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Hydrogen-rich pure water prevents superoxide formation in brain slices of vitamin C-depleted SMP30/GNL knockout mice

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ABSTRACT

Hydrogen is an established anti-oxidant that prevents acute oxidative stress. To clarify the mechanism of hydrogen's effect in the brain, we administered hydrogen-rich pure water (H_2) to senescence marker protein-30 (SMP30)/gluconolactonase (GNL) knockout (KO) mice, which cannot synthesize vitamin C (VC), also a well-known anti-oxidant. These KO mice were divided into three groups; recipients of H_2, VC, or pure water (H_2O), administered for 33 days. VC levels in H_2 and H_2O groups were <6% of those in the VC group. Subsequently, superoxide formation during hypoxia-reoxygenation treatment of brain slices from these groups was estimated by a real-time biography imaging system, which models living brain tissues, with Lucigenin used as chemiluminescence probe for superoxide. A significant 27.2% less superoxide formed in the H_2 group subjected to ischemia–reperfusion than in the H_2O group. Thus hydrogen-rich pure water acts as an anti-oxidant in the brain slices and prevents superoxide formation.

The production of reactive oxygen species (ROS) and reduction of anti-oxidant defense systems are major causes of oxidative stress [1]. Oxidative stress has been associated with the physiologic degeneration that accompanies Alzheimer's and Parkinson's diseases [2], cardiovascular disease [3], diabetes [4], and aging [5,6]. Exposure to ROS from a variety of sources has led organisms to develop multiple defense mechanisms such as enzymatic and non-enzymatic anti-oxidants [7]. Superoxide dismutase, glutathione peroxidase, and catalase are major enzymatic anti-oxidants, whereas vitamin C (VC, L-ascorbic acid), vitamin E (α-tocopherol), and glutathione are the main non-enzymatic anti-oxidants. However, VC is a particularly important anti-oxidant in the brain, which is richer in VC content than other tissues.

Although most organisms have developed anti-oxidant defense systems, not all excess ROS are detoxified. In 1997, Shirahata et al. [8] reported that electrolyzed-reduced water, which dissolved large amounts of hydrogen, had the ability to protect DNA from oxidative damage. Recently, Ohsawa et al. [9] found that hydrogen acts as a therapeutic anti-oxidant by selectively reducing hydroxyl radicals (·OH). We also described an improvement of lipid and glucose metabolism after supplementation with hydrogen-rich pure water in patients with type 2 diabetes or impaired glucose tolerance [10]. These findings strongly indicate that hydrogen has anti-oxidant ability *in vivo* and provides protection from the oxidative stress associated with numerous diseases. However, the specific mechanism of hydrogen's ability to scavenge ROS *in vivo* is still unclear.

Abbreviations: EDTA, ethylenediaminetetraacetic acid; GNL, gluconolactonase; HPLC, high-performance liquid chromatography; KO, knockout; ROS, reactive oxygen species; SMP30, senescence marker protein-30; SOD, superoxide dismutase; VC, vitamin C.

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In 1991, we originally identified senescence marker protein-30 (SMP30) as a distinctive protein, the expression of which decreases in an androgen-independent manner with aging [11]. Moreover, we established SMP30/gluconolactonase (GNL) knockout (KO) mice, which cannot synthesize VC *in vivo*, because SMP30 is an alternative name for GNL, a factor in the VC biosynthetic pathway [12,13]. Our recent study revealed that the SMP30/GNL knockout (KO) mouse develops scurvy when fed a VC-deficient diet [12]. Moreover, oxidative stress increases in brains from SMP30/GNL KO mice, without influencing the status of other anti-oxidant enzymes such as superoxide dismutase, glutathione peroxidase, and catalase [14]. Thus, the SMP30/GNL KO mouse is a powerful tool for investigating the mechanism used by anti-oxidant agents to scavenge ROS exclusively without affecting other anti-oxidant enzymes.

In this study, we found that hydrogen-rich pure water scavenges superoxide in the brain slices from VC-depleted SMP30/GNL KO mice. The real-time biography superoxide system we used indicated that hydrogen acts as an anti-oxidant that specifically eliminates superoxide in the brain *in vivo*.

Materials and methods

Hydrogen-rich pure water. Pure water was produced by the following processes: passage through (1) a reverse osmosis/ultrafiltration unit, (2) an ion-exchange resin, and (3) an ultrafiltration membrane (pure water: pH 6.9 ± 0.05; electric conductivity 0.7 ± 0.2 μS/cm). Hydrogen-rich pure water then resulted from dissolving hydrogen gas directly into pure water and had the following physical properties: pH 6.7 ± 0.1, low electric conductivity (0.9 ± 0.2 μS/cm), high content of dissolved hydrogen (1.2 ± 0.1 mg/L), low content of dissolved oxygen (0.8 ± 0.2 mg/L), and an extremely negative redox potential (−600 ± 20 mV). To prevent the loss of hydrogen, the hydrogen-rich pure water was sealed in 300 mL aluminum pouches and stored at room temperature.

SMP30/GNL KO mice. SMP30/GNL KO mice were generated with the gene targeting technique described previously [13]. Female KO (*SMP30/GNL*$^{-/-}$) mice were mated with male KO (*SMP30/GNL*$^{Y/-}$) mice to produce all male and female KO mice. Genotypes of SMP30/GNL KO mice were determined as described previously [13]. SMP30/GNL KO mice were weaned at 30 days of age, at which time they were divided into groups with free access to either hydrogen-rich pure water (H_2), VC water (VC), or pure water (H_2O) for 33 days. The H_2 group drank hydrogen-rich pure water the VC group drank pure water containing VC (1.5 g/L) and 10 μM ethylenediaminetetraacetic acid (EDTA), whereas the H_2O group drank pure water without H_2 and VC. Glass water bottles containing H_2, VC, and H_2O were changed twice daily until the experiment ended. All mice were fed a VC-deficient diet (CL-2, CLEA Japan, Tokyo, Japan). Throughout the experiment, 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 Toho University and Tokyo Metropolitan Institute of Gerontology.

Preparation of brain tissue and VC measurement. Brains were rapidly removed from all mice and placed on a tissue cutter. 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. For VC measurement, brain slices were homogenized in 10 mM Tris–HCl (pH 8.0) containing 1 mM PMSF by using glass-teflon homogenizer and centrifuged at 21,000*g* for 30 min at 4 °C. The supernatants obtained were immediately mixed with 5% metaphosphate and kept at −80 °C until use. Samples were treated with 0.1% dithiothreitol to reduce dehydroascorbic acid to ascorbic acid and analyzed by HPLC using an Atlantis dC18 5 μm column (4.6 × 150 mm, Nihon Waters, Tokyo, Japan). The mobile phase was 50 mM phosphate buffer (pH 2.8), 0.2 g/L EDTA, 2% methanol at a flow rate of 1.3 mL/min, and electrical signals were recorded by using an electrochemical detector with a glassy carbon electrode at +0.6 V [16,17]. Total VC in this preparation of brain tissue was measured by using a high-performance liquid chromatography (HPLC)-electrochemical detection method as described previously [15].

Dynamic chemiluminescence image of superoxide during hypoxia-reoxygenation in brain slices by real-time biography. To estimate the dynamic changes of superoxide radical formation during hypoxia-reoxygenation, we previously developed a real-time biography imaging system [18,19]. Here, we determined superoxide radical formation by chemiluminescence emission distribution imaging, for which the intact 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. After a 45 min pre-incubation, 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. Then the conditions were made hypoxic (95% N_2/5% CO_2) for 15 min, returned to an oxygenated environment, and incubated again for up to 120 min. Images of brain slices were acquired every 15 min during the intervals of oxygenation, hypoxia, and then reoxygenatation for up to 255 min (17 frames). Image brightness was represented by the same scale in all frames.

Statistical analysis. 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. For one- and two-way ANOVAs, we used KaleidaGraph software (Synergy Software, Reading, PA, USA). Statistical differences were considered significant at $p < 0.05$.

Results

Effect of hydrogen-rich pure water on body weight

SMP30/GNL KO mice were divided into three groups, mice fed hydrogen-rich pure water (H_2), VC water (VC), or pure water (H_2O) after weaning at 30 days of age. To investigate the effect of H_2, VC, or H_2O administration on growth, we compared body weight changes (Fig. 1). All three groups of SMP30/GNL KO mice gained the same amount of weight throughout the experiment. That is, the body weights of H_2, VC, and H_2O administration groups at 63 days of age were 26.1 ± 0.5, 25.0 ± 1.1, and 24.9 ± 0.9 g, respectively.

Total vitamin C levels in the brain after ingestion of hydrogen-rich pure water

Next, we determined the quantity of total VC in the brains of SMP30/GNL KO mice fed H_2, VC, or H_2O, until they reached 63 days of age. The brains from H_2 and H_2O administration groups had <6% of the VC values obtained for SMP30/GNL KO recipients of VC (Fig. 2). Total VC level in brain slices from H_2, VC, and H_2O administration groups were 0.3 ± 0.1, 5.5 ± 0.4, and 0.3 ± 0.1 μg/mg protein, respectively.

Superoxide formation during hypoxia-reoxygenation in a model of the living brain

To ascertain whether hydrogen-rich pure water protects the brain from ROS generation, we measured superoxide formation during hypoxia-reoxygenation treatment with a real-time

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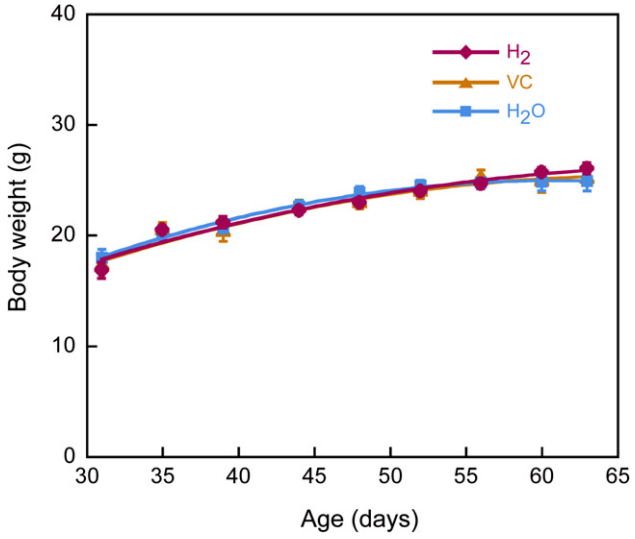

Fig. 1. Body weight changes of SMP30/GNL KO mice given either H_2, VC, or H_2O. SMP30/GNL KO mice weaned at 30 days of age were divided into three groups: recipients of either hydrogen-rich pure water (H_2), VC water (VC) or pure water (H_2O). Their body weights were measured, and the mean changes were plotted until the animals were 63 days of age. Values are expressed as a means ± SEM of 10 animals.

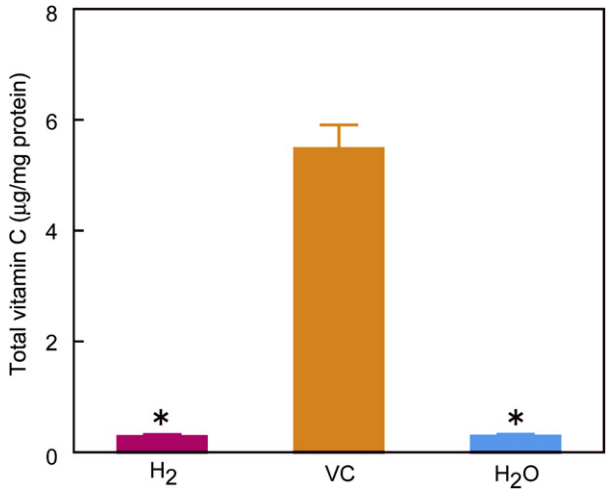

Fig. 2. VC levels in brains from the H_2, VC, and H_2O administration groups of SMP30/GNL KO mice. After weaning at 30 days, mice were grouped by those given free access to H_2, VC, or H_2O for 33 days. Glass water bottles containing H_2, VC, and H_2O were change twice daily until the experiment ended. Values are expressed as a means ± SEM of five animals. $^{*}p < 0.01$ as compared to the VC group.

biography imaging system using Lucigenin as chemiluminescence probe in brain tissues. Chemiluminescence emission images were obtained every 15 min from up the start of incubation until 255 min afterward, including the periods of oxygenation, hypoxia, and then reoxygenation. The time course of superoxide formation in the brain slices from H_2, VC, and H_2O administration groups of SMP30/GNL KO mice are shown in Fig. 3A. Superoxide formation was markedly decreased during the hypoxic condition (95% N_2/5% CO_2) and then increased during the oxygenated state lasting for 120 min. Superoxide formation in all three groups reached a maximum after 30 min of hypoxia, then gradually decreased and returned to the basal level. Superoxide formation at the maximal time of reoxygenation in H_2, VC, and H_2O administration groups was 7.45 ± 0.45, 5.36 ± 0.35, and 9.95 ± 0.74 counts/pixel for each 15 min reading, respectively. Superoxide formation during reoxygenation was calculated as an average over the 135–180 min time period (Fig. 3B). The H_2- and H_2O-only groups formed 1.9- and 1.4-fold higher levels of superoxide than the VC group did under the reoxygenation condition, respectively (Fig. 3B). Moreover, superoxide formation in the group given H_2 was a significant 27.2% lower than that in the H_2O administration group. A typical image of chemiluminescence in brain slices at basal, hypoxic, and reoxygenated conditions appear in Fig. 4. Superoxide formation was distributed heterogeneously throughout the brain regions and did not change significantly during hypoxia-reoxygenation treatment.

Discussion

In this study, we demonstrated that the administration of hydrogen-rich pure water created a pronounced decrease of superoxide formation in brain slices. These brain tissues came from VC-negative SMP30/GNL KO mice and were examined during hypoxia-reoxygenation treatment by using a real-time biographic system in which Lucigenin functioned as a chemiluminescence probe that detects superoxide. This outcome reflects a decrease of ROS generation in brain slices during ischemia and coincides with the report of Ohsawa et al. [9] indicating that hydrogen acts as a therapeutic anti-oxidant by selectively reducing ·OH. During ischemia, massive ATP consumption leads to accumulation of the urine catabolites hypoxanthine and xanthine, which upon subsequent reperfusion and influx of oxygen are metabolized by xanthine oxidase to produce enormous amounts of superoxide and ·OH [20]. We showed here that mice fed hydrogen-rich pure water formed 27.2% less superoxide when reoxygenated after an interval of hypoxia than mice fed pure water alone (Fig. 3). We speculate that the mechanism of this decrease in superoxide is the ability of hydrogen to reduce both ·OH and superoxide under specific conditions such as ischemia and reperfusion *in vivo*. Underlying this speculation is the fact that hydrogen can readily permeate the cell membrane and protect DNA from damage by ROS thereby influencing gene transcription [8,9]. An alternative possibility is that hydrogen permeates mitochondria and directly reduces the production of superoxide.

Recently we reported that superoxide-dependent chemiluminescent intensity in brain tissues from senescence accelerated mice (SAM) of the C57/BL6 strain, Wister rats, and pigeons clearly increased in an age-dependent manner [19]. The rate of this age-related increase in superoxide-dependent chemiluminescence was inversely related to the maximal lifespan of these animals; however, the activity of superoxide dismutase (SOD) in the brain was unchanged during the aging process. These findings strongly suggest that the reactive oxygen may be a signal that determines the aging process. Here, we used SMP30/GNL KO mice, which cannot synthesize VC *in vivo* [12], as a model of aging and oxidative stress and found that, in the absence of VC supplementation, superoxide generation in the brain slices increased during hypoxia-reoxygenation treatment (Figs. 2 and 3). VC is well known as a strong anti-oxidant that removes superoxide *in vitro* [21–24]; however, there is less evidence to prove that this effect actually occurs *in vivo*.

In our hands, reactive oxygen-dependent chemiluminescence was visible in a heterogeneous distribution throughout the brain (Fig. 4), although the intensity was greater in white matter than in gray matter. This heterogeneity did not significantly change during oxygenation and hypoxia-reoxygenation. However, since Okabe et al. [25] found less SOD activity in white matter than gray matter by histochemical localization analysis, the greater chemiluminescent intensity we noted in white matter could be explained by the latter's weaker SOD activity.

Subsequently, Fukuda et al. reported that inhalation of hydrogen gas suppressed hepatic injury caused by ischemia–reperfusion

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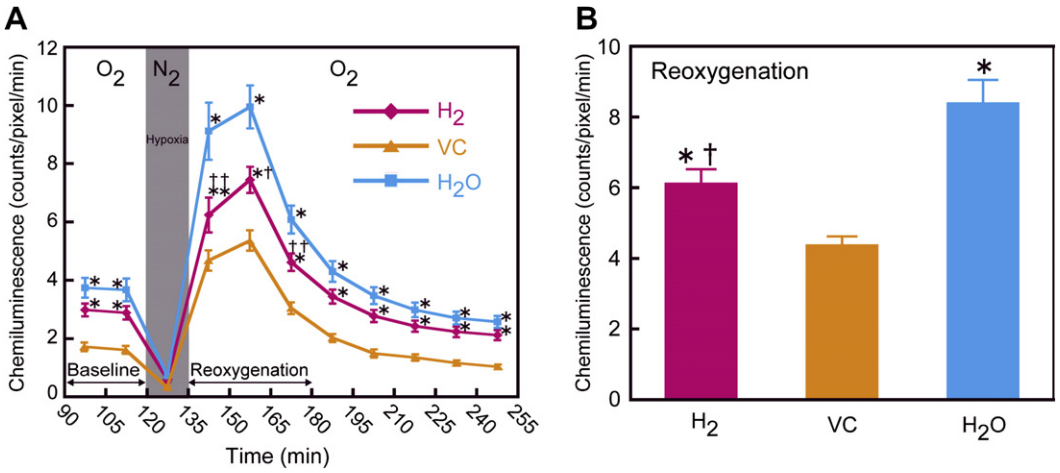

Fig. 3. Changes of chemiluminescent intensity in the brain slices during oxygenation and hypoxia-reoxygenation. (A) Brain slices from H_2, VC, and H_2O administration groups were incubated with 2 mM Lucigenin in oxygenated (95% O_2/5% CO_2) Krebs–Ringer medium in a chamber for 120 min (0–120 min). Then the slices were incubated in a hypoxic state (95% N_2/5% CO_2) for 15 min (120–135 min) and returned to oxygenated atmosphere for 120 min (135–255 min). Values for superoxide-dependent chemiluminescent intensity were acquired every 15 min and expressed as counts/pixel for each 15 min reading. (B) Superoxide formation during reoxygenation was calculated as averages from 135 to 180 min. Values are expressed as a means ± SEM of 10 animals. $^{*}p < 0.01$ and $^{**}p < 0.05$ as compared to the VC group. $^{\dagger}p < 0.01$ and $^{\dagger\dagger}p < 0.05$ as compared to the H_2O group.

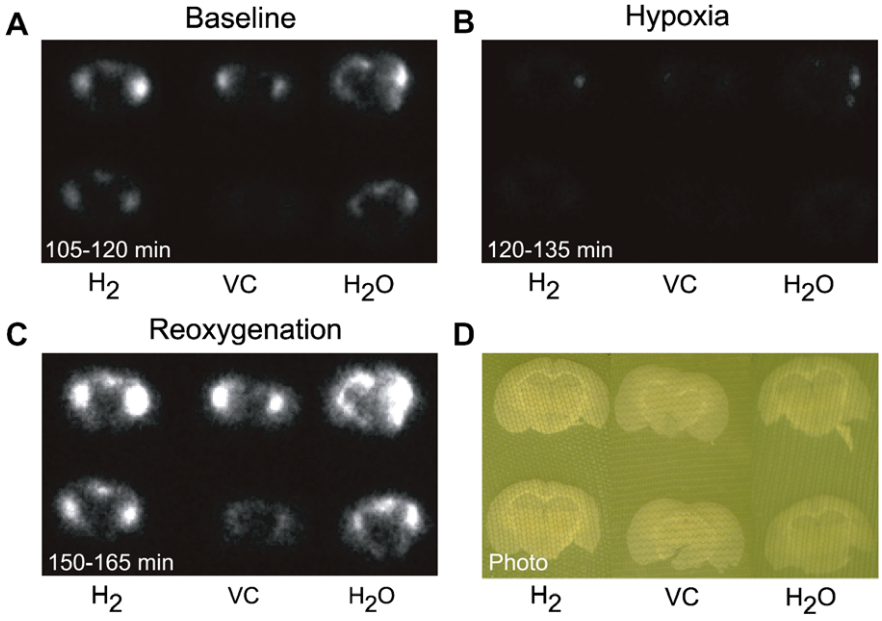

Fig. 4. Typical images of chemiluminescence in brain slices under basal, hypoxic, and reoxygenated conditions. Images were acquired during intervals of (A) oxygenation (105–120 min), (B) hypoxia (120–135 min), and then (C) reoxygenation (150–165 min). (D) Brightness is represented by the same area and scale in each image.

[26], and Hayashida et al. reported that inhalation of hydrogen gas limited the extent of myocardial infarction resulting from myocardial ischemia–reperfusion injury [27]. Our results support and extend those findings with the demonstration that hydrogen-rich pure water decreases superoxide formation caused by ischemia–reperfusion in the brain slices. Collectively, these data strongly suggest that hydrogen-rich pure water has beneficial anti-oxidant effects that increase resistance to the excessive oxidative stress prevalent in many states of physiologic degeneration.

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