Ascorbate Prevents the Interaction of Superoxide and Nitric Oxide Only at Very High Physiological Concentrations

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Abstract—The bioactivity of nitric oxide ($^{\bullet}$NO) depends, in part, on its interaction with superoxide. Usually, superoxide dismutase (SOD) preserves $^{\bullet}$NO bioactivity by limiting the availability of superoxide. Ascorbic acid also effectively scavenges superoxide, but the extent to which this interaction is necessary for intact $^{\bullet}$NO bioactivity is not known. Therefore, the present study examined the effect of ascorbic acid on $^{\bullet}$NO bioactivity with isolated rabbit arterial segments. A steady flux of superoxide (1.15 to 2.3 μmol · L^{-1} · min^{-1}) produced either by pyrogallol autoxidation or a hypoxanthine/xanthine oxidase system inhibited endothelium-derived $^{\bullet}$NO-mediated arterial relaxation elicited by acetylcholine. This effect of superoxide was completely blocked by SOD (300 IU/mL) and the manganese SOD mimic EUK-8 (300 μmol/L) and partially inhibited by ascorbic acid (10 mmol/L). Lower concentrations of ascorbic acid were ineffective despite scavenging >90% of superoxide. We increased the endogenous flux of superoxide (3.2±0.3-fold) by inhibiting vascular copper-zinc SOD with diethyldithiocarbamate. This increased endogenous flux of superoxide produced an impairment of $^{\bullet}$NO-mediated arterial relaxation that was reversed by EUK-8 (300 μmol/L) but not ascorbic acid (10 mmol/L) despite equivalent scavenging of the endogenous superoxide flux. We used 3-nitrotyrosine formation (from peroxynitrite) as an indicator of $^{\bullet}$NO interaction with superoxide and found that SOD and EUK-8 compete more effectively with $^{\bullet}$NO for superoxide than does ascorbic acid. These data indicate that preservation of $^{\bullet}$NO bioactivity by superoxide scavengers depends not only on superoxide scavenging activity, but also on the rate of superoxide scavenging. Normal extracellular concentrations of ascorbic acid (30 to 150 μmol/L) are not likely to prevent the interaction of $^{\bullet}$NO with superoxide under physiological conditions. **(*Circ Res*. 1998;83:916-922.)**

Key Words: antioxidant ■ free radical ■ blood vessel ■ oxidant ■ peroxynitrite

Normal vascular homeostasis depends on endothelial elaboration of paracrine factors that prevent both platelet adhesion to the endothelial surface and inappropriate vasospasm. One important endothelial product responsible for these functions is nitric oxide ($^{\bullet}$NO),[1] a free radical produced constitutively by the vascular endothelium. Abnormalities in $^{\bullet}$NO action and metabolism are known to develop in association with vascular disease and have been implicated in the development of clinically significant vascular events.[1]

In vivo, $^{\bullet}$NO is subject to rapid inactivation by the superoxide anion,[2–4] an obligate product of normal oxidative metabolism.[5] Endothelial cells constitutively produce both superoxide[6] and $^{\bullet}$NO,[7] suggesting that the effective release of $^{\bullet}$NO from the vascular endothelium depends on the relative concentrations of these 2 species.

Usually, the availability of superoxide in tissues is strictly limited by the abundant tissue concentration of superoxide dismutase (SOD) that may approach 10 μmol/L.[8] However, superoxide and $^{\bullet}$NO react rapidly with a bimolecular rate constant that approaches the diffusion limit (1.9×10^{10} mol · L^{-1} · s^{-1})[9] and is similar to the rate of superoxide dismutation by SOD (2×10^{9} mol · $^{-1}$ · s^{-1}).[8] These data indicate that $^{\bullet}$NO competes effectively with SOD for superoxide. Considerable data now exist to support this position. For example, inhibition of endothelial cell copper-zinc SOD impairs effective release of $^{\bullet}$NO from endothelial cells.[10,11] Intact copper-zinc SOD function is also required for smooth muscle cell relaxation in response to nitrovasodilators.[10] In addition, abnormalities in $^{\bullet}$NO-mediated arterial relaxation associated with hypercholesterolemia,[12,13] diabetes mellitus,[14] and hypertension[15] have been linked to excess vascular levels of superoxide. Thus, $^{\bullet}$NO-mediated arterial relaxation depends on SOD activity to limit the availability of superoxide.

The tissue availability of superoxide is limited by its interaction with other compounds in addition to SOD. Antioxidants such as α-tocopherol,[16] glutathione,[17] and ascorbic acid[18] are known to react with superoxide. Recent studies have demonstrated that acute treatment with ascorbic acid improves $^{\bullet}$NO-mediated arterial relaxation in patients with atherosclerosis,[19] but the mechanism of this effect is not clear. The action and metabolism of endothelium-derived $^{\bullet}$NO (EDNO) depends on vascular levels of superoxide[2,3,12,13]; thus, it is conceivable that ascorbic acid may exert some control over $^{\bullet}$NO-mediated arterial relaxation by preventing the interaction of $^{\bullet}$NO and superoxide. The purpose of the present study was to determine the extent to which physio-

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logically relevant concentrations of ascorbic acid influence arterial relaxation in response to EDNO.

Materials and Methods

Sodium pentobarbital was purchased from Anthony Products Co. Peroxynitrite was obtained from Alexis Corporation and diethylamine •NO (DEA-NO) was purchased from Cayman Chemical Corp. Xanthine oxidase was purchased from Boehringer-Mannheim. Acetylcholine hydrochloride, phenylephrine, diethylenetriamine pentaacetic acid (DTPA), SOD (copper-zinc form from bovine erythrocyte, 2500 to 7000 IU/mg), 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 $CaCl_2$, 1.2 mmol/L $MgSO_4$, 1.2 mmol/L KH_2PO_4, 25 mmol/L $NaHCO_3$, 11.1 mmol/L glucose, 10 μmol/L indomethacin, and 0.026 mmol/L Na_2EDTA. PBS consisted of 10 mmol/L NaH_2PO_4 and 0.15 mol/L NaCl (pH 7.4).

In Vitro Assay of Vascular Function

New Zealand White rabbits (2.5 to 3.5 kg) of either sex were used for the present study (Pine Acres Rabbitry, Vt). Animals consumed food and water ad libitum, and all animal studies were approved by the Boston University Medical Center Institutional Animal Care and Use Committee. The thoracic aorta was isolated from New Zealand White rabbits killed with pentobarbital (120 mg/kg) via a marginal ear vein. Vessel segments were prepared, suspended in organ chambers as previously described,[20] and gassed with 95% O_2/5% CO_2. After equilibration for 90 minutes, vessels were contracted with phenylephrine (1 μmol/L) and vascular tone was assayed in response to the addition of acetylcholine or the endothelium-independent, cGMP-dependent vasodilator, atrial natriuretic peptide (ANP). When pyrogallol was used as a source of superoxide it was added 1 minute before the assessment of vascular function. In some experiments, hypoxanthine (100 μmol/L) was added to the PSS and superoxide generation initiated with 0.02 IU/mL xanthine oxidase 1 minute before assessing vascular function. Ascorbic acid was dissolved in PSS and the pH adjusted with NaOH to produce a final pH of 7.4 in the organ chamber. Ascorbic acid was added to organ chambers 10 minutes before the assessment of arterial relaxation.

In some studies, vessels were treated for 30 minutes with 5 mmol/L diethyldithiocarbamate (DDC) to inhibit copper-zinc SOD.[21] Vessels treated with DDC were subsequently washed 3 times with PSS containing 100 μmol/L DTPA to remove any residual redox-active copper liberated by DDC treatment.

Quantification of Superoxide and •NO

The flux of superoxide from pyrogallol autoxidation was quantified as the reduction of cytochrome *c* inhibited by SOD with an extinction coefficient of 2.1×10^4 mol · L^{-1} · cm^{-1} at 560 nm.[22] The flux of •NO from DEA-NO decomposition was estimated spectrophotometrically at 250 nm (ϵ=6500 mol · L^{-1} · cm^{-1}) noting that each mole of DEA-NO produces ≈1.5 mol •NO.[23] Because ascorbate directly reduces cytochrome *c*,[24] scavenging of superoxide by ascorbate was estimated by the inhibition of pyrogallol autoxidation, which is superoxide dependent at pH<9.5.[25] Pyrogallol (200 μmol/L) was incubated in PBS, and autoxidation was estimated by monitoring the change in absorbance at 420 nm[25] with and without ascorbic acid or SOD. All additions of ascorbic acid were adjusted to achieve a final pH of 7.4.

Vascular SOD Activity

Segments of thoracic aorta were isolated as described above and incubated with PSS at 37°C gently bubbled with 95% O_2/5% CO_2. After 30 minutes, vessels were incubated for 10 minutes in 5-mL polyethylene tubes containing HEPES-buffered PSS (PSS containing 20 mmol/L HEPES) with 0.25 mmol/L lucigenin (bis-*N*-methylacridium nitrate). After equilibration with lucigenin, vascular superoxide levels were estimated from chemiluminescence recorded with a Turner Designs Model 20e luminometer at 37°C in a dark, light-sealed room. The integral of the chemiluminescence signal was recorded at 30-second intervals for 5 minutes, and the integral readings were combined. Background chemiluminescence was determined from identically processed vessel-free incubations and subtracted from the determinations with vessels. Chemiluminescence was converted to superoxide by a standard curve relating known quantities of superoxide (from a xanthine/xanthine oxidase system as determined by SOD-inhibited cytochrome *c* reduction) to chemiluminescence. To inactivate copper-zinc SOD, vessels were incubated for 30 minutes with 5 mmol/L DDC, washed 3 times (10 mL) with HEPES-buffered PSS containing DTPA (100 μmol/L), and superoxide determined as above. The effect of ascorbic acid, SOD, and EUK-8 (a cell-permeable manganese SOD [MnSOD] mimic; Evkaryon, Bedford, Mass)[26] on vascular superoxide scavenging activity was determined by adding these compounds directly to the chemiluminescence chamber and repeating the measurement. In the absence of such additions, the chemiluminescence signal was stable during the time of the assay.

Estimation of Peroxynitrite Formation

The interaction of •NO and superoxide results in the formation of peroxynitrite[27] that, in the presence of CO_2, spontaneously reacts with tyrosine to form 3-nitrotyrosine.[28] We estimated peroxynitrite formation as the production of 3-nitrotyrosine with a modification of the method described by van der Vliet et al.[29] *d,l*-Tyrosine (1 mmol/L) in 10 mmol/L phosphate buffer with 50 μmol/L DTPA was incubated with DEA-NO (20 μmol/L) and pyrogallol (200 μmol/L) with or without ascorbic acid (0 to 10 mmol/L), SOD (0.3 to 300 IU/mL), or EUK-8 (0.1 to 300 μmol/L) for 15 minutes. The formation of 3-nitrotyrosine was analyzed by UV detection at 274 nm after separation on an LC-18 column (25 cm×4.6 mm, Supelco) with a mobile phase of 50 mmol/L KH_2PO_4, pH 3 and methanol (92:8).[29] Ascorbic acid (0 to 10 mmol/L) had no effect on the yield of 3-nitrotyrosine when authentic peroxynitrite (10 mmol/L) was added to 2 mmol/L *d,l*-tyrosine (data not shown).

Data Analysis

All values are presented as mean±SEM. The vascular responses to acetylcholine and NO are reported as the percent reduction in tension (relaxation) compared with the contraction produced by 1 μmol/L phenylephrine. Dose responses to acetylcholine and ANP were compared within treatment groups with repeated-measures ANOVA and responses between treatment groups were compared with 2-way ANOVA with a post hoc Dunn's or Dunnett's test as appropriate. Statistical significance was accepted if the null hypothesis was rejected with a $P<0.05$.

Results

Effect of Superoxide and Ascorbic Acid on Arterial Relaxation

We observed dose-dependent arterial relaxation of the thoracic aorta in response to acetylcholine between the concentrations of 1 nmol/L and 10 μmol/L (Figure 1A). In contrast, superoxide (2.3±0.04 μmol · L^{-1} · min^{-1}) generated from autoxidation of pyrogallol (200 μmol/L) significantly inhibited the dose-dependent relaxation to acetylcholine with maximal relaxation reduced from 73±3% to 48±3% ($P<0.05$ by 2-way repeated-measures ANOVA). This effect of superoxide was completely reversed with 300 IU/mL SOD producing a maximal relaxation of 66±6% (P=NS versus control). To test the effect of superoxide on smooth muscle cell relaxation independent of •NO, we examined arterial relaxation in response to the cGMP-dependent vasodilator, ANP. As shown in Figure 1B, superoxide generated from pyrogallol (200 μmol/L) autoxidation had no effect on cGMP-dependent arterial relaxation in response to ANP.

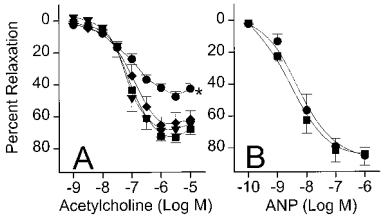

Figure 1. The effect of superoxide on •NO-dependent arterial relaxation. Segments of thoracic aorta were harvested from New Zealand White rabbits as described in Materials and Methods. A, Vessels were contracted with phenylephrine (1 μmol/L) and relaxation assayed in response to the indicated concentrations of acetylcholine in the presence of PSS containing no additions (■), 200 μmol/L pyrogallol (●), 200 μmol/L pyrogallol with 300 IU/mL SOD (♦), or 300 IU/mL SOD (▼). B, Vessels were contracted as mentioned above and relaxation to ANP assayed in the presence of PSS containing no additions (■) or 200 μmol/L pyrogallol (●). Data represent mean±SEM of 5 to 8 experiments. *$P<0.05$ vs control group by 2-way ANOVA.

To determine whether ascorbic acid can prevent the inactivation of •NO by superoxide, we incubated arterial segments with pyrogallol and increasing doses of ascorbic acid just before the assessment of arterial relaxation. As expected, a steady flux of superoxide from pyrogallol autoxidation produced significant inhibition of EDNO-mediated arterial relaxation ($P<0.05$ by 2-way repeated-measures ANOVA; Figure 2). The impairment of EDNO-mediated arterial relaxation by superoxide was prevented by ascorbic acid only at a concentration of 10 mmol/L (Figure 2A). We also sought to confirm these observations with another superoxide-generating system. We used a steady flux of superoxide (1.15 ± 0.2 μmol · L^{-1} · min^{-1}; n=3) from hypoxanthine (100 μmol/L) and xanthine oxidase (0.02 IU/mL) and observed significant inhibition of EDNO-mediated arterial relaxation that was reversed by SOD ($P<0.05$ by 2-way repeated-measures ANOVA; Figure 2B). Similar to the situation with pyrogallol, this impairment of EDNO-mediated arterial relaxation by superoxide was only partially prevented by ascorbic acid at a concentration of 10 mmol/L (Figure 2B).

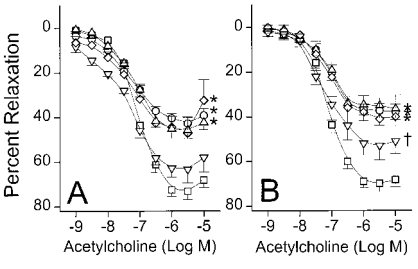

Figure 2. Ascorbic acid and the impairment of •NO-mediated arterial relaxation from superoxide. Segments of thoracic aorta were harvested from New Zealand White rabbits as described in Materials and Methods. A, Vessels were contracted with phenylephrine (1 μmol/L) and relaxation assayed in response to the indicated concentrations of acetylcholine in the presence of PSS containing no additions (❑), 200 μmol/L pyrogallol (○), or 200 μmol/L pyrogallol with 0.1 (△), 1.0 (♦), or 10 (▽) mmol/L ascorbic acid. B, Vessels were prepared as in panel A, except relaxation was assessed in the presence of PSS containing no additions (❑), 100 μmol/L hypoxanthine with 0.02 IU/mL xanthine oxidase (HX/XO) (○), or HX/XO with 0.1 (△), 1.0 (♦), or 10 (▽) mmol/L ascorbic acid. Values are plotted as mean±SEM and are derived from 5 to 7 experiments in each group. *$P<0.05$ vs PSS alone or †$P<0.05$ vs HX/XO alone by 2-way ANOVA.

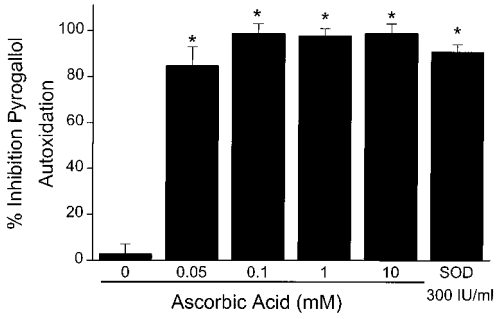

Figure 3. Superoxide scavenging by SOD and ascorbic acid. Pyrogallol (200 μmol/L) was incubated in PBS with or without the addition of SOD (300 IU/mL) or the indicated concentrations of ascorbic acid. Pyrogallol autoxidation was estimated by the change in absorbance at 420 nm during a 10-minute incubation.[25] Data are mean±SEM of 4 experiments. *$P<0.05$ vs no additions.

Ascorbate and Superoxide Scavenging

To quantify the extent of superoxide scavenging by ascorbate and SOD in our system, we determined the inhibition of pyrogallol autoxidation, which is superoxide-dependent at pH<9.5.[25] As presented in Figure 3, the autoxidation of pyrogallol was inhibited 91±3% by 300 IU/mL SOD ($P<0.05$ versus control, n=4) compared with 85±8%, 99±4%, 98±3%, and 99±4% by 0.05, 0.1, 1, and 10 mmol/L ascorbic acid, respectively (all $P<0.05$ versus control by 1-way ANOVA, n=4). Thus, ascorbate effectively scavenges superoxide at concentrations that are considerably lower than those needed to preserve •NO-mediated arterial relaxation.

Pyrogallol and Endothelial Cell Toxicity

To determine whether pyrogallol produced endothelial damage, we assessed acetylcholine-stimulated EDNO-mediated arterial relaxation in vessel segments before and after a 20-minute exposure to a flux of superoxide (2.3 μmol · L^{-1} · min^{-1}) from pyrogallol (200 μmol/L) autoxidation. As shown in Figure 4, endothelium-dependent arterial relaxation to

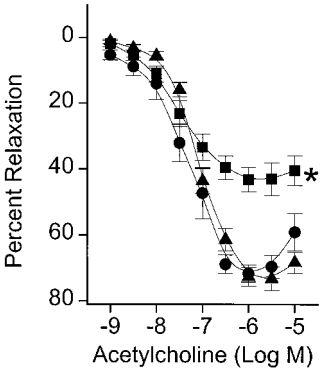

Figure 4. Arterial relaxation before and after exposure to superoxide. Segments of thoracic aorta were harvested from New Zealand White rabbits as described in Materials and Methods. After contraction with phenylephrine (1 μmol/L) arterial relaxation was assayed in response to acetylcholine before (▲), during (■), and after (●) exposure to a flux of superoxide (2.3 μmol · L^{-1} · min^{-1}) caused by pyrogallol (200 μmol/L) autoxidation. Values are plotted as mean±SEM and are derived from 6 experiments. *$P<0.001$ vs before pyrogallol by 2-way repeated-measures ANOVA.

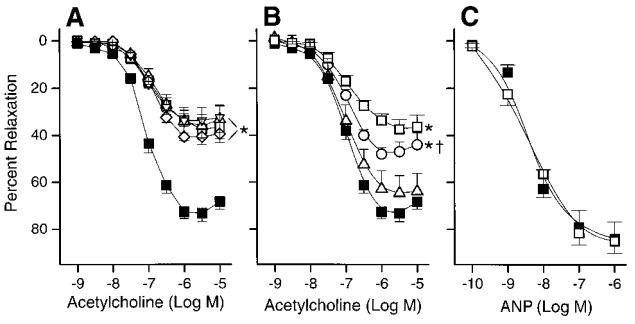

Figure 5. Arterial relaxation with an endogenous flux of superoxide. Segments of thoracic aorta were harvested from New Zealand White rabbits and prepared as described in Materials and Methods. After equilibration (90 minutes), arterial segments were incubated (30 minutes) with PSS alone (■) or PSS containing 5 mmol/L DDC (open symbols). A, Vessels were washed 3 times with PSS containing 100 μmol/L DTPA to remove copper liberated by DDC, and acetylcholine-mediated relaxation was assessed after contraction with phenylephrine in the presence of 0 (❑), 0.1 (△), 1.0 (♦), or 10 (▽) mmol/L ascorbic acid. B, Vessels were prepared identically as in panel A, and acetylcholine-mediated relaxation was assessed after contraction with phenylephrine in the presence of no additions (❑), 300 μmol/L EUK-8 (△), or 300 IU/mL SOD (○). C, Vessels were prepared identically as in panel A and contracted with phenylephrine (1 μmol/L); relaxation was assessed in response to ANP. Values are mean±SEM derived from 7 experiments. *$P<0.05$ vs PSS alone and †$P<0.05$ vs DDC alone both by 2-way repeated-measures ANOVA.

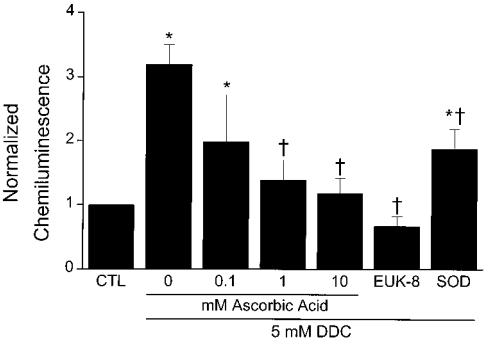

Figure 6. Vascular superoxide with various superoxide scavengers. Segments of thoracic aorta were harvested from New Zealand White rabbits and superoxide estimated by lucigenin chemiluminescence as described in Materials and Methods. Superoxide was determined with and without inactivation of copper-zinc SOD by a 30-minute exposure to DDC (5 mmol/L) followed by washing 3 times with PSS containing 100 μmol/L DTPA. The indicated concentrations of ascorbic acid, EUK-8 (300 μmol/L), or SOD (300 IU/mL) were added directly to the vessels in the luminometer for 10 minutes and chemiluminescence determined for an additional 5 minutes. The superoxide signal was stable throughout the duration of the assay. Data are mean±SEM representing 5 to 12 independent experiments and are normalized to the chemiluminescence signal obtained in the absence of DDC. *$P<0.05$ vs control; †$P<0.05$ vs 0 mmol/L ascorbic acid.

acetylcholine was the same before and after exposure to pyrogallol, indicating that short-term exposure to superoxide does not result in any permanent impairment in •NO-mediated arterial relaxation.

Effect of Ascorbic Acid on •NO-Mediated Arterial Relaxation With an Endogenous Flux of Superoxide

It is difficult to extrapolate observations with pyrogallol to events that are relevant in vivo. To generate a more relevant superoxide flux, we treated aortic segments with 5 mmol/L DDC, a copper chelator that inactivates endogenous copper-zinc SOD.[21] As shown in Figure 5A, treatment of aortic segments with DDC produces a significant impairment in EDNO-mediated arterial relaxation in response to acetylcholine ($P<0.001$ versus no DDC by 2-way ANOVA). This impairment in EDNO-mediated arterial relaxation was not mitigated by ascorbic acid in concentrations up to 10 mmol/L (all $P<0.05$ versus no DDC by 2-way ANOVA; Figure 5A). Authentic SOD (300 IU/mL) only partially restored EDNO-mediated arterial relaxation (Figure 5B), which was likely a result of its limited cellular access. In contrast, the MnSOD mimic EUK-8 (300 μmol/L) completely restored EDNO-mediated arterial relaxation in response to acetylcholine (Figure 5B). Endothelium-independent arterial relaxation to ANP was not impaired in DDC-treated vessels (Figure 5C).

Vascular Superoxide Scavenging With Endogenous Superoxide

To determine the extent of superoxide scavenging with an endogenous flux of superoxide, we estimated vascular superoxide in vessel segments treated with DDC, ascorbate, SOD, or EUK-8 as mentioned above. As shown in Figure 6, treatment of aortic segments with 5 mmol/L DDC resulted in a 3.2±0.3-fold increase in the superoxide signal by lucigenin chemiluminescence ($P<0.05$). This DDC-mediated increase in superoxide was reduced 55%, 81%, and 90% by 0.1, 1, and 10 mmol/L ascorbic acid, respectively ($P<0.001$ for dose response by ANOVA). In fact, treatment with either 10 mmol/L ascorbic acid, 300 μmol/L EUK-8, or 300 IU/mL SOD reduced the superoxide signal in DDC-treated aortic segments to near control levels (P=NS versus control). Thus, although ascorbic acid, EUK-8, and SOD all reduced vascular superoxide in DDC-treated aortic segments, only EUK-8 and SOD improved EDNO-mediated arterial relaxation.

Ascorbic Acid and Superoxide Competition for •NO

The interaction of •NO and superoxide results in the formation of peroxynitrite[27] that spontaneously reacts with tyrosine to form 3-nitrotyrosine.[28] Incubation of tyrosine (2 mmol/L) with an equimolar flux of superoxide (2.3 $\mu mol \cdot L^{-1} \cdot min^{-1}$from pyrogallol autoxidation) and •NO (2.1 $\mu mol \cdot L^{-1} \cdot min^{-1}$ from DEA-NO) for 15 minutes readily produced 3-nitrotyrosine (2.1±0.31 μmol/L; Figure 7) indicating the formation of peroxynitrite. Both SOD and EUK-8 were able to compete effectively with •NO for superoxide at concentrations exceeding 0.2 μmol/L and 1 μmol/L, respectively (both $P<0.05$ versus PSS alone by ANOVA; Figure 7). In contrast, ascorbic acid was only partially effective in competing with •NO for superoxide even at a concentration of 10 mmol/L (Figure 7). Thus, ascorbic acid is not as effective as SOD or EUK-8 in competing with •NO for superoxide.

Discussion

The data presented here indicate that ascorbic acid does not compete effectively with •NO for superoxide at physiologi-

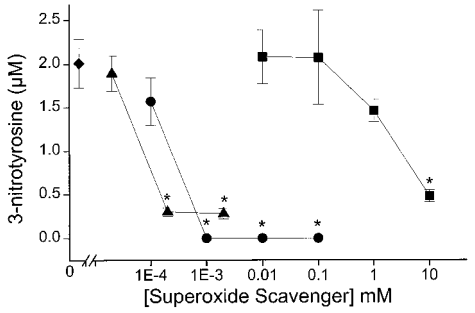

Figure 7. Competition with •NO for superoxide. Tyrosine (1 mmol/L) was incubated for 15 minutes with pyrogallol (200 μmol/L) and DEA-NO (20 μmol/L) producing an equimolar flux of superoxide (2.3 μmol · L^{-1} · min^{-1}) and •NO (2.1 μmol · L^{-1} · min^{-1}), respectively. Incubations were performed in PBS alone (♦) or in PBS containing the indicated concentrations of SOD (▲), EUK-8 (●), or ascorbic acid (■). After incubation, the formation of 3-nitrotyrosine was determined by HPLC as described in Materials and Methods. Data are mean±SEM derived from 3 independent experiments. *$P<0.05$ vs PBS alone by 1-way ANOVA.

cally relevant concentrations. With a flux of superoxide from pyrogallol autoxidation we found that superoxide inhibits EDNO-mediated arterial relaxation and that this effect is readily prevented by physiologically relevant concentrations of SOD (300 IU/mL or ≈2 μmol/L). In contrast, to achieve any effect with ascorbic acid in our system, a concentration of 10 mmol/L was required despite evidence of effective superoxide scavenging at much lower concentrations. This discrepancy between superoxide scavenging and the preservation of EDNO-mediated arterial relaxation was even more pronounced with an endogenous flux of superoxide induced by inhibition of vascular copper-zinc SOD with DDC. We used peroxynitrite formation as a marker for the interaction of •NO with superoxide and were able to demonstrate that high physiological concentrations of ascorbic acid are required to interrupt the bimolecular combination of •NO and superoxide.

In complex biologic systems such as the arterial wall, reactive species like •NO and superoxide are often produced within a milieu that contains several potential scavengers and reactants. Several potential chemical interactions are never realized because the rate at which they occur is too slow. The data presented here serve as a case in point. We found that both ascorbic acid (100 μmol/L) and SOD (300 IU/mL or ≈2 μmol/L) readily scavenged superoxide generated by pyrogallol autoxidation (Figure 3), yet only SOD prevented the inactivation of EDNO by superoxide (Figures 1 and 2). Similarly, in vessels treated with DDC to generate a (patho)-physiological superoxide flux, ascorbic acid (10 mmol/L), SOD (300 IU/mL), and EUK-8 (300 μmol/L) all demonstrated some superoxide scavenging (Figure 6), but only SOD and EUK-8 inhibited EDNO inactivation by superoxide (Figure 5). Thus, there appears to be a discrepancy between the capacity of ascorbic acid to scavenge superoxide and its ability to prevent the interaction of •NO with superoxide.

This discrepancy is not particularly surprising. Although ascorbic acid is an effective scavenger of superoxide, the bimolecular rate constant for this reaction is 2.7 to 3.3×10^5 mol · L^{-1} · s^{-1}[16,18] approximately 10^5-fold less than the rate at which superoxide reacts with either SOD (2×10^9 mol · L^{-1} · s^{-1})[8] or •NO (1.9×10^{10} mol · L^{-1} · s^{-1}).[4] Therefore, for ascorbic acid to compete effectively with •NO for any given concentration of superoxide, the concentration of ascorbic acid must exceed that of •NO by a factor of 10^5. Recent studies with a porphyrinic microsensor have estimated •NO concentrations of ≈0.1 to 1.0 μmol/L adjacent to endothelial cells in culture and rabbit aorta.[30,31] Based on this information, one would predict that a concentration of $10^5\times$(0.1 to 1.0) μmol/L ≅10 to 100 mmol/L ascorbic acid is required to prevent the interaction of •NO and superoxide, a value that is in excellent agreement with the data presented here (Figure 2A and 2B). These data indicate that although ascorbic acid is an efficient scavenger of superoxide, the rate of this reaction is insufficient to compete effectively with •NO for superoxide at anything less than supraphysiologic concentrations.

The effect of ascorbic acid on EDNO-mediated arterial relaxation has been examined in human subjects. Ting et al[32] found that EDNO-mediated forearm blood flow responses to methacholine were improved by a concomitant infusion of ascorbic acid in patients with type II diabetes or hypercholesterolemia.[33] In chronic smokers, Heitzer et al[34] found that an acute arterial infusion of ascorbic acid normalized EDNO-mediated forearm blow flow responses to acetylcholine. An improvement in EDNO-mediated brachial artery dilation has also been reported with ascorbic acid in patients with heart failure.[35] A common feature of these studies has been the implication that ascorbic acid improves EDNO-mediated responses through superoxide scavenging.[32–35] The arterial concentration of ascorbic acid in those studies was not determined directly but was estimated to be ≅10 mmol/L.[32,34] These prior observations agree with the results reported here, namely that plasma ascorbic acid concentrations of ≅10 mmol/L may support competition between ascorbic acid and •NO for superoxide (Figures 2 and 6) and thus impair superoxide-mediated EDNO inactivation.

Despite the potential of ascorbic acid to prevent the interaction of superoxide and •NO, plasma concentrations of ascorbic acid in the range of 10 mmol/L are not physiologically relevant. Ascorbic acid is the main water-soluble antioxidant in human plasma and extracellular fluids with normal concentrations in the range of 50 to 150 μmol/L.[36,37] We have recently examined the effect of physiologically relevant ascorbic acid concentrations on EDNO-mediated vasodilation.[19] In patients with documented coronary artery disease, a single oral dose of ascorbic acid (2 grams) reversed endothelial dysfunction in the brachial artery in 2 hours. Moreover, this dose of ascorbic acid was associated with an increase in plasma ascorbate within the normal range (46±8 to 114±11 μmol/L).[19] Therefore, it is unlikely that changes in plasma ascorbate within the normal range are sufficient to prevent superoxide-mediated EDNO inactivation in plasma (Figure 2). The beneficial effect of oral ascorbic acid on EDNO-mediated arterial relaxation in patients with atherosclerosis must occur at the intracellular level or involve mechanism(s) other than simple superoxide scavenging.

The antioxidant activity of ascorbic acid is not restricted to plasma and extracellular fluids. Ascorbate is actively trans-

ported into cells and along with glutathione is a major determinant of intracellular redox state and antioxidant defenses.[38] Intracellular concentrations of ascorbic acid have been reported in the range of 1.3 to 2.5 mmol/L,[39,40] and our data (Figures 2 and 7) suggest these concentrations just begin to support effective competition between ascorbic acid and •NO for superoxide. Therefore, it is not likely that the improvement in EDNO-mediated arterial relaxation that we previously observed with oral ascorbic acid[19] was purely a consequence of superoxide scavenging by increased intracellular ascorbate.

The source(s) of superoxide in the blood vessel wall remains unclear. In normal vessels, superoxide can be detected throughout the blood vessel wall although the endothelium and adventitia are most notable.[41] In hypercholesterolemia and atherosclerosis, there is evidence for increased activity of xanthine oxidase either within[12] or closely associated with[42] the endothelium. Because •NO is freely permeable in biologic tissues, any site of excess superoxide generation will have some impact on EDNO-mediated responses.[43] In contrast, superoxide is not membrane permeable, and our data suggest that competition with •NO for superoxide by ascorbic acid will be highly dependent on the site of superoxide generation. For example, impairment of EDNO-mediated arterial relaxation caused by extracellular superoxide generation by xanthine oxidase[42] is not subject to modification by ascorbic acid because the plasma and extracellular ascorbic acid concentration is typically <150 μmol/L.[37] Intracellular source(s) of superoxide that impair EDNO responses, however, may be subject to the action of ascorbic acid by virtue of its higher concentration (1 to 2.5 mmol/L)[39,40] in the cytosol (Figure 2).

We observed a discrepancy in the action of ascorbic acid that depended on the site of superoxide generation. In vessels treated with DDC to produce an intracellular superoxide flux, ascorbic acid (10 mmol/L) did not improve EDNO-mediated arterial relaxation (Figure 5). In contrast, 10 mmol/L ascorbic acid did improve EDNO responses with an extracellular superoxide flux from pyrogallol or hypoxanthine/xanthine oxidase. One potential explanation for these observations may relate to incomplete intracellular transport of ascorbic acid during the time course (20 minutes) of our experiment.[39] Another important point concerns the scavenging of •NO. The treatment of tissues with DDC leads to the formation of $Fe(DDC)_2$ and $Fe(DDC)_3$ complexes that bind •NO.[44] In this context, the results depicted in Figure 5 may reflect, in part, some component of •NO scavenging. It is unlikely, however, that •NO scavenging accounts for much of the response to DDC because treatment with EUK-8 normalizes arterial relaxation to acetylcholine.

In summary, the data presented here indicate that ascorbic acid is not likely to prevent the interaction of •NO and superoxide at concentrations that are routinely achieved in plasma or extracellular fluids (<150 μmol/L). These observations are supported by kinetic data indicating that superoxide reacts with •NO at a rate that is 10^5-fold greater than the rate at which superoxide reacts with ascorbic acid. Within the cell cytosol, however, ascorbic acid concentrations (1 to 2.5 mmol/L) begin to approach those needed to support ascorbic acid competition with •NO for superoxide. These data indicate that effects of ascorbic acid attributed to preventing the interaction of •NO with superoxide must be interpreted with some caution.

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