Ascorbic Acid and Glutathione Modulate the Biological Activity of S-Nitrosoglutathione

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Abstract—Ascorbic acid and glutathione (GSH) are important determinants of the intracellular redox state, and both are known to accelerate the decomposition of S-nitrosoglutathione (GSNO), an endogenous adduct of nitric oxide (NO). The implications of these observations for GSNO bioactivity are not yet clear. We investigated the effect of ascorbic acid and GSH on GSNO bioactivity by using a bioassay with isolated segments of guinea pig aorta suspended in organ chambers. Arterial segments demonstrated relaxation to GSNO (0.1 μmol/L) that was significantly enhanced by 300 μmol/L ascorbic acid (71±6% versus 53±6%, P<0.05) but not GSH. Both ascorbic acid and GSH significantly shortened the duration of arterial relaxation in response to 0.1 μmol/L GSNO (from >120 minutes to 22.5±3.5 and 36.3±4.3 minutes, respectively; P<0.05), consistent with accelerated decomposition of GSNO that was confirmed spectrophotometrically. The effect of ascorbic acid was abrogated by either DTPA or the copper(I)-specific agent bathocuproine but not deferoxamine, indicating a dependence on the availability of redox-active copper. Consistent with this notion, the action of ascorbic acid on GSNO bioactivity was also supported by copper-zinc superoxide dismutase, a physiologically relevant source of copper. In contrast, the effect of GSH on GSNO degradation and GSNO-mediated arterial relaxation was independent of transition metal ions, because DTPA had no effect. These data indicate that both ascorbic acid and GSH modulate GSNO bioactivity and suggest a distinction between the mechanism of GSNO degradation by ascorbic acid or GSH. Whereas both ascorbic acid and GSH accelerate the degradation of GSNO, only ascorbic acid is dependent on the presence of transition metal ions. (Hypertension. 2000;36:291-295.)

Key Words: antioxidants ■ oxidation-reduction ■ blood vessels ■ nitric oxide ■ S-nitrosoglutathione ■ relaxation

Vascular homeostasis is dependent, in part, on nitric oxide (NO), a free radical that is synthesized enzymatically from l-arginine and O₂ by an endothelial isofrom of nitric oxide synthase. NO contributes to resting vascular tone, impairs platelet activation, prevents leukocyte adhesion to the endothelium, and inhibits vascular smooth muscle migration and proliferation. Mice lacking endothelial nitric oxide synthase are hypertensive, and impaired NO bioactivity in diabetes and atherosclerosis is thought to contribute to vascular disease (reviewed in Reference 9). Thus, NO is an important mediator of vascular homeostasis.

Under aerobic conditions, NO can combine with O₂ to form S-nitrosothiols, which possess bioactivity similar to authentic NO. The intracellular space, the abundance of glutathione (GSH; 1 to 5 mmol/L) renders the formation of S-nitrosoglutathione (GSNO) kinetically feasible, and GSNO formation has been implicated in cellular functions such as neutrophil oxidant production and signal transduction. The precise metabolic fate of GSNO and its implications for NO bioactivity, however, remain unclear.

Recent evidence suggests that S-nitrosothiol metabolism is sensitive to the local reducing environment. Reduced transition metal ions such as Cu²⁺ catalyze the decomposition of S-nitrosothiols more effectively than do their oxidized forms (eg, Cu⁷⁺). Both ascorbic acid and GSH accelerate the decomposition of GSNO in vitro and may modulate the release of NO from S-nitrosothiols. Enhanced release of NO from S-nitrosothiols has been shown to augment the hypertensive response to these agents. However, the effect of ascorbic acid and GSH on the NO-like bioactivity of GSNO is not clear and serves as the purpose of the present study.

Methods

Materials
Sodium nitrite was purchased from Fisher Scientific. Sulfanilamide and N-(1-naphthyl)ethylene diamine dihydrochloride were obtained from Aldrich Chemical Co. Chelex-100 resin was purchased from Bio-Rad Laboratories, whereas DTPA, GSH, prostaglandin F₂α (PGF₂α), and all other compounds were purchased from Sigma Chemical Co.

Physiological salt solution (PSS) contained 118.3 mmol/L NaCl, 4.7 mmol/L KCl, 2.5 mmol/L CaCl₂, 1.2 mmol/L MgSO₄, 1.2 mmol/L KH₂PO₄, 25 mmol/L NaHCO₃, and 11.1 mmol/L glucose. All solutions were prepared with double-distilled water and treated with Chelex before use. All glassware was acid-washed followed by thorough flushing with Chelex-treated water. Solutions of NO and GSNO were synthesized and quantified immediately before use as described.

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Acid but not GSH enhances GSNO-mediated arterial relaxation. Segments of thoracic aorta were harvested from Hartley guinea pigs as described in Methods. Vessels were contracted with PGF$_2$-$\alpha$, and relaxation was assayed in response to the indicated concentrations of GSNO in the presence of PSS containing no additions (▵), or either ascorbic acid (A) or GSH (B) at concentrations of 0.1 (□), 0.3 (○), or 1 (▲) mmol/L. Data are derived from 5 to 7 experiments. *P<0.05 versus control by 2-way ANOVA.

**NO Bioassay**

The NO-like bioactivity of GSNO was assessed in a PSS buffer system with vascular relaxation as a bioassay with segments of Hartley guinea pig thoracic aorta. Animals (450 to 500 g) were killed with CO$_2$, and aortic ring segments (5 mm) without endothelium were prepared and suspended in organ chambers as described. Organ chambers were acid-washed followed by copious flushing with Chelex-treated water to minimize contamination with metal ions. With this treatment, the buffer metal ion content (as assessed by ascorbate oxidation) was reduced from 6 to <0.01 mmol/L (n=3). Animal studies were approved by the Boston University Medical Campus Institutional Animal Care and Use Committee.

**Quantification of GSNO Degradation**

Time-dependent changes in GSNO concentration were monitored continuously at 332 nm ($\varepsilon=750$ mol/L$^{-1}$·cm$^{-1}$) over a period of 100 minutes with the use of a Varian Cary 3 dual-beam spectrophotometer at 37°C. The rate of GSNO decay as a function of time ($d$[GSNO]/$dt$) was determined by the initial slope of the decay curve over the first 10 minutes of incubation.

Results

**Effect of Ascorbic Acid and GSH on GSNO-Induced Arterial Relaxation**

We used arterial relaxation as a marker for the NO-like bioactivity of GSNO. Extracellular GSNO added to organ chambers produced dose-dependent arterial relaxation that was mildly enhanced by physiologically relevant ascorbic acid (>$0.1$ mmol/L) in the buffer (Figure 1A; P<0.05 for ascorbate versus control by 2-way ANOVA). Higher concentrations of ascorbic acid produced no additional effect. In contrast, GSNO-mediated arterial relaxation was not materially altered by GSH (Figure 1B), and no vasodilation was observed with decomposed GSNO (data not shown). Thus, ascorbic acid but not GSH modestly augments the extent of GSNO-induced arterial relaxation.

Ascorbic acid and GSH had more uniform effects on the duration of GSNO-mediated arterial relaxation. Contracted arterial segments exposed to GSNO (0.1 mmol/L) demonstrated stable relaxation that was shortened considerably by 300 mmol/L ascorbic acid or GSH (Figure 2A). Quantitatively, the halftime of GSNO (0.1 mmol/L) arterial relaxation (ie, the time required to restore 50% of the tension reduction produced by a vasodilator) exceeded 120 minutes (Figure 2B) and decreased to 22.5±3.5 minutes and 36.3±4.3 minutes in the presence of ascorbic acid and GSH, respectively (both P<0.05 versus control by 1-way ANOVA; n=6). Thus, both ascorbic acid and GSH appear to accelerate GSNO decomposition manifested as a shorter duration of arterial relaxation.

**Transition Metal Ions and Ascorbic Acid–Induced GSNO Decomposition**

The decomposition of GSNO has been linked to reduced transition metal ions, and ascorbic acid is known to reduce the valence state of transition metal ions in solution. In the presence of DTPA, a strong metal chelator, we found that ascorbic acid had no effect on the duration of GSNO-mediated arterial relaxation (Figure 3). Specific chelation of Cu(I) with 100 mmol/L bathocuproine sulfonate also abrogated the effect of ascorbic acid, whereas the iron-specific chelator deferoxamine (100 mmol/L) was ineffective (Figure 3).

To examine the kinetics of GSNO decomposition, we followed GSNO decay as the change in absorbance at 332 nm. As shown in Figure 4, GSNO decay was significantly enhanced by ascorbic acid, and this effect was inhibited by chelation of transition metals with DTPA (Figure 4). The effect of ascorbic acid was specific for Cu(I), because it was inhibited by bathocuproine and not affected by deferoxamine (Figure 4). Individually, copper and ascorbic acid enhanced GSNO degradation, and their combination was additive.

Figure 1. Ascorbic acid (AA) and the duration of GSNO-mediated arterial relaxation. Segments of thoracic aorta were harvested from Hartley guinea pigs as described in Methods. A, Vessels were contracted with PGF$_2$-$\alpha$, and the duration of relaxation was assayed in response to 0.1 µmol/L GSNO in the presence or absence of 0.3 mmol/L ascorbic acid or GSH. B, Composite data on the duration of GSNO-mediated arterial relaxation derived from the half-time of arterial relaxation as described in Methods. Data are mean±SEM of 6 experiments. CTL indicates control. *P<0.05 vs no additions.
Thus, ascorbic acid enhances GSNO decomposition in a copper-dependent manner.

**Transition Metal Ions and GSH-Induced GSNO Decomposition**

Arterial relaxation to GSNO (0.1 μmol/L) was significantly shortened by GSH (Figures 5A and 5B), but this effect was only partially inhibited by DTPA (Figure 5A) and not materially altered in the presence of copper or deferoxamine (Figure 5B). Consistent with this observation, GSNO decay was significantly enhanced by GSH (Figure 6). However, the effect of GSH on GSNO decay was not significantly inhibited by chelation of transition metals with DTPA (Figure 6). In fact, GSH actually inhibited the action of copper to enhance GSNO decomposition (Figure 6). Thus, GSH accelerates GSNO decay through a mechanism that appears largely independent of transition metals.

The availability of redox-active copper in vivo is controversial. We sought to determine whether a physiologically relevant source of copper (eg, copper-zinc superoxide dismutase [Cu-Zn SOD]) would support the effects of ascorbic acid on GSNO-mediated arterial relaxation. As shown in Figure 7, Cu-Zn SOD enhanced the effect of ascorbic acid on GSNO-mediated arterial relaxation, and this effect was inhibited by DTPA. In contrast, Cu-Zn SOD had no effect on the ability of GSH to shorten the duration of GSNO-mediated arterial relaxation (Figure 7).

**Discussion**

The data presented here indicate that both ascorbic acid and GSH modulate the bioactivity of S-nitrosoglutathione.
ascorbic acid (50 to 100 μmol/L) induced a maximum extent of arterial relaxation. Specifically, the extent of arterial relaxation increased in the presence of ascorbic acid compared with authentic NO.38,39 With this scenario, one would predict that GSH-mediated GSNO decomposition would not lead to enhanced NO-like bioactivity, consistent with the results reported here (Figure 1B). Thus, metal ion–independent reaction of GSH with GSNO can account for the reduced GSNO-mediated arterial relaxation in the presence of millimolar concentrations of GSH.

In summary, the data presented here indicate that both ascorbic acid and GSH enhance the decomposition of GSNO. The proposed product of this reaction was peroxynitrite, a compound with limited vasodilating bioactivity compared with authentic NO.38,39 With this scenario, one would predict that GSH-mediated GSNO decomposition would not lead to enhanced NO-like bioactivity, consistent with the results reported here (Figure 1B). Thus, metal ion–independent reaction of GSH with GSNO can account for the reduced GSNO-mediated arterial relaxation in the presence of millimolar concentrations of GSH.

One important issue to consider is the relevance of our findings for S-nitrosothiol action and metabolism in vivo. Considerable evidence indicates that S-nitrosothiols are formed in vivo, and protein S-nitrosylation has been implicated in the modulation of energy metabolism,17 signal transduction,40 apoptosis,41 and even blood flow.42 For such events to be reversible, the cellular environment must provide some mechanism(s) to restore nitrosothiols back to their reduced form. Singh and colleagues43 have proposed that GSNO serves as a sink for functional protein NO− moieties, leading to the “repair” of S-nitrosylated proteins, and that GSH-mediated GSNO decomposition facilitates this repair mechanism. Our data and those of others19,39 indicate that ascorbic acid may also facilitate GSNO decomposition, perhaps through copper-containing enzymes such as SOD (Figure 7),43 and enhance the NO-like bioactivity of GSNO (Figure 1). Prior reports that ascorbic acid enhances NO bioactivity44 and reduces blood pressure45 have prompted speculation on the role of GSNO decomposition in these observations.

In summary, the data presented here indicate that both ascorbic acid and GSH enhance the decomposition of GSNO. The in vivo role of ascorbic acid, accelerated GSNO decomposition is metal ion dependent and associated with a modest increase in bioactivity (eg, vasodilation) that is reduced in duration. With GSH, however, accelerated GSNO decom-
sition is not metal ion dependent and does not seem to result in enhanced bioactivity. Instead, the main effect is a reduction in the duration of GSNO bioactivity. These data suggest that the reducing environment within the cell has important implications for S-nitrosothiol bioactivity.

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