

# Role of ascorbic acid in the metabolism of *S*-nitroso-glutathione

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Received 10 May 1996

**Abstract** Although nitric oxide (NO) has been known to generate *S*-nitroso-thiols (RSNOs), dynamic aspects of their metabolism remain to be elucidated. The present work reports the reactivity of *S*-nitroso-glutathione (GS-NO) with various compounds with reducing activity. Kinetic analysis revealed that among various reagents tested, ascorbic acid showed a potent activity to decompose GS-NO to glutathione and oxidized products of NO. During the reaction of GS-NO and ascorbic acid, monodehydroascorbate was found to appear as an intermediate. These results suggest that ascorbic acid might be an important modulator for RS-NO metabolism.

**Key words:** *S*-Nitroso-glutathione; Ascorbic acid; Nitric oxide; Thiol

## 1. Introduction

Nitric oxide (NO) has received much attention not only as an endothelium-derived relaxing factor (EDRF) [1] but also as a key molecule for a variety of biological events. NO exerts its biological function through interaction with a number of biomolecules, such as heme iron, nonheme iron [2], oxygen [3,4] and superoxide anion [5]. NO also generates *S*-nitroso-thiols (RS-NOs) including *S*-nitroso-glutathione (GS-NO) [6,7]. In this context, Stamler et al. [8,9] reported that plasma and bronchial lavage fluid from healthy human contained about 7 and 0.3  $\mu\text{M}$  of RS-NO and GS-NO, respectively.

RS-NO also exhibits various activities attributed to NO. In fact, GS-NO, *S*-nitrosocysteine and *S*-nitroso-albumin have been reported to possess EDRF-like activity [10–13]. Ignarro [14] and Craven [15] suggested that NO released from RS-NOs was responsible for their EDRF-like action. On the other hand, Mathews [16] suggested that chemical stability of various RS-NOs in vitro did not correlate with their depressor effect in vivo.

In contrast to the short lifetime of NO, RS-NO is fairly stable. For example, under physiological conditions, half-lives of GS-NO and *S*-nitroso-albumin in vitro are longer than 10 h, while that of *S*-nitroso-cysteine is about 90 min [17]. Since glutathione *S*-conjugates are degraded to their constituent amino acids and the corresponding cysteine *S*-conjugates by  $\gamma$ -glutamyltransferase and peptidases [18], the fate and biological function of GS-NO might be affected by its metabolism in vivo. Since sodium borohydride has been used for releasing

NO from RS-NO [19], other molecules with reducing activity may also affect the fate of RS-NO in vivo. It has been well documented that ascorbic acid and GSH are the naturally occurring major reducing compounds ( $\sim 10$  mM in cytosol and  $\sim 80$   $\mu\text{M}$  in plasma) [18,20]. The present work describes the reactivity of GS-NO with ascorbic acid.

## 2. Materials and methods

### 2.1. Materials

GSH, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), sodium nitrite and ascorbic acid were obtained from Wako Co. (Osaka, Japan). Diethylenetriamine-*N,N,N',N'',N'''*-pentaacetic acid (DETAPAC) was obtained from Dojin Chemical Co. (Kumamoto, Japan). Dehydroascorbic acid was obtained by oxidizing ascorbic acid in 20 mM phosphate buffer (pH 7.5) at 37°C for 5 days under an air stream. The oxidized sample contained no detectable level of the reduced form of ascorbic acid as detected by HPLC-electrochemical detection [21]. GS-NO was synthesized as reported by Saville [22]. Briefly, GSH was reacted with equimolar sodium nitrite in acidic medium (pH 2.0). After standing for 15 min at 25°C, 1 N NaOH was added to the reaction mixture to give a final pH of 7.5. To the mixture was added Tris-HCl buffer (pH 7.5) to give a final concentration of 20 mM. The extent of GS-NO formed was determined spectrophotometrically at 545 nm [23]. Disappearance of GSH and nitrite in the reaction mixture was confirmed by the methods of Ellman [24] and Green [25], respectively. The yield of GS-NO generated from GSH was higher than 96%. All other reagents were of the highest grade commercially available.

### 2.2. Measurement of GS-NO and glutathione

The concentration of GS-NO was determined spectrophotometrically as described previously [23] ( $\epsilon_{545} = 13.0 \text{ M}^{-1} \text{ cm}^{-1}$ ) and by the method of Saville [22]. Total glutathione (GSH + 2 GSSG) was measured by the method of Tietze [26]. Free sulfhydryl group was determined by using DTNB as described previously [24].

### 2.3. Measurement of nitrite and nitrate

The amount of NO formed from RS-NO was determined as nitrite plus nitrate ( $\text{NO}_x^-$ ). Nitrate in the reaction mixture was reduced to nitrite by the cadmium column method [25]. Nitrite was measured by the method of Griess [27].

### 2.4. Electron spin resonance (ESR) measurement

The reaction mixture contained, in a final volume of 0.2 ml, 20 mM phosphate buffer (pH 7.5), 10  $\mu\text{M}$  DETAPAC, 10 mM ascorbic acid and/or GS-NO. The reaction was started by adding GS-NO. The ESR spectrum for monodehydroascorbate was recorded in a flat cell using JEOL TES-TE200 at 37°C, 12 mW power, 0.1 mT modulation, 335 mT magnetic field strength, 100 mT scan range and time constant of 0.3 s.

## 3. Results

Fig. 1a shows the disappearance of GS-NO under air atmospheric conditions at 37°C and pH 7.5 in the presence or absence of various concentrations of ascorbic acid. In the absence of ascorbic acid, GS-NO disappeared from the incubation mixture extremely slowly: the half-life of GS-NO was longer than 10 h. In the presence of ascorbic acid, however,

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**Abbreviations:** NO, nitric oxide; RS-NO, *S*-nitroso-thiol; GS-NO, *S*-nitroso-glutathione; EDRF, endothelium-derived relaxing factor; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid);  $\text{NO}_x^-$ , nitrite and nitrate; GSH, reduced form glutathione; GSSG, oxidized form glutathione; ESR, electron spin resonance; LPS, lipopolysaccharide; DETAPAC, diethylenetriamine-*N,N,N',N'',N'''*-pentaacetic acid

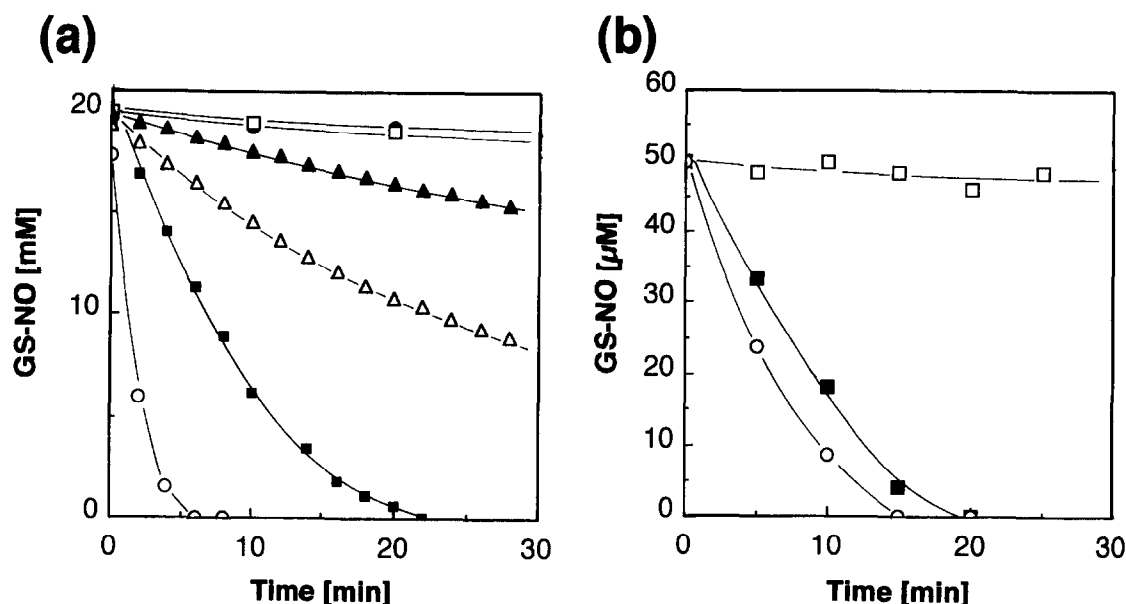


Fig. 1. Effect of ascorbic acid on *S*-nitroso-glutathione. The incubation mixture contained, in a final volume of 1 ml, 20 mM phosphate buffer (pH 7.5), 10 μM DETAPAC, 20 mM (a) or 50 μM (b) GS-NO and varying concentrations of ascorbic acid. The reaction was started by adding ascorbate at 37°C. In some experiments, the mixture contained either 20 mM of dehydroascorbate (●) or 7 mM GSH (▲). The concentrations of ascorbic acid used were 0 (□), 7 (Δ), 20 (■) and 200 (○) mM for (a) and 0 (□), 50 (■) and 100 (○) μM for (b).

GS-NO rapidly disappeared from the incubation mixture. The rate of GS-NO disappearance depended on the concentration of ascorbic acid. In contrast, incubation with dehydroascorbic acid had no effect on the rate of GS-NO disappearance. These results indicate that GS-NO reacted with ascorbic acid. In the presence of excess amounts of ascorbic acid, GS-NO disappeared with a first-order rate constant of  $1.02 \times 10^{-2} \text{ s}^{-1}$ . GS-NO also reacted with GSH though the reactivity with this

thiol was significantly lower than that with ascorbic acid. Fig. 1b also shows the disappearance of GS-NO in the presence of low concentrations of ascorbic acid. GS-NO also disappeared rapidly in the presence of physiologically low levels of ascorbic acid.

Fig. 2 shows the amount of GS-NO disappearing in the presence of various concentrations of ascorbic acid. These results show that one mol of GS-NO disappeared at the expense of 0.5 mol of ascorbic acid, indicating that the stoichiometry of ascorbic acid to GS-NO is 2. Table 1 summarizes the reaction products formed upon incubation of GS-NO and ascorbate. GS-NO disappeared with the concomitant appearance of glutathione. After incubation for 60 min at 37°C, no DTNB-titratable thiol was found in the mixture. At the same time, the nitrous moiety of GS-NO was recovered as nitrite and nitrate ( $\text{NOx}^-$ ); the recovery of  $\text{NOx}^-$  was about 80% of the GS-NO decomposed.

Fig. 3 shows the ESR spectra for GS-NO and ascorbic acid. GS-NO itself showed no ESR signal (Fig. 3a). In contrast, ascorbic acid revealed a small signal caused by the presence of monodehydroascorbate (Fig. 3b). In the presence of both GS-NO and ascorbic acid, the ESR signal for monodehydroascorbate increased markedly (Fig. 3c). Monodehydroascorbate increased immediately after mixing GS-NO with ascorbic acid. The signal slowly decreased during the incubation with concomitant formation of glutathione (data not shown). These observations suggested the formation of monodehydroascorbate as an intermediate during the reaction of GS-NO and ascorbic acid.

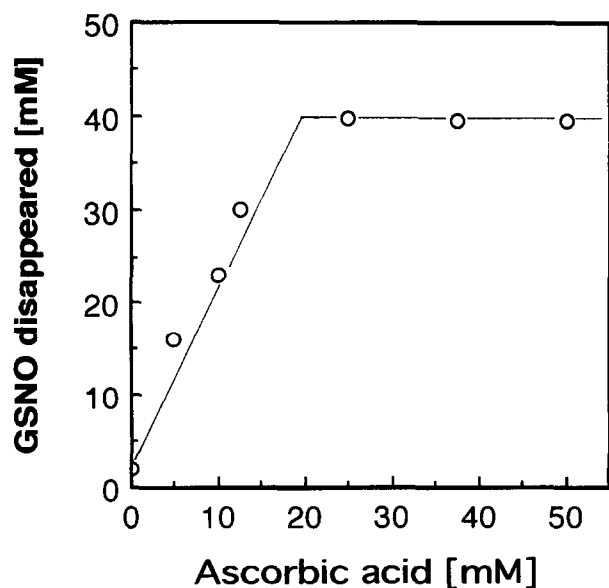


Fig. 2. Stoichiometric reaction of GS-NO and ascorbic acid. The incubation mixture contained, in a final volume of 1 ml, 20 mM phosphate buffer (pH 7.5), 10 μM DETAPAC, 40 mM GS-NO and varying concentrations of ascorbic acid. After incubation at 37°C for 60 min in air, the reaction mixture was rapidly cooled to 0°C. Then, the concentration of GS-NO consumed was determined spectrophotometrically.

#### 4. Discussion

The present work demonstrates that ascorbic acid and GS-NO stoichiometrically reacted and generated glutathione and  $\text{NOx}^-$ , oxidized products of NO. One mol of ascorbic acid reacted with two mol of GS-NO with concomitant formation

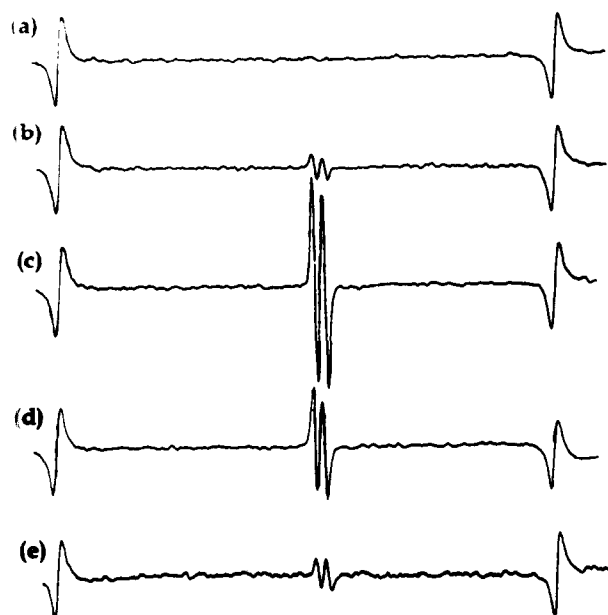
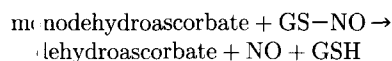
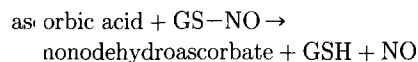


Fig. 3. ESR spectrum for GS-NO, ascorbic acid and their mixtures. The reaction mixture contained, in a final volume of 0.2 ml, 20 mM phosphate buffer (pH 7.5), 10  $\mu$ M DETAPAC and 10 mM GS-NO (a, c–e). The reaction was started by adding ascorbic acid. After incubation for 1 (c), 22 (d) and 40 min (e), the ESR signals were obtained in the absence (a) or presence (b–e) of 10 mM ascorbic acid. Other conditions were as described for Fig. 1.

of monodehydroascorbate and  $\text{NOx}^-$ . When Ellman's reagent (1 mM) was added to the reaction mixture, chemical reduction of this reagent occurred during the incubation (data not shown). This observation might suggest the formation of a free sulfhydryl group as an unstable intermediate. Thus, the following reaction might have occurred during the incubation:



In the presence of oxygen, NO is easily oxidized to  $\text{NOx}^-$  [4]. In fact, after incubation for 60 min, about 80% of the NO moiety of GS-NO was recovered as  $\text{NOx}^-$ . The remaining 20% of the NO moiety would be metabolized to other compound(s) than  $\text{NOx}^-$ . In this context, nitrous oxide has been known to appear during the reaction of NO and GSH [6,28].

Thus, nitrous oxide might possibly be formed during the incubation of GS-NO and ascorbic acid.

Steady-state levels of NO in and around cells which have NO synthase would be fairly high. For example, endothelial cells of the aorta generate NO at a rate of  $\sim 0.5$  nmol/min/g tissue [29]. In the presence of oxygen, NO is rapidly converted to  $\text{N}_2\text{O}_3$  at a rate constant of  $7 \times 10^6 \text{ M}^{-2} \text{ s}^{-1}$  which then reacts with thiols to form RS-NO [7]. Since the intracellular level of GSH is as high as  $\sim 10$  mM [18], significant fractions of NO would react with the SH group of this tripeptide. Assuming that NO exclusively reacted with GSH to form GS-NO, about 0.5 nmol/min of GS-NO might theoretically occur in and around 1 g of arterial walls. Intracellular concentrations of ascorbic acid are also high ( $\sim 10$  mM) [20]. Since GS-NO and ascorbic acid reacted stoichiometrically and form monodehydroascorbate as intermediary metabolite, this radical species would also occur in and around vascular walls. In fact, fairly high concentrations of monodehydroascorbate occurred in the circulation of normal rats [30,31]. It has been suggested that ascorbic acid also reacts with vitamin E radical on cell surface membranes [32]. Thus, interactions of ascorbic acid with vitamin E radical and RS-NO might underlie the mechanism for the formation of ascorbyl radical *in vivo* [30,31].

It has been reported that various thiols, such as GSH and cysteine, also react with RS-NO [22,33]. Thus, the lifetime of GS-NO would be fairly short in cells which are enriched with ascorbic acid and GSH. In contrast, plasma levels of ascorbic acid (40–80  $\mu$ M) and GSH (5–25  $\mu$ M) are significantly lower than those in cells ( $\sim 10$  mM). Therefore, the lifetime of GS-NO in the circulation would be longer in plasma than in cells.

In animals with septicemia, NO synthase (iNOS) is induced in the liver and other organs and, hence, significant amounts of NO (and GS-NO) would be generated in these tissues. Thus, the formation and degradation of GS-NO would be enhanced in endotoxemic subjects. Since plasma levels of ascorbic acid are fairly low, they might also be affected by the occurrence of GS-NO. In fact, plasma levels of ascorbic acid have been reported to decrease in lipopolysaccharide (LPS)-treated animals in which NO generation is significantly enhanced by iNOS [34]. Active oxygen species formed in various cells and tissues which were challenged with oxidative stress also react with ascorbic acid thereby decreasing its plasma levels. The enhanced production of both GS-NO and active oxygen species might decrease plasma levels of ascorbic acid *in vivo*. Thus, levels and redox status of ascorbic acid might principally determine the fate and function of GS-NO and related nitroso-thiols. The pathophysiological significance of

Table 1  
Reaction products from S-nitroso-glutathione and ascorbic acid

| Before     |                    | After incubation |                    |                     |                  |         |
|------------|--------------------|------------------|--------------------|---------------------|------------------|---------|
| GS-NO (mM) | Ascorbic acid (mM) | GS-NO (mM)       | Ascorbic acid (mM) | $\text{NOx}^-$ (mM) | Glutathione (mM) | SH (mM) |
| 40         | 0                  | $38 \pm 2.3$     | n.d.               | $1.3 \pm 0.3$       | $2.8 \pm 0.4$    | n.d.    |
| 40         | 20                 | n.d.             | n.d.               | $34 \pm 2.5$        | $42 \pm 4.0$     | n.d.    |
| 40         | 40                 | n.d.             | $18 \pm 2$         | $33 \pm 1.5$        | $40 \pm 2.6$     | n.d.    |
| 0          | 40                 | n.d.             | $38 \pm 0.9$       | n.d.                | n.d.             | n.d.    |

Reaction mixtures contained, in a final volume of 1 ml, 20 mM phosphate buffer (pH 7.5), 10  $\mu$ M DETAPAC, and varying concentrations of GS-NO and ascorbic acid. After incubation at 37°C for 60 min, the concentrations of various products were determined. Other conditions were as described in the text. Glutathione, GSH+2 GSSG; n.d., not detectable.

the interaction of RS-NO and ascorbic acid should be studied further.

**Acknowledgements** We thank Drs. T. Yoshimura and O. Pechanova for helpful suggestions. This work was supported by the Japan Heart Foundation and the Osaka City University Medical Research Foundation Fund for Medical Research.

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