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# 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 ( $\text{Asc}^{\bullet-}$ ) and  $\text{H}_2\text{O}_2$  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 ( $\approx 0.25\text{--}0.5$  mg per gram of body weight). After i.v. injection, ascorbate baseline concentrations of  $50\text{--}100$   $\mu\text{M}$  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  $<150$   $\mu\text{M}$  in both fluids. In blood,  $\text{Asc}^{\bullet-}$  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,  $\text{Asc}^{\bullet-}$  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,  $\text{H}_2\text{O}_2$  in extracellular fluid was detected only after parenteral administration of ascorbate and when  $\text{Asc}^{\bullet-}$  concentrations in extracellular fluid exceeded 100 nM. The data show that pharmacologic ascorbate is a prodrug for preferential steady-state formation of  $\text{Asc}^{\bullet-}$  and  $\text{H}_2\text{O}_2$  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

Ascorbic 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  $\text{Asc}^{\bullet-}$  and  $\text{H}_2\text{O}_2$  in cell culture media, with minimal  $\text{Asc}^{\bullet-}$  and no  $\text{H}_2\text{O}_2$  detectable in blood (14).  $\text{H}_2\text{O}_2$  concentrations  $\geq 25$   $\mu\text{M}$  *in vitro* were toxic to cancer cells (14). Based on these data, we propose *in vivo* (Fig. 1) that pharmacologic ascorbate concentrations selectively generate  $\text{Asc}^{\bullet-}$  in extracellular fluid but not in blood. The electron lost from ascorbate would reduce a protein-centered metal, selectively driving  $\text{H}_2\text{O}_2$  formation in extracellular fluid. In contrast, in blood pharmacologic ascorbate concentrations would produce low  $\text{Asc}^{\bullet-}$  concentrations compared with extracellular fluid, whereas any  $\text{H}_2\text{O}_2$  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  $\text{H}_2\text{O}_2$  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  $\text{Asc}^{\bullet-}$  and  $\text{H}_2\text{O}_2$  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  $\text{Asc}^{\bullet-}$  were measured in blood and extracellular fluid by using HPLC with coulometric electrochemical detection or EPR, respectively.  $\text{H}_2\text{O}_2$  formation in extracellular fluid was measured as a function of time and administered ascor-

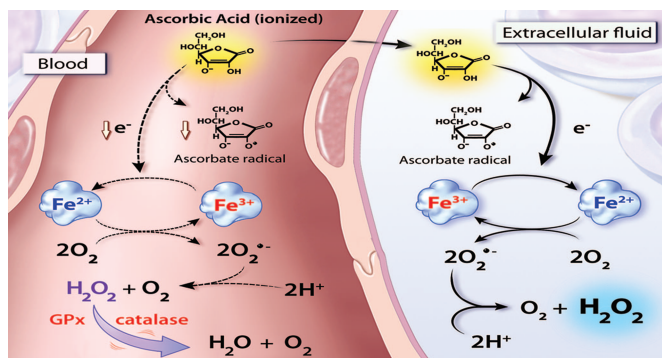
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The authors declare no conflict of interest.

Abbreviations:  $\text{Asc}^{\bullet-}$ , ascorbate radical; GSH, glutathione; GSSG, GSH disulfide; PARP, polyADP-ribose polymerase; PX1, peroxyxanthone.

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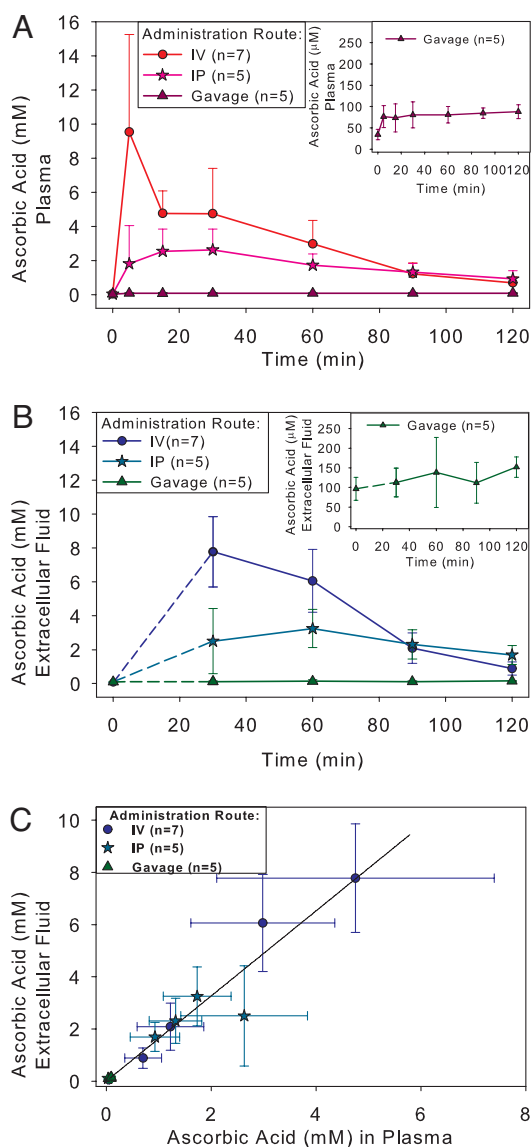


**Fig. 1.** Proposed mechanism of preferential formation of  $\text{Asc}^{\bullet-}$  and  $\text{H}_2\text{O}_2$  in extracellular fluid compared with blood. After oral and parenteral administration, ascorbic acid is proposed to achieve equivalent concentrations in blood (left side) and extracellular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

bate. Because ascorbate interferes with most peroxidase-based detection methods, we used an assay based on a modified synthesis of peroxyxanthone (PX1) (19). The data show that pharmacologic ascorbate concentrations produced  $\text{Asc}^{\bullet-}$  selectively in extracellular fluid compared with blood and that  $\text{H}_2\text{O}_2$  formation occurred when  $\text{Asc}^{\bullet-}$  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 ascorbate administration, and ascorbate was determined at the end of each time period. With oral (gavage) dosing, initial plasma ascorbate concentrations of  $50 \mu\text{M}$  did not increase to  $>100 \mu\text{M}$ , 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. Intraperitoneal injection achieved peak concentrations of  $\approx 3$  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

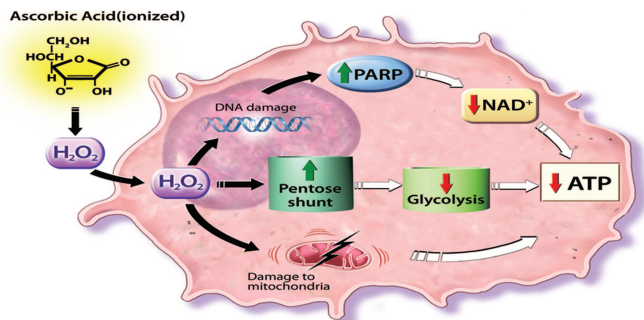


**Fig. 2.** Parenteral administration of ascorbic acid bypasses tight control of its intestinal absorption. A total dose of 0.5 mg of ascorbate per gram of body weight was given to rats by i.v. injection (circles) (two-thirds of the dose at 0 min and one-third at 30 min); by i.p. injection (stars) at 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 ( $R^2 = 0.93$ ,  $P < 0.0001$ ).

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. 5.** Pharmacologic ascorbic acid concentrations: mechanisms for selective cell death. Pharmacologic ascorbic acid concentrations produce extracellular H<sub>2</sub>O<sub>2</sub>, which can diffuse into cells, deplete ATP in sensitive cells, and thereby cause cell death. ATP may be depleted by three mechanisms. (i) DNA damage induced by H<sub>2</sub>O<sub>2</sub> activates PARP. Activated PARP catabolizes NAD<sup>+</sup>, thereby depleting substrate for NADH formation and consequent ATP synthesis. (ii) H<sub>2</sub>O<sub>2</sub> is catabolized by concurrent oxidation of GSH to GSSG. To reduce GSSG back to GSH, GSH reductase utilizes NADPH, which is provided by the pentose shunt from glucose. Glucose used to reduce NADP<sup>+</sup> to NADPH cannot be used for glycolysis or NADH production so that ATP generation is decreased. (iii) H<sub>2</sub>O<sub>2</sub> may directly damage mitochondria, especially ATP synthase, so that ATP production falls. Some cancer cells rely primarily on glycolysis rather than on oxidative phosphorylation for ATP production (the Warburg effect). Compared with oxidative phosphorylation, ATP generation by glycolysis is inefficient. In glycolysis-dependent cancer cells, decreased glycolysis may lower intracellular ATP. Cancer cells that are glycolysis-dependent may be particularly sensitive to pharmacologic ascorbic acid concentrations, compared with cells that use oxidative phosphorylation. See text for additional details.

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<sup>•−</sup> is detected. The concentration of the latter is 10<sup>−3</sup> to 10<sup>−4</sup> less than the former, and ascorbate oxidation is only detectable by measuring Asc<sup>•−</sup>. Asc<sup>•−</sup> measurements reflect a dynamic process of formation by ascorbate oxidation and disappearance by either dismutation or reduction. The lifetime of Asc<sup>•−</sup> depends on its own concentrations and on ascorbate concentrations and the milieu. In extracellular fluid, Asc<sup>•−</sup> likely degrades by dismutation, whereas blood red cells reduce Asc<sup>•−</sup>. As shown in Fig. 1, it is likely that the electron from ascorbate reduces a protein-centered metal and Asc<sup>•−</sup> appearance is a reaction indicator, although it remains possible that Asc<sup>•−</sup> itself provides an electron (25). *In vitro*, killing is mediated by H<sub>2</sub>O<sub>2</sub> rather than Asc<sup>•−</sup>. H<sub>2</sub>O<sub>2</sub> formation results in selective cytotoxicity. Tumor cells are killed with exposure to H<sub>2</sub>O<sub>2</sub> for ≤30 min (26–30).

With *in vivo* validation of ascorbate as a prodrug for selective H<sub>2</sub>O<sub>2</sub> 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 H<sub>2</sub>O<sub>2</sub> formation (14). We propose that external H<sub>2</sub>O<sub>2</sub> 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, H<sub>2</sub>O<sub>2</sub> may cause DNA

single-strand breaks, repaired by polyADP-ribose polymerase (PARP). Enhanced PARP activity may deplete NAD<sup>+</sup>, resulting in ATP depletion (27, 29). Second, H<sub>2</sub>O<sub>2</sub> 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<sup>+</sup> 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 H<sub>2</sub>O<sub>2</sub> (28, 34, 36). Mitochondria in such cells may be less efficient at baseline in generating ATP compared with normal cells. Enhanced mitochondrial sensitivity to H<sub>2</sub>O<sub>2</sub>, with or without inefficient generation of ATP at baseline, may result in decreased ATP production. These pathways for ATP depletion induced by H<sub>2</sub>O<sub>2</sub> 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 H<sub>2</sub>O<sub>2</sub> as those in some cancer cells.

Accumulating evidence indicates that H<sub>2</sub>O<sub>2</sub> is a signaling agent at intracellular concentrations of <1 μM (22, 24). Signaling actions result in proliferation and enhanced survival of some cells. However, H<sub>2</sub>O<sub>2</sub> concentrations generated by pharmacologic ascorbate injection are greater than H<sub>2</sub>O<sub>2</sub> 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 H<sub>2</sub>O<sub>2</sub> toxicity to cancer but not normal cells.

If pharmacologic parenteral ascorbate is a prodrug for selective H<sub>2</sub>O<sub>2</sub> delivery to the extracellular space, then therapeutic use should consider more broadly H<sub>2</sub>O<sub>2</sub> in applications where H<sub>2</sub>O<sub>2</sub> may have clinical benefit. In addition to cancer treatment, another potential therapeutic use is for treatment of infections. H<sub>2</sub>O<sub>2</sub> 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 H<sub>2</sub>O<sub>2</sub> 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 *Staphylococcal aureus*. H<sub>2</sub>O<sub>2</sub> 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 H<sub>2</sub>O<sub>2</sub> 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 ( $\approx 0.25\text{--}5$  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  $\mu\text{l}/\text{min}$ . Extracellular fluid was collected on ice at the same flow at 30-min intervals before and after ascorbate administration, analyzed immediately for  $\text{H}_2\text{O}_2$  and  $\text{Asc}^{\bullet-}$ , and then frozen at  $-70^\circ\text{C}$  for subsequent ascorbate analysis. Collection periods were 30 min because two microdialysis probes provided 60  $\mu\text{l}$ , a sufficient sample volume for all analyses. Membrane efficiencies were as follows: ascorbate, 30%;  $\text{Asc}^{\bullet-}$ , 50%;  $\text{H}_2\text{O}_2$ , 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  $\text{Asc}^{\bullet-}$  and then centrifuged at  $1,800 \times g$  for 15 min to obtain plasma. Plasma was frozen at  $-70^\circ\text{C}$  until analyzed for ascorbate.

**$\text{Asc}^{\bullet-}$  and Ascorbate Detection.**  $\text{Asc}^{\bullet-}$  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-carboxyproxyl 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  $\text{H}_2\text{O}_2$ , the boronate fluorophore PX1 was synthesized (19). The precursor 3,6-dihydroxyxanthone (42) was prepared in 95% yield from 2,2',4,4'-tetrahydroxyacetophenone by thermal cyclization of 250 mg in 15 ml of  $\text{H}_2\text{O}$  at  $200^\circ\text{C}$

for 3 h in a Biotage Initiator microwave reactor (Biotage, Uppsala, Sweden). The  $^1\text{H}$  NMR ( $\text{CD}_3\text{OD}$ , 300 MHz) parameters were as follows:  $\delta$ , 8.07 (2H, d,  $J = 8.73$  Hz); 6.85 (4H, m) ppm. MS analysis as calculated for  $[\text{MH}^+]$  was 229.040 (found, 229.041), which was converted to 3,6-bis(trifluoromethanesulfonyl)xanthone by reaction with *N*-phenyl bis(trifluoromethanesulfonylamide) (19). PX1 [3,6-bis(pinacolatoboron)xanthone] was obtained by palladium-catalyzed coupling of the bis-triflate with bis(pinacolato)diboron (19), using microwave heating with a Biotage Initiator at  $160^\circ\text{C}$  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  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 300 MHz) parameters were:  $\delta$ , 8.30 (2H, d,  $J = 7.82$  Hz); 7.94 (2H, s); 7.77 (2H, d,  $J = 7.82$  Hz), 1.39 (24H, s). MS analysis as calculated for  $[\text{MH}^+]$  was 449.220 (found, 449.230).

**$\text{H}_2\text{O}_2$  Detection.** Microdialysis eluate was collected into tubes containing 20  $\mu\text{M}$  PX1 (initial volume, 60  $\mu\text{l}$ ). Eluate was simultaneously collected from the opposite femoral muscle into tubes containing 20  $\mu\text{M}$  PX1 and 600 units/ml catalase (initial volume, 60  $\mu\text{l}$ ) to validate the  $\text{H}_2\text{O}_2$  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  $\text{H}_2\text{O}_2$  concentrations, determined from a standard curve comparing signals obtained in the presence and absence of exogenous catalase (Fig. 4A). 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  $\text{H}_2\text{O}_2$  formation in relation to  $\text{Asc}^{\bullet-}$  concentration in extracellular fluid was the inverse of the equation describing  $\text{Asc}^{\bullet-}$  formation as a function of ascorbate concentration in either extracellular fluid or plasma, such that  $\text{H}_2\text{O}_2$  formation was linearly related to ascorbate concentration in extracellular fluid and in plasma.

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