Oral ‘hydrogen water’ induces neuroprotective ghrelin secretion in mice

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The therapeutic potential of molecular hydrogen (H2) is emerging in a number of human diseases and in their animal models, including in particular Parkinson’s disease (PD). H2 supplementation of drinking water has been shown to exert disease-modifying effects in PD patients and neuroprotective effects in experimental PD model mice. However, H2 supplementation does not result in detectable changes in striatal H2 levels, indicating an indirect effect. Here we show that H2 supplementation increases gastric expression of mRNA encoding ghrelin, a growth hormone secretagogue, and ghrelin secretion, which are antagonized by the β1-adrenoceptor blocker, atenolol. Strikingly, the neuroprotective effect of H2 water was abolished by either administration of the ghrelin receptor-antagonist, D-Lys3 GHRP-6, or atenolol. Thus, the neuroprotective effect of H2 in PD is mediated by enhanced production of ghrelin. Our findings point to potential, novel strategies for ameliorating pathophysiology in which a protective effect of H2 supplementation has been demonstrated.

The therapeutic applications of molecular hydrogen (H2) have been reported in a variety of human diseases and their animal models1, including ischemia-reperfusion injury2–4, metabolic syndrome5, diabetes mellitus type 26, organ transplantation7–9, reduction of adverse effects of anti-tumor drug therapy10,11 and radiation therapy12,13. Although the mechanism of action of H2 has not been clearly demonstrated, it is assumed that its anti-oxidative properties, particularly against hydroxyl radical (·OH) and peroxinitrite (ONOO−), are likely to underlie therapeutic efficacy2. Unlike other medical-gas therapy, H2 can be applied in air for inhalation or in solution for drinking, intravenous injection or dialysis. Whereas intravenous injection or dialysis delivers H2 directly into the blood stream, oral hydrogen-supplemented water (hydrogen water, H2-water) must be absorbed into the circulation resulting in limited H2 concentrations in the blood and in target organs7,14.

Parkinson’s disease (PD) has been a major focus in the field of oxidative stress and disease, because it is thought that degeneration of dopaminergic neurons can be triggered and aggravated by the accumulation of oxidative damage. However, although antioxidant therapies have been assessed in PD patients, clinical efficacy has not been established15,16. In contrast, a pilot study of hydrogen water therapy in PD patients has shown promising results17, and it has been reported that hydrogen water exhibits neuroprotective effects18 in the murine MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)-induced PD model19. H2 levels were below measurable thresholds in the substantia nigra in PD model mice14, and hydrogen water, but not continuous inhalation of 2% H2, prevented the development of PD in a rat model19. These findings suggest that the therapeutic effects of hydrogen water may not require its anti-oxidant activity in the brain, and further that its efficacy requires processing that is consequent upon oral administration.

The purpose of the present study was to employ PD model mice to elucidate the underlying mechanism of the neuroprotective effects of oral H2-water. In particular, we hypothesized that oral H2 induces a messenger molecule, which travels to the brain and exerts neuroprotective activity.

Results

Oral hydrogen water induces ghrelin gene expression in the stomach. The stomach functions as an endocrine organ that secretes various peptide hormones with a broad range of physiological effects. We first focused on the stomach to investigate possible effects of oral H2-water at the level of gene induction. In a previous study24, it was reported that drinking H2-water for a period of 7 days prior to MPTP injection protected against MPTP toxicity. We administered oral H2-water for 4 consecutive days and analyzed expression in stomach tissue of gastrin,
somatostatin, and ghrelin by real-time PCR method. Levels of ghrelin mRNA increased 1.9-fold in H2-water-treated versus control mice (Figure 1), whereas no effects of hydrogen water were detected on expression of the somatostatin gene (expressed only at trace levels) or the gastrin gene (expression of which was highly variable from individual to individual) (not shown).

To examine the time-course of ghrelin induction by hydrogen water, mice were administered hydrogen water or control water once a day for 0 (control) 1, 2 or 4 days and ghrelin levels were measured by ELISA in plasma derived from blood obtained 4–5 hours after the final administration of H2- or control water. Only mice that received H2 water for 4-day exhibited a significant increase in plasma ghrelin level, although mice administered hydrogen water for 2-day showed a non-significant increase (Figure 2).

**β1-adrenergic receptor signaling mediates enhancement of ghrelin secretion by oral hydrogen water.** It has been shown that gastric secretion of ghrelin is regulated by local environmental cues including blood glucose, estrogen, insulin and catecholamines. In particular, it has been reported that β1-adrenergic receptor stimulation increases ghrelin secretion in vitro and in vivo. We verified the expression of β1- and β2-adrenergic receptors in stomach tissue samples by real-time PCR method, and determined that there were no significant changes in the levels of expression after administration of oral H2-water for 4 days (data not shown). The increase in plasma ghrelin levels by oral hydrogen water was eliminated by administration of the β1-adrenergic receptor-specific blocker, atenolol (10 mg/kg i.p.) injected 30 min prior to H2 water administration on each of four days (Figure 3). Thus, activation of β1-adrenergic receptors is required for hydrogen water-induced enhancement of circulating ghrelin.

**Blockades of ghrelin action abrogates the protective effect of H2 water in PD model mice.** In a previous report, oral H2-water exhibited significant protective effects in MPTP-induced PD model mice against the loss of dopaminergic neurons from the substantia nigra. To test directly the role of ghrelin, either the growth hormone secretagogue receptor antagonist, D-Lys3 GHRP-6 (100 nmol/day i.p.), or β1-adrenoceptor blocker, atenolol (10 mg/kg i.p.), was administered along with control or hydrogen water for 7 days prior to administration of MPTP. Loss of dopaminergic neurons from the substantia nigra was evaluated seven days following administration of MPTP. As shown in Figure 4, systemic administration of MPTP caused a significant loss of dopaminergic neurons from the pars compacta of the substantia nigra (SNpc) as assessed by immunohistochemical detection of tyrosine hydroxylase (TH) (a), and further confirmed by immunological detection of TH protein in...
the substantia nigra with actin as control (b), and stereological analysis (c). Whereas administration of H₂ water alone in control mice had no effect, the loss of dopaminergic neurons in MPTP-treated mice was significantly decreased by administration of hydrogen water, as previously reported. Strikingly, although administration of D-Lys³ GHRP-6 or atenolol in control mice had no effect alone and D-Lys³ GHRP-6 or atenolol did not affect MPTP-induced loss of dopaminergic neurons, the protective effects of hydrogen water were eliminated by either one of D-Lys³ GHRP-6 or atenolol. Thus, induction of gastric ghrelin production and subsequent activation of ghrelin-initiated signal transduction underlies the protective effects of hydrogen water in the MPTP model of PD.

**Discussion**

Our findings demonstrate that the neuroprotective effects of oral hydrogen water, which produces negligible levels of H₂ in the brain, result from gastric induction of the neuroprotective peptide hormone ghrelin and the subsequent activation of ghrelin receptors. In addition, we have shown an obligate role for β₁-adrenergic receptors in hydrogen water-induced ghrelin up-regulation in plasma, consistent with previous reports that adrenergic stimulation regulates ghrelin release in vitro and in vivo.

The neuroprotective effects of ghrelin in PD are well-established, and it has been demonstrated that the receptor for ghrelin, the growth hormone secretagogue receptor (GHSR), is highly expressed by dopaminergic neurons of the substantia nigra. It has been suggested that ghrelin protects nigrostriatal dopamine neurons via an uncoupling protein 2 (UCP2)-dependent mitochondrial mechanism. However, we found that the expression of neither UCP2 mRNA nor protein was significantly upregulated by administration of H₂ water drinking for 7 days (data not shown). This finding suggests an alternative signaling mechanism downstream of GHSR activation, perhaps involving PI3K/Akt.

It was reported that administration of saturated hydrogen water (approx. 0.8 mM) led to symptomatic improvement in PD patients. Administration of hydrogen water at about 0.05% saturation successfully maintained dopaminergic neurons in MPTP-induced PD model mice. We employed three different methods to prepare
hydrogen water (see Methods), which resulted in H₂ concentration of 0.04–0.8 mM, and we observed that the effects of hydrogen water on ghrelin induction and protection of dopamine neurons were dose-independent over this range. Thus, small amounts of oral H₂ are sufficient for gastric induction of ghrelin and subsequent neuroprotection, in the absence of detectable H₂ in the brain. Interestingly, gut microbes can produce molecular hydrogen constitutively and lactulose, a synthetic disaccharide, is an effective substrate to enhance bacterial H₂ production in the colon13. However, ingestion of lactulose had no significant effect on dopaminergic neuron survival in 6-OHDA-induced PD model rats (although alveolar H₂ concentrations were elevated)19. These results emphasize the importance of gastric ghrelin induction and a neuroprotective action of H₂. Oral H₂ water is being explored as a therapeutic for PD as well as a variety of other human pathophysiological conditions17,32,33 under the generally held assumption that the mechanism of action of supplemental H₂ is likely to reflect an antioxidative role. Our findings that oral H₂ water exerts a neuroprotective effect through activation of an endogenous, gastric ghrelin system that is tightly coupled to β-adrenergic receptor signaling suggests the possibility of novel applications of H₂ therapy for various diseases.

Methods

Animals. Procedures in animal experiments were approved by the university review board for animal care in Chiba University and Kyushu University, and performed in accordance with the guidelines established by the Science Council of Japan. All mice (C57BL/6J) were maintained in the animal facility under controlled temperature and lighting (12-hour light, 12-hour dark), and received a standard mouse chow diet and water ad libitum.

Preparation and administration of H₂ water. H₂ water was prepared by one of three methods. The first method utilized the spontaneous ionization reaction of magnesium in water14. A magnesium stick (AZ31, Nakagawa Metal, Japan), composed of 96% magnesium, 3% aluminum, and 1% zinc) was polished to remove the oxidized surface and wiped clean with 1 N hydrogen chloride before dipping into drinking water for 15–20 min at 25°C. The second method was based on the electrolysis of water with platinum electrodes at 150 V DC for 20 min (open-air water electrolysis method). The anode was placed inside a drinking straw to vent generated oxygen. In this method and kept in sealed drinking bottles remained saturated with H₂ for at least 24 hr.

In most experiments, each mouse received 0.8 ml of H₂ water (administered within 30 min of preparation) or control water (obtained by boiling H₂ water to degas) once every 3 days via feeding needle according to the experimental schedule. In MPTP-induced PD model mice, fresh H₂ water was supplied every 24 hr and water intake was ad libitum for the 7 days prior to MPTP administration.

Table 1 | Primer sets used for real-time PCR analyses

| RPL4.F/Rev | GCAAGACTTGGCAGCGGAAAT/GAACTTTGGGTGCTCTGTCTGTT
| RPL4.For/Rev | GTCAGCGTCGCGCTTCCGTT/CTCCTGACCCAGGCTAGTA
| Ghrelin.For/Rev | TCAAGGTCTCAGGAGCTGCTAGA/TTCTGAGCGTCGGCAGGCTGT
| Somatostatin.For/Rev | AGTACCTGGCAGGCTGCTTT/ATCCGGCTTTGCTGCTGTCTT
| Gastrin.For/Rev | CAGCGGCGATTCCAACCAGCT/ACCTGGCAGAGGAGAA
| b1AR.For/Rev | CACTTGGAGACCGAGGACTGA/ACCTGGACCTCGGAGGAA
| b2AR.For/Rev | ACAGGAATGCGCTGTTGAAGGAT/ACCGTAAAGGCTAAGGCACT

Measurements of ghrelin expression. Male and female mice aged between 41 and 48 weeks were chosen for the initial screening for the altered expression of gastric enzymes (n = 4 for each group), as they are relatively stable in dietary intake, which may influence mostly on the digestive enzyme expressions. Since the previous study was done with male mice14, the following experiments chose male mice. For the time-course analysis of ghrelin induction by hydrogen water, male mice (16–21 weeks, average 17.9 weeks, n = 5–10 in each group) were selected. Adrenergic mediated changes of ghrelin secretion was analyzed in male mice (age between 11–13 weeks, n = 4–5 for each group). Of note, there was no body weight change more than one gram during the period of experiment (4 days). These results are in line with the previous report with a recombinant ghrelin injection to mice, where the body weight change was below one gram in three days.

Mice were administrated H₂ water or control water for 4 days according to the experimental schedule. At the end of the experiment, mice were sacrificed by cervical dislocation and blood was collected by puncture with EDTA as anti-coagulant. Blood was centrifuged at 2000 g for 5 min at 4°C and the plasma was collected and mixed with 1 N hydrogen chloride (10% of plasma volume) to avoid inactivating deacylation of ghrelin20. Samples were stored at −80°C until analysis. Following blood collection, the stomach was removed intact and snap-frozen in liquid nitrogen for further analysis. For consistency, mice were killed 4–5 hours after the final administration of H₂ water and at the same time of day. At the time of blood sampling the stomach was almost full of chow, which suggests that the water administration at a time of ~ml/time) through the feeding needle did not cause the sustained reduction on appetite.

The active form of ghrelin was measured in plasma by ELISA (Active Ghrelin ELISA kit, Sceti, Japan) according to the manufacturer’s instructions. For semi-quantitative PCR, stomach tissue frozen in liquid nitrogen was crushed into fine granules and total RNA was extracted with RNAsio (Takara, Japan) according to the manufacturer’s instructions. Two μg of total RNA was then used to synthesize first strand cDNA with a SuperScript VILO cDNA Synthesis Kit (Invitrogen, USA). mRNA expression levels were quantified by real-time PCR with SYBR green dye (Thunderbird Sybr qPCR Mix, Toyobo, Japan) with the specific primer sets shown in Table 1, and normalized to ribosomal protein L4 (RPL4) mRNA15–17.

Administration of ghrelin receptor antagonist, β₁-adrenergic blocker, and MPTP. The ghrelin receptor antagonist, D-Lys³ GHRP-6 (Sigma-Aldrich, USA; 100 nmol/day), β₁-adrenergic blocker, atenolol (ICI, USA; 10 mg/kg), or saline was administered by i.p. injection daily for 7 days in conjunction with supplying fresh control or H₂ water. On day 7, MPTP-HCl (Sigma-Aldrich, USA; 15 mg/kg i.p. in 0.9% NaCl per injection) or saline as control was administered by i.p. injection four times at 2 hr intervals. Mice were supplied with untreated tap water for the next 7 days before removal of brains under deep anesthesia (pentobarbital, 50 mg/kg i.p.).

Stereological and immunological analyses of nigral dopaminergic neurons. Stereological analysis was carried out as described18–21. In brief, coronal sections (30 μm thickness) were obtained through SNpc (~ 2.70 mm to ~ 3.80 mm relative to bregma22) with a MICROM cryostat. Free-floating sections were incubated with Block Ace (Dainippon Pharmaceutics, Japan) for 30 min followed by incubation with anti-tyrosine hydroxylase (TH) antibody (Chemicon, USA, 1:300 in 10% Block Ace) for 2 days at 4°C. After rinsing, sections were immersed in a solution of 3% H₂O₂ in methanol/PBS (1:1) for 10 min at room temperature, followed by incubation for 2 hr in biotinylated goat anti-rabbit IgG (1:400, Vector, USA) and processing with a Vectastain ABC kit (Vector, USA) using 3'-diaminobenzidinetetrahydrochloride (DAB, Vector, USA) as peroxidase chromogen.

Unbiased stereological counts of TH-immunoreactive cell bodies in the SNpc were obtained using an optical fractionator method23 and Stereo Investigator software (Stereo Investigator 8, MicroBrightfield Inc., USA). The boundary of SNpc was delineated under 100 × magnification and immunopositive neurons were counted in every third section (eight sections per brain) at 400 × magnification. All sections were acquired using an Axioskop ECLIPSE 80 i microscope using a grid of 70 × 70 μm on a counting grid (25 × 25 μm) with 2 μm upper and lower guard zones. Gundersen’s coefficient of error in all samples was < 0.07.

Immunological detection and quantification of TH expression in the substantia nigra were performed by western blotting with actin as control. Animals were prepared identically to those of the stereological analysis. The substantia nigra was removed and stored at ~80°C until use. Samples were lysed on ice in hypotonic buffer (20 mM Heps pH 7.6, 10 mM NaCl, 1.5 mM MgCl₂, 1 mM EDTA, 0.1% Triton X-100) with protease inhibitor cocktail (Roche, USA), and cleared by a centrifugation at 20,000 g for 15 min at 4°C. The supernatant was fractionated into protein concentration using the BCA method with BSA as control, five μg of tissue lysate reduced in sample buffer was resolved by 10% SDS-PAGE, transferrt to nitrocellulose membrane, and probed with anti-TH antibody (1:2000) overnight at 4°C. Proteins were visualized using anti-rabbit secondary antibody conjugated to HRP and a chemiluminescence detection system (Immobilon Western Chemiluminescent HRP Substrate, Millipore, USA). Chemiluminescence image was quantified by Gauge application (Fuji Film, Japan) and the value was normalized to the level of actin band on the same sample specified by anti-Actin monoclonal antibody (Clone C4, Millipore, USA).

Statistical analysis and data managing. The statistical significance of data was assessed by unpaired Student t-test (two-tailed) unless otherwise mentioned and results were considered significant at *p ≤ 0.05.

1. Ohta, S. Recent Progress Toward Hydrogen Medicine: Potential of Molecular Hydrogen for Preventive and Therapeutic Applications. Curr. Pharm. Des. 17, 2241–2252 (2011).
2. Ohsawa, I. et al. Hydrogen acts as a therapeutic antioxidant by selectively reducing cytotoxic oxygen radicals. Nat. Med. 13, 688–94 (2007).
5. Kamimura, N., Nishimaki, K., Ohsawa, I. & Ohta, S. Molecular hydrogen alleviates nephrotoxicity induced by cisplatin in rats. Kidney Int. 77, 101–109 (2005).
6. Kavara, T. et al. Inhaled hydrogen gas therapy for prevention of lung transplant-induced ischemia/reperfusion injury in rats. Transplantation 90, 1344–51 (2010).
9. Buchholz, B. M. & et al. Hydrogen inhalation ameliorates oxidative stress in transplantation induced intestinal graft injury. Am. J. Transplant 8, 2015–24 (2008).
11. Matsushita, T., Kusakabe, Y., Kitamura, A., Okada, S. & Murase, K. Investigation of protective effect of hydrogen-rich water against cisplatin-induced nephrotoxicity in rats using blood oxygenation level-dependent magnetic resonance imaging. Jpn. J. Radiol. 29, 503–12 (2011).
12. Qian, L. et al. Radioprotective effect of hydrogen in cultured cells and mice. Free Radic. Res. 44, 275–82 (2010).
14. Schoenfeld, M. P. et al. Hydrogen therapy may reduce the risks related to radiation-induced oxidative stress in space flight. Med. Hypotheses 76, 117–8 (2011).
15. Fujita, K. et al. Hydrogen in drinking water reduces dopaminergic neuronal loss in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of Parkinson’s disease. PLoS One 4, e7247 (2009).
16. Dexter, D. T. & et al. Oxidative stress in Parkinson’s disease. Ann. Neurol. 53 Suppl 3, S26–36; discussion S36–8 (2003).
17. Yorita, A. et al. Pilot study of H2 therapy in Parkinson’s disease: A randomized, double-blind placebo-controlled trial. Mov. Disord. 28, 836–9 (2013).
19. Ito, M. et al. Drinking hydrogen water and intermittent hydrogen gas exposure, but not lactulose or continuous hydrogen gas exposure, prevent 6-hydroxydopamine-induced Parkinson’s disease in rats. Med. Gas. Res. 2, 15 (2012).
20. Kojima, M. & et al. Ghrelin: structure and function. Physiol. Rev. 85, 495–522 (2005).
21. Kamegai, J. et al. Effects of insulin, leptin, and ghrelin on ghrelin secretion from isolated perfused rat stomachs. Regul. Pept. 119, 77–81 (2004).
22. Zhao, T. et al. Ghrelin secretion stimulated by β-3-adrenergic receptors in cultured ghrelinoma cells and in fasted mice. Proc. Natl. Acad. Sci. U. S. A. 107, 15868–73 (2010).
23. Gagnon, J. & et al. Insulin and norepinephrine regulate ghrelin secretion from a rat primary stomach cell culture. Endocrinology 153, 3646–56 (2012).
24. Gagnon, J. & et al. Glucagon stimulates ghrelin secretion through the activation of MAPK and ERK and potentiates the effect of norepinephrine. Endocrinology 154, 666–74 (2013).
25. Iwakura, H. et al. Oxytocin and dopamine stimulate ghrelin secretion by the ghrelin-producing cell line, MG-63, in vitro. Endocrinology 152, 2619–25 (2011).
26. Andrews, Z. B. et al. The extra-hypothalamic actions of ghrelin on neuronal function. Trends Neurosci. 34, 31–40 (2011).
27. Sigman, J. M., Jones, J. E., Lee, C. E., Saper, C. B. & Elmquist, J. K. Expression of ghrelin receptor mRNA in the rat and the mouse brain. J. Comp. Neurol. 494, 528–48 (2006).
28. Andrews, Z. B. et al. UCP2 mediates ghrelin’s action on NPV/AgRP neurons by lowering free radicals. Nature 454, 846–51 (2008).
29. Andrews, Z. B. et al. Ghrelin promotes and protects nigrostriatal dopamine function via a UCP2-dependent mitochondrial mechanism. J. Neurosci. 29, 14057–65 (2009).

Acknowledgments

The authors are grateful to Dr. Douglas T. Hess (Case Western Reserve Univ.) for valuable advice and critical reading of the manuscript, and Dr. Yoshinori Tanaka (Corporate Engineering Division, Appliances Company, Panasonic Corporation, Japan) for help with hydrogen measurement. The authors also acknowledge the technical support of Mr. Yuichiro Kojima (Kyushu Univ.). This work was partly performed in the Cooperative Research Project Program of the Medical Institute of Bioregulation, Kyushu University. This work is supported in part by Grant-in-Aid for Scientific Research on Innovative Areas (MEET 20117008 to A.M.), Grant-in-Aid for Exploratory Research (ISPS 24659111 to A.M.), Grant-in-Aid for Scientific Research (B) (ISPS 23390053 to H.N.), Grant-in-Aid for Scientific Research (S) (ISPS 22221004 to Y.N.), Grant-in-Aid for Scientific Research (C) (ISPS 22598084 to M.N.) and Academic Challenge in Robert T. Huang Entrepreneurship of Kyushu University (to M.Y.).

Author contributions

A.M., Y.N., and M.N. conception and design of research; A.M., M.Y., T.T. performed experiments; A.M., M.Y., and M.N. analyzed data; A.M., M.Y., Y.N., and M.N. interpreted results of experiments; A.M. and M.Y. prepared figures; A.M., M.Y., M.N., and H.N. wrote and revised manuscript; A.M., T.T., Y.N., M.N., and H.N. approved final version of manuscript.

Additional information

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Matsumoto, A. et al. Oral ‘hydrogen water’ induces neuroprotective ghrelin secretion in mice. Sci. Rep. 3, 3273; DOI:10.1038/srep03273 (2013).

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