Effects of Ascorbic Acid Deficiency on Protein and Lipid Oxidation in Livers from SMP30/GNL Knockout Mice

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Summary Ascorbic acid (AA) functions as an electron donor and scavenges reactive oxygen species such as superoxide, singlet oxygen, and hydroxyl radicals in vitro. However, little is known about the effect of an AA deficiency on protein and lipid oxidation levels in the liver. Therefore, we measured the levels of protein carbonyl and thiobarbituric acid reactive substances (TBARS) in livers from senescence marker protein-30 (SMP30)/gluconolactonase (GNL) knockout (KO) mice. These mice are deficient in AA, because they lack the SMP30/GNL gene, which is essential for the biosynthesis of AA in vivo. To track the effect of an AA deficiency, at 30 d of age, mice were divided into the following four groups: AA (−) SMP30/GNL KO, AA (+) SMP30/GNL KO, AA (−) wild type (WT), and AA (+) WT. The AA (−) groups were given water containing 1.5 g/L AA, whereas the AA (−) groups received water without AA for 57 d. All mice were fed an AA-free diet. Subsequently, protein carbonyl levels in livers from AA (−) SMP30/GNL KO mice were significantly higher than those from the other three groups; however, TBARS levels were not significantly different among the four groups. Therefore, AA must act as an anti-oxidant for proteins but might not directly protect lipid oxidation in the liver.

Key Words ascorbic acid, protein carbonyl, TBARS, gluconolactonase, SMP30

1-Ascorbic acid (AA) is a hexonic sugar acid that has two dissociable protons (1). At physiological pH, AA exists as the monovalent anion, ascorbate. Ascorbate is an electron donor and, as observed in vitro, scavenge reactive oxygen species such as superoxide (2), singlet oxygen (3), and hydroxyl radicals (4). Physiologically, superoxide is generated mainly from the mitochondrial electron-transport chain (5). During normal respiration, a small amount of electron flow through the mitochondrial electron-transport chain results in only partial reduction of oxygen, generating superoxide. On the other hand, mitochondrial manganese superoxide dismutase (Mn-SOD) and cytosolic copper, zinc superoxide dismutase (Cu.Zn-SOD) eliminate superoxide by catalyzing dismutation to hydrogen peroxide (6). Then, hydrogen peroxide is inactivated by catalase. Excess superoxide leads to hydroxyl radical formation through hydrogen peroxide formation. These oxygen radicals, such as superoxide and hydroxyl radicals, can react with almost all cellular components, i.e., lipids (7), DNA (8), and proteins (9, 10). This oxidation of biomolecules results in the cell and tissue damage of age-associated disorders such as Alzheimer’s disease (11, 12) and cardiovascular disease (13).

AA is essential for post-translational proline and lysine hydroxylation of collagen molecules (14). Long-term AA deficiency leads to scurvy that is caused by defective collagen synthesis (15). Primates, a few rodents such as the osteogenic disorder (ODS) rat (16), and guinea pigs (17) have lost the ability to synthesize AA because of mutations in the l-gulonolactone oxidase gene, which is essential for the AA biosynthetic pathway (18, 19). Therefore, they must obtain AA from dietary sources.

Senescence marker protein-30 (SMP30) was first discovered as an age-associated protein whose content decreases in the liver and kidney with age (20). Previously, we found that SMP30 is a lactone-hydrolyzing enzyme, gluconolactonase (GNN) (EC 3.1.1.17) and is an essential component for the AA biosynthetic pathway to unfold (21). Therefore, SMP30/GNL knockout (KO) mice lack any ability to synthesize AA in vivo (21, 22). Formerly, we reported that SMP30/GNL KO mice with a prolonged AA deficiency developed an increased...
rate of pulmonary emphysema (23), susceptibility to ultraviolet radiation (UVR)-induced cataracts (24), epidermal atrophy, and extensive ultraviolet B (UVB)-induced skin pigmentation (25) as well as a decrease in skin collagen content, and hair growth (26). Moreover, the expression of sodium-dependent vitamin C transporter (SVCT) 1 and 2 in the liver increased (27). Using this SMP30/GNL KO murine model, we also reported that an AA deficiency increased superoxide formation in the brain (28, 29), but those values in other tissues are still unknown. Therefore, in this study, we examined the effects of an AA deficiency on protein and lipid oxidation and antioxidant enzyme levels in livers from SMP30/GNL KO mice.

MATERIALS AND METHODS

Animals. SMP30/GNL KO mice were generated by the gene targeting technique as described previously (22). Male KO (SMP30/GNL<sup>−/−</sup>) mice were mated with female KO (SMP30/GNL<sup>−/−</sup>) mice to produce KO offspring. Only SMP30/GNL KO male mice were used in this study. Male wild-type (WT) (SMP30/GNL<sup>+/+</sup>) mice (C57BL/6Cr slc) were purchased from Japan SLC, Inc. (Shizuoka, Japan). At 30 d of age, these mice were divided into the following four groups: AA (−) SMP30/GNL KO, AA (+) SMP30/GNL KO, AA (−) WT, and AA (+) WT mice. The AA (+) group had free access to water containing 1.5 g/L AA and 10 μM edetaminedione tetraacetic acid (EDTA), whereas the AA (−) group had free access to water without AA until 87 d of age, when the experiment ended. Water bottles were changed every 3 or 4 d during the experiment. All mice were fed an AA-free diet (CL-2, CLEA Japan, Inc., Tokyo, Japan).

Typical experiments, animals were maintained on a 12-h light/dark cycle in a controlled environment. All experimental procedures using laboratory animals were approved by the Animal Care and Use Committee of the Tokyo Metropolitan Institute of Gerontology (No. 12016).

Measurement of AA and dehydroascorbic acid (DHA). AA and DHA, an oxidized form of AA, was measured by using high-performance liquid chromatography (HPLC) and an electrochemical detector (ECD) as described previously (30). Livers were homogenized in 14 volumes of 5.4% metaphosphoric acid (Wako Pure Chemical Industries, Ltd., Osaka, Japan) using a Potter-Elvehjem teflon homogenizer and centrifuged at 21,000 ×g for 10 min at 4°C. Then, 10 μL of centrifugal supernatants was added to 175 μL of solution containing 0.8% butylated hydroxytoluene/acetate acid, 8.1% SDS and acetate buffer (pH 3.5) (1 : 4 : 33). To this mixture we added 17.6 mM 2-thiobarbituric acid followed by incubation in an ice-water bath for 60 min. Then, the mixture was boiled for 60 min. After cooling, 0.78 mM n-butyl alcohol and 52 mM pyridine were added before centrifugation at 21,000 ×g for 5 min at 4°C. The upper layer of centrifugal supernatants was dispensed onto microplates. The amounts of TBARS were measured fluorometrically at 515 nm excitation and 553 nm emission on EnVision Multilabel Plate Readers (PerkinElmer, Santa Clara, CA) using 1,1,3,3-tetraethoxypropane as a standard.

SOD activity. SOD activity was measured by an SOD Assay Kit-WST (Dojin Laboratories, Kumamoto, Japan) according to the manufacturer’s instructions. Briefly, livers were homogenized with 0.25% sucrose, 10 mM Tris–HCl (pH 7.4), and 1 mM EDTA using a Polytron homogenizer and centrifuged at 21,000 ×g for 60 min at 4°C. The centrifugal supernatant was used for this assay.

Western blot analysis. Livers were homogenized with 0.1% SDS using a Potter-Elvehjem teflon homogenizer and centrifuged at 21,000 ×g for 10 min at 4°C. The centrifugal supernatants were electrophoresed on a 10% polyacrylamide gel by the method of Laemmli (33). Proteins in the gel were transferred onto a polyvinylidene fluoride membrane (Merck Millipore, Bedford, MA) by the method of Towbin et al. (34). The membranes were incubated with 5% skim milk in 0.01 M Tris-HCl (pH 7.5), 0.14 M NaCl, 0.1% Tween 20 and primary antibodies: Mn-SOD (1 : 5,000, Upstate Biotechnology, Billerica, MA), Cu/Zn-SOD (1 : 4,000, Calbiochem,
San Diego, CA), catalase (1:4,000, Sigma), or actin (1:1,000, Santa Cruz Biotechnology, CA). The membranes were successively incubated with horseradish peroxidase-conjugated anti-rabbit, anti-mouse or anti-sheep secondary antibodies. Chemiluminescence signals were detected with a LAS-3000 imaging system (Fujifilm, Tokyo, Japan) using ECL Western Blotting Detection Reagents (GE Healthcare UK, Buckinghamshire, England). The band intensity was quantitated with the imaging software of MultiGauge version 3.0 (Fujifilm). Mn-SOD, Cu,Zn-SOD, and catalase protein levels were corrected by actin.

**Statistical analysis.** Results are expressed as means±SE. The probability of statistical differences among experimental groups was determined by analysis of variance (ANOVA) followed by post-hoc Tukey’s honestly significant difference test. ANOVAs were performed using Kaleidagraph software (Synergy Software, Reading, PA). Statistical differences were considered significant at p<0.05.

**RESULTS**

**Body weights**

We measured the body weights among the following four groups of mice to assess the effect of AA depletion on growth: AA (−) SMP30/GNL KO, AA (+) SMP30/GNL KO, AA (−) WT, and AA (+) WT mice (Fig. 1). Initially, mice of all four groups gained weight to the same degree. However, the mean body weight of AA (−) SMP30/GNL KO mice gradually decreased starting at 56 d of age. At 87 d of age, the mean body weights of the AA (−) SMP30/GNL KO, AA (+) SMP30/GNL KO, AA (−) WT, and AA (+) WT mice were 18.7±0.9, 27.9±1.0, 25.1±0.7, and 27.2±1.5 g, respectively. Notably, in AA (−) SMP30/GNL KO mice, the body weight was a significant 23–25% less than the weights of the other three groups.

**AA levels in liver**

To investigate the effect of dietary AA depletion, we compared AA levels in livers from AA (−) SMP30/GNL KO mice, AA (+) SMP30/GNL KO, AA (−) WT, and AA (+) WT mice at 87 d of age. The AA (−) group had free access to water containing 1.5 g/L AA and 10 μM EDTA, whereas the AA (−) group had free access to water without AA until the end of the experiment. Values are expressed as means±SE of 4 animals. *p<0.05, for AA (−) SMP30/GNL KO mice versus the other three groups of mice.

**Protein carbonyl and TBARS levels in AA-Deficient Liver**

Fig. 3. Protein carbonyl levels in livers of AA (−) SMP30/GNL KO, AA (+) SMP30/GNL KO, AA (−) WT, and AA (+) WT mice at 87 d of age. Values are expressed as means±SE of 4 animals. *p<0.01, for AA (−) SMP30/GNL KO mice versus the other three groups of mice.

Fig. 4. TBARS levels in livers of AA (−) SMP30/GNL KO, AA (+) SMP30/GNL KO, AA (−) WT, and AA (+) WT mice. Values are expressed as means±SE of 4 animals.
mice were 0.00±0.00, 0.84±0.05, 0.79±0.06, and 1.07±0.04 μmol/g tissue, respectively. On the other hand, DHA levels of all four groups were all less than 1% of total AA (AA plus DHA).

**Protein carbonyl levels in liver**

Protein carbonyl levels in livers of AA (−) SMP30/GNL KO, AA (+) SMP30/GNL KO, AA (−) WT, and AA (+) WT mice at 87 d of age were then determined. These levels in livers of AA (−) SMP30/GNL KO mice were a significant 25–27% higher than in the other three groups (Fig. 3). That is, protein carbonyl levels of AA (−) SMP30/GNL KO, AA (+) SMP30/GNL KO, AA (−) WT, and AA (+) WT mice were 3.0±0.0, 2.4±0.0, 2.2±0.0, and 2.3±0.1 nmol/mg protein, respectively.

**TBARS levels in liver**

To assess the effect of AA depletion on lipid peroxidation, we determined TBARS levels in livers from AA (−) SMP30/GNL KO, AA (+) SMP30/GNL KO, AA (−) WT, and AA (+) WT mice at 87 d of age. However, there were no statistically significant differences of TBARS levels among the four groups (Fig. 4). TBARS levels in livers of AA (−) SMP30/GNL KO, AA (+) SMP30/GNL KO, AA (−) WT, and AA (+) WT mice were 1.5±0.1, 1.6±0.0, 1.3±0.3, and 1.2±0.0 nmol/mg protein, respectively.

**SOD activity**

SOD activity in livers from all four groups of mice at 87 d of age were then determined. An SOD activity of 1.943±104 U/mg protein in livers of AA (−) SMP30/GNL KO mice was 25–38% higher than that of the other three groups (Fig. 5A). Comparatively, the respective SOD levels for AA (+) SMP30/GNL KO, AA (−) WT, and AA (+) WT mice were 1.348±56, 1.544±55, and 1.403±61 U/mg protein.

**Mn-SOD, Cu,Zn-SOD, and catalase protein levels**

When Mn-SOD, Cu,Zn-SOD, and catalase protein levels in livers from AA (−) SMP30/GNL KO, AA (+) SMP30/GNL KO, AA (−) WT, and AA (+) WT mice at 87 d of age were then determined, there were no statistically significant differences for Mn-SOD (Fig. 5B). On the other hand, Cu,Zn-SOD protein levels in livers of AA (−) SMP30/GNL KO mice were 32–39% higher than those of the other three groups (Fig. 5C). Catalase protein levels in livers of AA (−) SMP30/GNL KO mice were 23–32% lower than those of the other three groups (Fig. 6).

**DISCUSSION**

In the present study, we showed that an AA deficiency caused protein carbonyl levels to rise in the livers of mice, although there were no significant differences in TBARS levels. Moreover, SOD activity and Cu,Zn-SOD protein levels were heightened by the AA deficiency, whereas catalase protein levels were reduced.

The fact that protein carbonyl levels in liver of AA (−) SMP30/GNL KO mice were higher than those of the other three groups reflects the greater extent of oxidative stress. This intensified protein oxidation leads to a wide variety of diseases such as diabetes and cataracts (9, 35, 36). Therefore, AA must be an antioxidant that protects the mouse liver from the damage of protein oxidation.
In this study, AA levels in the livers of AA (+) WT mice were significantly higher than those of AA (−) WT mice; however, their protein carbonyl levels were not significantly different. These results might suggest that AA in the liver does not act to reduce the protein carbonyl levels in a concentration-dependent manner. That is, AA in the liver might suppress only the extent of oxidative stress.

In our previous report, the AA deficiency increased superoxide formation in the brain (28, 29). In this study, SOD activity in the livers of AA (−) SMP30/GNL KO mice exceeded that of the other three groups. Similarly, cytosolic Cu,Zn-SOD protein levels in livers from AA (−) SMP30/GNL KO mice were also higher than those in the other three groups. However, there were no significantly different mitochondrial Mn-SOD protein levels in the livers among the four groups. Therefore, the levels of superoxide in the livers of AA (−) SMP30/GNL KO mice must be increased. The current results strongly suggest that SOD activity and Cu,Zn-SOD protein levels must be up-regulated as a compensatory mechanism in the liver.

In view of the increased SOD activity in livers of AA (−) SMP30/GNL KO mice, it is possible that hydrogen peroxide formation also increased in the liver because SOD eliminated superoxide by catalyzing dismutation to hydrogen peroxide. Then, hydrogen peroxide was inactivated by catalase. The catalase protein levels in the livers of AA (−) SMP30/GNL KO mice were lower than in the other three groups. Catalase protein is known to be inactivated by hydrogen peroxide (37). Moreover, Min et al. reported that prolonged exposure to reactive oxygen species down-regulated catalase expression at the transcriptional level in hepatocellular carcinomas (38). Therefore, reduced catalase protein levels must result from an increase of reactive oxygen species in the livers of AA (−) SMP30/GNL KO mice promoted by the AA deficiency.

Moreover, hydrogen peroxide is also inactivated by glutathione peroxidase (GPx) at which point glutathione (GSH) is oxidized to glutathione disulfide (GSSH). Tokumaru et al. reported that GSH and GSSG were decreased; however, the activity of GPx was not changed in the livers of ODS rats subjected to an AA-deficient status when compared to AA-sufficient groups (39).

Further, hydrogen peroxide can be broken down to a highly reactive hydroxyl radical in the presence of transition metals such as iron and copper (40). The hydroxyl radical might then produce carbonylated proteins.

In contrast, there were no significant differences in TBARS levels in the liver among the four groups in this study. Although Tokumaru et al. reported that TBARS levels were elevated in the hearts of ODS rats subjected to an AA-deficient status when compared to AA-sufficient groups, there were no differences in the brain, lung, kidney, or liver from AA-deficient ODS rats (39). That outcome is consistent with our results in the mouse liver. Moreover, since Tveden-Nyborg et al. reported that a chronic AA deficiency did not change lipid oxidation levels in livers from guinea pigs (41), an AA deficiency may specifically affect the oxidation of proteins but not lipids in the liver.

Tanaka et al. described the interactions between AA and vitamin E after using ODS rats fed an AA-free and vitamin E-free diet (42). TBARS levels in the livers of the AA-deficient ODS rats were significantly higher than those of the AA-sufficient and the vitamin E-deficient groups. Moreover, TBARS levels in the livers of ODS rats deficient in both AA and vitamin E were significantly higher than those of the AA-deficient or the vitamin E-deficient groups, suggesting an additive effect of the deficiencies of AA and vitamins E on hepatic TBARS. In our study, there was no difference in TBARS levels in the livers of AA (−) compared to AA (+) SMP30/GNL KO mice. The cause of this discrepancy is still unclear but may be from the difference in dietary composition used in the two experiments. The AA-free diet (CL-2) used in our study contains 378 mg vitamin E per 1 kg; in contrast, the vitamin E content of the AA-free diet in the former group’s experiment was considerably lower (only 50 mg/kg) (42). In addition, Vergely et al. reported that decreased AA levels in myocardial tissues from ODS rats were not associated with an increase of malondialdehyde, which is one of the final products of certain primary and secondary lipid peroxidation processes. Moreover, at that time, levels of vitamin E had not changed according to a comparison between AA-deficient mice and those in AA-sufficient groups (43). Therefore, vitamin E levels in the livers of our AA (−) SMP30/GNL KO mice might have been similarly unchanged, although that possibility is still uncertain.

In conclusion, SMP30/GNL KO mice made deficient in AA by excluding this vitamin from their diet had a notable increase of protein carbonyl levels in their livers; however, TBARS levels were not changed. Therefore, AA must be an anti-oxidant agent that defends against damage to proteins but presumably does not directly protect lipid oxidation in the livers of mice.

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Conflict of interest
The authors declare that they have no conflict of interest.

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