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Consumption of hydrogen water prevents atherosclerosis in apolipoprotein E knockout mice

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ABSTRACT

Oxidative stress is implicated in atherogenesis; however most clinical trials with dietary antioxidants failed to show marked success in preventing atherosclerotic diseases. We have found that hydrogen (dihydrogen; H_2) acts as an effective antioxidant to reduce oxidative stress [I. Ohsawa, M. Ishikawa, K. Takahashi, M. Watanabe, K. Nishimaki, K. Yamagata, K. Katsura, Y. Katayama, S, Asoh, S. Ohta, Hydrogen acts as a therapeutic antioxidant by selectively reducing cytotoxic oxygen radicals, Nat. Med. 13 (2007) 688–694]. Here, we investigated whether drinking H_2-dissolved water at a saturated level (H_2–water) *ad libitum* prevents arteriosclerosis using an apolipoprotein E knockout mouse (apoE$^{-/-}$), a model of the spontaneous development of atherosclerosis. ApoE$^{-/-}$ mice drank H_2–water *ad libitum* from 2 to 6 month old throughout the whole period. Atherosclerotic lesions were significantly reduced by *ad libitum* drinking of H_2–water ($p = 0.0069$) as judged by Oil-Red-O staining series of sections of aorta. The oxidative stress level of aorta was decreased. Accumulation of macrophages in atherosclerotic lesions was confirmed. Thus, consumption of H_2-dissolved water has the potential to prevent arteriosclerosis.

Atherosclerosis is a multifactorial and long-lasting process, and atherosclerosis and related cardiovascular diseases represent a state of inflammation and heightened oxidative stress characterized by the accumulation of macrophages and oxidized products of low-density lipoprotein in affected blood vessels [1–3]. Oxidation of low-density lipoprotein is considered an early event; however, most clinical trials supplying a single dietary antioxidant have not resulted in great success in preventing atherosclerotic diseases [1,4–7].

We have reported that molecular hydrogen is an efficient antioxidant by gaseous rapid diffusion into tissues and cells [8]. This finding was soon confirmed by several laboratories [9–12]. Moreover, consumption of water with dissolved molecular hydrogen to a saturated level (hydrogen water) prevents stress-induced cognitive decline in mice [13], and the superoxide formation in mice [14]. A clinical trial showed the decrease in modifying low-density lipoprotein by drinking hydrogen water [15].

Here, we show that consumption of hydrogen dissolved in water has the potential to prevent atherosclerosis using apolipoprotein E knockout (apoE$^{-/-}$) mice, which show impaired clearing of plasma lipoproteins and which develop atherosclerosis in a short time [16,17].

Materials and methods

Animals. Apolipoprotein E-deficient mice (apoE$^{-/-}$) were purchased at the age of 2 months from Taconic. The care and treatment of experimental animals were in accordance with institutional guidelines. This study was approved by the Animal Care and Use Committee of Nippon Medical School.

Hydrogen water administration. Molecular hydrogen (H_2) was dissolved in water under high pressure (0.4 MPa) to a supersaturated level using hydrogen water-producing apparatus (ver. 2) produced by Blue Mercury Inc. (Tokyo, Japan). The saturated hydrogen water was stored in an aluminum bag. Hydrogen water was freshly prepared every week, which ensured that a concentration of more than 0.6 mM was maintained. We confirmed the hydrogen content with a hydrogen electrode (ABLE). Each day, hydrogen water from the aluminum bag was placed in a closed glass vessel (70 mL) equipped with an outlet line containing two ball bearings, which kept the water from being degassed. This vessel ensured that the hydrogen concentration was more than 0.4 mM after one day. Hydrogen water degassed by gentle stirring was used for control

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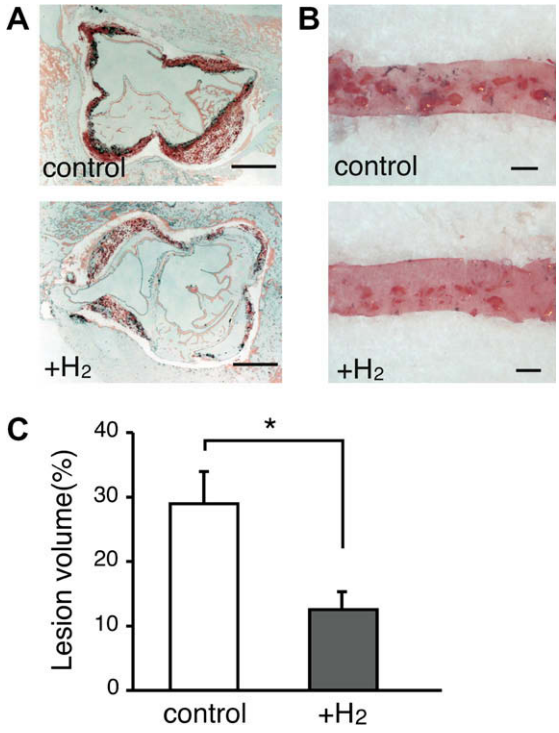

Fig. 1. Consumption of hydrogen water decreased atherosclerotic lesion. ApoE$^{-/-}$ mice drank water containing hydrogen (+H_2) or degassed water (control) for 6 months from the age of 2 months old. Representative microscopic pictures of horizontal sections of the proximal aorta attached to the heart (A) and vertical sections of the distal aorta (2 mm from the heart) (B) are shown by Oil-Red-O staining. Scale bar; 100 μm (for A) and 1 mm (for B). (C) Lesion volume was estimated by Oil-Red-O staining of a series of 30 sections (mean value ± SEM, $n = 10$, $p = 0.0069$).

animals; the complete removal of hydrogen gas was confirmed with a hydrogen electrode.

Quantification of atherosclerotic lesions in the aorta. The proximal aorta attached to the heart was used to prepare cross-sections. After fixation with 4% paraformaldehyde, cryosections (8 μm) were cut from the site where the aorta valve cups appear at the aorta root. All other sections were collected and stained with Oil-Red-O [18]. The volume of stained lipid (%) was calculated from eight sections for each mouse. The distal aorta (2 mm from the heart) was fixed with 4% paraformaldehyde, opened longitudinally using microscissors and stained with Oil-Red-O.

Immunocytochemistry. After fixation of the proximal aorta with 4% paraformaldehyde, cross-sections (6 μm) were cut with a cryostat, incubated with either an antibody against mouse macrophage (MOMA-2, AbD Serotec), anti-iNOS (BIOMOL), and anti-4-hydroxyl-2-nonenal (HNE) antibody (JaICA, Japan) [19–21]. After washing, the sections were then exposed to a biotinylated second antibody and avidin–peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories Inc.). Sections were developed with DAB as a substrate. One section from each mouse was stained with hematoxylin and eosin (HE).

Statistical analysis. We performed statistical analysis using StatView software (SAS Institute) by applying an unpaired two-tailed Student's *t*-test and ANOVA followed by Fisher's exact test.

Results

It is easy to consume molecular hydrogen by drinking water containing dissolved molecular hydrogen (hydrogen water). Thus, we examined whether consumption of hydrogen water prevents atherosclerosis using apoE$^{-/-}$ mice. Mice drank nearly the same volume of hydrogen water as control water [4.3 ml/day/mouse(0.1(SD)(hydrogengroup) vs. 4.0 ml/day/mouse(0.1(SD)(controlgroup)]. The amount of food eaten per mouse was also the same in both groups [3.56 ± 0.3 g/day (hydrogen group) vs. 3.28 ± 0.6 g/day (control group)]. After 6 months, we removed the aorta to stain with Oil-Red-O staining. As expected, atherosclerotic lesions were found in 6-month-old apoE$^{-/-}$ mice. In contrast, in mice that had drunk hydrogen water, the volume

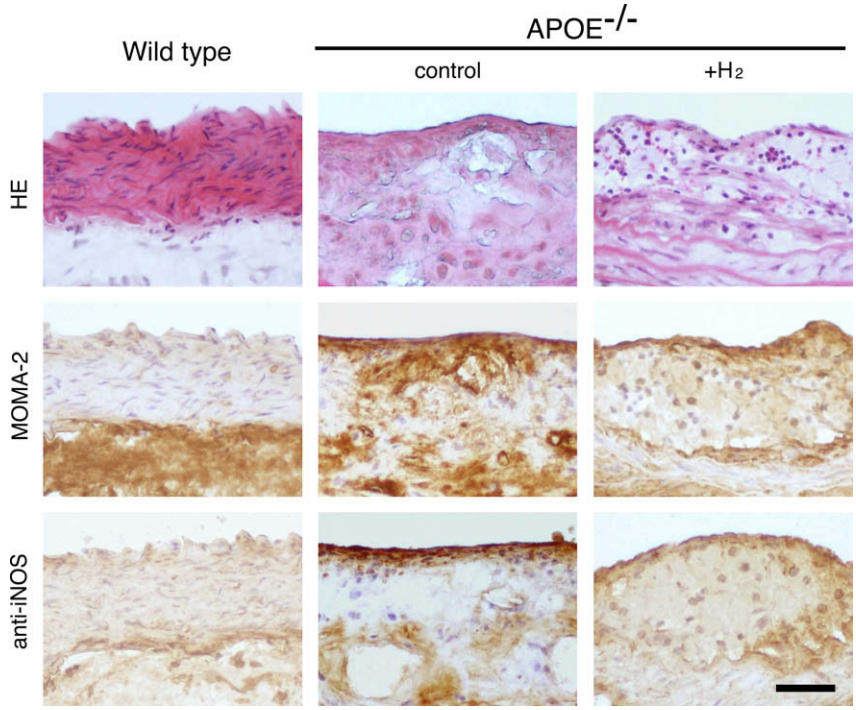

Fig. 2. Representative histochemical or immunostaining of the aorta. ApoE$^{-/-}$ mice drank hydrogen or control water throughout the 6-month period from 2 months old. The proximal aorta attached to the heart was sectioned and stained with HE staining, anti-MOMA-2 immunostaining and anti-iNOS immunostaining. Scale bar: 250 μm.