Vitamin C suppresses oxidative lipid damage in vivo, even in the presence of iron overload

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1The Evans Memorial Department of Medicine, Whitaker Cardiovascular Institute, Boston University School of Medicine, Boston 02118; 2The Core Laboratory, Beth Israel Hospital and Deaconess Medical Center, Boston, Massachusetts 02115; 3The Linus Pauling Institute, Oregon State University, Corvallis, Oregon 97331; and 4Departments of Medicine and Pharmacology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

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Chen, Kent, Jung Suh, Anitra C. Carr, Jason D. Morrow, John Zein, and Balz Frei. Vitamin C suppresses oxidative lipid damage in vivo, even in the presence of iron overload. Am J Physiol Endocrinol Metab 279: E1406–E1412, 2000.—Ascorbate is a strong antioxidant; however, it can also act as a prooxidant in vitro by reducing transition metals. To investigate the in vivo relevance of this prooxidant activity, we performed a study using guinea pigs fed high or low ascorbate doses with or without prior loading with iron dextran. Iron-loaded animals gained less weight and exhibited increased plasma \( \beta \)-N-acetyl-\( N \)-glucosaminidase activity, a marker of tissue lysosomal membrane damage, compared with control animals. The iron-loaded animals fed the low ascorbate dose had decreased plasma \( \alpha \)-tocopherol levels and increased plasma levels of triglycerides and \( \text{F}_2 \)-isoprostanes, specific and sensitive markers of in vivo lipid peroxidation. In contrast, the two groups of animals fed the high ascorbate dose had significantly lower hepatic \( \text{F}_2 \)-isoprostane levels than the groups fed the low ascorbate dose, irrespective of iron load. These data indicate that 1) ascorbate acts as an antioxidant toward lipids in vivo, even in the presence of iron overload; 2) iron loading per se does not cause oxidative lipid damage but is associated with growth retardation and tissue damage, both of which are not affected by vitamin C; and 3) the combination of iron loading with a low ascorbate status causes additional pathophysiological changes, in particular, increased plasma triglycerides.

antioxidant; ascorbate; \( \text{F}_2 \)-isoprostanes; guinea pigs; lipid peroxidation

OXIDATIVE STRESS, defined as an imbalance in prooxidant vs. antioxidant species in favor of the former, results in oxidative damage to biological macromolecules (13, 26). Increased oxidative stress has been implicated in iron overload conditions, such as homozygous hemochromatosis and treatment of \( \beta \)-thalassemia (24, 43), and is thought to be due to iron-catalyzed generation of hydroxyl and alkoxyl radicals through Fenton chemistry (reactions 1 and 2; Ref. 19). Ascorbate (vitamin C) is a strong antioxidant capable of scavenging a wide variety of reactive oxygen and nitrogen species (19). Ascorbate is the most effective water-soluble antioxidant in human plasma against lipid peroxidation induced by aqueous peroxyl radicals, activated neutrophils, or the gas-phase of cigarette smoke (15–17). However, under certain in vitro conditions, ascorbate can act as a prooxidant by reducing transition metal ions (reaction 3), thereby driving the Fenton reaction (19).

\[
\begin{align*}
\text{H}_2\text{O}_2 + \text{Fe}^{2+} & \rightarrow \text{HO}^• + \text{Fe}^{3+} + \text{OH}^- \quad (1) \\
\text{LOOH} + \text{Fe}^{2+} & \rightarrow \text{LO}^• + \text{Fe}^{3+} + \text{OH}^- \quad (2) \\
\text{AH}^- + \text{Fe}^{3+} & \rightarrow \text{A}^{•−} + \text{Fe}^{2+} + \text{H}^+ \quad (3)
\end{align*}
\]

where \( \text{HO}^• \) is hydroxyl radical, \( \text{LOOH} \) is lipid hydroperoxide, \( \text{LO}^• \) is alkoxyl radical, \( \text{AH}^− \) is ascorbate, and \( \text{A}^{•−} \) is ascorbyl radical.

Contrary to its putative prooxidant role in the presence of redox-active transition metal ions, we have found that ascorbate not only effectively protects low-density lipoprotein from metal ion-dependent oxidation (35, 36) but also acts as an antioxidant toward lipids in iron-overloaded human plasma in vitro (3). Furthermore, ascorbate did not increase lipid peroxidation in 3T3 fibroblasts incubated with iron (10), and the addition of ascorbate to the diet of guinea pigs supplemented with iron inhibited, rather than promoted, lipid peroxidation in an ex vivo microsomal system (9). Given this mounting evidence for an antioxidant role of ascorbate in the presence of excess iron in vitro and ex vivo, the question arises whether ascorbate acts as a prooxidant or an antioxidant in vivo under conditions of iron overload.

Therefore, we conducted a study in a well-characterized guinea pig model of iron overload (1, 37) by use of a two-by-two factorial design of iron loading and ascorbate feeding. The guinea pig is a physiologically relevant model to study the effects of dietary ascorbate...
manipulation, because it is one of the few animals that, like humans, lack a functional enzyme, l-gulono-γ-lactone oxidase, required for de novo biosynthesis of ascorbate. We assessed oxidative damage by measuring F$_2$-isoprostanes, which are specific and sensitive markers of oxidative lipid damage (29).

METHODS

Iron loading. Fifty-two female albino guinea pigs (Charles River Scientific, Wilmington, MA), ~21 days old and weighing between 200 and 250 g, were housed in groups of three animals in stainless steel cages and given a commercial diet containing 1 mg of ascorbate/g of chow (Purina Guinea Pig Chow #5025, St. Louis, MO) and water ad libitum. The guinea pigs were weighed twice weekly throughout the study. Twenty-six guinea pigs were iron loaded over the course of 4 wk by intraperitoneal injections of iron (Fe$^{3+}$) dextran (Sigma, St. Louis, MO) on alternate days (83 mg Fe/kg body wt) (37). The remaining 26 guinea pigs were injected on alternate days with the equivalent volume of dextran (Sigma). At the end of 4 wk and an additional 1 wk of recovery, six guinea pigs from each group were killed, and their tissues were collected for iron analysis.

Ascorbic acid manipulation. The diet of the remaining 40 guinea pigs was switched to a casein-based test diet (Purina Mills #5710C-6, Richmond, IN) containing no detectable ascorbate. To achieve chronic hypovitaminosis C without symptoms of scurvy, one-half of the animals (10 iron-loaded, 10 controls) were given a maintenance dose of 1.0 mg ascorbate (Sigma) every other day by gastric gavage, while the other 20 guinea pigs (10 iron-loaded, 10 controls) were given 100 mg ascorbate every other day. This regimen was continued for 4 wk, after which the guinea pigs were killed and the tissues collected for analysis. Ascorbate was withheld 2 days before death, and animals were killed after an overnight fast. Two guinea pigs in each of the iron-loaded groups (given either the high or low ascorbate dose) died before completion of the study. Necropsies showed severe inflammation, especially of the small bowel, and deposits of hemosiderin in several tissues, most markedly in the liver, as well as brown pieces, which were either snap frozen in liquid nitrogen and stored at −70°C until analysis, or homogenized in ice-cold phosphate-buffered saline and further processed for ascorbate or α-tocopherol analysis.

Measurements. Ascorbate and α-tocopherol were analyzed by reversed phase HPLC with electrochemical detection as described (15, 40). Total cholesterol, triglycerides, and serum iron levels were determined spectrophotometrically using Sigma kits #352, #339, and #565, respectively. Total iron levels in liver, heart, and spleen were measured by atomic absorption spectroscopy. Protein levels were determined spectrophotometrically using the Lowry assay (Sigma procedure P5656) or the Bradford assay (Bio-Rad, Hercules, CA). The determination of β-N-acetyl-d-glucosaminidase activity was adapted from a method by Skrha et al. (38). Briefly, 100 µl of plasma were added to 1.25 ml of sodium citrate buffer (100 mM, pH 4.4), which was then incubated for 5 min at 37°C with 0.25 ml of 10 mM p-nitro-phenyl-β-N-acetyl-d-glucosaminide (Sigma). The reaction was terminated by adding 1.5 ml sodium carbonate (200 mM, pH 10.4), and the absorbance was measured at 405 nm. For analysis of free and acylated F$_2$-isoprostanes, lipids in plasma or liver homogenates were extracted by a modified Folch procedure and base hydrolyzed (27). The resulting free F$_2$-isoprostanes were measured after purification and derivatization by capillary gas chromatography-negative ion chemical ionization mass spectrometry (GC-MS) as described (28).

Statistical analysis. For comparisons involving three or more groups, the Kruskal-Wallis nonparametric ANOVA test was used to determine whether the medians of each group were different. If they were different, the Dunn multiple comparison test was performed to determine which pairs were different. For comparisons involving only two groups, the Wallace nonparametric two-tailed t-test was performed. For comparisons of groups with two levels of independent variables, the two-way ANOVA test was performed with the Dunn multiple comparison test included to determine which pairs were different. The level of statistical significance was set at P < 0.05 for all tests.

RESULTS

Iron loading of the guinea pigs was accomplished by intraperitoneal injections of iron dextran over the course of 4 wk to achieve a final load of 1.5 g iron/kg body wt. Control animals were injected with equal volumes of a dextran solution. After a 1-wk recovery period, ascorbate was fed to the guinea pigs by gastric gavage for another 4 wk, either at a high dose (50 mg/day) or a low, maintenance dose (0.5 mg/day) causing chronic hypovitaminosis without scurvy (18). Thus the 9-wk study protocol produced four groups of guinea pigs: high ascorbate/control (n = 10); low ascorbate/control (n = 10); high ascorbate/iron-loaded (n = 8); and low ascorbate/iron-loaded (n = 8).

At the end of the study period, the animals in the iron-loaded groups weighed significantly less than the animals in the control groups (Table 1), indicating that iron loading, but not ascorbate dose, adversely affected the animals' growth rate. Growth retardation has been observed previously in guinea pigs (37) and humans suffering from iron overload (14, 44). Plasma β-N-acetyl-d-glucosaminidase activity, a marker of tissue lysosomal membrane damage (1), was about fourfold higher in the iron-loaded animals compared with the control animals, irrespective of ascorbate dose (Table
1). This result is in agreement with earlier findings of severe tissue damage in iron-loaded guinea pigs (1, 37).

Plasma total cholesterol levels were 20% lower (\(P < 0.05\)) in the iron-loaded compared with the control animals (Table 1), in agreement with previous observations in iron-loaded rabbits (12). In contrast, plasma triglyceride levels were more than doubled in the iron-loaded animals fed the low dose of ascorbate compared with the other three groups (Table 1). Interestingly, in the same group of animals, lipid-standardized plasma \(\alpha\)-tocopherol levels were decreased significantly (Table 1), suggesting the presence of increased oxidative stress. However, hepatic \(\alpha\)-tocopherol levels were not affected by either ascorbate feeding or iron loading (Table 1).

The iron-loaded guinea pigs had 30-fold elevated hepatic iron levels compared with control animals (Fig. 1A). Significant increases in iron levels by 10- and 20-fold were also observed, respectively, in heart and spleen, which had the highest iron levels of the tissues examined (not shown). Interestingly, there was no effect of ascorbate dose on hepatic iron levels (Fig. 1A).

No significant differences were observed in serum iron levels between control and iron-loaded animals fed the high or low dose of ascorbate (Fig. 1B).

Hepatic levels of ascorbate were significantly higher in the animals fed the high compared with the low dose of ascorbate (Fig. 1C, Table 2). Significant ascorbate accumulation was also observed in spleen, heart, and adrenals (Table 2). The highest tissue ascorbate levels were found in adrenals, followed by spleen, liver, and heart, in agreement with previous studies (21). Interestingly, iron load did not have a significant effect on ascorbate levels in the liver (Fig. 1C) or any of the other tissues examined (Table 2). Plasma ascorbate concentrations also were significantly higher in the animals fed the high compared with the low dose of ascorbate but were not affected by iron status (Fig. 1D).

To determine the extent of oxidative lipid damage, levels of \(F_2\)-isoprostanes were measured in liver and plasma using GC-MS. As shown in Fig. 2A, hepatic \(F_2\)-isoprostane levels were significantly elevated in the animals fed the low ascorbate dose (control 7.6 ± 2.8, and iron-loaded 9.4 ± 2.6 ng/g) compared with the high

Table 1. Selected measurements in guinea pig cohorts

<table>
<thead>
<tr>
<th></th>
<th>HiAA (n = 10)</th>
<th>LoAA (n = 10)</th>
<th>HiAA/Fe (n = 8)</th>
<th>LoAA/Fe (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight, g</td>
<td>497 ± 64</td>
<td>490 ± 66</td>
<td>435 ± 32*</td>
<td>435 ± 41*</td>
</tr>
<tr>
<td>Plasma NAGase activity, (\mu)M/min</td>
<td>2.8 ± 0.9</td>
<td>3.6 ± 1.9</td>
<td>14.4 ± 2.6*</td>
<td>13.2 ± 5.5*</td>
</tr>
<tr>
<td>Plasma cholesterol, mM</td>
<td>0.89 ± 0.16</td>
<td>0.96 ± 0.40</td>
<td>0.81 ± 0.12*</td>
<td>0.71 ± 0.01*</td>
</tr>
<tr>
<td>Plasma triglycerides, mM</td>
<td>1.2 ± 0.8</td>
<td>1.2 ± 0.5</td>
<td>1.1 ± 0.4</td>
<td>2.4 ± 0.5*</td>
</tr>
<tr>
<td>Plasma (\alpha)-tocopherol, (\mu)M/M total lipid</td>
<td>0.28 ± 0.30</td>
<td>0.25 ± 0.35</td>
<td>0.25 ± 0.18</td>
<td>0.07 ± 0.01*</td>
</tr>
<tr>
<td>Liver (\alpha)-tocopherol, pmol/mg protein</td>
<td>108.3 ± 60.0</td>
<td>148.4 ± 94.3</td>
<td>133.5 ± 55.5</td>
<td>130.3 ± 58.4</td>
</tr>
</tbody>
</table>

Values are means ± SD; \(n = \) no. of animals. HiAA, high ascorbate/control; LoAA, low ascorbate/control; HiAA/Fe, high ascorbate/iron-loaded; LoAA/Fe, low ascorbate/iron-loaded. Weight was used as a marker of growth rate and general well-being. Plasma \(\beta\)-N-acetyl-D-glucosaminidase (NAGase) activity was used as a marker of tissue damage. *Significantly different from unmarked groups, \(P < 0.05\), by two-way ANOVA.
ascorbate dose (control 5.9 ± 2.2, and iron-loaded 4.9 ± 2.6 ng/g; P < 0.05). Interestingly, comparison of the liver F2-isoprostane levels in iron-loaded animals with control animals revealed no significant differences. Plasma F2-isoprostane levels were significantly decreased and increased, respectively, in iron-loaded compared with control animals fed the high or low ascorbate dose (Fig. 2B), indicating that ascorbate feeding lowers oxidative lipid damage in the plasma of iron-loaded animals.

In Fig. 3, the liver F2-isoprostane levels of all 36 animals in the study are plotted against liver iron (Fig. 3A) and ascorbate levels (Fig. 3B). In contrast to iron levels (r² = 0.003, P = 0.73), ascorbate levels were significantly (r² = 0.32, P = 0.003) inversely correlated with F2-isoprostane levels. These data indicate that ascorbate protects against oxidative lipid damage in the liver, irrespective of iron status, and that iron loading itself does not cause oxidative lipid damage.

**DISCUSSION**

This study represents the first demonstration that ascorbate exhibits antioxidant activity, rather than prooxidant activity, in vivo toward lipids in the presence of iron overload. Our results show the following. 1) Iron loading per se does not cause oxidative lipid damage but nevertheless is associated with growth retardation, tissue damage, and hypocholesterolemia. This systemic dysfunction with iron overload is not affected by vitamin C. 2) Iron loading combined with a low ascorbate status results in decreased lipid-standardized plasma α-tocopherol levels and substantially increased plasma triglyceride levels. Vitamin C deficiency in guinea pigs has been previously shown to be associated with increased plasma triglycerides (4), and this may be exacerbated by iron loading. 3) Ascorbate protects against oxidative lipid damage in vivo, independent of iron status. This finding is best illustrated in Fig. 3, which shows that tissue ascorbate levels, in contrast to iron levels, are significantly inversely correlated with F2-isoprostanes.

Oxidative damage in the present study was assessed by measuring esterified and free F2-isoprostanes in, respectively, liver and plasma. F2-isoprostanes are sta-
ble, specific, and sensitive markers of oxidative lipid damage that are found in almost every tissue and biological fluid (29). Esterified F₂-isoprostanes are formed in vivo on arachidonyl-containing lipids by a free radical-catalyzed mechanism that is independent of cyclooxygenase; these esterified F₂-isoprostanes are released into plasma following hydrolysis by phospholipases (29). Elevated levels of F₂-isoprostanes have been detected under conditions of increased oxidative stress (13, 29), and administration of antioxidants, in particular ascorbate and α-tocopherol, has been shown to reduce their levels (26, 29).

The finding that iron load is not correlated with F₂-isoprostane levels in guinea pigs (Fig. 3A) raises the interesting question of whether iron per se causes tissue damage (as indicated by increased plasma activity of β-N-acetyl-β-glucosaminidase activity) through oxidative mechanisms. Dabbagh et al. (12), in a previous study carried out in iron-loaded rabbits, also showed no increase in F₂-isoprostanes in plasma and liver, while in a third animal study (11), slightly increased hepatic, but not plasma, F₂-isoprostanes were observed in iron-loaded rats. Several other studies investigating the effects of iron overload in rats found increased markers of lipid oxidation, i.e., thiobarbituric acid-reactive substances and conjugated dienes (2, 5, 11, 25). It should be noted, however, that rats synthesize vitamin C and as such may not be relevant models for iron overload in humans. Most importantly, unlike F₂-isoprostanes, thiobarbituric acid-reactive substances and conjugated dienes are nonspecific markers of oxidative lipid damage, particularly in complex biological samples (13, 19). Finally, some of the inconsistent results may be explained by the fact that some studies have employed intraperitoneal iron dextran injection (this study and Ref. 12) and others iron carbonyl feeding (2, 5, 11, 25); the former regimen leads to the accumulation of iron primarily in macrophages and Kupffer cells (22, 37), while the latter delivers iron to parenchymal cells (31).

Increased levels of lipid and protein oxidation products together with decreased levels of ascorbate and α-tocopherol have been observed in the serum of hemochromatosis and β-thalassemia patients (24, 43). These findings were attributed to the iron overload conditions; however, they do not demonstrate a prooxidant role of ascorbate. In the present study, high levels of ascorbate suppressed, rather than promoted, the formation of F₂-isoprostanes, while low levels of ascorbate were associated with increased oxidative stress, as indicated not only by increased F₂-isoprostane levels but also decreased plasma α-tocopherol levels. In addition, our earlier work showed that F₂-isoprostane and protein carbonyl levels were not significantly different in plasma of preterm infants containing nonprotein-bound, bleomycin-detectable iron compared with plasma of preterm infants devoid of bleomycin-detectable iron, despite the presence of high levels of ascorbate (3). Finally, a recent report (42) in which healthy volunteers were supplemented with both iron (14 mg/day) and ascorbate (60 or 260 mg/day) for 12 wk showed a modest reduction of ex vivo low-density lipoprotein oxidizability and beneficial effects on platelet function; no evidence for a prooxidant effect of vitamin C and iron cosupplementation was observed.

It is possible that changes in oxidative damage to lipids differ from oxidative damage to other biological macromolecules. For example, oxidative damage may depend on iron binding sites present on proteins or DNA but not lipids (20). A study (34) investigating the effects of ascorbate and iron cosupplementation on 13 different oxidative DNA damage products in human leukocytes found that, although some oxidative products increased and others decreased, total base damage increased after 6 wk of supplementation and returned to baseline after 12 wk. Similarly, supplementation with ascorbate alone was found to increase 8-oxodenoine levels in human leukocytes, while also decreasing 8-oxoguanine levels (32). However, the findings from both of these studies (32, 34) are likely confounded by artifactual ex vivo DNA oxidation during GC-MS analysis, and other problems with these studies have been identified (23, 33).

In addition to addressing oxidative lipid damage, the present study also provides some information on the possible interactions between ascorbate and iron metabolism. The data indicate that in guinea pigs iron overload does not affect tissue and plasma ascorbate levels and, vice versa, ascorbate feeding does not affect serum and liver total iron levels, as reported previously (7, 39). Similar results were also observed recently in ascorbate-requiring osteogenic disorder Shionogi rats injected intraperitoneally with 0.5 g iron dextran/kg body wt and fed 150 or 900 parts per million ascorbate (N. Gorman and P. Sinclair, personal communication). In these rats, iron loading only slightly lowered hepatic ascorbate levels and ascorbate dose did not affect hepatic nonheme iron levels. In contrast, iron overload in humans is associated with significantly decreased leukocyte, platelet, and plasma ascorbate levels, suggesting that high iron levels enhance the rate of ascorbate catabolism (24, 41, 43). Furthermore, vitamin C administration to patients with β-thalassemia major enhances the removal of iron by the chelator deferoxamine and markedly increases transferrin saturation and serum iron and ferritin levels (8, 30). These data suggest that ascorbate, most likely through its reducing capacity, can mobilize excess tissue iron in humans. The discrepancy between our and other researchers’ data in experimental animals may be explained by the fact that iron dextran is deposited primarily in Kupffer cells (37), while in humans excess iron is localized mainly to parenchymal cells (31).

In summary, the results from the present in vivo study, in agreement with previous in vitro and ex vivo studies (3, 9, 10, 36, 42), demonstrate that ascorbate does not promote oxidative lipid damage but instead acts as an antioxidant toward lipids in vivo, even in the presence of iron overload. Further studies are needed to evaluate the effects of dietary and supplemental vitamin C on iron redistribution and oxidative damage.
in humans suffering from iron overload before the data of the present study can be applied clinically.

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REFERENCES


