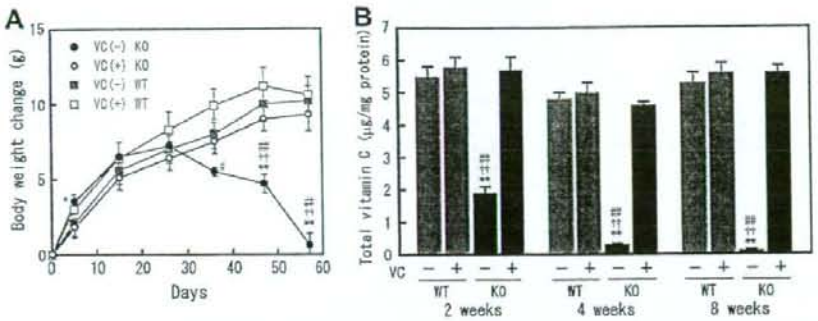

Fig. 1. (A) Body weight changes in groups of VC(+) and VC(−) WT and SMP30/GNL KO mice. After the mice were weaned at 30 days of age (indicated at day 0), their body weights were measured for 57 days, and the mean body weight changes (difference from the mean body weight at day 0) were plotted. The final 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.

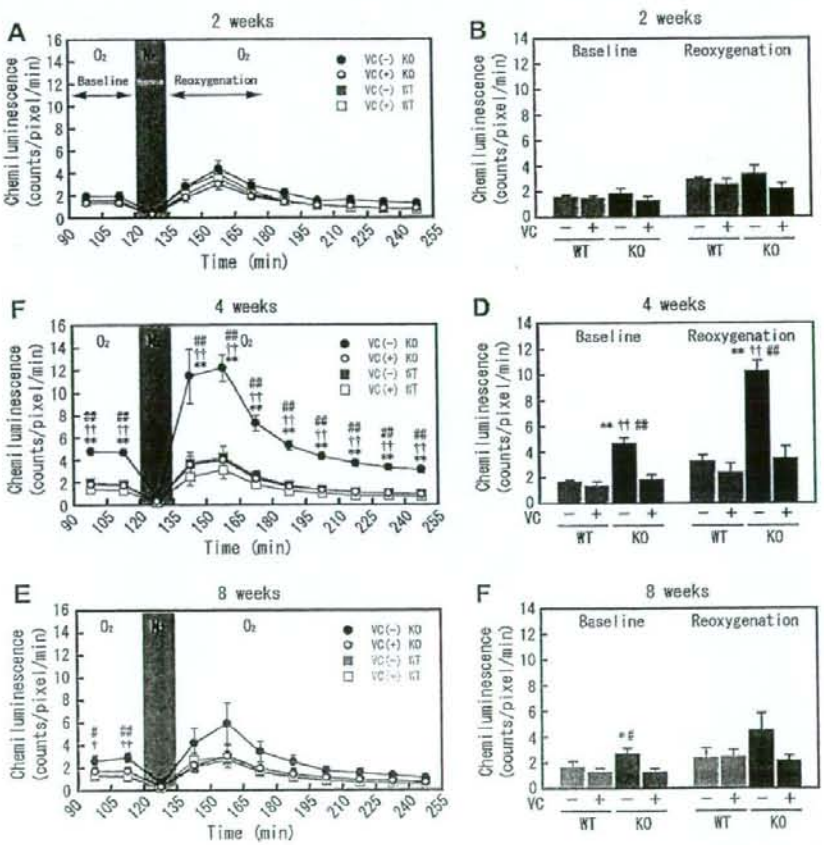

Fig. 2. Superoxide formation in brain slices estimated by imaging of chemiluminescence distribution. Brain slices at 2 (A), 4 (C), and 8 (E) weeks after weaning from VC(+) and VC(−) WT and SMP30/GNL KO mice were incubated with 2 mM Lucigenin in oxygenated (95% O_2/5% CO_2) Krebs-Ringer medium in a chamber 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

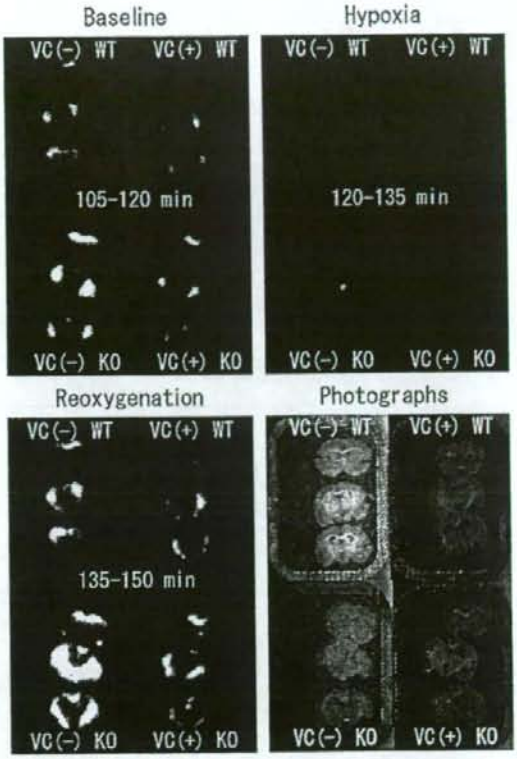

Fig. 3. Typical chemiluminescence images in brain slices at experimental week 4 from VC(+) and VC(−) groups composed of WT and SMP30/GNL KO mice during hypoxia-reoxygenation treatment. Images were acquired during oxygenated (105–120 min), hypoxic (120–135 min), and then reoxygenated (135–150 min) conditions. 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

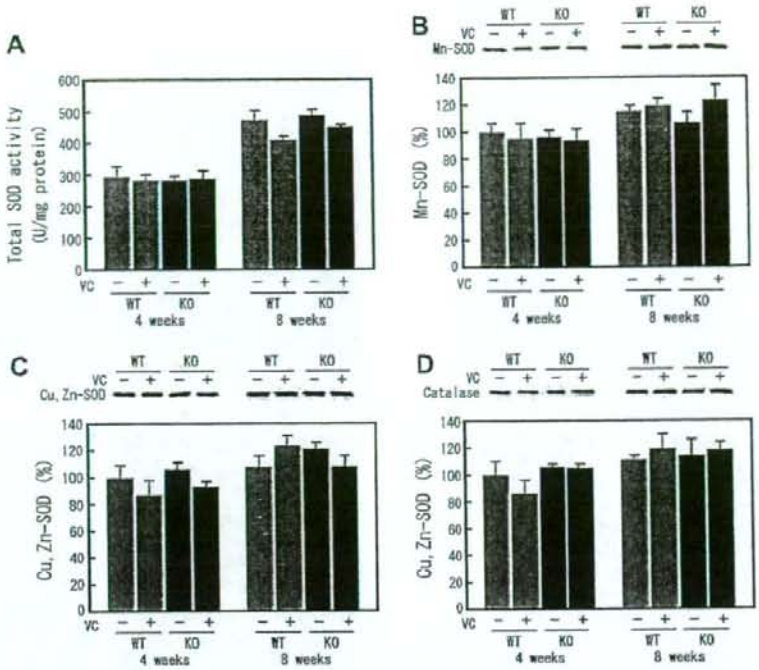

Fig. 4. Antioxidant activity and protein levels in brain slices at experimental weeks 4 and 8 from VC(+) and VC(−) groups composed of WT and SMP30/GNL KO mice. (A) Total SOD activity and protein levels of (B) Mn-SOD, (C) Cu, Zn-SOD and (D) catalase were determined as described in Materials and methods. 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*.

Acknowledgments

This study is supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture, Japan (to A.I., S.H. and N.S.), an award from Health Science Research Grants for Comprehensive Research on Aging and Health supported by the Ministry of Health, Labor, and Welfare, Japan (to N.M.), and Grant-in-Aid from the Asahi Breweries Foundation, Japan (to A.I.) and Smoking Research Foundation, Japan (to A.I.). We thank Ms. P. Minick for the excellent English editorial assistance. Vitamin C powder was kindly provided by DSM Nutrition Japan.

References

[1] M.E. Rice, Ascorbate regulation and its neuroprotective role in the brain, Trends Neurosci. 23 (2000) 209–216.
[2] M. Nishikimi, Oxidation of ascorbic acid with superoxide anion generated by the xanthine–xanthine oxidase system, Biochem. Biophys. Res. Commun. 63 (1975) 463–468.
[3] R.S. Bodannes, P.C. Chan, Ascorbic acid as a scavenger of singlet oxygen, FEBS Lett. 105 (1979) 195–196.
[4] B.H. Bielski, H.W. Richter, P.C. Chan, Some properties of the ascorbate free radical, Ann. NY Acad. Sci. 258 (1975) 231–237.
[5] E.J. Lesnefsky, S. Moghaddas, B. Tandler, J. Kerner, C.L. Hoppel, Mitochondrial dysfunction in cardiac disease: ischemia—reperfusion, aging, and heart failure, J. Mol. Cell. Cardiol. 33 (2001) 1065–1089.
[6] A. Okado-Matsumoto, I. Fridovich, Subcellular distribution of superoxide dismutases (SOD) in rat liver: Cu, Zn-SOD in mitochondria, J. Biol. Chem. 276 (2001) 38388–38393.
[7] I. Emerit, S.H. Khan, H. Esterbauer, Hydroxynonenal, a component of clastogenic factors?, Free Radic. Biol. Med. 10 (1991) 371–377.
[8] R.A. Floyd, M.S. West, K.L. Eneff, J.E. Schneider, P.K. Wong, D.T. Tingey, W.E. Hogsett, Conditions influencing yield and analysis of 8-hydroxy-2′-deoxyguanosine in oxidatively damaged DNA, Anal. Biochem. 188 (1990) 155–158.
[9] E.R. Stadtman, Protein oxidation and aging, Science 257 (1992) 1220–1224.
[10] P.M. Kidd, Parkinson's disease as multifactorial oxidative neurodegeneration: implications for integrative management, Altern. Med. Rev. 5 (2000) 502–529.
[11] S.C. Barber, R.J. Mead, P.J. Shaw, Oxidative stress in ALS: a mechanism of neurodegeneration and a therapeutic target, Biochim. Biophys. Acta 1762 (2006) 1051–1067.
[12] Y. Christen, Oxidative stress and Alzheimer disease, Am. J. Clin. Nutr. 71 (2000) 621S–629S.
[13] S. Tokumaru, S. Takeshita, R. Nakata, I. Tsukamoto, S. Kojo, Change in the level of vitamin C and lipid peroxidation in tissues of the inherently scorbutic rat during ascorbate deficiency, J. Agric. Food Chem. 44 (1996) 2748–2753.
[14] H. Furusawa, Y. Sato, Y. Tanaka, Y. Inai, A. Amano, M. Iwama, Y. Kondo, S. Handa, A. Murata, M. Nishikimi, S. Goto, N. Maruyama, R. Takahashi, A. Ishigami, Vitamin C is not essential for carnitine biosynthesis in vivo: verification in vitamin C-depleted senescence marker protein-30/gluconolactonase knockout mice, Biol. Pharm. Bull. 31 (2008) 1673–1679.
[15] A. Ishigami, N. Maruyama, Significance of SMP30 in gerontology, Geriatr. Gerontol. Int. 7 (2007) 316–325.
[16] A. Ishigami, T. Fujita, S. Handa, T. Shirasawa, H. Koseki, T. Kitamura, N. Enomoto, N. Sato, T. Shimosawa, N. Maruyama, Senescence marker protein-30 knockout mouse liver is highly susceptible to tumor necrosis factor-α- and Fas-mediated apoptosis, Am. J. Pathol. 161 (2002) 1273–1281.
[17] A. Ishigami, Y. Kondo, R. Nanba, T. Ohsawa, S. Handa, S. Kubo, M. Akita, N. Maruyama, SMP30 deficiency in mice causes an accumulation of neutral lipids and phospholipids in the liver and shortens the life span, Biochem. Biophys. Res. Commun. 315 (2004) 575–580.
[18] Y. Kondo, Y. Inai, Y. Sato, S. Handa, S. Kubo, K. Shimokado, S. Goto, M. Nishikimi, N. Maruyama, A. Ishigami, Senescence marker protein 30 functions as gluconolactonase in L-ascorbic acid biosynthesis, and its knockout mice are prone to scurvy, Proc. Natl. Acad. Sci. USA 103 (2006) 5723–5728.
[19] T. Sasaki, A. Iwamoto, H. Tsuboi, Y. Watanabe, Development of real-time bioradiographic system for functional and metabolic imaging in living brain tissue, Brain Res. 1077 (2006) 161–169.
[20] J.R. Vanfleteren, A. De Vreese, Rate of aerobic metabolism and superoxide production rate potential in the nematode Caenorhabditis elegans, J. Exp. Zool. 274 (1996) 93–100.
[21] Y. Li, H. Zhu, P. Kuppusamy, V. Roubaud, J.L. Zweier, M.A. Trush, Validation of lucigenin (bis-*N*-methylacridinium) as a chemilumigenic probe for detecting superoxide anion radical production by enzymatic and cellular systems, J. Biol. Chem. 273 (1998) 2015–2023.
[22] G. Barja, M. Lopez-Torres, R. Perez-Campo, C. Rojas, S. Cadenas, J. Prat, R. Pamplona, Dietary vitamin C decreases endogenous protein oxidative damage, malondialdehyde, and lipid peroxidation and maintains fatty acid unsaturation in the guinea pig liver, Free Radic. Biol. Med. 17 (1994) 105–115.
[23] H. Kimura, Y. Yamada, Y. Morita, H. Ikeda, T. Matsuo, Dietary ascorbic acid depresses plasma and low density lipoprotein lipid peroxidation in genetically scorbutic rats, J. Nutr. 122 (1992) 1904–1909.
[24] K. Tanaka, T. Hashimoto, S. Tokumaru, H. Iguchi, S. Kojo, Interactions between vitamin C and vitamin E are observed in tissues of inherently scorbutic rats, J. Nutr. 127 (1997) 2060–2064.
[25] T. Sasaki, K. Unno, S. Tahara, A. Shimada, Y. Chiba, M. Hoshino, T. Kaneko, Age-related increase of superoxide generation in the brains of mammals and birds, Aging Cell 7 (2008) 459–469.
[26] M. Okabe, S. Saito, T. Saito, K. Ito, S. Kimura, T. Niioka, M. Kurasaki, Histochemical localization of superoxide dismutase activity in rat brain, Free Radic. Biol. Med. 24 (1998) 1470–1476.
[27] R.A. Floyd, K. Hensley, Oxidative stress in brain aging. Implications for therapeutics of neurodegenerative diseases, Neurobiol. Aging 23 (2002) 795–807.
[28] S. Kc, J.M. Carcamo, D.W. Golde, Vitamin C enters mitochondria via facilitative glucose transporter 1 (Glut1) and confers mitochondrial protection against oxidative injury, FASEB J. 19 (2005) 1657–1667.
[29] K. Sas, H. Robotka, J. Toldi, L. Vecsei, Mitochondria, metabolic disturbances, oxidative stress and the kynurenine system, with focus on neurodegenerative disorders, J. Neurol. Sci. 257 (2007) 221–239.