Bioactivation of Nitroglycerin by Ascorbate

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ABSTRACT

Bioactivation of nitroglycerin (GTN) into an activator of soluble guanylate cyclase (sGC) is essential for the vasorelaxant effect of the drug. Besides several enzymes that catalyze GTN bioactivation, the reaction with cysteine is the sole nonenzymatic mechanism known so far. Here we show that a reaction with ascorbate results in GTN bioactivation. In the absence of ascorbate, GTN did not affect the activity of purified sGC. However, the enzyme was activated to ~20% of maximal NO-stimulated activity by GTN in the presence of 10 mM ascorbate with an EC_{50} value of 27.3 ± 4.9 μM GTN. The EC_{50} value of ascorbate was 0.11 ± 0.011 mM. Activation of sGC was sensitive to oxyhemoglobin, superoxide, and a heme-site enzyme inhibitor. GTN had no effect when ascorbate was replaced by 1000 U of superoxide dismutase per milliliter. Ascorbate is known to reduce inorganic nitrite to NO. In the presence of 10 mM ascorbate, ~30 μM nitrite caused the same increase in sGC activity as 0.3 mM GTN. Determination of ascorbate-driven 1,2- and 1,3-glycerol dinitrate formation indicated that a 140 nM concentration of products was generated from 0.3 mM GTN within 10 min, excluding nitrite as a relevant intermediate. Our results suggest that a reaction between GTN and ascorbate or an ascorbate-derived species yields an activator of sGC with NO-like chemical properties. This reaction may contribute to GTN bioactivation in blood vessels under conditions of GTN tolerance and ascorbate supplementation.

In line with the complex redox chemistry of ascorbate and its actions as both anti- and pro-oxidant, the potential interactions of ascorbate with vascular NO/cGMP signaling are manifold. According to the available literature, ascorbate seems to affect this pathway at virtually all levels, including the biosynthesis and biological half-life of NO, the activation state of sGC, and the release of NO from precursors, in particular inorganic nitrite, which is reduced to NO by ascorbate (May, 2000).

Despite a bulk of literature on the effects of ascorbate on endothelial function and cGMP-mediated vasodilatation (May, 2000), there are hardly any reports on the effects of ascorbate on purified sGC. The early studies (Craven and DeRubertis, 1978; Ignarro and Gruetter, 1980) are difficult to interpret because ascorbate triggers NO release from various precursors (see below), and clean NO-releasing drugs were not available before 1991 (Maragos et al., 1991). Thus, it is hard to distinguish between direct effects on sGC and effects of ascorbate on NO release from donor compounds that exhibit rather complex chemical reactivity (Wang et al., 2002). More recently, we reinvestigated the effect of ascorbate on sGC using highly purified protein and DEA/NO as NO donor compound. It was surprising to find that ascorbate inhibits cGMP formation, presumably by two distinct mechanisms: 1) superoxide-mediated scavenging of NO; and 2) inactivation of sGC by a product of ascorbate autoxidation. However, both effects were largely prevented by GSH and the metal chelator DTPA (Schrammel et al., 2000), indicating that ascorbate should not considerably affect NO-stimulated sGC activity under physiological conditions.

Ascorbate is known to trigger the release of NO from various endogenous precursors. The reduction of nitrite to NO was first described as a protective mechanism preventing the formation of carcinogenic nitrosamines (Archer et al., 1975) and later shown to result in pronounced activation of sGC (Ignarro et al., 1980). Low and high molecular weight S-nitrosothiols are believed to have important biological functions as relatively stable storage and transport forms of NO.
(Singel and Stamler, 2005; Zhang and Hogg, 2005). Because cleavage of the S-NO bond is catalyzed by reduced trace metal ions, the reaction is essentially dependent on the presence of redox agents like GSH or ascorbate (Gorren et al., 1996; Kashiba-Iwatsuki et al., 1996; Singh et al., 1996). A similar mechanism may be involved in the release of NO from furanox derivatives of aspirin that are being developed to overcome the gastric side effects of nonsteroidal anti-inflammatory drugs (Turnbull et al., 2006).

The first evidence that GTN does not directly activate sGC was obtained by Murad and coworkers, who found that the effect of the organic nitrate was fairly poor and tissue-specific (Katsuki et al., 1977). In the meantime, it is well-established that vasodilation to GTN requires bioactivation of the drug that is catalyzed by several enzymes, including GSH-S-transferase, cytochrome P450, xanthine oxidase, and mitochondrial aldehyde dehydrogenase. For a recent review on the various mechanisms of GTN bioactivation and the development of nitrate tolerance see Fung (2004). Nonenzymatic metabolism of GTN to yield 1,2- and 1,3-GDN together with nitrite is triggered by a wide variety of thiols, whereas bioactivation specifically requires cysteine or cysteine derivatives (Ignarro and Gruetter, 1980; Bennett et al., 1989). The chemistry of the GTN/cysteine system is not well understood (Thatcher and Weldon, 1998), and the identity of the bioactive reaction product is still a matter of debate (Artz et al., 2001; Gorren et al., 2005). Besides the GTN/cysteine reaction, no other chemical reaction is known that causes GTN bioactivation. In the present study, we describe a new ascorbate-driven mechanism that results in formation of a GTN-derivated activator of sGC with NO-like reactivity.

Materials and Methods

Materials. Bovine lung sGC was purified as described previously (Russwurm and Koesling, 2005). Rat lung fibroblast cells (RFL-6, number CCL-192; American Type Culture Collection, Manassas, VA) were purchased from LGC Promochem GmbH (Wesel, Germany). [α-32P]GTP (400 Ci/mmol; NEN Radiochemicals) was obtained from PerkinElmer Life and Analytical Sciences (Vienna, Austria). Nitrone ampolles (G. Pohl-Boskamp GmbH and Co., Hohenlockstedt, Germany) were obtained from a local pharmacy and diluted in TEA/HCl. DEA/NO and ODQ (Alexis Corporation, Lausanne, Switzerland) were obtained via Eubio (Vienna, Austria). DEA/NO was dissolved and diluted in 10 mM NaOH. Stock solutions of ODQ (10 mM) were prepared in dimethyl sulfoxide and diluted with 25% dimethyl sulfoxide in H2O (v/v). Oxyhemoglobin was prepared by reduction of bovine hemoglobin (Sigma, Vienna, Austria) with sodium dithionite as described previously (Schmidt et al., 1994). All other chemicals were from Sigma.

Determination of cGMP Accumulation in Cultured RFL-6 Cells. RFL-6 cells cultured in 24-well plastic plates (2 × 10^6 cells/dish) were preincubated for 7 h in Ham's F-12 medium (37°C, 5% CO2), containing 20% (v/v) heat-inactivated fetal calf serum, 100 U/ml penicillin, 1 mg/ml streptomycin, 1.25 mg/ml amphotericin B, and, where indicated, 0.1 to 10 mM ascorbate. Before experiments, cells were washed and equilibrated in 50 mM Tris buffer (pH 7.4, 37°C) containing 100 mM NaCl, 5 mM KCl, 1 mM MgCl2, 3 mM CaCl2, 1 mM 3-isobutyl-1-methylxanthine, and 1000 U/ml SOD. After 15 min, GTN (final concentration, 0.1 mM) or DEA/NO (final concentration, 1 μM) was added, and cells were incubated for further 10 min at 37°C followed by cell lysis with 0.01 M HCl and determination of cGMP by radioimmunoassay.

Determination of sGC Activity. Purified bovine lung sGC (50 ng) was incubated at 37°C for 10 min in a final volume of 0.2 ml with the indicated concentrations of GTN, sodium nitrite, or DEA/NO in the presence of 50 mM TEA/HCl, pH 7.4, 0.5 mM [α-32P]GTP, −250,000 cpm), 3 mM MgCl2, 1 mM cGMP, and 0.1 mM DTPA. Reactions were terminated by the addition of 450 μl of zinc acetate (120 mM) and 450 μl of sodium bicarbonate (120 mM) followed by isolation of [32P]cGMP as described previously (Schultz and Böhme, 1984).

Determination of 1,2- and 1,3-GDN by Radio Thin-Layer Chromatography. Radiolabeled GTN (6, 100, and 300 μM, −150,000 dpm) was incubated with 10 mM ascorbate at 37°C for 3 h in a total volume of 0.2 ml of 50 mM phosphate buffer, pH 7.4. Products were extracted, separated by thin-layer chromatography, and quantified by liquid scintillation counting of the radioactive spots as described previously (Kollau et al., 2005). Blank values were determined in the absence of ascorbate under identical conditions and subtracted from the values obtained with ascorbate.

Results

Accounting for the complex chemistry of ascorbate, we performed all assays in a "minimally essential" reaction mixture (i.e., without thiols and bovine serum albumin, which are often included in sGC assays for optimal enzyme activity). Under these conditions, purified sGC exhibited a basal activity of 0.13 μmol/min/mg that was increased approximately 60-fold (to −8 μmol/min/mg) in the presence of 0.1 to 1 μM DEA/NO. The addition of 2 mM GSH had no significant effect on sGC activation by DEA/NO or GTN in the absence or presence of ascorbate (data not shown). The iron chelator DTPA (0.1 mM) was present throughout the study to minimize ascorbate autoxidation (Schrammel et al., 2000).

As shown in Fig. 1A, up to 1 mM GTN did not activate sGC. However, in the presence of 10 mM ascorbate, the organic nitrate caused a pronounced increase in enzyme activity with an EC50 value of 27.3 ± 4.9 μM (mean ± S.E.; n = 3). Maximal sGC activity in the presence of 0.3 mM GTN was 1.34 ± 0.05 μmol/min/mg, corresponding to 18% of the Vmax measured in the presence of 1 μM DEA/NO under identical conditions (7.3 ± 0.2 μmol/min/mg). Neither inorganic nitrate (up to 10 mM) nor other organic nitrites (isosor- bide dinitrate and pentaerythritol tetranitrate, 0.3 mM each) activated sGC above basal levels in the presence of ascorbate (n = 3, data not shown). As shown in Fig. 1B, ascorbate increased the EC50 value of DEA/NO from 12.2 ± 1.7 to 95.5 ± 28.3 nM. Because ascorbate did not affect the rate of DEA/NO decay measured by UV/Vis spectroscopy (data not shown), this effect was presumably caused by residual NO scavenging by ascorbate in the presence of DTPA (Schrammel et al., 2000). Figure 1C shows that inorganic nitrite caused a pronounced activation of sGC in the presence of 10 mM ascorbate, approaching the maximal activity of the DEA/NO-stimulated enzyme at 1 mM. Comparison with the GTN data (Fig. 1A) shows that ~30 μM (28–34 μM) nitrite was equieffective to 0.3 mM GTN. As shown in Fig. 2A, ascorbate alone only slightly increased sGC activity (approximately 2-fold at 10 mM) in the absence of GTN but triggered pronounced enzyme stimulation in the presence of 0.3 mM GTN with a sharp maximum at 1 mM and an EC50 value of 0.11 ± 0.011 μM. Up to 10 mM dehydroascorbate, which may account for sGC inactivation under conditions of ascorbate autoxidation (Schrammel et al., 2000), slightly inhibited basal sGC activity and did not support sGC activation by GTN (data not shown).
It is conceivable that the effect of ascorbate could have been caused by scavenging of superoxide generated from GTN in the assay mixtures. However, as shown in Fig. 2B, up to 1 mM GTN had no effect on sGC activity when ascorbate was replaced by 1000 U/ml SOD or 0.1 mM Trolox.

We attempted to measure NO release with an NO-sensitive electrode but obtained no signal upon incubation of up to 1 mM GTN with 10 mM ascorbate (data not shown). The detection limit of the method is 10 to 20 nM (peak concentration), but accumulation of 2 to 3 nM NO would have been sufficient to account for sGC activation by GTN/ascorbate, according to a rough estimate based on the DEA/NO data shown in Fig. 1B. Therefore, we took an indirect approach and tested the effects of the superoxide-generating agent FMN (0.1 mM), the NO scavenger oxyhemoglobin (20 μM), and the heme-site sGC inhibitor ODQ (30 μM). As shown in Fig. 3, all three agents had virtually identical effects on sGC activation by DEA/NO and GTN, suggesting the involvement of an identical reactive intermediate, presumably an NO radical. It should be noted that FMN was significantly less effective in the presence of ascorbate than under control conditions (virtually complete inhibition of sGC stimulation by DEA/NO; data not shown). It is possible that FMN-triggered NO inactivation was partially overcome by the superoxide scavenging activity of ascorbate.

Because the sGC data argued against NO sensitization of sGC and superoxide scavenging as potential mechanisms of ascorbate action, we speculated that ascorbate may react with GTN to yield a bioactive product and determined the formation of 1,2- and 1,3-GDN from radiolabeled GTN by thin-layer chromatography. However, after 10 min of incubation of GTN with 10 mM ascorbate, we did not observe the formation of radioactive products above blank levels (data not shown).

Fig. 1. Effects of GTN, DEA/NO, and nitrite on sGC activity in the absence and presence of ascorbate. Purified sGC (50 ng) was incubated at 37°C for 10 min in a final volume of 0.2 ml with the indicated concentrations of GTN (A), DEA/NO (B), and nitrite (C) in the absence (unfilled) or presence (filled) of 10 mM ascorbate. Assay mixtures contained 50 mM TEA/HCl, pH 7.4, 0.5 mM [α-32P]GTP (~250,000 cpm), 3 mM MgCl2, 1 mM cGMP, and 0.1 mM DTPA. Samples were analyzed for 32P-cGMP as described under Materials and Methods. Data are mean values ± S.E. of three experiments. Asc, ascorbate.

Fig. 2. Effect of ascorbate and superoxide scavengers on sGC activation by GTN. A, purified sGC (50 ng) was incubated at 37°C for 10 min in a final volume of 0.2 ml with the indicated concentrations of ascorbate and 0.3 mM GTN in the presence of 50 mM TEA/HCl, pH 7.4, 0.5 mM [α-32P]GTP (~250,000 cpm), 3 mM MgCl2, 1 mM cGMP, and 0.1 mM DTPA. Samples were analyzed for [32P]cGMP as described under Materials and Methods. Data are mean values ± S.E. of three experiments. B, GTN concentration-response curve in the presence of 10 mM ascorbate, 1000 U/ml SOD, or 0.1 mM Trolox. Experimental conditions as described in A. Data (mean values ± S.E.; n = 3) are expressed as a percentage of sGC activity measured in the absence of GTN to account for changes in basal enzyme activity.
not shown). Considering that even a very slow reaction could generate sufficient NO to activate sGC, we incubated increasing concentrations of GTN (6, 100, and 300 μM) with 10 mM ascorbate for 38 h at 37°C and determined the ascorbate-driven formation of 1,2- and 1,3-GDN. In the absence of ascorbate, nonenzymatic breakdown of 6, 100, and 300 μM GTN, respectively, led to the formation of 0.92 ± 0.10, 10.45 ± 0.14, and 26.1 ± 0.77 μM 1,2-GDN after 38 h. The rate of 1,3-GDN formation was virtually identical. As shown in Table 1, product yield was significantly higher in the presence of ascorbate, and ascorbate-driven product formation increased with increasing GTN concentration. Assuming linear reaction rates, the amount of 1,2- and 1,3-GDN generated by ascorbate within 38 h (31.4 μM) implicates the formation of 140 nM total products in the sGC assays (10-min incubation time).

To see whether cellular bioactivation of GTN is enhanced by ascorbate, we loaded RFL-6 cells for 7 h with 0.1 to 10 mM ascorbate and determined the accumulation of intracellular cGMP in response to GTN and DEA/NO. The experiments were performed in the presence of 1000 U/ml SOD to account for potential superoxide scavenging by ascorbate. As shown in Table 2, preincubation with ascorbate had no effect on DEA/NO-induced cGMP accumulation but significantly enhanced the effect of GTN, indicating that ascorbate contributes to GTN bioactivation in cells.

Discussion

The present study shows that GTN stimulates sGC in the presence of ascorbate. In a study published more than two decades ago, it was reported that ascorbate triggers bioactivation of nitrite but not of GTN (Ignarro and Gruetter, 1980). The lack of effect of ascorbate in the earlier study may be related to the low sGC activity and/or the presence of interfering components in crude blood vessel extracts. The effect of ascorbate observed here does not necessarily implicate the involvement of a chemical reaction with GTN. Although ascorbate decreased the potency of DEA/NO to stimulate sGC (Schrammel et al., 2000 and Fig. 1B of the present study), the bioavailability of GTN-derived NO could have been increased by scavenging of superoxide and/or by reduction of GTN-derived nitrite. However, our data seem to exclude both alternatives. GTN did not stimulate cGMP formation in the presence of SOD or Trolox, excluding superoxide scavenging as a relevant mechanism of ascorbate action. Nitrite reduction cannot account for sGC activation because formation of GTN-derived nitrite (based on the total amount of 1,2- and 1,3-GDN formation) was 2 orders of magnitude lower than required. Thus, by exclusion of other possibilities, we propose that sGC becomes activated by a product of a reaction between GTN and ascorbate or an ascorbate-derived species (see below).

We observed a slight but consistent increase in basal sGC activity in the presence of ascorbate (maximally 2-fold at 10 mM; see Fig. 2A). The mechanism underlying this effect is unclear. It could be caused by the reduction of critical sulfhydryl residues of the enzyme or keeping the heme iron in the reduced ferrous redox state (Murphy, 1999). Considering that ascorbate triggers homolytic cleavage of S-nitrosothiols (Gorren et al., 1996), an anonymous reviewer of this article suggested that ascorbate may trigger the release of NO from nitrosated cysteine residues of the enzyme. Although there is no evidence for S-nitrosation of sGC, this proposal is interesting and is worth pursuing. However, it should be mentioned that basal sGC activity is affected by slight changes in incubation conditions and even by environmental factors (Friesie et al., 1996). Therefore, we refrained from further investigating the relatively minor direct effect of ascorbate.

The ascorbate-driven formation of GTN metabolites was slow, yielding ~30 μM total products from 0.3 mM GTN within 38 h. Assuming that 1) only 1,2-GDN formation reflects GTN bioactivation (Bennett et al., 1989), 2) stoichiometric amounts of NO were released, and 3) NO autoxidation is negligible at low NO concentrations (Schmidt et al., 1997), 70 mM NO should have accumulated in the sGC assays (10-min incubation). Thus, sGC should have been maximally activated by GTN/ascorbate, and NO should have been detected.

![Fig. 3. Effects of ODQ, oxyhemoglobin, and FMN on sGC activation by GTN/ascorbate. Purified sGC (50 ng) was incubated at 37°C for 10 min in a final volume of 0.2 ml with 0.3 μM DEA/NO or 0.3 mM GTN in the presence of 10 mM ascorbate. ODQ (30 μM), oxyhemoglobin (OxyHb; 20 μM), or FMN (0.1 mM) was present as indicated. Data are expressed as the percentage of controls determined in the absence of these agents (mean values ± S.E.; n = 3).](image)

### Table 1

| Ascorbate-driven formation of 1,2-GDN and 1,3-GDN from GTN Radiolabeled GTN (−150,000 dpm) was incubated with 10 mM ascorbate at 37°C for 38 h. Formation of 1,2- and 1,3-GDN was determined by radio thin-layer chromatography as described under Materials and Methods. Values are mean values ± S.E. of three independent duplicate determinations and are corrected for blanks determined under identical conditions in the absence of ascorbate. The sum of 1,2-GDN plus 1,3-GDN was calculated from individual experiments. |
|----------------|----------------|----------------|----------------|
| GTN            | 1,2-GDN        | 1,3-GDN        | Total          |
| μM             | μM             | μM             | μM             |
| 6 μM           | 0.52 ± 0.13    | 0.55 ± 0.09    | 1.07 ± 0.22    |
| 100 μM         | 4.13 ± 0.43    | 3.57 ± 0.17    | 7.71 ± 0.60    |
| 300 μM         | 16.01 ± 0.15   | 15.34 ± 0.06   | 31.41 ± 0.21   |

### Table 2

<table>
<thead>
<tr>
<th>Ascorbate</th>
<th>GTN (0.1 mM)</th>
<th>DEA/NO (1 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mM</td>
<td>131 ± 12.3</td>
<td>651 ± 22.6</td>
</tr>
<tr>
<td>0.1 mM</td>
<td>152 ± 18.4</td>
<td>616 ± 40.4</td>
</tr>
<tr>
<td>1 mM</td>
<td>189 ± 14.96</td>
<td>628 ± 66.5</td>
</tr>
<tr>
<td>10 mM</td>
<td>182 ± 19.8*</td>
<td>670 ± 66.2</td>
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</tbody>
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*P < 0.05 versus preincubation in the absence of ascorbate (analysis of variance, Fisher’s protected least significant difference test).
tectable by the NO electrode. Because this was not the case, 1) the bioactive product is either not identical with NO, 2) the decay of GTN-derived NO is faster than autoxidation in the presence of GTN/ascorbate, or 3) release of NO accounts for only a fraction of ascorbate-driven product formation. The data in Fig. 3 show that DEA/NO- and GTN-stimulated cGMP formation was equally sensitive to ODQ and NO scavengers, suggesting the formation of intermediates with identical chemical reactivity. Considering this circumstantial evidence for NO-mediated sGC activation together with the lack of conceivable alternatives, we prefer the NO hypothesis for the sake of simplicity. Thus, the failure to detect GTN-derived NO in the presence of ascorbate suggests that NO accumulation is lower than predicted by the rate of GTN metabolism. At present, we cannot distinguish between decay and fractional release of NO, but the more relevant question is whether NO release could account for the observed activation of sGC. Assuming that DEA/NO was decomposed completely and NO decay was negligible during incubation, the DEA/NO concentration-response curve shown in Fig. 1B indicates that accumulation of 2 to 3 nM NO over 10 min would be sufficient for sGC activation by GTN/ascorbate. Such low NO concentrations are lower than the detection limit of our NO sensor.

The mechanism of the GTN/ascorbate reaction is unknown. Although ascorbate is very stable in the presence of DTPA (half-life, ~21 h; A. C. F. Gorren and B. Mayer, unpublished observations), the involvement of ascorbate autoxidation cannot be excluded because of the high concentrations of ascorbate applied. Because dehydroascorbate, the product of ascorbate autoxidation, did not support sGC activation by GTN, the organic nitrate must react either with ascorbate itself or a reactive autoxidation intermediate.

Oxidation of sGC-bound heme by GTN results in impaired NO stimulation of the enzyme (Waldman et al., 1986; Schröder et al., 1998), and we have recently shown that GTN-triggered heme oxidation counteracts the stimulation of sGC by GTN/cysteine-derived NO (Gorren et al., 2005). The same may apply to the GTN/ascorbate system, explaining submaximal sGC activation. We speculated that the rate-limiting effect of heme oxidation might be overcome by increasing the concentration of ascorbate, but the concentration-response to ascorbate was biphasic with a sharp maximum at 1 mM. The decrease of cGMP formation at higher ascorbate concentrations could be caused by residual NO scavenging or a competitive effect of heme oxidation. The mechanism of the GTN/ascorbate reaction is unknown. Although ascorbate is very stable in the presence of DTPA (half-life, ~21 h; A. C. F. Gorren and B. Mayer, unpublished observations), the involvement of ascorbate autoxidation cannot be excluded because of the high concentrations of ascorbate applied. Because dehydroascorbate, the product of ascorbate autoxidation, did not support sGC activation by GTN, the organic nitrate must react either with ascorbate itself or a reactive autoxidation intermediate.