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Notes:
Ascorbate in pharmacologic concentrations selectively generates ascorbate radical and hydrogen peroxide in extracellular fluid in vivo

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Ascorbate (ascorbic acid, vitamin C), in pharmacologic concentrations easily achieved in humans by i.v. administration, selectively kills some cancer cells but not normal cells. We proposed that pharmacologic ascorbate is a prodrug for preferential steady-state formation of ascorbate radical (Asc−) and H2O2 in the extracellular space compared with blood. Here we test this hypothesis in vivo. Rats were administered parenteral (i.v. or i.p.) or oral ascorbate in typical human pharmacologic doses (~0.25–0.5 mg per gram of body weight). After i.v. injection, ascorbate baseline concentrations of 50–100 μM in blood and extracellular fluid increased to peaks of >8 mM. After i.p. injection, peaks approached 3 mM in both fluids. By gavage, the same doses produced ascorbate concentrations of <150 μM in both fluids. In blood, Asc− concentrations measured by EPR were undetectable with oral administration and always <50 nM with parenteral administration, even when corresponding ascorbate concentrations were >8 mM. After parenteral dosing, Asc− concentrations in extracellular fluid were 4- to 12-fold higher than those in blood, were as high as 250 nM, and were a function of ascorbate concentrations. By using the synthesized probe peroxyxanthone, H2O2 in extracellular fluid was detected only after parenteral administration of ascorbate and when Asc− concentrations in extracellular fluid exceeded 100 nM. The data show that pharmacologic ascorbate is a prodrug for preferential steady-state formation of Asc− and H2O2 in the extracellular space but not blood. These data provide a foundation for pursuing pharmacologic ascorbate as a prooxidant therapeutic agent in cancer and infections.

ascorbic acid | cancer | vitamin C | pharmacokinetics

A scorbic acid (ascorbate, vitamin C) has a controversial history in cancer treatment (1). Observational studies, initiated by Cameron and Campbell (2) and expanded in collaboration with Pauling (3, 4), suggested that ascorbate doses of 10 g daily prolonged survival. These studies had multiple uncertainties, including use of retrospective controls, lack of independent pathologic confirmation, and no blinding or placebo use (1, 5–7). In two double-blind, placebo-controlled trials, investigators at the Mayo Clinic found that 10 g of ascorbate had no effect on cancer survival (5, 6). Ascorbate was dismissed as a therapeutic agent in cancer treatment (7), but its use continues by practitioners of complementary and alternative medicine (8–10).

Emerging evidence indicates that ascorbate in cancer treatment deserves reexamination. Pharmacokinetics studies in healthy men and women show that ascorbate concentrations in plasma and tissue are tightly controlled as a function of oral dose (11–13). Intravenous injection of ascorbate bypasses tight control and produces plasma concentrations as much as 70-fold greater than those produced by maximal oral dosing (13). These data demonstrate that oral and i.v. ascorbate administration are not comparable. Surprisingly, it was unrecognized for years that the observational Cameron studies used both oral and i.v. administration, but the Mayo Clinic studies used oral dosing only. Thus, these outcome studies are also not comparable (1, 8, 13). New in vitro studies using ascorbate at pharmacologic concentrations only achievable by i.v. administration show that ascorbate is selectively toxic for some cancer but not normal cells (14). Clinical case reports also suggest that i.v. ascorbate might have a role in treating some cancers and that further investigation is warranted (9, 10).

Although many hypotheses could be tested to explain ascorbate action on cells, it is an essential prerequisite to investigate whether reaction products obtained from ascorbate in vitro are found in vivo. In vitro, pharmacologic ascorbate concentrations mediated selective cancer cell toxicity via formation of Asc− and H2O2 in cell culture media, with minimal Asc− and no H2O2 detectable in blood (14). H2O2 concentrations ≥25 μM in vitro were toxic to cancer cells (14). Based on these data, we propose in vivo (Fig. 1) that pharmacologic ascorbate concentrations selectively generate Asc− in extracellular fluid but not in blood. The electron lost from ascorbate would reduce a protein-centered metal, selectively driving H2O2 formation in extracellular fluid. In contrast, in blood pharmacologic ascorbate concentrations would produce low Asc− concentrations compared with extracellular fluid, whereas any H2O2 formed in blood would be immediately destroyed (14–18).

Based on the proposed reactions, if the predicted products are formed in vivo, then many next steps are justified, including determining molecular mechanisms of ascorbate action, isolation of proteins that mediate H2O2 formation, full characterization of ascorbate’s preferential action on malignant but not normal cells, and animal and clinical trials. If the predicted reaction products are not formed in vivo, then a potential role of ascorbate in cancer treatment would require an entirely new explanation or may have to be discarded.

Here, we tested in vivo the hypothesis that ascorbate is a prodrug for selective delivery of Asc− and H2O2 to the extracellular space. Ascorbate in rats was administered parenterally (by i.v. or i.p. injection) or by oral gavage, and extracellular fluid was obtained by microdialysis. Ascorbate and Asc− were measured in blood and extracellular fluid by using HPLC with coulometric electrochemical detection or EPR, respectively. H2O2 formation in extracellular fluid was measured as a function of time and administered asc-
bate. Because ascorbate interferes with most peroxidase-based detection methods, we used an assay based on a modified synthesis of peroxynitrite (PNX) (19). The data show that pharmacologic ascorbate concentrations produced Asc\(^{\text{ox}}\) selectively in extracellular fluid compared with blood and that H\(_2\)O\(_2\) formation occurred when Asc\(^{\text{ox}}\) concentrations were >100 nM in extracellular fluid. These data validate the hypothesis that ascorbate is a prodrug for selective delivery of reactive species to the extravascular space (Fig. 1) and provide the foundation for rational exploration of pharmacologic ascorbate as a prooxidant drug for therapeutic use.

**Results**

**Pharmacologic Ascorbate Concentrations Achieved in Blood and in Extracellular Fluid by i.v. or i.p. Administration.** We first tested whether parenteral (i.v. or i.p.) injection but not oral administration by gavage could achieve pharmacologic ascorbate concentrations in vivo both in blood and in extracellular fluid. Rats received doses similar to pharmacologic doses in humans (0.5 mg per gram of body weight) (8–10, 13). Blood was sampled at 0, 5, 15, 30, 60, 90, and 120 min, and plasma was separated for ascorbate analyses using HPLC with coulometric electrochemical detection. Extracellular fluid was collected by microdialysis at 30-min intervals before and after oral and parenteral administration, and ascorbate was determined at the end of each time period. With oral (gavage) dosing, initial ascorbate administration, and ascorbate was determined at 120 min, and plasma was separated for ascorbate analyses using HPLC with coulometric electrochemical detection. Extracellular fluid was collected by microdialysis at 30-min intervals before and after ascorbate administration, and ascorbate was determined at the end of each time period. With oral (gavage) dosing, initial plasma ascorbate concentrations of 50 \(\mu\)M did not increase to >100 \(\mu\)M, similar to findings of tight control of ascorbate concentrations in humans mediated by intestinal absorption (11–13). Intravenous administration of the same dose resulted in peak plasma concentrations of >8 mM, 80-fold higher than that produced by oral dosing. Intrapерitoneal injection achieved peak concentrations of 3 mM, 30-fold higher than concentrations produced by oral dosing (Fig. 2A). In extracellular fluid, ascorbate concentrations produced by the different administration routes mirrored the findings for plasma. Both i.v. and i.p. administration produced pharmacologic ascorbate concentrations, whereas oral dosing did not (Fig. 2B). For all dose routes and all time points, ascorbate concentrations in extracellular fluid were a highly correlated function of ascorbate concentrations in plasma (Fig. 2C). Furthermore, the correlation underestimated the coupled relationship between plasma and extracellular fluid concentrations as a consequence of extracellular fluid collection. To obtain enough fluid for analyses, it was necessary to collect it over 30-min intervals; values are for the whole collection time. Plasma values are point values at the end of 30-min intervals. Considered together, these data show that parenteral injection bypassed the tight control of oral ascorbate administration, pharmacologic concentrations were established both in blood and in extracellular fluid by parenteral injection but not oral dosing, and ascorbate was distributed similarly in plasma and extracellular fluid for all dosing routes.

Fig. 1. Proposed mechanism of preferential formation of Asc\(^{\text{ox}}\) and H\(_2\)O\(_2\) in extracellular fluid compared with blood. After oral and parenteral administration, ascorbic acid is proposed to achieve equivalent concentrations in blood (left side) and extracellular fluid (right side). In extracellular fluid, pharmacologic concentrations of ascorbic acid lose one electron and form Asc\(^{\text{ox}}\). The electron reduces a protein-centered metal: An example reaction is shown as reduction of Fe\(^{3+}\) to Fe\(^{2+}\). Fe\(^{2+}\) donates an electron to oxygen, forming active oxygen including superoxide (O\(_2^\bullet^-\)) with subsequent dismutation to H\(_2\)O\(_2\) (17). In blood (left side), it is proposed that these reactions are damped or inhibited (dashed lines). Asc\(^{\text{ox}}\) appearance will be inhibited by red blood cell membrane-reducing proteins (18) and by large plasma proteins that do not distribute to the extracellular space. Any formed H\(_2\)O\(_2\) will be immediately destroyed by plasma catalase and red blood cell GSH peroxidase, so that no H\(_2\)O\(_2\) will be detectable (14–16). The identities of the metal-centered proteins are unknown.

Fig. 2. Parenteral administration of ascorbic acid bypasses tight control of its intestinal absorption. A total dose of 0.5 mg of ascorbate per gram of body weight was given to rats by i.v. injection (circles) (two-thirds of the dose at 0 min and one-third at 30 min); by i.p. injection (stars) at 0 min; or by gavage (oral administration) (triangles) at 0 min. Blood was taken at each indicated time point. Extracellular fluid at the end of 30-min intervals was collected for ascorbic acid measurement (see Materials and Methods). Numbers of rats for each administration route are indicated. All data are displayed \(\pm\) SD. (A and B) Ascorbic acid concentration in plasma (A) and extracellular fluid (B), measured in millimolar as a function of time in minutes. (A Inset and B Inset) Gavage administration of ascorbic acid, displayed as plasma concentration (micromolar) as a function of time (minutes). (C) Ascorbic acid concentration in extracellular fluid (millimolar) as a function of ascorbic acid concentration in plasma (millimolar) for all administration routes, all animals, and all time points \((R^2 = 0.93, P < 0.0001)\).
Preferential Formation of Asc$^{-}$ in Extracellular Fluid Compared to Blood with Parenteral Administration of Ascorbate. Before and after i.v. administration of ascorbate, we investigated whether Asc$^{-}$ formation occurred in blood and extracellular fluid. Asc$^{-}$ was measured by EPR in whole blood and extracellular fluid obtained by microdialysis. As a control, Asc$^{-}$ measurements were performed after ascorbate was added to the perfusate buffer used with the microdialysis pump, with no extracellular fluid present. After i.v. administration of ascorbate at 0.5 mg per gram of body weight, EPR spectra showed preferential formation of Asc$^{-}$ in extracellular fluid compared to blood, and the signal was present for at least 2 h (Fig. 3A). The spectra shown are a 4-fold underestimate of the difference in the magnitude of the Asc$^{-}$ signal from extracellular fluid compared to blood. The spectra for extracellular fluid are underestimates because extracellular fluid had to be diluted 1:1 with buffer for collection and because efficiency of Asc$^{-}$ transit across the microdialysis membrane was 50% (data not shown). In contrast, blood and saline samples were measured without dilution. The Asc$^{-}$ signal was not an artifact, because no Asc$^{-}$ was observed when ascorbate was added to the perfusate buffer without extracellular fluid present.

We studied Asc$^{-}$ formation in extracellular fluid and blood (Fig. 3B) as a function of time. Each dosing route was used to administer a pharmacologic dose of ascorbate, 0.5 mg per gram of body weight. For extracellular fluid, Asc$^{-}$ concentrations approaching 250 nM were detected after i.v. administration, corresponding to peak ascorbate concentrations of 6–8 mM in extracellular fluid (see Fig. 2 for ascorbate concentrations). With i.p. injections, peak Asc$^{-}$ concentrations in extracellular fluid were ~150 nM, corresponding to lower peak ascorbate concentrations of ~3 mM. With gavage, Asc$^{-}$ concentrations in extracellular fluid did not exceed 50 nM, consistent with minimal elevations in ascorbate concentrations from oral dosing. Findings for Asc$^{-}$ in extracellular fluid after ascorbate administration stand in contrast to findings in blood. With i.v. or i.p. injection, Asc$^{-}$ concentrations in blood maximized at ~30–40 nM. With oral administration, Asc$^{-}$ in blood was undetectable.

Data were combined from all doses, time points, and administration routes. Asc$^{-}$ formation in extracellular fluid was expressed as a function of ascorbate concentration in this fluid, and Asc$^{-}$ formation in blood was expressed as a function of ascorbate concentration in plasma (Fig. 3C). Asc$^{-}$ formation in extracellular fluid was exponentially correlated with ascorbate concentration in this fluid. For example, with 6 mM ascorbate in plasma, 220 nM of Asc$^{-}$ was formed in extracellular fluid. However, in blood, Asc$^{-}$ concentrations were all <50 nM, even when plasma ascorbate concentration was 9 mM. As expected, Asc$^{-}$ formation in extracellular fluid was similarly correlated with ascorbate concentration in plasma (data not shown). Together, these findings support the hypothesis that pharmacologic ascorbate concentrations from parenteral administration selectively generate Asc$^{-}$ in extracellular fluid, with minimal formation of Asc$^{-}$ in blood.

H$_2$O$_2$ Formation in Extracellular Fluid with Parenteral Administration of Ascorbate. H$_2$O$_2$ in extracellular fluid was measured by an assay using a synthesized boronate fluorophore PX1 (see Materials and Methods and Fig. 4A) (19). PX1 reacted with H$_2$O$_2$ and produced blue fluorescent 3,6-dihydroxyxanthone, with peak fluorescence at an emission wavelength of 450 nm. Catalase was added to samples to account for H$_2$O$_2$-specific fluorescence and background. With background subtraction, H$_2$O$_2$ concentrations were linearly correlated with fluorescence intensity. This assay was used to measure H$_2$O$_2$ in extracellular fluid before and after ascorbate administration by all dose routes as a function of time (Fig. 4B). After i.v. administration, H$_2$O$_2$ concentrations increased from undetectable to ~20 µM, but no change occurred after oral administration. With i.p. administration, H$_2$O$_2$ concentrations were lower than those from i.v. administration, consistent with concentrations of ascorbate and Asc$^{-}$ produced by i.p. dosing.

H$_2$O$_2$ concentrations in extracellular fluid produced by all dosing routes and at all time points are displayed as functions of Asc$^{-}$ concentration in extracellular fluid (Fig. 4C), ascorbate concentration in extracellular fluid (Fig. 4D), and ascorbate concentration in blood.
correlation of H$_2$O$_2$ concentration with ascorbic acid in extracellular fluid (concentrations in plasma were determined as in Fig. 2. and oral (triangles) ascorbic acid administration. (Fig. 4)). These concentrations only occurred with parenteral administration. Furthermore, there was a linear correlation between H$_2$O$_2$ concentrations in extracellular fluid and ascorbate concentrations in blood or in extracellular fluid (Fig. 4D). These data indicate that the higher the ascorbate concentration in either fluid, the higher the attained H$_2$O$_2$ concentration from parenteral administration.

In control animals, pharmacologic ascorbate concentrations added exogenously to collected extracellular fluid but not buffer induced formation of Asc$^-$ and H$_2$O$_2$ (data not shown), indicating that extracellular fluid components are required for reactions.

Discussion

Recently we reported that pharmacologic ascorbic acid concentrations produced H$_2$O$_2$ concentrations of ≥25 μM, causing cancer cell death in vitro (14). Building on these results, here we tested and validated in vivo the hypothesis that parenteral administration of ascorbate in pharmacologic doses produces millimolar concentrations in blood and extracellular fluid, with preferential generation of Asc$^-$ and H$_2$O$_2$ in extracellular fluid but not blood. We found that H$_2$O$_2$ concentrations generated in vivo were those that caused cancer cell death in vitro (14). When ascorbate was given parenterally, Asc$^-$, the product of a loss of one electron from ascorbate, was detected preferentially in extracellular fluid compared with blood. Asc$^-$ generation in extracellular fluid depended on the ascorbate dose and the resulting concentrations. With i.v. administration of ascorbate, Asc$^-$ concentrations were as much as 12-fold greater in extracellular fluid compared to blood and approached 250 nM. Asc$^-$ concentrations of >100 nM in extracellular fluid were the threshold concentration for detectable production of H$_2$O$_2$. In blood, such Asc$^-$ concentrations were never produced and were always <50 nM. Even if Asc$^-$ concentrations had reached the 100 nM threshold for H$_2$O$_2$ production, H$_2$O$_2$ in blood is immediately destroyed by plasma and red blood cell proteins (14–16). These data are all consistent with the hypothesis that pharmacologic ascorbate concentrations in vivo serve as a produg for selective delivery of H$_2$O$_2$ to the extracellular space.

The experiments in this paper are based on principles of tight control of ascorbate in humans (11–13). After oral ingestion, control of intracellular and extracellular ascorbate concentrations is mediated by three mechanisms: intestinal absorption, tissue transport, and renal reabsorption. First, intestinal absorption, or bioavailability, declines at doses >200 mg, corresponding to plasma concentrations of ~60 μM. Second, at approximately this concentration, ascorbate is excreted in urine (11, 12). These three mechanisms work coordinately, ensuring that ascorbate is tightly controlled. Parenteral administration bypasses tight control, which is restored as kidneys excrete ascorbate when concentrations are more than those corresponding to $V_{\text{max}}$, and tissues appear to be saturated (11, 12, 20). Third, also at ~60 μM, renal reabsorption approaches saturation, and excess ascorbate is excreted in urine (11, 12). These three mechanisms work coordinately, ensuring that ascorbate is tightly controlled. Parenteral administration bypasses tight control, which is restored as kidneys excrete ascorbate when concentrations are more than those corresponding to $V_{\text{max}}$ of the reabsorptive transporters. Tight control principles in humans are based both on clinical and modeled data (11–13, 21). Here, we provide in vivo evidence that validates the modeled values and confirms principles of tight control. With an oral pharmacologic dose of 0.5 mg per gram of body weight in animals, plasma concentrations and extracellular fluid concentrations did not exceed 150 μM, whereas the same i.v. pharmacologic dose produced plasma concentrations up to 60-fold higher.

The data here offer an attractive explanation as to why tight control occurs. When tight control is bypassed, H$_2$O$_2$ forms in the extracellular space. As tight control is restored, H$_2$O$_2$ formation ceases. If tight control did not exist, H$_2$O$_2$ formation and exposure could be constant, with untoward consequences on cell division and growth (22–24). Tight control provides a mechanism preventing continuous tissue exposure to high concentrations of H$_2$O$_2$. Temporally bypassing tight control with parenteral administration of ascorbate allows H$_2$O$_2$ to form in discrete time periods only, decreasing likelihood of harm, and provides a pharmacologic basis for therapeutic use of i.v. ascorbate.

Fig. 4. H$_2$O$_2$ formation in extracellular fluid with parenteral administration of ascorbic acid. Administered doses were 0.5 mg per gram of body weight for all routes and were given as described for Fig. 2. Extracellular fluid at the end of 30-min intervals was collected for H$_2$O$_2$, ascorbic acid, and Asc$^-$ measurement (see Fig. 2 and Materials and Methods). Numbers of rats for each administration route are indicated. All data are displayed ± SD. (A) Fluorescent spectra of PX1 in normal saline with added H$_2$O$_2$ at the indicated concentrations. Catalase (600 units/ml) was added to parallel samples to account for non-H$_2$O$_2$ fluorescence background. (Left Inset) Chemical structure of PX1 and its product after reaction with H$_2$O$_2$. (Right Inset) Typical H$_2$O$_2$ standard curve. (B) H$_2$O$_2$ formation in extracellular fluid as a function of time before and after i.v. (circles), i.p. (stars), and oral (triangles) ascorbic acid administration. (C) Correlation of H$_2$O$_2$ concentration with Asc$^-$ concentration in extracellular fluid ($R^2 = 0.77, P < 0.0015$). (D) Correlation of H$_2$O$_2$ concentration with ascorbic acid in extracellular fluid ($R^2 = 0.84, P < 0.0001$) or in plasma (Inset; $R^2 = 0.87, P < 0.0001$). Ascorbic acid concentrations in plasma were determined as in Fig. 2.
We also provide data about ascorbate distribution in plasma and extracellular fluid with both oral and parenteral dosing. Although ascorbate extracellular fluid values were somewhat higher than those for plasma, this is likely due to the collection procedure. Plasma values, collected at the end of each 30-min collection period, are point values. To obtain enough volume for analyses of extracellular fluid, it was necessary to collect it for 30 min. Reported values represent averages for this time. Due to pharmacokinetics of ascorbate renal clearance, average values in extracellular fluid should be higher than point values in plasma at the end of each collection period. We interpret the data to show that in vivo extracellular fluid and plasma ascorbate concentrations are similar and that ascorbate diffuses from plasma to the extravascular space.

Pharmacologic ascorbate concentrations in extracellular fluid are stable for at least 1 h at 4°C (data not shown). Ascorbate must oxidize because Asc− is detected. The concentration of the latter is 10−3 to 10−4 less than the former, and ascorbate oxidation is only detectable by measuring Asc+. Asc− measurements reflect a dynamic process of formation by ascorbate oxidation and disappearance by either dismutation or reduction. The lifetime of Asc− depends on its own concentrations and on ascorbate concentrations and the milieu. In extracellular fluid, Asc− likely degrades by dismutation, whereas blood red cells reduce Asc−. As shown in Fig. 1, it is likely that the electron from ascorbate reduces a protein-centered metal and Asc− appearance is a reaction indicator, although it remains possible that Asc− itself provides an electron (25). In vitro, killing is mediated by H2O2 rather than Asc−. H2O2 formation results in selective cytotoxicity. Tumor cells are killed with exposure to H2O2 for ≤30 min (26–30).

With in vivo validation of ascorbate as a prodrug for selective H2O2 formation, we can now suggest mechanisms to account for selective ascorbate action. In vitro data indicate that external pharmacologic ascorbate concentrations are required for external H2O2 formation (14). We propose that external H2O2 formed from pharmacologic ascorbate concentrations diffuses into cells (31) and mediates toxicity in sensitive cells by ATP depletion (23) via one or more of three pathways (Fig. 5). First, H2O2 may cause DNA single-strand breaks, repaired by polyADP-ribose polymerase (PARP). Enhanced PARP activity may deplete NAD+, resulting in ATP depletion (27, 29). Second, H2O2 removal within cells may be mediated in part by glutathione (GSH) peroxidase. GSH peroxidase has an essential requirement for GSH, which, upon enzyme activity, is oxidized to GSH disulfide (GSSG). GSSG is regenerated to GSH with reducing equivalents from NADPH, which in turn is regenerated from glucose via the pentose shunt. Glucose used to reduce NADP+ to NADPH is not available for ATP generation (26). In cancer cells that depend on anaerobic metabolism for ATP generation (the Warburg effect), loss of glucose to the pentose shunt may result in decreased ATP, leading to cell death (32–35). Third, mitochondria in some cancer cells may have increased sensitivity to H2O2 (28, 34, 36). Mitochondria in such cells may be less efficient at baseline in generating ATP compared with normal cells. Enhanced mitochondrial sensitivity to H2O2 with or without inefficient generation of ATP at baseline, may result in decreased ATP production. These pathways for ATP depletion induced by H2O2 are independent, and more than one could be responsible for cell death in sensitive cells (28, 34). Pharmacologic ascorbate concentrations should not impair normal cells because their primary ATP generation is via aerobic metabolism and because their mitochondria may not be as sensitive to H2O2 as those in some cancer cells.

Accumulating evidence indicates that H2O2 is a signaling agent at intracellular concentrations of <1 μM (22, 24). Signaling actions result in proliferation and enhanced survival of some cells. However, H2O2 concentrations generated by pharmacologic ascorbate injection are greater than H2O2 concentrations that enhance survival and instead are in the range that induce cell death. Other consequences of such oxidative stress, distinct from effects on ATP concentrations (Fig. 5), might also induce selective H2O2 toxicity to cancer but not normal cells.

If pharmacologic parenteral ascorbate is a prodrug for selective H2O2 delivery to the extracellular space, then therapeutic use should consider more broadly H2O2 in applications where H2O2 may have clinical benefit. In addition to cancer treatment, another potential therapeutic use is for treatment of infections. H2O2 concentrations of 25–50 μM are bacteriostatic (37), and as we show here these concentrations are generated in vivo by pharmacologic ascorbate administration. We need to learn whether some bacteria are especially sensitive to clinically possible H2O2 concentrations, whether there is synergy with antibiotic therapy, and whether such synergy can be used to treat problematic resistant species, such as Acinetobacter or methicillin-resistant Staphylococcus aureus. H2O2 concentrations only slightly higher than those presented in this paper are selectively toxic to hepatitis C virus replication in cell culture models (38). Other virally infected cells may also be candidates (14) and should be investigated, particularly where there are no current therapies. Pharmacologic ascorbate as a prodrug for H2O2 generation offers potential promise in clinical treatment of some cancers and infections with minimal harm. We advocate enhanced basic and clinical research in these areas to advance possibilities quickly so that patients might benefit.

Materials and Methods

Animals. Using general anesthesia, terminal experiments were performed on Wistar rats (17 males; Charles River Laboratories, Wilmington, MA) and Sprague–Dawley rats (six females; Taconic Laboratories, Rockville, MD) at ages of 10–22 weeks. Animals were euthanized at the end of experiments, which were approved by the Animal Care and Use Committee.

Ascorbate Administration. Anesthesia was initiated by 5% isoflurane via nose cone and maintained by using inhaled 1–2% isoflurane (balance compressed air). Rats were supine and warmed on a 37°C water-jacketed heat pad. Ascorbate solutions were prepared for each experiment and adjusted to pH 7 with NaOH. Ascorbate
dosages (~0.25–5 mg per gram of body weight) were administered by tail vein injection, i.p. injection, or gavage. Doses for tail vein injections were divided to lessen osmotic load: two-thirds of the dose was given at 0 min, and the remaining third was given 30 min later. For i.p. injections and gavages, the full dose was given at 0 min.

**Microdialysis and Blood Sampling.** Microdialysis was performed as described (39). Briefly, after establishing maintenance isoflurane anesthesia, two CMA/20 microdialysis probes with 10-mm membrane lengths, 20-kDa cutoffs, and 0.5-mm outer diameters (CMA/Microdialysis, North Chelmsford, MA) were implanted into each hind limb by femoral muscle dissection. Each probe was connected to a pump for perfusion and sample collection. Before sample collection, implanted probes were equilibrated with normal saline for 30 min at a flow rate of 1 μl/min. Extracellular fluid was collected on ice at the same flow at 30-min intervals before and after ascorbate administration, analyzed immediately for H2O2 andAsc−, and then frozen at −70°C for subsequent ascorbate analysis. Collection periods were 30 min because two microdialysis probes provided 60 μl, a sufficient sample volume for all analyses. Membrane efficiencies were as follows: ascorbate, 30%; Asc−, 50%; H2O2, 85% (39).

Whole-blood samples were collected before and after ascorbate administration from femoral veins at the indicated time points. Whole blood was used immediately to measure Asc− and then centrifuged at 1,800 × g for 15 min to obtain plasma. Plasma was frozen at −70°C until analyzed for ascorbate.

**Asc− and Ascorbate Detection.** Asc− was measured by X-band EPR (40, 41). Spectrometer (E9 series; Varian) settings were as follows: microwave power, 20 mW; modulation amplitude, 1.0 G; time constant, 0.5 s; and scan rate, 80 G per 8 min. Radical quantitation was performed by using 3-carboxyprolyl as a standard (40). The coefficient of variation was 4%.

Ascorbate was measured by HPLC with coulometric electrochemical detection (12), with a coefficient of variation of 3%. The results for microdialysis were corrected by the recovery rate of the microdialysis membrane and sample dilution from fluid collection.

**PX1 Synthesis.** For detection of H2O2, the boronate fluorophore PX1 was synthesized (19). The precursor 3,6-dihydroxyxanthone was converted to 3,6-bis(trifluoromethanesulfonyl)xanthone by reaction with N-phenyl bis(trifluoromethanesulfonamide) (19). PX1 [3,6-bis(pinacolatoboron)xanthone] was obtained by palladium-catalyzed coupling of the bis-trflate with bis(pinacolato) diboron (19), using microwave heating with a Biogene Initiator at 160°C for 16 min as a modification. The solution of the crude PX1 in warm toluene was first decolorized with activated carbon before continuing the procedure. Pure PX1 was obtained in a yield of 35%. The 1H NMR (CDCl3, 300 MHz) parameters were: δ, 8.07 (2H, d, J = 8.73 Hz); 6.85 (4H, m ppm). MS analysis was calculated for [MH+H2O2] = 229.040 (found, 229.041), which was converted to 3,6-bis(trifluoromethanesulfonyl)xanthone by reaction with N-phenyl bis(trifluoromethanesulfonamide) (19). PX1 [3,6-bis(pinacolatoboron)xanthone] was obtained by palladium-catalyzed coupling of the bis-trflate with bis(pinacolato) diboron (19), using microwave heating with a Biogene Initiator at 160°C for 16 min as a modification. The solution of the crude PX1 in warm toluene was first decolorized with activated carbon before continuing the procedure. Pure PX1 was obtained in a yield of 35%. The 1H NMR (CDCl3, 300 MHz) parameters were: δ, 8.07 (2H, d, J = 8.73 Hz); 7.92 (2H, s); 7.77 (2H, d, J = 7.82 Hz), 1.39 (24H, s). MS analysis as calculated for [MH+H2O2] = 229.040 (found, 229.041).

**H2O2 Detection.** Microdialysis eluate was collected into tubes containing 20 μM PX1 (initial volume, 60 μl). Eluate was simultaneously collected from the opposed femoral muscle into tubes containing 20 μM PX1 and 600 units/ml catalase (initial volume, 60 μl) to validate the H2O2 signal and determine background fluorescence. Samples were collected at 30-min intervals. Spectra were attained on a fluorescent spectrophotometer (PerkinElmer, Shelton, CT) at an excitation wavelength of 350 nm. A peak area between 420 and 500 nm was used for calculating H2O2 concentrations, determined from a standard curve comparing signals obtained in the presence and absence of exogenous catalase (Fig. 4). Results were corrected by the throughput (recovery) rate of the microdialysis membrane and a dilution factor from addition of PX1 with or without catalase. The assay coefficient of variation was 7%.

**Statistics.** Statistical analyses and curve fitting were performed with SigmaPlot 10 (Systat, San Jose, CA). Results were independent of animal sex and age (data not shown). All error bars represent standard deviation. The equation describing H2O2 formation in relation to Asc− concentration in extracellular fluid was the inverse of the equation describing Asc− formation as a function of ascorbate concentration in either extracellular fluid or plasma, such that H2O2 formation was linearly related to ascorbate concentration in extracellular fluid and in plasma.

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