Antioxidant-independent ascorbate enhancement of catecholamine-induced contractions of vascular smooth muscle

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Submitted 13 October 2003; accepted in final form 5 February 2004

Dillon, P. F., R. S. Root-Bernstein, and C. M. Lieder. Antioxidant-independent ascorbate enhancement of catecholamine-induced contractions of vascular smooth muscle. Am J Physiol Heart Circ Physiol 286: H2353–H2360, 2004.—Ascorbate reduces the oxidation rate of catecholamines and, by an independent mechanism, enhances rabbit aortic ring contractions initiated by catecholamines. The largest significantly different fractional increases in force produced by ascorbate enhancement of norepinephrine (NE), epinephrine, phenylpropanolamine (PPA), and ephedrine (Eph) are 5.5, 1.8, 1.6, and 1.3 times, respectively. In physiological salt solutions bubbled with 95% O2 at 37°C, NE, PPA, and Eph have oxidation rate constants of 1.24, 247, and 643 h, respectively. Ascorbate significantly enhances 100 nM NE contractions by at least twofold at all ascorbate concentrations >15 μM, including the entire physiological range of 40–100 μM. Ascorbate preloading and washout followed by NE exposure produces significantly greater contractions than NE without ascorbate preloading but significantly lower than NE simultaneously with ascorbate. Ascorbate does not enhance K+ or angiotensin II-induced contractions. Ascorbate enhancement of catecholamine contractions occurs in addition to the reduction in oxidation rate, because the increases in force occur faster than oxidation can occur, the increases occur with compounds that have negligible oxidation rates, and the increases occur when ascorbate and NE are not physically present together. These results are consistent with ascorbate acting on the adrenergic receptor. Ascorbate may play a role in shock and asthma treatments and potentiate the cardiovascular health consequences of PPA and Eph (Ephedra).

Because the reduced oxidation rate of NE in the presence of ascorbate would result in higher NE concentrations during prolonged contractions and, thus, higher forces, it was necessary to perform multiple experiments in which the further hypothesis that the ascorbate enhancement of NE contraction is independent of the ascorbate effect on NE oxidation was tested. These experiments included measurements of the oxidation rates of NE and epinephrine (Epi) in the presence and absence of ascorbate; measurement of the oxidation rates of the NE-related compounds phenylpropanolamine (PPA) and ephedrine (Eph); estimates of the NE concentration during activation of the aortic rings; ascorbate concentration dependence of NE-induced contractions; temporal and concentration effects of ascorbate preload/washout on subsequent NE-induced contractions, in which ascorbate and NE were never simultaneously present; effects of ascorbate on Epi-, PPA-, and Eph-induced contractions, the latter two of which are so slowly oxidized that the antioxidant effects of ascorbate will not come into play; the ascorbate effects on non-catecholamine-related aortic ring contractions induced by high K+ and angiotensin II (ANG II); and the effect of a different antioxidant, EDTA, on NE contractions.

As our results will show, ascorbate has a profound effect on catecholamine-induced contractions of rabbit aortic smooth muscle. This effect is independent of the direct antioxidant effect of ascorbate on NE and Epi. We will discuss the possible mechanism for this effect on the basis of the multiple states that the adrenergic receptor has been shown to have (19). The reduction of this receptor by ascorbate may lead to the greater sensitivity to catecholamines found in our experiments. Potential physiological effects of this finding on blood flow and bronchodilation are also discussed.

METHODS

Solutions. Physiological salt solution (PSS) contained (in mM) 116 NaCl, 5.4 KCl, 19 NaHCO3, 1.1 NaH2PO4, 2.5 CaCl2, 1.2 MgSO4, and 5.6 glucose. PSS was aerated with 95% O2-5% CO2 to maintain pH 7.4 and warmed to 37°C before addition to tissue baths. Distilled and filtered water with a resistance of 17 MΩ was used for all experiments. Isosmolar high-K+ PSS was made by reducing the NaCl concentration to 46 mM and increasing KCl to 75.4 mM. Solutions with 40.4 and 22.9 mM K+ were made using 1:1 and 3:1 mixtures of PSS and high-K+ PSS. NE, PPA, Eph, Epi, and ANG II were obtained from Sigma. Ascorbate was obtained from Aldrich. Solutions of ascorbate, NE, PPA, Eph, Epi, and ANG II were prepared fresh from powder on the day of the experiment as a concentrated, refrigerated stock and serially diluted in PSS for each experiment 10 min (to allow warming to 37°C) before each contraction. EDTA was prepared in isosmolar high-K+ PSS.

WE PREVIOUSLY DISCOVERED that norepinephrine (NE) and ascorbate bind to each other with a dissociation constant of 37 μM (4). This discovery led to the hypothesis that ascorbate may directly affect NE-induced contractions of vascular smooth muscle. It has been known that ascorbate reduces the oxidation rate of catecholamines (7, 12, 13), although no direct quantitative measurement of this observation has been reported. Our hypothesis that the direct binding of NE with ascorbate could alter the NE receptor binding was based on the reduction in free NE that could occur when ascorbate is present in sufficient concentration. Such a reduction could reduce the contractile effect of NE, although dissociation of the complex by the membrane electric field would negate this reduction (4). Alternatively, ascorbate-bound NE could bind more strongly to its receptor; thus the contractile effect of NE would be enhanced.

Initial experiments showed that ascorbate produced a pronounced enhancement of NE contractions of rabbit aortic rings.

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PSS from a 10 mM stock solution. All components were kept separate and refrigerated before they were mixed and added to the prewarming chambers.

**Oxidation rates.** NE, PPA, and Eph oxidation rates were measured by preparing 0.1 mM solutions in PSS. NE oxidation was measured in the presence or absence of different ascorbate concentrations. The oxidation rate of ascorbate was also measured. The solutions were placed in the same baths used for the tissue contractions, including the stainless steel-Plexiglas clips used to hold the tissue. Samples of these solutions were measured using capillary electrophoresis (4). Samples were introduced into an ISCO 3850 electropherometer for 2 s at 8.6 nl/s into a 100-μm-diameter, 94-cm-long uncoated glass capillary. The samples were exposed to 20 kV (≈80 μA) in a carrier buffer of 20 mM sodium borate, pH 9.4, and measured at a window 66 cm from the injection site at 195 nm. The rate of disappearance of NE (or ascorbate) was plotted as a function of time, and the rate constant was calculated with the fractional concentration c/t = (exp − (−ct)), where c is the concentration relative to the initial concentration c₀, at time t and τ is the calculated rate constant. PPA and Eph oxidation were measured under the same conditions. These compounds were measured multiple times on consecutive days, with the tissue baths sealed to prevent evaporation overnight. The change in peak height over ∼24 h was measured, and τ was calculated using the above equation.

The PPA-ascorbate and Eph-ascorbate dissociation constants were measured using UV spectrophotometry. Solutions of 0.002 M ascorbate and 0.1 M PPA or 0.1 M Eph were prepared in pH 7.0 phosphate buffer (Fisher Scientific). Serial dilutions of PPA were then made. In a crystal 96-well plate, an aliquot of 150 μl of each dilution of the PPA was added to an aliquot of 150 μl of ascorbate solution to make the mixtures; 150 μl of each dilution of the PPA was also added to 150 μl of buffer solution. Also, 150 μl of ascorbate were diluted with 150 μl of buffer solution. All samples were run in triplicate. Data were collected for binding analysis using a SpectraMax Plus scanning spectrophotometer (Molecular Devices) set to record the absorbance at 260 nm. The average absorbance of ascorbate alone was added to the average absorbance of PPA alone to predict an expected spectral value that was compared with the experimentally obtained value for the mixture.

**Tissue procedures.** The rabbits were kept in university-approved facilities before experimental use. All proper procedures were followed according to a protocol approved by the Michigan State University All-University Committee on Animal Use and Care. Adult New Zealand White rabbits of either gender were relaxed with ketamine (55 mg/kg im). After 15 min, the rabbits were anesthetized with pentobarbital sodium (Nembutal; 50 mg/kg ip). When the rabbits were unresponsive to toe pinch, the abdomen was opened and the abdominal aorta was exposed. The aorta was teased from the vena cava and clamped at the rostral and caudal ends. The aorta was removed using surgical scissors and placed in a 4°C PSS. The aortic clamps were removed to induce euthanasia.

The tissues were prepared for mechanical measurements using the procedures previously used for tissue rings (3). The aorta was debried of excess connective tissue, flushed of any remaining blood, and placed in fresh PSS. Aortic rings (3 mm long) were cut using a single-edged razor blade and placed in fresh PSS. The scissors-cut ends were not used. A pair of stainless steel loops with a flat, straight central section were passed through the lumen of each aortic ring. Upper and lower loops were secured to Plexiglas-stainless steel clamps with stainless steel screws. The lower clamp was attached to a micrometer (Newport) for length adjustment. The upper clamp was connected to a 50-g force transducer (Kulite Semiconductor) with a gold chain. The force transducers were interfaced with an eight-channel signal conditioning instrument.

The rings were immersed in 20- or 25-ml aerated, jacketed tissue baths (Harvard Apparatus) and maintained at 37°C using a Haake circulator. After the rings were mounted, each ring was stretched to 5 g and allowed to stress relax for 2 h before activation. If stress relaxation reached 0 g, the ring was stretched to 2 g and allowed to stress relax until the passive force was stable. The rings had a stretched linear length of 3–4 mm. This procedure leaves the rings near their optimal length for force development.

The tissues were activated with K⁺, NE, PPA, Eph, or ANG II. Tests of ascorbate alone were also done and showed no contractile effects of ascorbate alone. Individual contractions were generated by replacing PSS in the tissue baths with prewarmed PSS containing the stimulating agent. An initial K⁺ contraction was made on each ring before any catecholamine contractions. Each contraction lasted ≈10 min, at which time prewarmed PSS was used to wash out the contracting solution. Relaxation to baseline force typically took 10 min. In addition, either a standard nanomolar NE-PSS or Eph-PSS solution was used at the beginning and end of a day’s experiments to ensure that the force generated by the standard was retained throughout the set of experiments. At the conclusion of the experiment, the rings were removed from the baths, blotted dry, and weighed on a Mettler balance to the nearest 0.1 mg. For the 51 rings in these experiments, the average weight was 7.2 ± 0.3 (SE) mg. To minimize error that can be introduced by percent comparisons in dose-response curves, the contractions were also normalized to the weight of the ring (g force/mg tissue).

**Ascorbate washout.** The washout of any compound from a tissue bath will always leave some residual concentration. For the experiments in which ascorbate was washed out of the baths before the introduction of NE, knowing the residual ascorbate allows a comparison with the known ascorbate dependence of NE contractions when both are present. A previously developed capillary electrophoresis method (4) to measure ascorbate was used to make a standard curve of ascorbate absorbance at 195 nm between 0 and 50 μM ascorbate in PSS. The lowest measurable ascorbate in this system is 2 μM. PSS containing 500 μM ascorbate was placed in the tissue bath with all the mechanics apparatus at 37°C and bubbled with 95% O₂-5% CO₂ and allowed to equilibrate for 20 min. This solution was then rinsed from the bath and replaced with ascorbate-free PSS, which was allowed to equilibrate for 5 min. No measurable ascorbate was found in samples of this fresh solution. A higher concentration of ascorbate, 50 mM, was then added to the bath and rinsed out, and fresh ascorbate-free solution was allowed to equilibrate for 5 min. This solution contained 18.3 μM ascorbate, the residual ascorbate from the 50 mM ascorbate PSS, or a reduction of 2,728-fold. With use of this factor, it is estimated that the washout of 500 μM ascorbate leaves a residual concentration of 183 nM and the washout of 50 μM ascorbate leaves a residual concentration of 18.3 nM, both of which are far below the amount of ascorbate needed to enhance adrenergic activity.

**Statistics.** Comparisons between different samples were made by using Student’s two-tailed t-test. P < 0.05 was considered as indicating a significant difference. The Axxum data-processing program was used to calculate the half-maximal dose-response values (using the Hill equation) and the rate constants of NE and ascorbate oxidation.

**RESULTS**

Figure 1 shows the dose-response relations for NE, PPA, and Eph. NE was more sensitive than PPA or Eph, which required concentrations of ≥10 μM to produce significant contractions of the rabbit aortic rings. In this range, NE reached its maximum force production.

Despite this difference in sensitivity, PPA and Eph can bind ascorbate with dissociation constants similar to the dissociation constant for NE and ascorbate (37 μM). Figure 2 shows the changes in (measured − predicted) absorbance as different concentrations of ascorbate were added to PPA. The calculated dissociation constant of 27 μM was similar to the Eph-ascorbate constant of 15 μM (R² = 0.99).
The similarity in ascorbate binding did not predict relative oxidation rates of the different compounds. The oxidation of PPA and Eph is very slow, and over an ~24-h period only a small fraction of each compound was oxidized. PPA oxidation was measured using initial control measurements of 1.0 ± 0.06 (SD, n = 4) for PPA in PSS at 37°C bubbled with 95% O₂-5% CO₂. After 20.6 ± 0.5 (SD) h (n = 3), PPA had fallen to 0.92 ± 0.03 (n = 3) of control, for an extrapolated rate constant of 247.2 h. In similar experiments with Eph, the value decreased from 1.0 ± 0.7 (n = 7) to 0.96 ± 0.03 (n = 3) after 26.3 ± 1.3 h (n = 3), for an extrapolated rate constant of 643.2 h. The extrapolated values for the PPA and Eph rate constants will have a relatively high degree of quantitative uncertainty but a high qualitative value that these compounds oxidize over the course of weeks, not minutes or hours. The oxidation of NE and ascorbate alone occurs in a few hours under the high-O₂ conditions in the tissue baths. The time constants for oxidation of NE and ascorbate were measured by following the decay in the peak height at different times (Fig. 3) and using a least-squares best fit to the equation c/c₀ = (exp) - (t/τ). The rate constant for NE oxidation in PSS at 37°C was 1.24 h (n = 6, R² = 0.92). The rate constant for ascorbate in PSS at 37°C was 1.55 h (n = 7, R² = 0.88). The n values indicate the number of measurements used to measure the oxidation rates, in which 65% of NE and ascorbate was oxidized. Both showed that significant oxidation occurs in a relatively short time. Figure 3 shows the effect of ascorbate on the oxidation rate of NE. Concentrations of ascorbate in the physiological range (100 μM) more than doubled the rate constant for NE oxidation.

Figure 4 shows the effect of ascorbate on vascular smooth muscle contractions induced by NE. At almost every submaximal NE concentration, ascorbate statistically enhanced the contractions. A physiological concentration of NE produced virtually no contraction, whereas substantial contractions were produced when ascorbate was present. On the basis of the oxidation rate constants measured above, after 10 min of prewarming and 10 min of contraction, 76% of the NE should remain in the absence of ascorbate, a change unlikely to produce the difference in Fig. 4. Compare the peak 3 nM NE + 500 μM ascorbate force with the peak 30 nM NE force. These values are not statistically different (P = 0.92). If ascorbate protects NE, then the putative 30 nM NE should actually have a concentration of ~3 nM. Because the peak force occurs ~3 min after the introduction of NE, or ~13 min after warming begins, 90% oxidation of the NE would have to occur in ~13 min. With use of the measured NE oxidation rate constant from above, only 16% of the NE is oxidized by this time. Removal of 90% of the NE would actually require 2.3 times the measured rate constant or, in this case, >3 h. Although ascorbate

![Graph](image-url)
does reduce the rate of NE oxidation, this reduction cannot explain the increases in force seen with ascorbate in Fig. 4.

Figure 5 shows how ascorbate enhances the maintenance of NE contractions. For all but the highest concentrations, force induced by NE alone falls to 50–60% of the peak force (3 min) at 10 min. Prevention of NE oxidation will play a part in this, but catecholamine antioxidant mechanisms cannot be solely responsible. On the basis of the NE oxidation rate above, the NE concentration should fall from 84% to 76% of the labeled concentration in those 7 min in the absence of ascorbate. For the force to fall to 50–60% of the peak with such a small change in concentration and over that wide a range of concentrations, the dose-response curve would have to be very steep locally to accommodate the force change and that steep at every concentration over 2 orders of magnitude. Such a dose-response curve is not consistent with that shown in Fig. 1.

Figure 6 addresses the range over which ascorbate enhances NE contractions. Ascorbate has a range of 40–100 μM in plasma (1). The enhancement of NE by ascorbate, regardless of the mechanism, occurs over the physiological range. Ascorbate at >15 μM produced significant increases in NE-generated force. At no ascorbate concentration tested was force produced in the absence of an agonist such as NE.

The results in Fig. 7 show that ascorbate and NE need not be present simultaneously to enhance NE contractions. Ascorbate was preloaded and then washed from the tissue bath before the introduction of NE. Preloading for 2 min with a physiological
concentration of ascorbate, 50 μM, produced a significant increase in the NE-induced contraction, even though the ascorbate had been washed from the bath for 8 min before the NE was introduced. Ascorbate remaining after the washout of 50 μM, estimated from the washout of 50 mM ascorbate to 18 μM, should be ~18 nM, which is well below the 15 μM ascorbate needed to enhance ascorbate contractions (Fig. 6). In this case, it is not possible that ascorbate prevented the oxidation of NE, because they were never in the same solution. The enhancement effect peaked at 4 min of preloading, with 6 min of preloading showing no additional enhancement.

The concentration-dependent preloading experiments are shown in Fig. 8. Preloading and washout of 50, 150, and 500 μM ascorbate significantly enhanced the NE contractions under conditions in which the residual ascorbate was well below 1 μM. Moreover, the addition of 50 μM ascorbate simultaneously with the NE produced a significantly higher contraction than all the preload/washout conditions. The ascorbate enhancement of NE contractions had a limited temporal range.

Figure 9 shows the ascorbate enhancement of PPA and Eph contractions. PPA and Eph oxidize very slowly, with time constants of many days. Both showed a significant increase in force development over the physiological ascorbate range. In contrast to the NE enhancement in which 500 μM ascorbate was not different from 50 μM ascorbate (Fig. 6), 500 μM ascorbate produced a significant increase in force not only over 0 μM ascorbate, but also over 50 μM ascorbate additions to PPA and Eph.
Figure 10 shows that the dose-response curve for Epi was shifted by ascorbate in a manner that was qualitatively similar to the ascorbate-induced shift in NE contractions. Ascorbate did not enhance the Epi contractions at the top of the dose-response curve, which is similar to the findings with NE.

Ascorbate does not enhance contractions generated by high K+ or ANG II. Figure 11 shows that when these agonists are titrated to produce forces similar to those that produce 2.5-fold NE enhancement (Fig. 6), ascorbate produces no increase in force at any concentration.

Table 1 shows that the enhancement of NE force development is not limited to ascorbate. The metal chelator and antioxidant EDTA also produced a significant increase in the force produced by 100 nM NE. The force enhancement was similar to that produced by ascorbate.

**DISCUSSION**

The results shown above demonstrate a substantial ascorbate alteration in the force generation produced by NE and related compounds. These results impose significant limitations on the possible mechanisms of this enhancement.

Ascorbate does enhance the submaximal contractions of NE, Epi, PPA, and Eph. Figures 4–10 demonstrate this point in a wide range of experiments. In all cases, physiological and pharmacological concentrations of ascorbate resulted in longer and stronger contractions. What are the reasons behind this enhancement?

It has been qualitatively known for some time (7, 12, 13) that ascorbate is capable of reducing the oxidation rate of catecholamines. Our measurements above and in Fig. 3 demonstrate this in quantitative terms. NE and ascorbate oxidize over a time scale of hours when placed in a 95% O2 PSS at 37°C. PPA and Eph do not significantly oxidize over this time scale, instead having calculated time constants for oxidation 10 days. The calculations included with Fig. 5 show that, although ascorbate will retard the oxidation of NE (and, presumably, Epi in Fig. 10), this slowing of oxidation cannot account for all the force enhancement shown. No calculations are needed for the ascorbate enhancement of PPA and Eph. For these compounds, days are required for any significant oxidation to occur, even in the absence of ascorbate. Ascorbate enhancement of their contractions cannot in any way be due to the reduction in the rate of oxidation, inasmuch as the enhancement occurs in minutes. Other ascorbate effects must be at work.

PPA and Eph are α- and β-agonists, using the same receptors as NE (9). PPA and Eph bind to ascorbate with dissociation constants similar to that of NE.

**Table 1. Comparative effects of 150 μM ascorbate and 10 μM EDTA on 100 nM NE contractions of rabbit abdominal aortic rings**

<table>
<thead>
<tr>
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<th>100 μM NE</th>
<th>100 nM NE + 150 μM AA</th>
<th>100 nM NE + 10 μM EDTA</th>
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<tr>
<td>Force, g/mg</td>
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<tr>
<td>Peak</td>
<td>0.30±0.06</td>
<td>0.68±0.07*</td>
<td>0.52±0.07*</td>
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<tr>
<td>10 min</td>
<td>0.17±0.05</td>
<td>0.66±0.06*</td>
<td>0.051±0.07*</td>
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Values are means ± SE; NE, norepinephrine; AA, ascorbate. *Significantly different, P < 0.05.
tion constants of 27 and 15 μM, respectively (Fig. 2), at which these compounds start to produce significant contractions (Fig. 1). The ascorbate enhancement of PPA and Eph occurs at ≥50 μM. With concentrations of ascorbate and the agonist at or greater than the dissociation constant, most of the PPA and Eph will be bound to ascorbate under enhancement conditions. This, if anything, would lower the free concentration of PPA and Eph, making contractile enhancement less, not more, likely. As we previously showed (4), however, the electric field produced by the cell membrane generates a natural electrophoresis that will separate noncovalently bound compounds, such as NE and ascorbate, within 10 nm of the cell membrane. This separation presumably keeps PPA and Eph, as well as NE and Epi, available to bind to the adrenergic receptor at the membrane. It is most likely that PPA and Eph work by the same ascorbate enhancement mechanism as NE. Both bind to the adrenergic receptor (9). In addition, Eph is known to cause NE release from nerve terminals (17), which might suggest that ascorbate enhancement of Eph occurs via increased NE release followed by ascorbate enhancement of NE. Because there are no NE additions during these experiments, the residual nerve terminals would become depleted of endogenous NE during repeated contractions, and the Eph effect would fade over time. Eph contractions do not fade over time. NE release is not, therefore, a plausible mechanism by which ascorbate enhances Eph activity. Further evidence against an NE release mechanism comes from studies of PPA activity. PPA is not known to release NE from nerve terminals, but PPA contractions are enhanced by ascorbate. PPA does decrease reuptake of NE at nerve terminals (10), suggesting that decreased NE uptake may be the mechanism for ascorbate enhancement of PPA contractions. This is unlikely, because no exogenous NE is present during the PPA experiments. Also, if endogenous reuptake into nerve terminals were a significant factor in the mechanism, PPA activity would fade with time as the endogenous NE was depleted. PPA activity does not fade with time. Logically then, because PPA and Eph produce responses similar to Epi and NE (Fig. 9) and because their enhancement by ascorbate is similar to that of NE, they are likely to use a common adrenergic receptor-mediated mechanism.

If the direct binding of ascorbate to these compounds does not lower the force generation by reducing the free concentration of agonist or work through the reduction in oxidation rate, then the ascorbate effect may occur independent of physical interaction with the agonist. Figures 7 and 8 demonstrated this. Measurement of the temporal and concentration dependence of ascorbate enhancement during ascorbate preload/washout experiments showed that enhancement occurs even when the ascorbate and the agonist are never simultaneously present. In these cases, there can be no prevention of oxidation, nor can there be direct binding. In addition, Fig. 8 showed that 50 μM ascorbate in the presence of NE produced a greater force than NE alone after 2 min of 500 μM ascorbate followed by 8 min of washout. The ascorbate effect does not last for an extended time when the catecholamines are not present. It is reversible. The time course of the rapid onset (minutes) and reversal (within 20 min) of the ascorbate effect eliminates ascorbate enhancement of protein synthesis as a possible mechanism. Also, although the residual concentration of ascorbate in the tissue cannot be measured directly, the rapid changes in force in the presence of NE and ascorbate indicate rapid equilibration between the PSS and the tissue. The ascorbate washout measurements above indicate such low levels of ascorbate that the residual amounts cannot be the cause of the postwashout enhancement. Ascorbate must be producing a tissue alteration that outlasts its removal, at least for some minutes.

The K+ and ANG II experiments further narrow the range of possible mechanisms. K+ contractions occur when the cell membrane depolarizes and Ca2+ channels open. The absence of any ascorbate effect on K+ contractions makes the possibility of ascorbate opening of Ca2+ channels unlikely. Furthermore, because ascorbate itself, at any concentration up to and including 500 μM, does not produce any measurable contraction, it is unlikely that ascorbate has any direct effect on the intracellular Ca2+ concentration. Also, ascorbate cannot have its enhancement effect downstream from the increase in Ca2+. Any mechanism here, such as increasing myosin light chain kinase activity or altering the latch mechanism, would be a common element for K+ and NE and, thus, is ruled out by the lack of an ascorbate effect on K+ contractions.

Similarly, NE and ANG II use inositol trisphosphate mechanisms to trigger contraction (2, 16). Because ANG II does not show the ascorbate enhancement, an inositol trisphosphate mechanism is unlikely. Mechanisms involving the adrenergic receptor and the enzymes it activates directly are the best candidates for the ascorbate effect. The adrenergic receptor has been shown to exist in two states with a fivefold difference in sensitivity to catecholamines (19), with the basic state more sensitive than the acidic state. If ascorbate can interact with the receptor and reduce it from the acidic to the basic state, the increase in sensitivity would explain our findings. The amino acids producing the pH sensitivity have been hypothesized to be a cysteine and an aspartic acid (19), which in the α-receptor occupy positions 99 and 106, respectively (8). Similarly, ascorbate could be altering the redox state of the iron atom in the adrenergic receptor (15). The ascorbate-iron complex has been shown to alter enzyme activity (18). It is also possible that the G protein connected to the adrenergic receptor is activated by ascorbate, although no putative mechanism for this, analogous to the two-state adrenergic receptor, is available.

The force enhancement produced by EDTA shows that the findings generated by ascorbate may be generalized to other antioxidants. EDTA, in addition to reducing the oxidation rate of NE, significantly enhances its force generation. This enhancement, as shown in Table 1, is on the same order as the enhancement produced by ascorbate. This implies that the rate arguments made above for the effect of ascorbate on NE contractions are also applicable to EDTA. Furthermore, the highly charged EDTA molecule is unlikely to diffuse through the cell membrane in any significant amount. This would indicate that its effect must occur extracellularly. Reducing the adrenergic receptor into the high-affinity state would be consistent with an extracellular effect for ascorbate and EDTA.

There are several potential applications of these findings, regardless of the mechanism. Increased sensitivity in the presence of ascorbate could enhance and prolong the effects of catecholamines in clinical situations. The use of catecholamines to treat shock, for example, could be enhanced by ascorbate, as could inhaler treatments for asthma. Furthermore, our findings may shed light on the harmful side effects found with PPA and Eph use. Because ascorbate is not used in testing catecholamines and related compounds for drug safety, the
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enhancement effect, produced at physiological levels of ascorbate, would have been missed. Also, because pharmacological concentrations of ascorbate produce greater force than physiological concentrations for PPA and Eph (but not NE) stimulation, ingestion of excessive ascorbate could exacerbate the enhancement. This enhancement could play a role in the adverse cardiovascular consequences produced by PPA (6, 11, 14, 20) and, potentially, by Eph. Our findings present a new role for ascorbate that had previously been masked by its direct effect in reducing the oxidation rate of catecholamines and may provide a new tool for maximizing the effectiveness of catecholamine treatments.

Experiments to elicit the mechanism behind these findings will need to include 1) measurements of the ascorbate effect on the oxidation state of the adrenergic receptor using radiolabeled catecholamines, 2) the effect of ascorbate on the number of available adrenergic receptors using radiolabeled and immunological agents, 3) the effect of ascorbate on the second messenger activity of the adrenergic receptor and the second messenger effects on Ca\(^{2+}\) release from the sarcoplasmic reticulum and the opening of Ca\(^{2+}\) channels, and 4) the binding of adrenergic compounds to their receptor when there is an adrenergic-ascorbate complex. The washout experiments indicate that the binding of adrenergic compounds to their receptor when there is an adrenergic-ascorbate complex cannot account for the entire enhancement, yet these mechanisms are not mutually exclusive, and all may play some role in ascertaining the cause(s) of this intriguing phenomenon. Other considerations include whether other antioxidants such as EDTA work through the same mechanisms as ascorbate; whether acidosis, known to alter adrenergic receptor affinity, alters the ascorbate effects; and the degree to which unanticipated mechanisms may be operating. Overall, the demonstration that ascorbate enhances adrenergic activity independent of the oxidation of the adrenergic agent leaves many unanswered questions.

GRANTS
This study was supported in part by a grant from Acorn Technologies.

DISCLOSURES
Acorn Technologies has licensed patent rights from Michigan State University based on the work of P. F. Dillon and R. S. Root-Bernstein have stock options from Acorn Technologies.

REFERENCES