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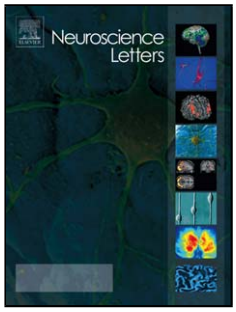

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Molecular hydrogen is protective against 6-hydroxydopamine-induced nigrostriatal degeneration in a rat model of Parkinson's disease

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Abstract

Molecular hydrogen serves as an antioxidant that reduces hydroxyl radicals, but not the other reactive oxygen and nitrogen species. In the past year, molecular hydrogen has been reported to prevent or ameliorate eight diseases in rodents and one in human associated with oxidative stress. In Parkinson's disease, mitochondrial dysfunction and the associated oxidative stress are major causes of dopaminergic cell loss in the substantia nigra. We examined effects of ~50%-saturated molecular hydrogen in drinking water before or after the stereotactic surgery on 6-hydroxydopamine-induced nigrostrital degeneration in a rat model of Parkinson's disease. Methamphetamine-induced behavioral analysis showed that molecular hydrogen prevented both the development and progression of the nigrostrital degeneration. Tyrosine hydroxylase staining of the substantia nigra and striatum also demonstrated that pre- and post-treatment with hydrogen prevented the dopaminergic cell loss. Our studies suggest that hydrogen water is likely able to retard the development and progression of Parkinson's disease.

Key words: Parkinson's disease, molecular hydrogen, oxidative stress

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder after Alzheimer's disease, affecting ~0.3% of the population over age 50. PD is characterized by resting tremor, bradykinesia, and rigidity. PD is caused by loss of dopaminergic neurons in the substantia nigra pars compacta. Although the pathomechanims of PD remain mostly unknown, oxidative stress to dopaminergic neurons is one of the major causes leading to dopaminergic neuronal cell loss [33]. Mitochondrial dysfunction and the associated oxidative stress in PD are directly or indirectly supported by studies of genetic forms of PD including *PINK1* [2], *DJ1* [19], and *HTRA2* [38], as well as by mitochondrial toxins including 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) [15] and rotenone [3].

Molecular hydrogen (H_2) can reduce only hydroxyl radicals (•OH), but not superoxide (O_2^-•), hydrogen peroxide (H_2O_2), or nitric oxide (NO•) [5, 25]. To date, H_2 has no known side effects in rodents or humans. Being prompted by these unique features of H_2, studies of molecular hydrogen for oxidative stress-associated disorders have flourished this past year. Molecular hydrogen in the form of gas reduces the cerebral infarction volume in rats [25], suppresses hepatic ischemia/reperfusion injury in mice [12], reduces the infarct size of myocardial ischemia/reperfusion injury in rats [16], reduces apoptosis in neonatal hypoxic brain injury in rats [8], and mitigates small intestinal transplantation-induced inflammation in rats [4]. Effects on the neonatal hypoxic brain injury are also shown in peritoneal injection of H_2-saturated saline [7]. Molecular hydrogen dissolved in drinking water similarly prevents stress-induced learning impairment in mice [23], improves lipid and glucose metabolism in type 2

diabetes and impaired glucose tolerance in humans [18], reduces atherosclerotic lesions in mice [26], and prevents cisplatin-induced nephrotoxicity [24].

Intrastriatal injection of the catecholaminergic neurotoxin 6-hydroxydopamine (6-OHDA) exerts its toxic effect by increasing oxidative stress in dopaminergic neurons [30]. The technique has been widely used to evaluate neuroprotective effects of therapeutic modalities. For example, the rat PD model has been treated with vitamin E [6], serofendic acid [17], estrogen [28], and insulin-like growth factor-1 (IGF-1) [27]. Compared to MPTP, 6-OHDA triggers more prominent dopaminergic cell loss that cannot be readily prevented by neuroprotective methodologies [1].

In an effort to examine a neuroprotective effect of molecular hydrogen for PD, 6-OHDA-induced PD rats were given free access to ~50%-saturated hydrogen water starting either before or after the stereotactic surgery. Behavioral and pathological analyses demonstrated that molecular hydrogen efficiently prevents both the development and progression of the nigrostriatal degeneration in rats.

Materials and Methods

Experimental design

Seven-week-old male Sprague–Dawley rats, ranging from 245 g to 255g, were purchased from Charles River Laboratories (Osaka, Japan). Rats were housed in a room temperature-controlled environment at 25˚C under a 12-hour light/dark cycle with *ad libitum* access to food and water. The animal studies were approved by the Animal Care and Use Committee of the Nagoya University Graduate School of Medicine.

Fifteen rats were randomly divided into three groups: a control group (Ctr, n = 5) with unlimited access to dehydrogenized water, a pretreatment group (PreH, n = 5) with unlimited access to hydrogen water starting from seven days before surgery, and a posttreatment group (PostH, n = 5) with unlimited access to hydrogen water starting from three days after surgery. Rats were supplied with fresh hydrogen water seven days a week. We also confirmed before surgery that the weight gains and the amounts of drinking water were not statistically different between the Ctr and PreH groups.

As the immunostaining of 8-hydroxy-dG (8-OHdG) and 4-hydroxy-2-nonenal (4-HNE) failed to stain any cells in the substantia nigra and striatum four weeks after surgery (data not shown), we examined the rat brains 48 hours after surgery. We randomly divided six additional rats into three groups. The Ctr and PreH groups were essentially the same as above. The third group was a sham operation group that was infused with saline into the striatum and supplied with dehydrogenized water.

Hydrogen Water

Hydrogen water was provided by Blue Mercury (Tokyo, Japan) or TYK (Tokyo, Japan). For both suppliers, we measured that the hydrogen concentration was more than 0.4 mM (equivalent to 50% saturation at room temperature) immediately before transferring to a 50-ml closed glass vessel equipped with an outlet line having two ball bearings. With the glass vessel, the hydrogen concentration remained more than 0.2 mM after 24 hours. We measured the hydrogen concentrations with a hydrogen electrode (ABLE, Tokyo, Japan). To make dehydrogenized water, we left hydrogen water uncovered overnight at room temperature and confirmed the complete removal of hydrogen gas.

Stereotactic surgery to infuse 6-OHDA into striatum

We administered 6-OHDA (Sigma-Aldrich Japan, Tokyo, Japan) steretotactically into the right striatum as previously described [22]. Briefly, each rat was anesthetized by an intraperitoneal injection of chloral hydrate (400 mg/kg) and was placed in a stereotactic frame. We infused 20 μg of 6-OHDA in 2 μl of saline containing 0.02% ascorbic acid each into two sites of the right striatum that corresponded to the following coordinates: AP 1.6 mm, ML 2.4, and DV 4.2; and AP 0.2, ML 2.6, and DV 7.0.

Methamphetamine-induced rotation test

On days 3, 7, 14, 21, and 28 after surgery, rats were intraperitoneally injected with 5.0 mg/kg of methamphetamine (Dainippon Sumitomo Pharma, Osaka, Japan) to provoke dopamine release from the dopaminergic nerve terminals. We started counting turns at ten minutes and counted the total number of turns in the following 30 minutes.

We only counted turns with a diameter of 20 cm or less in order not to include counts when the rats walked along the edge of a 40-cm round platform. The investigator was blinded whether the rats were taking dehydrogenized or hydrogen water.

Histological Assays

The rats were deeply anesthetized with chloral hydrate and transcardially perfused with 4% paraformaldehyde. The brains were frozen at -80˚C, and cut into coronal sections. After blocking the sections with horse serum, we incubated the sections either with an anti-TH rabbit polyclonal antibody (Biomol International, Plymouth Meeting, PA) at a dilution of 1:200, an anti-8-OHdG monoclonal antibody (JaICA, Nikken SEIL, Shizuoka, Japan) at 1:100, or an anti-4-HNE monoclonal antibody (JaICA, Nikken SEIL) at 1:40 at 4˚C overnight. We next incubated the sections with the biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) at a dilution of 1:200, followed by incubation with the avidin-biotin-horseradish peroxidase complex (the Vectastain ABC kit, Vector Laboratories) and the ImmPACT DAB Substrate (Vector Laboratories). The images of the striatum were reconstructed by the BZ-8000 microscope (Keyence, Woodcliff Lake, NJ). Two blinded investigators counted the numbers of TH-positive cells.

Results

Behavioral analysis reveals improvement of the methamphetamine-induced rotational asymmetry by H_2

We counted the number of clockwise turns in 30 min in response to an intraperitoneal injection of methamphetamine (Fig. 1). In two rats before surgery, we confirmed that methamphetamine induced no turn with a diameter of 20 cm or less. The Ctr group exhibited gradual development of hemiparkinsonism over the course of four weeks. On the contrary, the PreH group demonstrated a gradual and significant decrease of the number of turns over the course (Fig. 1A). In the PostH group, the number of turns was similar to that of the Ctr group on day 3. After the rats started drinking hydrogen water on day 3, however, the number of turns gradually decreased in two weeks, and the improved state persisted up to the end of our observation period of four weeks (Fig. 1B).

TH staining of the substantia nigra and striatum reveals amelioration of the dopaminergic cell loss by H_2

The rats were sacrificed four weeks after surgery. We then immunostained the substantia nigra and striatum for TH (Fig. 2). TH is a rate-limiting enzyme in catecholamine synthesis, and is a marker for dopaminergic neurons in the central nervous system [9]. We counted the number of TH-positive cells at the nigra and found that the 6-OHDA-treatment reduced the number of TH-positive cells to 40.2 ± 10.6% (mean ± SD, n = 5) in the Ctr group, whereas the pre- and post-treatment with hydrogen water increased the ratios to 83.0 ± 10.2% and 76.3% ± 7.0%, respectively (Fig. 2J). We did not observe TH-positive nerve terminals in the striatum in the Ctr group, whereas in the PreH

and PostH groups we observed patchy sparing of TH-positive nerve terminals (Fig. 2C, 2F, and 2I).

Oxidative stress is observed only in the striatum 48 hours after surgery and H_2 exhibits no distinct protection in the acute phase

The rats were sacrificed 48 hours after surgery, and the substantia nigra and striatum were immunostained for TH, 8-OHdG, and 4-HNE (Fig. 3). We observed patchy loss of TH staining (Fig. 3A and 3B), as well as appearance of 8-OHdG- (Fig. 3D and 3E) and 4-HNE-positive cells (Fig. 3G and 3H) in the ipsilateral striatum in the Ctr and PreH groups. We observed no effects on the substantia nigra in 48 hours after surgery. Smith and Cass similarly report that 4-HNE-positive cells and reduced dopamine concentrations are observed in the striatum but not in the substantia nigra in day 1 after surgery [36]. Forty-eight hours were likely to be too short for 6-OHDA to exert its toxic effect on the substantia nigra.

In the substantia nigra, the oxidative damage and the loss of TH-staining were similar between the Ctr and PreH groups, and H_2 showed no discernible protection against oxidative stress in 48 hours.

Discussion

Our current studies demonstrate that molecular hydrogen in drinking water before the stereotactic surgery efficiently prevents development of PD in a 6-OHDA rat model. In humans, parkinsonian symptoms appear after 80% reduction of striatal dopamine concentration [29] or 50-70% dopaminergic cell loss at the substantia nigra [11]. We observed a loss of 17.0% TH-positive cells in the substantia nigra in the PreH group, which was likely to be below the threshold of the development of PD.

The formation of hydroxyl radical and the subsequent lipid peroxidation and protein oxidation maximize 48 hours after 6-OHDA administration and persist for seven days [30]. We thus examined the oxidative stress in 48 hours after surgery, and observed appearance of 8-OHdG-positive and 4-HNE-positive cells, as well as loss of TH-staining, in the ipsilateral striatum. Pretreatment with H_2, however, exhibited no apparent protective effect. This is likely because the acute toxicity of 6-OHDA has exceeded the capacity that molecular hydrogen in drinking water can scavenge. Alternatively, as the treated rats were scarcely capable of drinking water 24 hours after surgery, our experimental protocol of *ad libitum* administration of hydrogen water might not be suitable for protecting the acute oxidative stress posed by 6-OHDA. The protective effect against the dopaminergic cell loss in the substantia nigra is thus not likely due to immediate extinction of 6-OHDA in the striatum, but to prevention of the delayed toxic effect of 6-OHDA on the dopaminergic neurons in the substantia nigra. This can also explain why we observed the similar but less efficient effect with the PostH group.

When we administered 3 ml of 0.4 mM hydrogen water directly into the rat stomach, the H_2 concentration in the carotid artery increased by 0.011 mM in 10 min

(unpublished data), which was similar to an increase by 0.009 mM when rats were placed in 2% H_2 gas [25]. Hydrogen in drinking water, however, should not stay in the body as long as the inhaled hydrogen. In addition, the scavenging activity of hydroxyl radicals is observed at 0.2 mM or higher concentrations of H_2 in cell-free systems, and is demonstrated at 0.6 mM H_2 in culture cells [25]. The amount of H_2 taken by the rats was likely to be too low to reduce a large amount of hydroxyl radicals generated by 6-OHDA. One possible explanation would be that H_2 activates yet unidentified pathways that culminate in activation of the antioxidant activities. Indeed, H_2 prevents formation of O_2^{-}• in brain slices [31]. As H_2 cannot directly reduce O_2^{-}• [5, 25], a subtle reduction of hydroxyl radicals might have exerted beneficial effects on the integrity of mitochondria and led to reduced production of O_2^{-}•, but the exact molecular mechanisms remain to be elucidated.

α-Tocopherol demonstrates a neuroprotective effect for PD in rats [6], but not in humans in a double-blind placebo-controlled study [14, 34]. Coenzyme Q_{10} similarly exerts a neuroprotective effect in cultured cells and in rodents [20]. Coenzyme Q_{10}, however, may [35] or may not [37] be beneficial in humans in double-blind placebo-controlled trials. Neuroprotective effects of vitamin C and β-carotene are not observed in patients with PD even in open trials [10]. In contrast to the other radical scavengers, molecular hydrogen can reduce only hydroxyl radicals (•OH), but not the other reactive oxygen and nitrogen species including superoxide (O_2^{-}•), hydrogen peroxide (H_2O_2), and nitric oxide (NO•) [5, 25]. Reactive oxygen and nitrogen species other than the hydroxyl radical play essential roles in biological processes including cell proliferation [32], defense against bacterial infection [39], neurotransmission [13], and vasodilation [21].

Lack of therapeutic effects of α-tocopherol, coenzyme Q_{10}, vitamin C, and β-carotene in PD may be owing to aberrations of the biological processes mediated by the reactive oxygen species. On the other hand, hydroxyl radical has no known biological activities. The ability of molecular hydrogen to exclusively reduce hydroxyl radical may hold the greatest promise for a dependable and efficient neuroprotective modality for PD.

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Figure legends

Figure 1. Behavioral assays of 6-OHDA-treated rats. **A.** Temporal profiles of the Ctr (open bars) and PreH (closed bars) rats. **B.** Temporal profiles of the Ctr (open bars) and PostH (closed bars) rats. Vertical bars represent means ± SEM of the number of turns in 30 min after intraperitoneal injection of methamphetamine in five rats. Student's t-test results are indicated by asterisks: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$; ****, $p < 0.001$.

Figure 2. Representative TH staining of the substantia nigra and striatum of 6-OHDA-treated rats. **A, B and C**, Ctr; **D, E, and F**, PreH; **G, H, and I**, PostH. **A, D, and G,** Untreated left side of the nigra; **B, E, and H,** 6-OHDA-treated right side of the nigra; **C, F, and I,** the striatum. Arrows point to bleeding scars by the stereotactic needle. **J.** The number of TH-positive cells at the substantia nigra on the treated side is divided by that of the untreated side to calculate the ratio of the remaining dopaminergic cells (mean ± SD, n = 5 for each group). Asterisks indicate $p < 0.001$ compared to Ctr (Student's t-test). Bar = 200 μm for **A, B, D, E, G, and H**; bar = 1 mm for **C, F, and I**.

Figure 3. Representative TH, 8-OHdG, and 4-HNE staining of the substantia nigra and striatum of 6-OHDA-treated and sham-operated rats at 48 hours after surgery. **A, B, and C**, TH staining; **D, E, and F**, 8-OHdG staining; **G, H, and L**, 4-HNE staining and nuclear staining with hematoxylin. **A, D and G**, Ctr; **B, E, and H**, PreH; **C, F, and I**, sham-operation. For each panel, the upper part shows the striatum and the lower part shows the substantia nigra. High magnification images are attached on the right side of each panel,

and the stars point to the magnified positions. The ipsilateral side is shown on the right side. Arrows point to bleeding scars by the stereotactic needle, where cells are positive for 8-OHdG and 4-HNE even in the sham-operated brain. Bars = 1 mm for low magnification images on the left; 10 μm for high magnification images on the right.