Scurvy Leads to Endoplasmic Reticulum Stress and Apoptosis in the Liver of Guinea Pigs

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ABSTRACT Insufficient ascorbate intake causes scurvy in certain species. Beyond its known functions, it has been suggested that ascorbate participates in oxidative protein folding in the endoplasmic reticulum (ER). Because redox imbalance in this organelle might cause ER stress and apoptosis, we hypothesized that this might contribute to the pathology of scurvy. Guinea pigs were divided into 7 groups: the control group was fed a commercial guinea pig food containing 0.1 g/100 g ascorbate for 4 wk, 5 groups consumed an ascorbate-free food for 0, 1, 2, 3, or 4 wk and 1 group was fed this scorbutic diet for 2 wk and then the commercial food plus 1 g/L ascorbate in drinking water for 2 wk. TBARS generation and the expression of some ER chaperones and foldases were determined in hepatic microsomes. The apoptotic index was assessed in histological sections. Although ascorbate, measured by HPLC, was undetectable in the livers of the guinea pigs after they had consumed the scorbutic diet for 2 wk, the microsomal TBARS level was elevated relative to the initial value only at wk 4. Western blot revealed the induction of GRP78, GRP94, and protein disulfide isomerase at wk 3 and 4. Apoptosis was greater than in the control, beginning at wk 3. None of the alterations occurred in the groups fed the commercial guinea pig food or ascorbate-free food followed by ascorbate supplementation. Therefore, persistent ascorbate deficiency leads to ER stress, unfolded protein response, and apoptosis in the liver, suggesting that insufficient protein processing participates in the pathology of scurvy. J. Nutr. 135: 2530–2534, 2005.

KEY WORDS: • scurvy • ascorbate • endoplasmic reticulum stress • apoptosis • chaperone

Ascorbate is produced in the liver or kidney of most animals but some species (including humans and guinea pigs) are deficient in gulonolactone oxidase, the enzyme that catalyzes the last step of the synthesis; hence, they need to ingest ascorbate (vitamin C) (1). Insufficient intake of vitamin C causes scurvy, a potentially fatal disease characterized primarily by extreme weakness and various skin and gum abnormalities (2). The multitude of seemingly unrelated symptoms that occur in scurvy indicates that ascorbic acid is involved in many cellular reactions. It is a major water-soluble antioxidant that protects the tissues by scavenging harmful oxidizing agents, in cooperation with vitamin E and glutathione (3), and is a cosubstrate of several oxygenases. The known functions of ascorbate in the biosynthesis of collagen, catecholamines, and carnitine explain most symptoms of scurvy. Two post-translational modifications in procollagen are catalyzed by ascorbate-dependent enzymes (prolyl hydroxylase and lysyl hydroxylase). The weakened collagen structure is responsible for the majority of bone and joint abnormalities, including the loss of teeth, observed in vitamin C deficiency (4). Conversion of dopamine to norepinephrine by dopamine β-monoxygenase also requires ascorbate for maximal activity. This might be the basis of the development of depression and lethargy, and the mood changes that occur frequently in scurvy (5). Ascorbate is a cofactor for 2 enzymes (ε-N-trimethyllysine hydroxylase and γ-butyrobetaine hydroxylase) in the pathway of carnitine biosynthesis. Because carnitine is essential for fatty acid utilization, fatigue, and weakness in scurvy may be attributed to its deficient synthesis (5). In addition, there are some other ascorbate-dependent enzymes that may contribute to the symptoms of scurvy, but their pathogenic role has not yet been clarified. They are involved in peptide amidation, tyrosine metabolism, as well as in bile acid and steroid synthesis (5).

Previous studies indicated the role of ascorbate in oxidative protein folding in the endoplasmic reticulum (ER) (6). The protein or glutathione thiol groups are more oxidized (to disulfide bonds) in the ER lumen than in the cytosol (7). The way in which the luminal thiol oxidizing environment is generated and maintained has not been elucidated. Some protein components of the thiol oxidizing electron transfer chain were identified, e.g., Ero1p and protein disulfide isomer-
ase (PDI) (8), but some links are missing. The addition of ascorbate to hepatic microsomes in vitro leads to enzymatic ascorbate consumption (9) accompanied by an enhanced oxidation of protein thiol groups (10). The process was shown to involve a microsomal ascorbate oxidase activity, PDI, and tocopherol (vitamin E) (11).

On the basis of these observations, ascorbate deficiency (scurvy) can be expected to impair protein maturation in the ER. The defective protein folding, in turn, activates a signaling network called the unfolded protein response (UPR). The UPR is a complex defense mechanism against the excessive accumulation of faulty polypeptides in the secretory pathway (12). It includes upregulation of ER chaperones (e.g., GRP78, GRP94) and foldases (e.g., PDI, ERP72). When these mechanisms do not ameliorate the stress situation, apoptosis is initiated in higher eukaryotic organisms, presumably to eliminate unhealthy cells.

MATERIALS AND METHODS

Induction of ascorbate deficiency in guinea pigs. Male Hartley guinea pigs (Charles River Hungary; body weight 500–600 g) were housed in plastic cages in a room with controlled lighting (12 h/d), constant temperature (20°C), and relative humidity (55–65%). For 1 wk after their arrival in our laboratory, they were fed a pelleted commercial guinea pig food (containing an adequate amount of vitamin C) plus 1 g/L vitamin C in drinking water for an additional 2 wk. All other groups were switched to a scorbutic (ascorbate-free) food, Altromin C3015 (14) (Charles River Hungary) containing 0.1 g/100 g ascorbate and then were divided randomly into 7 groups of 4 guinea pigs. One group (control) was fed the regular guinea pig food for another 4 wk. One group was killed at the beginning of the study (wk 0). All other groups were switched to a scorbutic (ascorbate-free) food, Altromin C3015 (14) (Charles River Hungary). Four groups consumed this food for 1, 2, 3, or 4 wk. Group 7 consumed the scorbutic diet for 2 wk and then the commercial guinea pig food (containing an adequate amount of vitamin C) plus 1 g/L vitamin C in drinking water for an additional 2 wk. All guinea pigs had free access to food and water. The guinea pigs were decapitated, and the body and liver weights were measured. The applied protocols complied with the NIH guidelines.

Preparation of hepatic microsomal vesicles. The livers were homogenized in sucrose-HEPES buffer (0.3 mol/L sucrose, 0.02 mol/L HEPES, pH 7.2) with a glass-Teflon homogenizer. The microsomal fraction was then isolated using fractional centrifugation (15). After centrifugation of liver homogenates for 10 min at 1000 × g, the supernatant was spun for 10 min at 12,000 × g. The 12,000 × g supernatant was spun for 60 min at 100,000 × g. The sediment (microsomes) was resuspended in the homogenization medium to give ~50 g/L protein concentration, then immediately frozen in liquid nitrogen and kept in liquid nitrogen until use (within 2 mo). The protein concentration in microsomal samples was determined using the BioRad microprotein assay kit according to the manufacturer’s instructions with bovine serum albumin as a standard.

Measurement of ascorbate content and lipid peroxidation. Ascorbate was measured in the liver tissue by HPLC. Samples were prepared from the livers by homogenizing them in a solution containing 0.05 mol/L metaphosphoric acid, 0.005 mol/L Na3EDTA, and 1.7 g/L K2O2S2. The homogenates were then centrifuged (20,000 × g, 10 min) and the supernatants were filtered through cellulose acetate filters (0.2-μm pore size; Schleicher, Schuell). Ascorbate was then detected in the protein-free samples by HPLC analysis (16). The degree of lipid peroxidation was assessed by the measurement of TBARS according to Wills (17).

Western blot analysis. Equal amounts of microsomal proteins were separated by 9% SDS-PAGE and transferred to polyvinylidene fluoride filter membranes by electroph blotting. Anti-ERP72 rabbit polyclonal antibody was purchased from Calbiochem. Anti-GRP94 rabbit, anti-GRP78 goat, and anti-PDI rabbit polyclonal antibodies, as well as the horseradish peroxidase–conjugated anti-rabbit Ig and anti-goat Ig secondary antibodies were purchased from Santa Cruz Biotechnology. Each primary antibody labeled a band of the expected molecular weight (72, 94, 78, 55 kDa, respectively), and unspecific bands were not seen.

Redox Western blot. The thiol redox state of ER frac k HD dances were investigated by alkylation with 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS; Molecular Probes) as described previously (18). For the reduced and oxidized controls, microsomal samples corresponding to 0.04 g protein were incubated at 37°C for 15 min with 0.05 mol/L diithiothreitol or with 0.025 mol/L diamide, respectively.

Monitoring apoptosis in the liver. Apoptotic cells/bodies were counted independently by 2 observers in the hematoxylin and eosin–stained histological sections prepared from the same lobe of each liver. The results were validated by the terminal deoxynucleotidyl transferase dUTP nick-end labeling assay, using the ApopTag Peroxidase Kit (Chemicon), according to the manufacturer’s instructions. The apoptotic index was calculated as the number of apoptotic cells/bodies in 1000 cells.

Statistical method. Independent measurements were performed in triplicate using separate samples prepared from the 4 guinea pigs in each group. The quantified results are shown as means ± SEM and were compared using ANOVA with Tukey’s multiple comparison post hoc test. Differences with \( P < 0.05 \) were considered significant.

### RESULTS

The ascorbate concentration in the liver tissue did not differ between wk 0 and 4 in guinea pigs that consumed the commercial diet (control group), but it was quickly decreased by the scorbutic diet. Ascorbate was not detectable after wk 2, whereas it was effectively replenished in the group that was supplemented with ascorbate; in fact, the concentration was greater than the control level during the 2 wk of vitamin C administration (Table 1). Microsomal lipid peroxidation, a characteristic of oxidative injury, was greater than in controls.

### Table 1

<table>
<thead>
<tr>
<th>Duration of scorbutic diet, wk</th>
<th>Control (0)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>2 + 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver ascorbate, μmol/g tissue</td>
<td>1.91 ± 0.08</td>
<td>1.86 ± 0.06</td>
<td>1.07 ± 0.07</td>
<td>ND</td>
<td>ND</td>
<td>2.69 ± 0.01</td>
</tr>
<tr>
<td>TBARS, μmol/g protein</td>
<td>119 ± 14</td>
<td>114 ± 11</td>
<td>128 ± 11</td>
<td>121 ± 15</td>
<td>117 ± 9</td>
<td>194 ± 9</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>728 ± 32</td>
<td>550 ± 40</td>
<td>588 ± 25</td>
<td>627 ± 22</td>
<td>670 ± 17</td>
<td>612 ± 78</td>
</tr>
<tr>
<td>Liver weight, g</td>
<td>25.8 ± 0.9</td>
<td>19.8 ± 1.0</td>
<td>23.5 ± 2.4</td>
<td>21.1 ± 0.8</td>
<td>21.2 ± 0.4</td>
<td>20.5 ± 1.1</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM, \( n = 4 \). Means in a row with superscripts without a common letter differ, \( P < 0.001 \).
2 Not detectable (<10 nmol/g tissue).
only in the group that consumed the scorbutic diet for 4 wk. The TBARS concentrations were not increased when guinea pigs consumed the commercial diet or in the group that was switched from the scorbutic diet to the antiscorbutic treatment (Table 1).

The body and liver weights indicated that the guinea pigs were thriving and were not affected significantly by ascorbate deficiency. In fact, the body weights were within the range expected at the corresponding age (19).

The Western blot analysis of hepatic microsomal proteins revealed a progressive induction of 2 major ER chaperones (GRP78 and GRP94). The protein levels were significantly greater than in controls in the guinea pigs that consumed the scorbutic diet for 3 wk, i.e., after the development of complete ascorbate deficiency (Fig. 1). The chaperone levels were similar to those seen in control samples (or at wk 0) when the 2-wk long diet was followed by the same period of ascorbate treatment.

The expressions of the 2 investigated ER foldases differed during the course of ascorbate depletion. PDI was induced similarly to ER chaperones, whereas the amount of ERP72 was not affected by ascorbate deficiency (Fig. 2). The redox Western blot with AMS did not show any difference between the thiol redox states of the 2 foldases prepared from guinea pigs at wk 0 and after 4 wk of consuming the commercial diet (not shown) or between the samples prepared at wk 0 and 4 from guinea pigs that consumed the scorbutic diet (Fig. 3).

Very few apoptotic cells/bodies were seen in the histological tissue sections prepared from control livers or from the livers prepared before the scorbutic diet (wk 0); this also did not change in the first 2 wk of the scorbutic diet. In contrast, there were 2- and 3-fold elevations in the apoptotic index in the livers prepared after wk 3 and 4, respectively (Fig. 4). The apoptotic index was not elevated compared with controls in the guinea pigs that were switched from the scorbutic diet to the ascorbate treatment.

**DISCUSSION**

One of the major functions of the ER is the synthesis and processing of secretory and plasma membrane proteins. Disul-
Ascorbate deficiency could cause either over- or underoxidation of the ER proteins. On the one hand, it is one of the major water-soluble antioxidants and its shortage is expected to cause a general oxidative challenge. On the other hand, it can react with oxygen in a one-electron transfer, which generates reactive oxygen species. Our previous in vitro experiments indicated that this prooxidant feature of ascorbate contributes to the generation and maintenance of the oxidative environment in the lumen of the ER. It was logical, therefore, to suppose that the shortage of ascorbate in vivo should be accompanied by an ER stress caused by an insufficient luminal protein processing and manifested as UPR and/or increased apoptosis. To test our hypothesis, scorbutic guinea pigs were used and the liver was studied because its abundant ER is involved in the early protein folding machinery and leads to the accumulation of un-/misfolded proteins in the organelle. Such an imbalance between protein synthesis and folding is a stress for the ER and initiates a compensatory mechanism referred to as unfolded protein response (UPR). It includes the inhibition of overall protein synthesis to decrease the protein-load, as well as the induction of ER chaperones and foldases, by which the cell attempts to increase the folding capacity. In case the UPR is insufficient, the persistent ER stress can lead to a programmed cell death via activation of the apoptotic cascade.

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These observations agree with the presumed disturbance of the ER protein processing. In fact, our hypothesis was based on the assumption that the shortage of ascorbate should alter the oxidation of thiol groups in the lumen, which was expected to also affect the ER foldases (PDI and ERP72). The lack of a detectable redox shift, nevertheless, does not strongly contradict our hypothesis because the gradual development of ascorbate deficiency in vivo allows the cells to respond with compensatory mechanisms (including the UPR).

Because there was no alteration in the redox state of the foldases, it cannot be decided unequivocally whether the lack of the antioxidant or the prooxidant function of ascorbate is the major cause of ER stress in scurvy. However, based on the time courses, we favor the latter option, because the missing antioxidant function was manifested only in enhanced lipid peroxidation in wk 4 of the diet, whereas the elements of UPR and the increased apoptosis were detected earlier.

Ascorbate functions as a cosubstrate in the hydroxylation of prolyl and lysyl residues of procollagen. Thus, the development of ER stress due to ascorbate deficiency could be expected in fibroblasts or in cells overexpressing procollagen. Our results, in turn, suggest that ascorbate participates in the general protein maturation; that is, its function is not restricted to procollagen synthesis. The development of ER stress in ascorbate deficiency is a novel element of scurvy, which could contribute to a better understanding of its pathomechanism and symptoms.

**FIGURE 3** Redox state of 2 major foldases, PDI (upper panel) and ERP72 (lower panel), of the ER in the liver of guinea pigs fed an ascorbate-free food. Ox, oxidized; Red, reduced. Numbers show the duration (wk) of the scorbutic diet. This is a typical result of 4 analyses.

**FIGURE 4** Apoptotic index in the liver of guinea pigs fed a commercial (ascorbate-containing) guinea pig food for 4 wk (control, C), an ascorbate-free food for 0–4 wk, or that diet for 2 wk followed by 2 wk of ascorbate supplementation (2+2). Results are shown as means ± SEM, n = 4. Means without a common letter differ, P < 0.05.
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LITERATURE CITED