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Vitamin C depletion increases superoxide generation in brains of SMP30/GNL knockout mice

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ABSTRACT

Vitamin C (VC) has a strong antioxidant function evident as its ability to scavenge superoxide radicals *in vitro*. We verified that this property actually exists *in vivo* by using a real-time imaging system in which Lucigenin is the chemiluminescent probe for detecting superoxide in senescence marker protein-30 (SMP30)/gluconolactonase (GNL) knockout (KO) mice, which cannot synthesize VC *in vivo*. SMP30/GNL KO mice were given 1.5 g/L VC [VC(+)] for 2, 4, or 8 weeks or denied VC [VC(−)]. At 4 and 8 weeks, VC levels in brains from VC(−) KO mice were <6% of that in VC(+) KO mice. Accordingly, superoxide-dependent chemiluminescence levels determined by ischemia-reperfusion at the 4- and 8 weeks test intervals were 3.0-fold and 2.1-fold higher, respectively, in VC(−) KO mice than in VC(+) KO mice. However, total superoxide dismutase activity and protein levels were not altered. Thus, VC depletion specifically increased superoxide generation in a model of the living brain.

Vitamin C (VC, L-ascorbic acid) is a water-soluble, hexonic sugar acid that has two dissociable protons [1]. At physiological pH, VC exists as the monovalent anion, ascorbate. Ascorbate is an electron donor and, as observed *in vitro*, scavenges free radicals such as superoxide [2], singlet oxygen [3], and hydroxyl radical [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]. This oxidation of biomolecules results in cell and tissue damage.

Damage from oxygen radicals is considered a major source of the neuronal destruction that accompanies ischemic stroke and several neurodegenerative diseases, including Parkinson's disease [10], amyotrophic lateral sclerosis [11], and Alzheimer's disease [12]. Because the brain contains more VC than such tissues as the liver, kidney, heart, and skeletal muscle [13,14], presumably, VC provides a neuroprotective function in brain. However, whether VC actually scavenges oxygen radicals in the brain *in vivo* remains obscure.

Senescence marker protein-30 (SMP30) is an age-associated protein, that is, its production decreases in the liver, kidneys, and lungs with aging [15]. To clarify the relationship between age-associated decreases of SMP30 and age-associated organ disorders, we established SMP30 knockout (KO) mice [16]. These KO

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Abbreviations: *EDTA*, ethylenediaminetetraacetic acid;GNL, gluconolactonase;HPLC, high-performance liquid chromatography;KO, knockout;ODS, osteogenic disorder Shionogi;ROS, reactive oxygen species;SDS, sodium dodecyl sulfate;SMP30, senescence marker protein-30;SOD, superoxide dismutase;VC, vitamin C;WT, wild type.

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mice are viable and fertile but lower in body weight and shorter in life span than the wild type (WT) [17]. Throughout our experiments *in vitro* and *in vivo*, the livers of SMP30 KO mice were far more susceptible to TNF-α- and Fas-mediated apoptosis than those from the WT mice [16]. Recently, we identified SMP30 as the lactone-hydrolyzing enzyme gluconolactonase (GNL) of animal species [18]. GNL is a key enzyme involved in VC biosynthesis. We found that SMP30/GNL KO mice developed symptoms of scurvy when fed a VC-deficient diet, verifying the pivotal role of SMP30 in VC biosynthesis. Thus, SMP30/GNL KO mice lack the ability to synthesize VC *in vivo*.

In the present study, we utilized VC-depleted SMP30/GNL KO mice to examine whether this absence of VC results in increased generation of reactive oxygen species (ROS) in the brain. To clarify how much levels of oxygen radicals change in VC-depleted brains during oxygenation and hypoxia-reoxygenation, we used 'real-time biography,' a newly developed photonic imaging system, with the chemilumigenic probe, Lucigenin, to detect superoxide anion radicals [19]. This method enabled us to assess dynamic changes of superoxide generation in a model of the living brain.

Materials and methods

Animals. SMP30/GNL KO mice were generated previously by the gene targeting technique, as described [16]. Heterozygous female mice (*SMP30/GNL*$^{+/i}$) were mated with male KO mice (*SMP30/GNL*$^{Y/i}$) to produce male KO (*SMP30/GNL*$^{Y/i}$) and male WT (*SMP30/GNL*$^{Y/+}$) littermates. SMP30/GNL KO and WT mice were weaned at 30 days of age, at which time they were divided into the following four groups: VC [VC(+)], VC-free [VC(−)], WT, and SMP30/GNL KO mice. The VC(+) group had free access to water containing 1.5 g/L VC and 10 μM ethylenediaminetetraacetic acid (EDTA), whereas the VC(−) group had free access to water without VC. After weaning, all mice were fed a VC-deficient diet (CL-2, CLEA Japan, Tokyo, Japan). All experimental procedures using laboratory animals were approved by the Animal Care and Use Committee of the Toho University and the Tokyo Metropolitan Institute of Gerontology.

Preparation of brain tissue. Brains were rapidly removed from mice in all four groups sacrificed by decapitation and placed on a tissue cutter (Microslicer DTK-3000 W; Dosaka EM, Kyoto, Japan). Coronal slices cut 300 μm thick were transferred into ice-cold Krebs–Ringer solution (124 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1.2 mM KH_2PO_4, 26 mM $NaHCO_3$ and 10 mM glucose) equilibrated with 95% O_2/5% CO_2.

Measurement of total VC level in the brain. Total VC was measured by using a high-performance liquid chromatography (HPLC)-electrochemical detection method as described previously [14]. Briefly, brain slices were homogenized with 10 mM Tris–HCl (pH 8.0) containing 1 mM PMSF and centrifuged at 21,000*g* for 15 min at 4 °C. The supernatants obtained were immediately mixed with 5% metaphosphate and kept at −80 °C until use. Samples were analyzed by HPLC using an Atlantis dC18 5 μm column (4.6 × 150 mm, Nihon Waters, Tokyo, Japan). The protein concentration was determined by BCA protein assay (Pierce Biotechnology Inc., Rockford, IL, USA) using bovine serum albumin as a standard. Total VC levels in the brain were normalized by protein concentration.

Superoxide generation in the brain estimated by real-time biography. To estimate the dynamic changes of superoxide radical generation, we developed a real-time biography imaging system [19]. First, brain slices were pre-incubated in a chamber filled with oxygenated Krebs–Ringer solution with 2 mM *N*,*N*′-dimethyl-9,9′-biacridinium dinitrate (Lucigenin) (Sigma, St. Louis, MO, USA) for 45 min at 34 °C. Then, to determine superoxide radical generation by chemiluminescence emission distribution imaging, the brain slices were incubated for an additional 120 min in the same oxygenated environment (95% O_2/5% CO_2) in the imaging chamber at 34 °C. Next, the conditions were made hypoxic (95% N_2/5% CO_2) for 15 min before a return to the oxygenated environment, and incubation continued for up to 120 min. Images of brain slices were acquired every 15 min during the oxygenated, hypoxic, and then reoxygenated conditions for up to 255 min (17 frames). Each value of chemiluminescence was expressed as emission per unit area in a 15 min period.

Total SOD activity in the brain. Total SOD activity was measured by using the SOD Assay Kit-WST (DOJINDO Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. Briefly, brain tissues were homogenized with 0.25 M sucrose, 10 mM Tris–HCl (pH 7.4) and 1 mM EDTA, and centrifuged at 21,000 g for 30 min at 4 °C. The supernatant was used for this assay. SOD activity was expressed as U/mg protein.

Western blot analysis of Mn-SOD, Cu, Zn-SOD and catalase. Brain tissues were homogenized with 0.1% sodium dodecyl sulfate (SDS) and centrifuged at 21,000*g* for 10 min at 4 °C. The supernatants were boiled for 5 min with a lysis buffer containing 0.125 M Tris–HCl (pH 6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.2% bromophenol blue in a ratio of 1:1. Total protein (6.8 μg) equivalents for each sample were separated on SDS–polyacrylamide gels and transferred to a poly vinilidene difluoride (PVDF) membrane. The membrane was successively incubated with 5% skim milk in 10 mM Tris–HCl (pH 7.5), 0.14 M NaCl, 0.1% Tween 20 and then primary antibodies; Mn-SOD (1:5000, Upstate Biotechnology, Billerica MA, USA), Cu, Zn-SOD (1:2000, Calbiochem, San Diego, CA, USA) or catalase (1:2000, Sigma). Incubation with horseradish peroxidase-conjugated secondary antibodies followed: anti-rabbit (1:5000), anti-mouse (1:2000) or anti-sheep antibody (1:2000). Chemiluminescence signals were detected with a LAS-3000 imaging system (FUJIFILM, Tokyo, Japan) using ECL™ Western Blotting Detection Reagents (Amersham Bioscience, Piscataway, NJ, USA). Signal intensity of Mn-SOD, Cu, Zn-SOD and catalase were analyzed by using Multi Gauge software (FUJIFILM).

Statistical analysis. The results are expressed as means ± SEM. The probability of statistical differences between experimental groups was determined by Student's *t*-test or ANOVA as appropriate. One and two-way ANOVAs were performed using KareidaGraph software (Synergy Software, Reading, PA, USA).

Results

Body weight change during VC depletion

To investigate the effect of VC depletion on growth, we compared SMP30/GNL KO mice fed drinking water containing 1.5 g/L VC [VC(+)] with an identical group given water without VC [VC(−)]. Both groups initially gained equal amounts of weight; however, the mean body weight of VC(−) KO mice gradually decreased starting at 26 days after weaning (Fig. 1A). The mean body weights of the VC(+) and VC(−) KO mice at 57 days after weaning were 27.9 ± 1.0 and 18.7 ± 0.9 g, respectively, the weight of VC(−) KO mice being 33% less than that of VC(+) KO mice. The VC(−) KO mice seemed to lose appetite starting at 26 days after weaning, followed by a reduction in their locomotion activity. However, none of the VC(−) mice died until the experiment ended at 57 days after weaning. Throughout the experiment, the increase with time in body weights of VC(+) SMP30/GNL KO mice was similar to those of VC(+) and VC(−) WT mice tested for comparison.

Total VC levels in the brain during VC depletion

For confirmation of the VC-related differences in growth, we then measured VC levels in the brains of all four test groups. After weaning at 30 days, mice from all groups received or were deprived of VC in drinking water for 2, 4, or 8 weeks before their

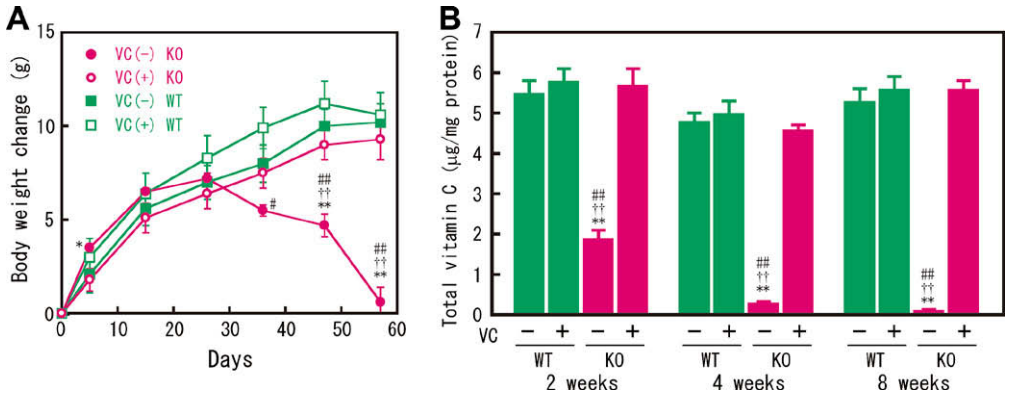

Fig. 1. (A) Body weight changes in groups of VC(+) and VC(−) WT and SMP30/GNL KO mice. After the mice were weaned at 30 days of age (indicated at day 0), their body weights were measured for 57 days, and the mean body weight changes (difference from the mean body weight at day 0) were plotted. The final body weights of VC(+) SMP30/GNL KO, VC(−) SMP30/GNL KO, VC(+) WT and VC(−) WT mice at day 57 were 27.9 ± 1.0, 18.7 ± 0.9, 26.4 ± 0.9 and 27.2 ± 0.7 g, respectively. Values are expressed as means ± SEM of five animals. (B) Total VC levels in the brains from VC(+) and VC(−) groups of WT and SMP30/GNL KO mice. Mice were supplied with or deprived of VC in drinking water for 2, 4, and 8 weeks, starting when they were weaned at 30 days of age. Values of total VC are expressed as means ± SEM of five animals. $^{*}p < 0.05$ and $^{**}p < 0.01$ as compared to VC(+) SMP30/GNL KO, $^{\dagger\dagger}p < 0.01$ as compared to VC(−) WT, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ as compared to VC(+) WT.

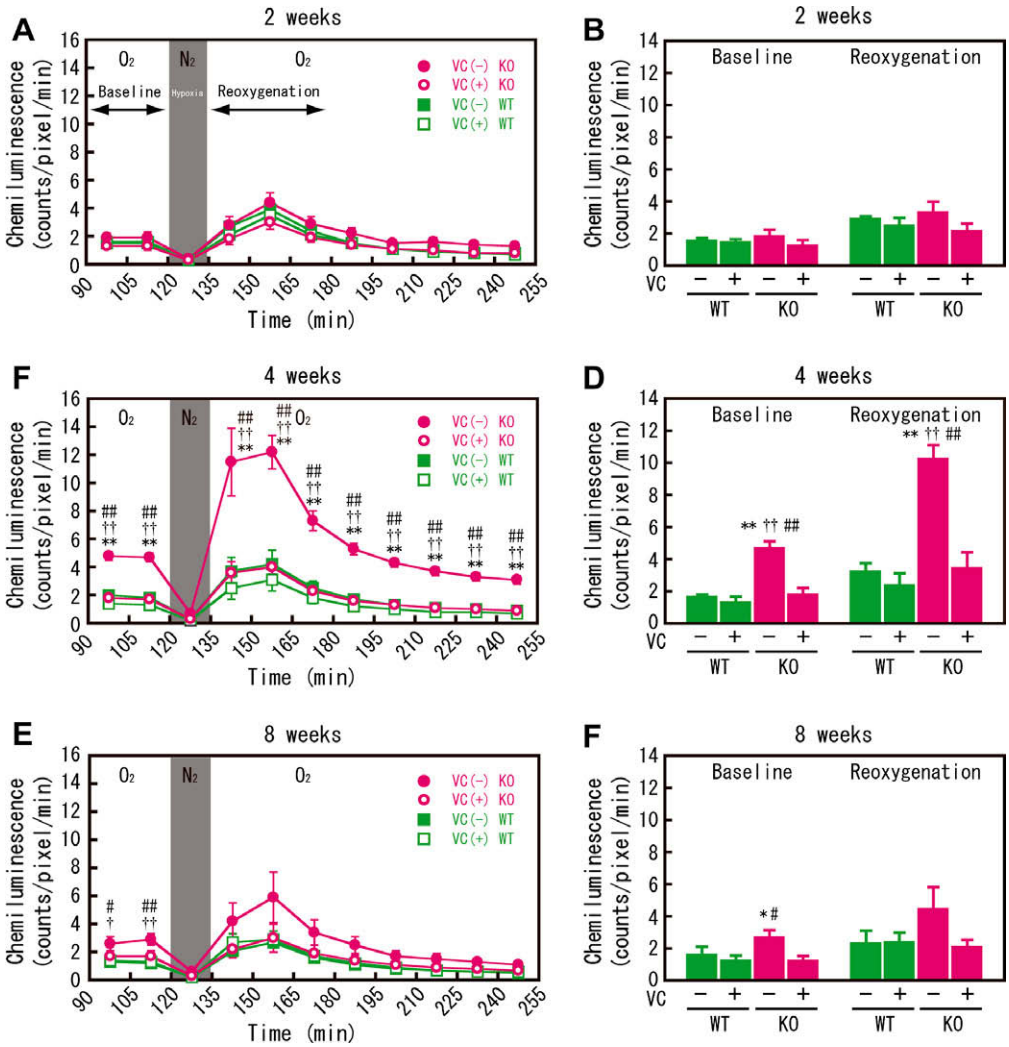

Fig. 2. Superoxide formation in brain slices estimated by imaging of chemiluminescence distribution. Brain slices at 2 (A), 4 (C), and 8 (E) weeks after weaning from VC(+) and VC(−) WT and SMP30/GNL KO mice were incubated with 2 mM Lucigenin in oxygenated (95% O_2/5% CO_2) Krebs-Ringer medium in a chamber for 120 min (0–120 min). Then the slices were incubated under hypoxic conditions (95% N_2/5% CO_2) for 15 min (120–135 min) and returned to the oxygenated condition for 120 min (135–255 min). Superoxide-dependent chemiluminescent intensities were acquired every 15 min and expressed as 'counts/pixel/min'. Superoxide formation of baseline and reoxygenation conditions at 2 (B), 4 (D), and 8 (F) weeks from VC(+) and VC(−) KO and WT mice were calculated as averages from 90 to 120 min and from 135 to 180 min, respectively. Values are expressed as means ± SEM of five animals. $^{*}p < 0.05$ and $^{**}p < 0.01$ as compared to VC(+) SMP30/GNL KO, $^{\dagger}p < 0.05$ and $^{\dagger\dagger}p < 0.01$ as compared to VC(−) WT, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ as compared to VC(+) WT.

preparation as the source of brain tissue. Thereafter, brains from VC(−) SMP30/GNL KO mice from the 2-, 4-, and 8-week experimental groups had total VC levels of 1.9 ± 0.2, 0.29 ± 0.03, and 0.12 ± 0.02 μg/mg protein, respectively (Fig. 1B). These values differed significantly from 2% to 34% levels in VC(+) SMP30/GNL KO mice. Most of the latter values resembled those of VC(+) and VC(−) WT mice.

Increased superoxide-dependent chemiluminescent intensity in brains from VC-depleted SMP30/GNL KO mice

To determine whether VC depletion affects ROS generation, we modeled conditions in the living brain by using a real-time biography imaging system. Here, Lucigenin acted as a chemiluminescence probe to measure superoxide formation during hypoxia-reoxygenation treatment. Chemiluminescence emission images were obtained every 15 min from the start of incubation and throughout the 255 min period that included the oxygenated, hypoxic, and then reoxygenated conditions. The time courses of superoxide formation in the brain slices from VC(+) and VC(−) groups of SMP30/GNL KO and WT mice are shown in Fig. 2. The intensity of chemiluminescence reached a steady-state (baseline) by 120 min after the start of oxygenation treatment. A decrease followed under hypoxic conditions (95% N_2/5% CO_2) for 15 min (from 120 to 135 min) and then increased during reoxygenation to reach a maximum at 15–30 min (from 150 to 165 min) after the hypoxic treatment. The intensity then decreased slowly and returned to the baseline after 255 min. Overall, the intensity of superoxide-dependent chemiluminescence during hypoxia-reoxygenation treatment at the experiment's 2-week-mark was not significantly different for VC(−) KO mice from that for the other three groups (Fig. 2A and B). However, at 4 weeks, the intensity of chemiluminescence under basal and reoxygenation conditions for VC(−) KO mice was 2.6- to 3.5-fold and 3.0- to 4.2-fold higher than that for the other three groups, respectively (Fig. 2C and D). The intensity level for VC(−) KO mice during basal and reoxygenation conditions at 8 weeks was also 1.6- to 2.1-fold and 1.9- to 2.1-fold higher, respectively, than levels for the other three groups, but levels during reoxygenation did not differ significantly (Fig. 2E and F). Typical images of chemiluminescence in brain slices under basal, hypoxic, and reoxygenated conditions from VC(+) VC(−) KO and WT mice at 4 weeks appear in Fig. 3. Superoxide formation was distributed heterogeneously throughout the brain regions and did not change significantly during hypoxia-reoxygenation treatment.

Antioxidant levels in the brain during VC depletion

Finally, to assess whether VC depletion affects antioxidant levels in the brain, we measured the SOD activity and protein levels of several antioxidative enzymes, including Mn-SOD, Cu, Zn-SOD, and catalase in the brains from VC(+) and VC(−) KO and WT mice. Total SOD activity at 4 and 8 weeks was not significantly different among the four groups (Fig. 4A). Similarly, the protein levels of Mn-SOD, Cu, Zn-SOD, and catalase at weeks 4 and 8 of experimentation did not vary significantly for any of the groups (Fig. 4B–D).

Discussion

The present study is the first report to prove that VC depletion results in an increase of superoxide generation. In the living brain modeled here, ischemia-reperfusion of brain slices from VC-depleted SMP30/GNL KO mice showed that the latter's superoxide levels were significantly higher than those of matched controls with a normal VC content and of their WT counterparts. *In vitro*, VC is known to scavenge superoxide generated by the xanthine-xanthine oxidase system [2], singlet oxygen generated photochemically

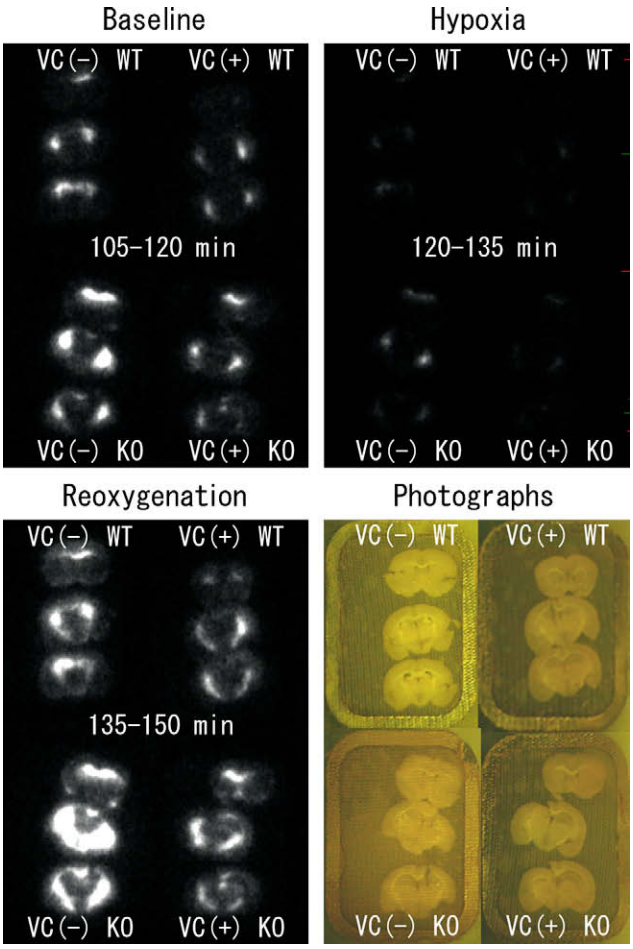

Fig. 3. Typical chemiluminescence images in brain slices at experimental week 4 from VC(+) and VC(−) groups composed of WT and SMP30/GNL KO mice during hypoxia-reoxygenation treatment. Images were acquired during oxygenated (105–120 min), hypoxic (120–135 min), and then reoxygenated (135–150 min) conditions. Brightness was represented by the same area and scale in each image. Superoxide-dependent chemiluminescence showed a heterogeneous distribution among the brain regions.

by using ultraviolet light and hematoporphyrin as a sensitizer [3], and hydroxyl radicals generated by exposure to ionizing radiation [4]. Measuring the ROS accurately in living tissues and whole animals is very difficult, because ROS is highly reactive and has an extremely short life span. Therefore, little direct evidence exists to verify that VC actually scavenges ROS in a physiologic setting. Here, we overcame this problem by using a real-time biographic system [19] in which Lucigenin is a chemiluminescence probe that detects superoxide anion radicals. Lucigenin represent superoxide production within cells and tissues at physiological pH [20,21].

Previously, Tokumaru et al. reported that the lipid hydroperoxide level was increased in the brains of VC-deficient rats with the genetically scorbutic osteogenic disorder, Shionogi (ODS) [13]. Others have also shown the antioxidative effects of VC; for example, VC supplementation reduced endogenous levels of the lipid peroxidation marker malondialdehyde, thiobarbituric acid reactive substances, and a protein oxidation marker, i.e., protein carbonyls, in various tissues from guinea pigs and ODS rats [22–24]. These earlier studies performed *in vivo* strongly support our present results showing that VC can actually scavenge ROS in the living brain.

Although the total VC levels in the brains from VC(−) KO mice were <6% of the values obtained for the VC(+) KO mice (the latter given 4 and 8 weeks of VC supplementation) (Fig. 1B), the total SOD

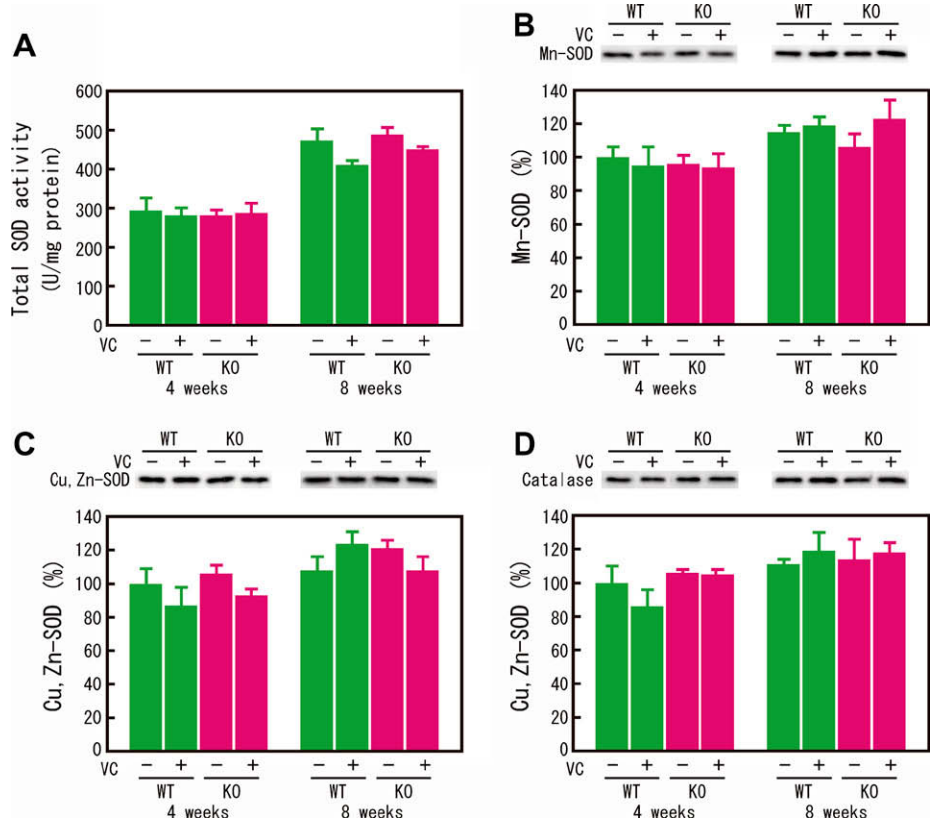

Fig. 4. Antioxidant activity and protein levels in brain slices at experimental weeks 4 and 8 from VC(+) and VC(−) groups composed of WT and SMP30/GNL KO mice. (A) Total SOD activity and protein levels of (B) Mn-SOD, (C) Cu, Zn-SOD and (D) catalase were determined as described in Materials and methods. Hundred percent has been adjusted according to the 4-week-value of VC(−) WT mice. Typical signals of Mn-SOD, Cu, Zn-SOD, and catalase were represented in Western blot analysis. Values are expressed as means ± SEM of five animals.

activity and protein levels of Mn-SOD, Cu, Zn-SOD and catalase were not altered in VC(−) KO mice (Fig. 4). We recently reported that superoxide-dependent chemiluminescent intensity in brain tissues from senescence accelerated mice (SAM) of the C57/BL6 strain as well as Wister rats and pigeons clearly increased in an age-dependent manner [25]. However, SOD activity in their brains was unchanged during the aging process. Thus, the antioxidative defense system in the brain must be very weak even in a state of high oxidative stress.

Superoxide-dependent chemiluminescence showed a heterogeneous distribution among the brain regions (Fig. 3). That is, chemiluminescent intensity in white matter was more vigorous than in gray matter. Okabe et al. [26] reported that less SOD activity was found in white matter than gray matter by histochemical localization analysis. Thus, weaker SOD activity in the white matter could account for the strong chemiluminescent intensity at those sites.

The brain needs a great deal more oxygen to produce high energy per unit mass than other organs [27], and this feature of brain metabolism translates into extremely high oxidative phosphorylation accompanied by a correspondingly large amount of electron leakage. Mitochondria are a major source of ROS generation and are implicated in the production of oxidative stress. Dehydroascorbic acid, which is an oxidative form of ascorbic acid, is known to enter mitochondria via facilitative glucose transporter 1 and then evolve into a reduced form, VC [28]. VC quenches ROS in the mitochondria to protect the mitochondrial genome from damage and prevent depolarization of the mitochondrial membrane. In the present study, VC depletion did not alter the scavenging capability represented by the protein levels of Mn-SOD, Cu, Zn-SOD, and catalase. Therefore, VC depletion in the brain must increase ROS generation within the cells, especially in their mitochondria, by causing a loss of VC's scavenging capability. An increase of oxidative stress in mitochondria is associated with mitochondrial dysfunction resulting from oxidative damage and, finally, induces cell death [11,29]. Here, we found that the intensity of superoxide-dependent chemiluminescence in the brain after 8 weeks of VC deprivation in KO mice was approximately one-half the intensity at 4 weeks (Fig. 2C and E). Histochemical analysis revealed numerous dead cells in the cerebral cortex of VC(−) KO mice at 8 weeks, but not at 4 weeks of VC deprivation (data not shown). Thus these outcomes suggest that long-standing ROS generation during VC deficiency in the brain must cause mitochondrial dysfunction and induce cell death, which would in turn decrease superoxide generation, as we noted during hypoxia-reoxygenation treatment. Finally, we verified that VC depletion increased superoxide generation in the brain during hypoxia-reoxygenation treatment. This result in our VC-depleted SMP30/GNL KO mice demonstrates the usefulness of this human-like animal model for the evaluation of antioxidants as scavengers of superoxide radicals *in vivo*.

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