε_{545} = 13.0 $M^{-1}\cdot cm^{-1}$), and by the method of Saville (*26*).

Preparation of SDS Micelles—α-Tocopherol micelles were prepared by vigorously mixing appropriate amounts of α-tocopherol and a 0.5 M SDS solution for 2 min (*31*).

Preparation of Tumor Cells—Ehrlich ascites tumor cells (EATC) were supplied by the Japanese Cancer Research Resources Bank (Tokyo), and inoculated into the peritoneal cavities of male ddY mice. Seven to 9 days after inoculation, EATC-containing ascites fluid was obtained. After washing 3 times by centrifugation in a Ca^{2+}-free Krebs-Ringer phosphate buffer (KRP), EATC were resuspended in KRP at 1×10^8 cells/ml.

Measurement of Respiration—The oxygen consumption by EATC was measured polarographically at 37°C using a Clark-type oxygen electrode fitted to a 2 ml water-jacketed closed chamber equipped with a magnetic stirrer (*32*). EATC were used at a final concentration of 5×10^6/ml in KRP containing 1 mM $CaCl_2$.

Measurement of Blood Pressure—Under urethane anesthesia, the right femoral artery was cannulated with polyethylene tubing and then the blood pressure was recorded with a polygraph (RM-6200; Nihon Koden, Tokyo). The right femoral vein was also cannulated with polyethylene tubing for the administration of various agents.

RESULTS

Effects of Plasma and Tissue Homogenates on the Stability of GS-NO—Figure 1 shows the effects of plasma and hepatorenal homogenates on the fate of GS-NO under air atmospheric conditions. GS-NO incubated in KRP buffer disappeared from the mixture with a half-life of longer than 10 h. Although the rate of GS-NO disappear-

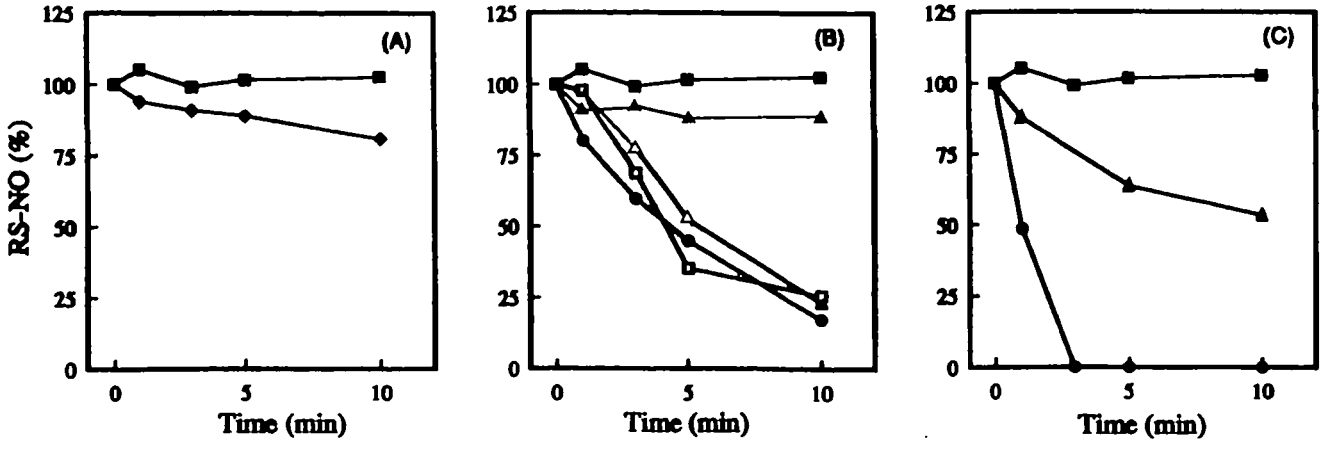

Fig. 1. **Effects of plasma (A), liver (B), and kidney (C) homogenate on the fate of GS-NO.** The incubation mixtures contained, in a final volume of 1 ml, 50 μM GS-NO and 20 mM EDTA. Each mixture were incubated at 37°C in the presence of either plasma (A), or various fractions of liver (B) and kidney (C) homogenates. During incubation at 37°C under air atmospheric conditions, the changes in the concentrations of RS-NO were determined. ◆, 900 μl of plasma; ▲, particulate fraction of homogenate (12 mg protein/ml); ●, supernatant fraction of homogenate (10 mg protein/ml); △, filtrated supernatant; □, acid-treated fraction of supernatant; ■, control.

ance was enhanced only slightly in the presence of plasma, it was enhanced significantly in the presence of the liver homogenate. Although the rate of GS-NO disappearance was enhanced more markedly by the supernatant fraction of the homogenate, it was not affected by the particulate fraction. The enhancing activity of the supernatant remained unaffected after the removal of proteins and other large molecular weight components ($M_r > 10,000$) by ultrafiltration or by treatment with trichloroacetic acid (TCA). These results suggest that the supernatant fraction of the liver contains some low molecular weight component(s) which enhances the decomposition of GS-NO. The supernatant fraction of the kidney homogenate enhanced the disappearance of GS-NO more strongly than the liver supernatant. In contrast to the particulate fraction of the liver, that of the kidney also exhibited a significantly high ability to decompose GS-NO.

Effects of Reducing Agents on the Stability of GS-NO—Figure 2 shows the effects of various reducing agents on the stability of GS-NO under air atmospheric conditions. Consistent with previous observations (*14, 18-20*), AsA, GSH, and cysteine enhanced the disappearance of GS-NO; the order of their potency was AsA > CySH ≫ GSH. α-Tocopherol containing micelles did not enhance the decomposition of GS-NO. The rates of decomposition remained unaffected even after removal of oxygen from the incubation medium. The enhancing effects of AsA, cysteine, and GSH increased dose dependently (Fig. 3).

Effects of Metals on the Decomposition of GS-NO—Because copper ions enhanced the decomposition of RS-NO (*16, 17, 34*), contamination heavy metals may underlie the mechanism for the enhancement by these reducing agents. To examine this possibility, the effects of chelating agents on the rate of GS-NO decomposition were examined under physiological pH and temperature. As shown in Fig. 4, the presence of diethylenetriamine-*N,N,N′,N″,N″*-pentaacetic acid (DTPA) did not affect the rate of RS-NO decomposition. Neither EDTA (1 mM) nor bathocuproine (100 μM) affected the rate of RS-NO decomposition (data not shown). These observations suggested that the enhanced decomposition by these reducing agents principally occurred through a metal-independent mechanism. However, in the presence of 10 μM $CuCl_2$, AsA and cysteine increased the rate of decomposition significantly (Fig. 5). $CuCl_2$ itself enhanced the disappearance of GS-NO only slightly. The GSH-enhanced decomposition of GS-NO was not affected by $CuCl_2$.

Effect of AsA on RS-NO Decomposition—The cellular concentrations of both AsA and GSH are higher than those of cysteine. Because the enhancing effect of AsA as to decomposition of GS-NO was significantly stronger than those of other reducing agents, the effect of AsA on the decomposition of various RS-NO was studied. As shown in Fig. 4, AsA also enhanced the rate of decomposition of both L-Cys-NO and D-Cys-NO. The disappearance of NAC-NO was also enhanced by AsA in both the presence and absence of DTPA.

Effects of Reducing Agents and RS-NO on Cellular Respiration—EATC show spontaneous respiration without the addition of any substrates. Consistent with previous studies (*21-25, 35*), the respiration of EATC was reversibly inhibited by low concentrations of NO; the inhibitory effect was stronger under low oxygen tension than under high tension (Fig. 6). GS-NO also inhibited the respiration of EATC, although its activity was lower than that of NO. However, the inhibitory effect of GS-NO seemed not to be affected by oxygen tension. L-Cys-NO, D-Cys-NO, and NAC-NO also inhibited the respiration of EATC. The order of their potency was NO > L-Cys-NO > D-Cys-NO > NAC-NO > GS-NO. Although the inhibitory effect of GS-NO was fairly smaller than that of NO, it was enhanced by either cysteine, AsA, or GSH. In the presence of equimolar

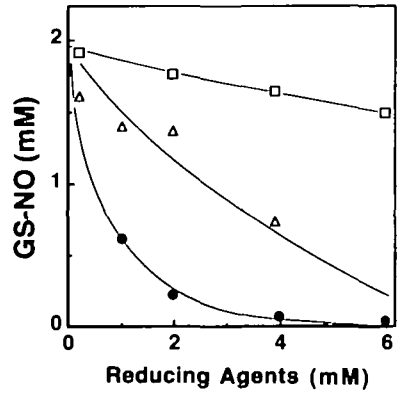

Fig. 3. **Dose-dependent decomposition of RS-NO by reducing agents.** Reaction mixtures containing 2 mM GS-NO, 20 mM phosphate buffer (pH 7.5) and various concentrations of reducing agents were incubated at 37°C for 30 min. After rapidly cooling to 0°C, the remaining RS-NO was determined spectrophotometrically. □, GSH; ●, AsA; △, cysteine.

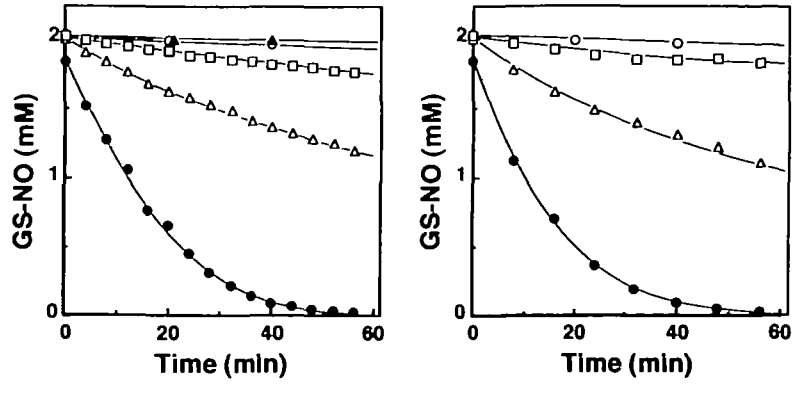

Fig. 2. **Effects of reducing agents on RS-NO decomposition.** The incubation mixtures contained, in a final volume of 1 ml, 20 mM phosphate buffer (pH 7.5) and 2 mM GS-NO in the absence (○) or presence of 2 mM GSH (□), AsA (●), cysteine (△), or α-tocopherol SDS micelles (▲). The reaction was started by adding GS-NO at 37°C under air (left) or a N_2 stream (right).

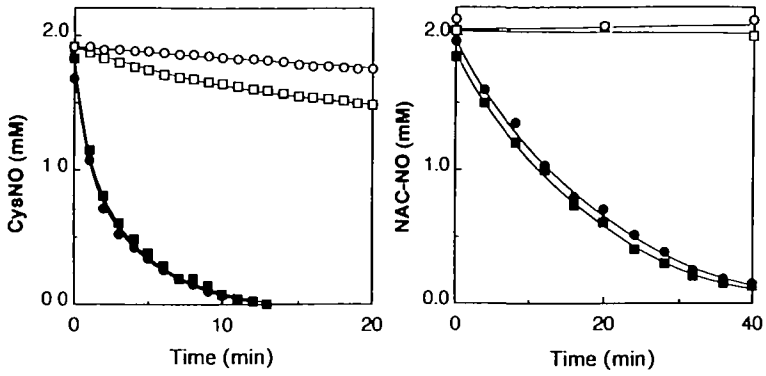

Fig. 4. **Effect of AsA on RS-NO decomposition.** The reaction mixtures contained, in a final volume of 1 ml of 20 mM phosphate buffer (pH 7.5), 2 mM either L-Cys-NO (left) or NAC-NO (right). Experiments were performed in the absence (open symbols) and presence of 2 mM AsA (close symbols). Circles show the experiment performed in the presence of 10 μM DTPA.

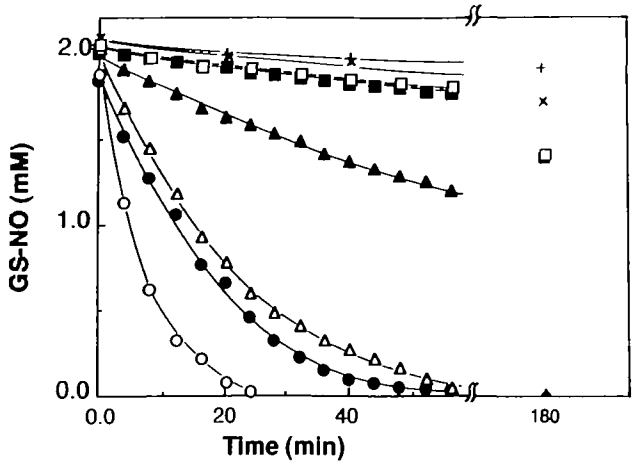

Fig. 5. **Effects of copper ion and reducing agents on GS-NO decomposition.** The reaction mixtures contained, in a final volume of 1 ml, 20 mM phosphate buffer (pH 7.5), and 2 mM GS-NO. The reaction was started in the absence (+) or presence of 2 mM GSH (squares), AsA (circles), or cysteine (triangles) under air. The mixtures were incubated with 10 μM $CuCl_2$ (open symbols) in the presence or absence (×) of a reducing agent.

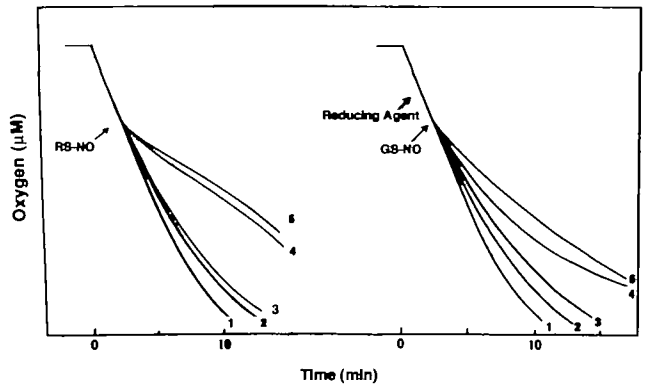

Fig. 7. **Effects of GS-NO and reducing agents on the respiration of EATC.** EATC (5×10^6 cells/ml) were incubated in a closed chamber containing 2 ml of KRP containing 10 μM DTPA at 37°C. In the left panel, 50 μM RS-NO was added to the mixture at the indicated times (arrows). 1, control; 2, GS-NO; 3, NAC-NO; 4, D-Cys-NO; 5, L-Cys-NO. In the right panel, at the indicated times (arrows), 50 μM GS-NO was added to the mixture. Five millimolar reducing agents were also added 1 min before the addition of GS-NO. During the incubation, the oxygen concentration in the medium was monitored. 1, control; 2, 50 μM GS-NO; 3, GS-NO+5 mM GSH; 4, GS-NO+5 mM AsA; 5, GS-NO+5 mM cysteine.

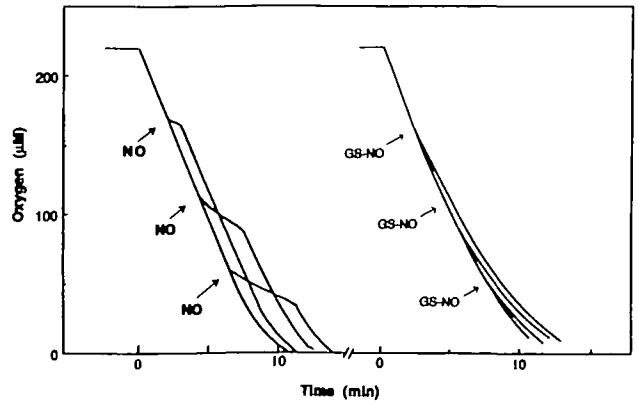

Fig. 6. **Effects of NO and GS-NO on the respiration of EATC.** EATC (5×10^6 cells/ml) were incubated in a closed chamber containing 2 ml of KRP containing 10 μM DTPA at 37°C. At the indicated times (arrows), either 10 μM NO (left) or 50 μM GS-NO (right) was added to the mixture. During the incubation, the oxygen concentration in the medium was monitored.

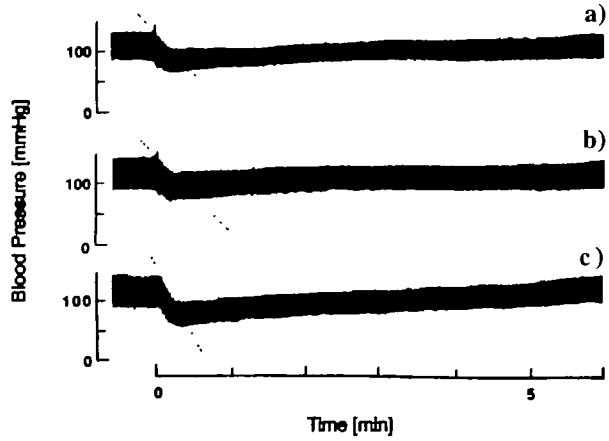

Fig. 8. **Effects of RS-NO on blood pressure.** Blood pressure was monitored under urethane anesthesia. At the indicated time (arrow), 0.5 μmol/kg of RS-NO was administered intravenously. (A) Control. (B) Saline was infused intravenously (0.1 ml/min) for 10 min before GS-NO was administered. (C) AsA (600 μmol/kg) was infused intravenously for 10 min before GS-NO administration.

TABLE I. **Effect of ascorbic acid on the hypotensive effect of GS-NO.** Under urethane anesthesia, animals were intravenously infused with 1 ml of either saline or AsA (0.6 mmol/kg) over 10 min. Then a GS-NO solution (0.5 μmol/kg) was administrated intravenously over a period of 3 s. $^*p<0.05$ compared to control.

	Rate of decrease in blood pressure (mmHg/min)	Change in blood pressure (mmHg)
Control	208±36	32±2.1
Saline	186±35	33±5.7
AsA	271±18*	39±4.3

concentrations of these reducing agents, the enhancing effect was strongest with cysteine (cysteine > AsA > GSH) (Fig. 7).

Effect of RS-NO on Blood Pressure—To determine the role of RS-NO in the regulation of vascular resistance, their effect on blood pressure was studied in the rat. A bolus injection of GS-NO rapidly and transiently decreased the blood pressure (Fig. 8). The depressor effect of GS-NO was enhanced by intravenous infusion of AsA. The rate of onset of the hypotensive response induced by GS-NO is faster in AsA-treated rats than in control animals (Table I).

DISCUSSION

The present work demonstrates that the decomposition of GS-NO is enhanced by low molecular weight compound(s) in liver and kidney but not fresh plasma. Consistent with previous observations (*14, 18-20*), AsA, cysteine and GSH also enhanced the decomposition of GS-NO (AsA > cysteine ≫ GSH). Because these compounds enhanced the decomposition in the presence of chelating agents, their effects might principally occur *via* some metal-independent mechanism. The hepato-renal levels of AsA, GSH, and cysteine are about 1-3, 3-6, and ~0.01 mM, respectively (*36, 37*). Considering their potency and tissue levels, AsA might predominantly be responsible for the enhancement of the decomposition. In fact, a preliminary experiment revealed that the rate of GS-NO decomposition markedly decreased on treatment of a liver homogenate with ascorbate oxidase.

The NO moiety of RS-NO could be transferred to other thiols through a transnitrosation mechanism (*29, 38, 39*). Because the method used in the present study does not distinguish GS-NO from other RS-NO, the apparent stability of GS-NO in fresh plasma may reflect the transnitrosation between GS-NO and plasma thiols, such as GSH (~25 μM) and mercaptalbumin (~0.5 mM). However, ultrafiltration analysis revealed that, after incubation of 50 μM GS-NO with fresh plasma at 37°C for 10 min, about 80% of RS-NO was recovered from the filtrated fraction (M_r < 10,000). Thus, transnitrosation between GS-NO and mercaptalbumin might be negligible in fresh plasma.

The present work also shows that, in the presence of a fairly high concentration of copper, AsA, and cysteine but not GSH synergistically enhanced the decomposition of GS-NO. In this context, Gorren *et al.* (*17*) reported that 10 μM $CuCl_2$ enhanced the decomposition of RS-NO only at GSH concentrations lower than 10 μM. Because the cellular level of GSH is extremely high (2-10 mM), the synergistic effect of copper and thiols might occur minimally in tissues.

GSH metabolism occurs *via* inter-organ cycles in which the liver, kidneys, bile trees and small intestine play important roles (*40, 41*). The apical membranes of these tissues are highly enriched with γ-glutamyltransferase (γ-GTP), a rate-limiting enzyme for GSH degradation, and peptidases, and hence the lumenal concentration of cysteine is fairly high. Because cysteine enhanced the decomposition of RS-NO as effectively as AsA did, this amino acid may also play a role in the release of NO in these tissues. It should be noted that both the supernatant and particulate fraction of kidney significantly enhanced the decomposition of GS-NO. Because both GSH and GS-NO serve as substrates for γ-GTP (*42*), they might be rapidly degraded to cysteine and Cys-NO. Thus, the rapid decomposition of GS-NO by the renal homogenate might reflect its conversion to unstable Cys-NO and the production of cysteine from endogenous GSH.

Both NO and GS-NO inhibited the respiration of EATC. Because NO is unstable under high oxygen tension while the decomposition of GS-NO occurs *via* an O_2-independent mechanism (Fig. 2), the inhibitory effect of NO but not that of GS-NO depended on the oxygen concentration. NO reacts with O_2 at a rate constant of $6\times10^6\ M^{-2}\cdot s^{-1}$ (*2*). Thus, under air atmospheric conditions (220 μM O_2), the lifetime of a low concentration of NO slowly released from RS-NO would be significantly longer than that of 10 μM NO used in Fig. 6. This might be one of the reasons why the inhibitory effect of NO but not that of GS-NO depended on the oxygen tension in the medium. Cys-NO inhibited the respiration more strongly than GS-NO and NAC-NO did. However, the D- and L-forms of Cys-NO exhibited similar abilities to inhibit the respiration. Because the *S*-conjugates of L-cysteine but not D-cysteine is actively taken up by cells (*43*), the similar inhibition by the two isomers might suggest the occurrence of NO outside the cells. Preliminary experiments revealed that a significant amount of AsA comes out from EATC into the medium during incubation. Thus, the extracellular release of NO from RS-NO might depend, at least in part, on the rate of AsA secretion into the medium. Because the decomposition of GS-NO was enhanced by AsA more strongly than that of Cys-NO, the secreted AsA might enhance the release of NO extracellularly more effectively from the L- and D-forms of Cys-NO than from GS-NO.

It should be noted that the GS-NO-dependent inhibition of EATC respiration was enhanced more effectively by cysteine than by AsA. Because the decomposition of GS-NO was enhanced by AsA 2-3 times more effectively than by cysteine, some mechanism other than a chemical reaction between GS-NO and the two reducing agents might operate in the presence of EATC. The mechanism by which cysteine enhanced the inhibitory effect of GS-NO more effectively than AsA should be studied further.

GS-NO rapidly and transiently decreased the blood pressure of animals, although its decomposition occurred extremely slowly in fresh plasma, in which the levels of AsA, cysteine, and GSH are fairly low. This suggested that the rate of NO release from GS-NO was significantly enhanced *in vivo*. Because GSH metabolism occurs extremely rapidly *in vivo*, predominantly due to the presence of its inter-organ cycle (*41*), the net amounts of GSH and cysteine that appear in the circulation would be significantly large. AsA is also metabolized rapidly *via* some inter-organ cycle (*44, 45*). Thus, the decomposition of GS-

NO through a γ-GTP-catalyzed mechanism and/or the cysteine/AsA-enhanced mechanism might occur significantly rapidly *in vivo*, and the released NO transiently exhibits its depressor action. Consistent with this notion, Scharfstein *et al.* reported that infusion of L-cysteine enhanced the depressor effect of *S*-nitroso-albumin (*38*). Thus, cross-talk between RS-NO and reducing compounds, such as AsA, cysteine, and related thiols, might play important roles in the regulation of the fates and functions of NO and RS-NO.

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