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Review

Molecular hydrogen is a novel antioxidant to efficiently reduce oxidative stress with potential for the improvement of mitochondrial diseases ☆

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ABSTRACT

Background: Mitochondria are the major source of oxidative stress. Acute oxidative stress causes serious damage to tissues, and persistent oxidative stress is one of the causes of many common diseases, cancer and the aging process; however, there has been little success in developing an effective antioxidant with no side effect. We have reported that molecular hydrogen has potential as an effective antioxidant for medical applications [Ohsawa *et al.*, *Nat. Med.* 13 (2007) 688–694].
Scope of review: We review the recent progress toward therapeutic and preventive applications of hydrogen. Since we published the first paper in *Nature Medicine*, effects of hydrogen have been reported in more than 38 diseases, physiological states and clinical tests in leading biological/medical journals. Based on this cumulative knowledge, the beneficial biological effects of hydrogen have been confirmed. There are several ways to intake or consume hydrogen, including inhaling hydrogen gas, drinking hydrogen-dissolved water, taking a hydrogen bath, injecting hydrogen-dissolved saline, dropping hydrogen-dissolved saline into the eyes, and increasing the production of intestinal hydrogen by bacteria. Hydrogen has many advantages for therapeutic and preventive applications, and shows not only anti-oxidative stress effects, but also has various anti-inflammatory and anti-allergic effects. Preliminary clinical trials show that drinking hydrogen-dissolved water seems to improve the pathology of mitochondrial disorders.
Major conclusions: Hydrogen has biological benefits toward preventive and therapeutic applications; however, the molecular mechanisms underlying the marked effects of small amounts of hydrogen remain elusive.
General significance: Hydrogen is a novel antioxidant with great potential for actual medical applications. This article is part of a Special Issue entitled Biochemistry of Mitochondria.

1. Introduction

Mitochondria are central to oxidative phosphorylation and much of the metabolism, and are also involved in many aspects of cell death [1]. Consequently, mitochondrial dysfunction contributes to a wide range of human pathologies. In many of these, excessive oxidative damage is a major factor because the mitochondrial respiratory chain is a significant source of damaging reactive oxygen species (ROS), superoxide ($\bullet O_2^-$), hydrogen peroxide (H_2O_2), and hydroxyl radicals (•OH) [2], as illustrated in Fig. 1; however, despite the clinical importance of mitochondrial oxidative damage, antioxidants have had limited therapeutic success [3–8].

Oxidative stress arises from the strong cellular oxidizing potential of excess ROS. Acute oxidative stress may arise from a variety of situations: inflammation, heavy exercise, cardiac infarction, cessation of operative bleeding, organ transplantation, and others [6]. On the other hand, persistent oxidative stress is accepted as one of the causes of many common diseases, cancer and the aging process; however, most clinical trials with dietary antioxidants failed to show marked success in preventing oxidative stress-related diseases [9]. Thus, it is very important to find an effective antioxidant with no side effects.

We found that molecular hydrogen (H_2) has potential as a "novel" antioxidant in preventive and therapeutic applications [10]. In the three and a half years since the first hydrogen paper was published in *Nature Medicine*, hydrogen effects have been reported in 38 diseases and physiological states [11], establishing overwhelming support for the beneficial biological effects of hydrogen have been established. There are several ways to ingest or consume hydrogen, including inhaling hydrogen gas, drinking hydrogen-dissolved water (hydrogen water), taking a hydrogen-dissolved bath, injecting hydrogen-dissolved saline (hydrogen saline), dropping hydrogen saline onto the eyes, and increasing the production of intestinal hydrogen by bacteria. Furthermore, H_2 exhibits not only anti-oxidative stress effects, but also has various anti-inflammatory and anti-allergic effects.

H_2 has a number of advantages as a potential antioxidant: it is mild enough not to disturb metabolic redox reactions or to affect ROS,

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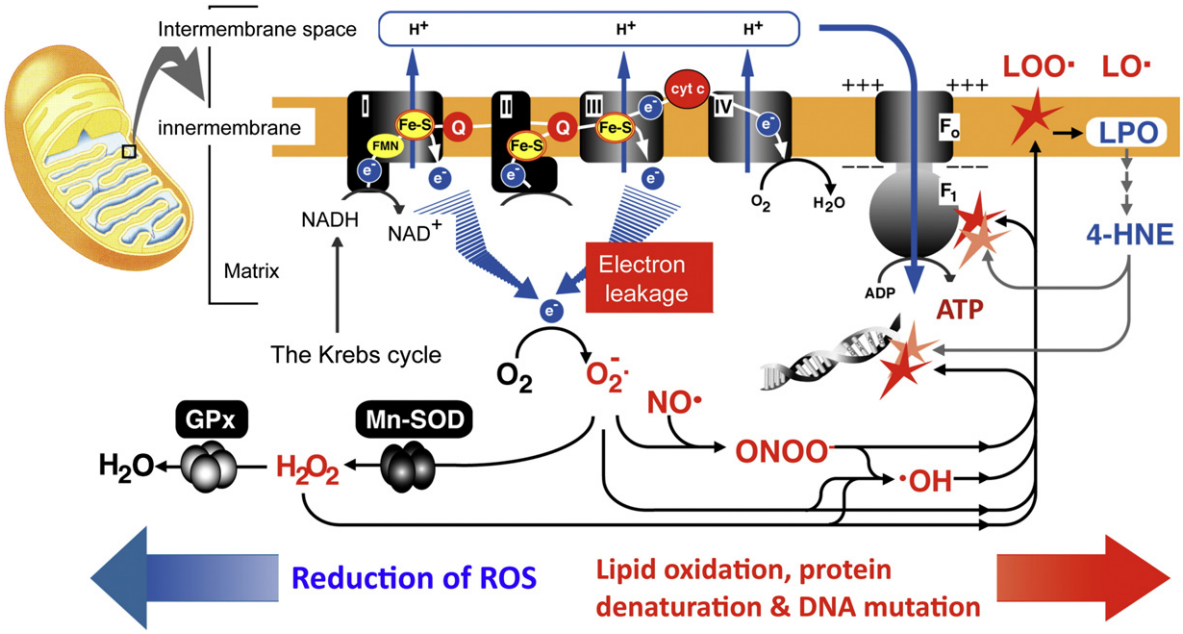

Fig. 1. Illustration of the generation and scavenging systems of reactive oxygen species (ROS) in mitochondria. Superoxide radical anions ($\bullet O_2^-$) are generated by the reaction of oxygen (O_2) with an electron leaked from the electron transport chain of mitochondria. $\bullet O_2^-$ is non-enzymatically or enzymatically converted to hydrogen peroxide (H_2O_2) with Mn-superoxide dismutase (Mn-SOD), and detoxified with Glutatione peroxidase (GPx) to water. Some H_2O_2 are converted to the most reactive ROS, hydroxyl radicals (•OH) by the Fenton reaction. •OH damages DNA, proteins and membranes. Additionally, nitric oxide (NO•) reacts with $\bullet O_2^-$ to generate peroxynitrite ($ONOO^-$). Molecular hydrogen (H_2) cannot directly react with $\bullet O_2^-$, H_2O_2 and NO, but can with •OH.

which functions in cell signaling [12–14] and has favorable distribution characteristics in its own physical ability to penetrate biomembranes and diffuse through barriers into cellular components.

Here, we review the recent progress toward therapeutic and preventive applications of hydrogen.

2. ROS as one of the major causes of acute and chronic diseases

Oxidative stress arises from an excess of free oxidizing radicals. As the first step in generating ROS, the majority of $\bullet O_2^-$ is generated in mitochondria by electron leakage from the electron transport chain [2,4–6].

Acute oxidative stress may arise from a variety of different situations: inflammation, heavy exercise, cardiac infarction, cessation of operative bleeding, organ transplantation, and others. The accelerated generation of ROS by reperfusion of the ischemic myocardium is a potential mediator of reperfusion injury [15–18]. During myocardial reperfusion, $\bullet O_2^-$ is generated within the injured mitochondria via electron leakage from the electron transport chain. Superoxide dismutase converts $\bullet O_2^-$ to H_2O_2, which is metabolized by glutathione peroxidase or catalase to generate H_2O. Highly reactive •OH is generated from H_2O_2 via the Fenton or Weise reaction in the presence of catalytically active metals, such as Fe^{2+} and Cu^+ [19,20].

These ROS mediate myocardial injury by inducing mitochondrial permeability transition pore (PTP) opening, causing a loss of mitochondrial membrane potential, and leading to mitochondrial swelling with membrane rupture [21]. Many attempts have been made to inhibit ROS production to limit the extent of reperfusion injury. The administration of ROS scavengers at the time of reperfusion has produced conflicting results that can be partially explained by the dual role of ROS in ischemia–reperfusion hearts [22,23]. The majority of detrimental effects associated with lethal reperfusion injury are attributed to •OH. By comparison, $\bullet O_2^-$ and H_2O_2 have less oxidative energy and, paradoxically, are implicated as crucial signaling components in the establishment of tolerance to oxidative stress. The inhibition of both pathways may be deleterious since ROS signaling during the first few minutes of myocardial reperfusion is essential for beneficial ischemic post-conditioning [24,25].

It is widely accepted that persistent oxidative stress is one of the causes of lifestyle-related diseases, aging, and cancer. ROS are generated inside the body in various situations in daily life, such as hard exercise, smoking, being exposed to ultraviolet rays or air pollution, aging, stress, and so on [26–29]. Inside the body of every aerobic organism, ROS are generated when breathing activity consumes oxygen. These ROS are generated under the condition of excessively high membrane potential at the mitochondrial inner membrane. Thereby, uncoupling proteins control the membrane potential to suppress the production of ROS and then consequently to repress diabetes [30–32].

3. Characteristics of molecular hydrogen

Hydrogen is the most abundant element in the universe, constituting nearly 75% of the universe's mass; however, hydrogen is absent on the earth in its monoatomic form and found in water and organic or inorganic compounds. Hydrogen gas, with the molecular formula H_2, is a colorless, odorless, tasteless and highly combustible diatomic gas. The earth's atmosphere contains less than 1 ppm of hydrogen gas [33].

Molecular hydrogen is rather less active and behaves as an inert gas in the absence of catalysts or at body temperature. H_2 does not react with most compounds, including oxygen gas at room temperature. Hydrogen gas is flammable only at temperature higher than 527 °C, and explodes by the rapid chain reaction with oxygen only in the explosive range of the H_2 concentration (4–75%, vol/vol).

Although H_2 has had a reputation for being a highly flammable diatomic gas since the explosion of the Hindenburg airship in 1937, there is no risk of combustion at levels less than 4%. Furthermore, safety standards are established for high concentrations of H_2 gas for inhalation since high pressure H_2 gas is used in deep diving gas mixes to prevent decompression sickness and arterial gas thrombi [34]. The safety of hydrogen for humans is demonstrated by its application in Hydreliox, an exotic, breathing gas mixture of 49% hydrogen, 50% helium and 1% oxygen, which is used for the prevention of

decompression sickness and nitrogen narcosis during very deep technical diving [34–37].

H_2 can be dissolved in water up to 0.8 mM (1.6 ppm, wt/vol) under atmospheric pressure. H_2 penetrates the glass and plastic walls of any vessels in a short time, while aluminum containers are able to retain hydrogen gas.

H_2 has a number of advantages as a potential antioxidant: it has favorable distribution characteristics with its own physical ability to penetrate biomembranes and diffuse into the cytosol, unlike most known antioxidants, as illustrated in Fig. 2. Excessive oxidative damage is a major factor because the mitochondrial respiratory chain is a significant source of damaging reactive oxygen species; however, despite the clinical importance of mitochondrial oxidative damage, antioxidants have had limited therapeutic success. This may be because the antioxidants are not selectively taken up by mitochondria [7,8,38]. Since H_2 effectively reaches the nucleus and mitochondria, the protection of nuclear DNA and mitochondria suggests preventive effects on lifestyle-related diseases, cancer and the aging process [10]. Moreover, H_2 passes through the blood brain barrier, although most antioxidant compounds cannot; this is also an advantage of H_2.

4. Gaseous diffusion of molecular hydrogen

The gaseous diffusion of H_2 can be proven by monitoring its concentration inside various tissues. H_2 can be detected with specific electrodes. In fact, the H_2 concentration has been monitored within a rat myocardium. The electrodes were inserted into the non-ischemic myocardium of the left ventricle. The incremental rate of H_2 saturation for the non-ischemic myocardium and arterial blood was similar. Electrodes were inserted into the 'at risk' area for infarction to investigate the diffusion of H_2 into the ischemic myocardium, induced by coronary artery occlusion. Of note, H_2 concentration was increased even in the ischemic myocardium (Fig. 3). Although the incremental rate of H_2 saturation was slower in the ischemic myocardium than in the non-ischemic myocardium, the peak level of H_2 in the ischemic myocardium was approximately two thirds of the value observed for the non-ischemic myocardium. After restoration of coronary artery blood flow, the level of H_2 in the ischemic myocardium immediately increased to the level observed in the non-ischemic myocardium [39].

Moreover, we devised eye drops with dissolved H_2 (H_2 eye drops) to directly administer H_2 to the retina, and monitored the time-course of changes in H_2 levels using a needle-shaped hydrogen sensor electrode inserted through the sclera to the vitreous body in rats (Fig. 3). H_2 was able to reach the vitreous body by administering H_2 saturated in normal saline saturated with H_2 (0.8 mM). When H_2 eye drops were administered continuously, approximately 0.5 mM H_2 was detected on the ocular surface. Two minutes after the start of administration, H_2 concentration in the vitreous body started to increase and reached a maximum level after 15 min. At that time, H_2 concentration was approximately 20% that in the eye-drops (0.16 mM). The maximum concentration of H_2 in the vitreous body reached approximately one third of the value observed on the ocular surface [40].

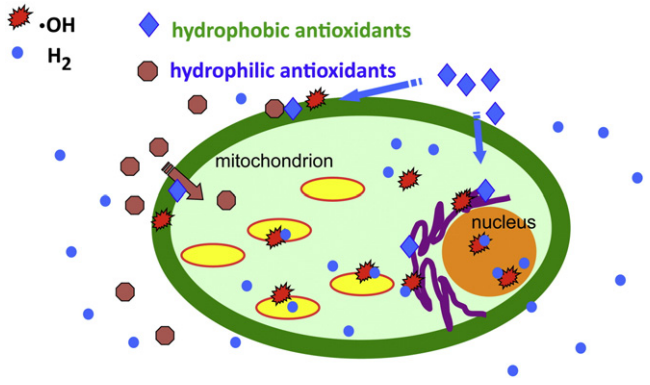

Fig. 2. Illustration of gaseous diffusion of molecular hydrogen (H_2) in the cell. Most hydrophilic compounds retain at membranes and cannot reach the cytosol, whereas most hydrophic ones cannot penetrate biomembranes in the absence of specific carriers. In contrast, H_2 can rapidly distribute into cytosol and organelles.

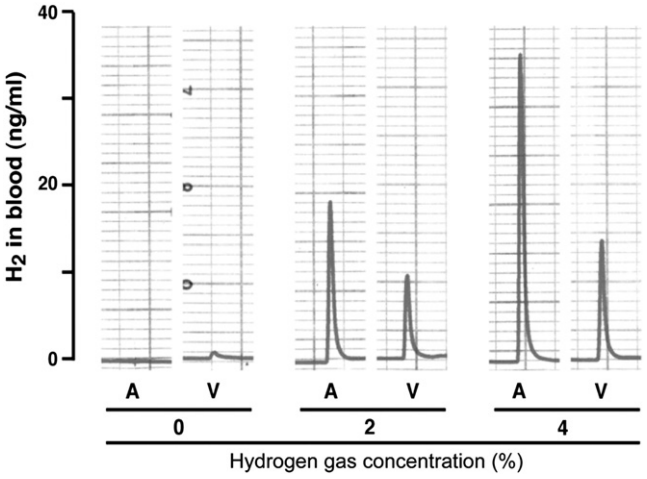

Fig. 3. Diffusion of molecular hydrogen (H_2) even without blood flow. A needle-type hydrogen electrode was inserted into the indicated tissues and the H_2 levels monitored. (A) H_2 gas at 2% was administered by respiration to intubated rats receiving mechanical ventilation. Change in the concentration of H_2 in 'at risk' intramyocardial areas for infarction during ischemia and reperfusion was monitored. (B) H_2-loaded eye drops increased intravitreal H_2. The concentrations of H_2 on the ocular surface and in the vitreous body were monitored with a needle-type H_2 sensor.

5. Molecular hydrogen as a medical gas

Gases possess the ability to diffuse readily in different materials and become uniformly distributed within a defined space. "Biologic gases" are assumed to diffuse freely across biologic membranes, acting in a variety of functional capacities [41]; hydrogen gas is an example of this, as mentioned earlier.

Gas inhalation as disease therapy has received recent interest. Novel medical gases are expected to provide more effective therapeutic interventions and preventive medicine. In past decades, there has been extraordinary, rapid growth in our knowledge of gaseous molecules, including nitric oxide (NO), carbon monoxide (CO), and hydrogen sulfide (H_2S), which have been known to play important roles in biological systems [42,43].

The increased production of these gasses under stress conditions may reflect the active involvement of these gasses in the protective response. In pre-clinical experimental models of disease, including ischemia–reperfusion injury, the inhalation of exogenous CO or H_2S has produced a favorable outcome for most vital organs [44–47]. In particular, NO has been approved as a therapeutic agent in clinical practice; however, the inherent toxicity of these gasses must be investigated for gas inhalation to be considered an effective therapeutic strategy because these gasses are highly toxic at considerable concentrations. Additionally, NO enhances oxidative stress by the reaction with $\bullet O_2^-$ by the production of highly oxidative peroxynitrite ($NO + \bullet O_2^- \rightarrow ONOO^-$). It is unknown if the therapeutically effective threshold for CO or H_2S can be attained locally in target organs without delivering a potentially toxic level of the gasses via the lungs.

CO, NO and H_2S are generated by endogenous enzymatic systems with complex interrelationships. Pharmaceutical development has taken advantage of these systems to design exogenous molecules to simulate those generated endogenously; however, mammals lack their own enzyme to produce H_2.

It is interesting that mitochondria are the common organelle targeted by all three gasses, which also modulate oxygen (O_2) reactivity and

consumption [42,44,48]. These gasses are bound to heme in the electron transport chain of mitochondria. Alternatively, the oxidative state of the heme iron in hemoglobin shifts between Fe^{2+} (functional) and Fe^{3+} (dysfunctional) states. The ferrous form (Fe^{2+}) of hemoglobin prefers to bind ligands such as O_2, CO, and NO. Conversely, ferric heme (Fe^{3+}) prefers to bind water, H_2S, and anions such as CN^-, N^{3-}, and OH^- [42].

Heme oxygenase-1 (HO-1), a microsomal enzyme degrading heme to carbon monoxide, free iron, and biliverdin, participates in the cell defense against oxidative stress and it has been speculated that it might be a new therapeutic target [49]. Notably, H_2 modulates HO-1 expression, which is commonly up-regulated by these medical gasses [33,50].

All four gasses, including H_2, may modulate signaling pathways and have some potentially therapeutic effects. In particular, H_2 has more advantages from the aspect of toxicity: H_2 has no cytotoxicity even at high concentration.

6. Delivery systems to ingest or consume molecular hydrogen

6.1. Inhalation of hydrogen gas

Inhalation of hydrogen gas is a straightforward therapeutic method. Hydrogen gas can be easily inhaled by delivering hydrogen gas through a ventilator circuit, facemask or nasal cannula. Hydrogen gas poses no risk of explosion in air and in pure oxygen when present at concentrations <4%, as mentioned earlier. However, safety is still a concern and the desired concentration of hydrogen must be monitored and maintained with an approved and commercially available tool.

Rats inhaled H_2 in a mix of nitrous oxide (N_2O) (for anesthesia), O_2, and N_2. The inhalation of H_2 actually increased H_2 dissolved in arterial blood depending upon the H_2 gas concentrations, and H_2 levels in venous blood were lower than in arterial blood, suggesting the incorporation of H_2 into tissues [10] (Fig. 4).

6.2. Oral intake by drinking hydrogen water

Since inhaled hydrogen gas acts more rapidly, it may be suitable for defense against acute oxidative stress. In particular, inhalation of gas does not affect blood pressure; blood pressure increased by infusion may be serious in myocardial infarction; however, inhalation of hydrogen gas may be unsuitable as continuous hydrogen consumption for preventive use. Inhalation of hydrogen gas is not practical in daily life for preventive use. In contrast, solubilized hydrogen (hydrogen-dissolved water; namely, hydrogen water) may be beneficial since it is a portable, easily administered and safe means of delivering H_2 [51]. H_2 can be dissolved in water up to 0.8 mM under atmospheric pressure at room temperature as mentioned earlier. Unexpectedly, drinking H_2 water has comparable effects to hydrogen inhalation [52].

When water saturated with hydrogen was placed in the stomach of a rat, hydrogen was detected at several μM in blood [51,52]. Moreover, hepatic hydrogen was monitored with a needle-type hydrogen electrode, and H_2 accumulated after oral administration of H_2 water, explaining why consumption of even a small amount of H_2 over a short dwell time could efficiently improve various disease models. An in vitro experiment confirmed that polymers of carbohydrates, including glycogen or starch, have an affinity for H_2 [52].

Hydrogen water can be made by several methods, including dissolving hydrogen gas in water under high pressure, dissolving electrolyzed hydrogen in water, and by the reaction of magnesium metal with water. The method of dissolving hydrogen gas under high pressure has an advantage because it is applicable not only in using water but also in any other solvents.

6.3. Injection or eye-dropping of hydrogen saline

Even though oral administration is safe and convenient, hydrogen in water tends to escape over time and some hydrogen is lost in the stomach or intestine, making it difficult to control the concentration of hydrogen administered. Administration of hydrogen via an injectable hydrogen saline (H_2-dissolved saline) vehicle may allow the delivery of more accurate concentrations of hydrogen [53].

Sun's group administered H_2-saturated saline by peritoneal injection to various model animals with great success. Thus, hydrogen saline has potential in actual clinical treatment. For example, injection of hydrogen saline showed neuroprotective effects in a neonatal hypoxia–ischemia rat model [53]. Moreover, H_2 saline was applied to an Alzheimer's disease model mouse, which was generated by intracerebroventricular injection of the Aβ1-42 peptide. H_2-treatment decreased the level of oxidative stress and inflammation markers and prevented memory dysfunction and motor dysfunction, respectively [54].

Alternatively, H_2-loaded eye drops were prepared by dissolving H_2 in saline and directly administered to the ocular surface [40,55].

6.4. Taking a hydrogen bath

Hydrogen easily penetrates the skin and distributes throughout the body via blood flow. Thus, taking a warm water bath dissolving H_2 is a method of incorporating H_2 into the body in daily life, especially in Japan. It takes only 10 min to distribute to the whole body as judged by measuring H_2 gas in expiration (unpublished results).

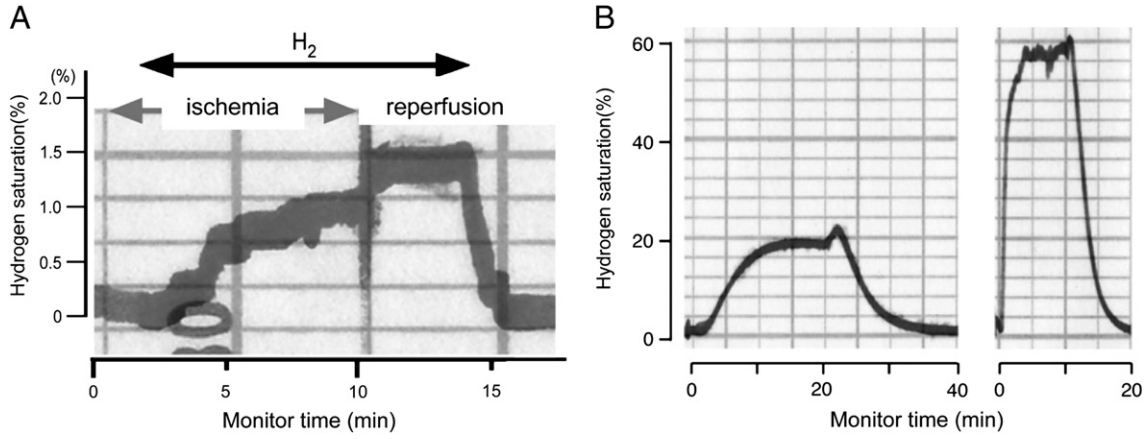

Fig. 4. Incorporation of H_2 after the inhalation of hydrogen gas. Rats inhaled H_2 and 30% O_2 for 1 h with the anesthetics N_2O and halothane. Arterial (A) and venous (V) blood was collected, and the amount of H_2 was examined by gas chromatography.

6.5. Increase in intestinal hydrogen

The spontaneous production of H_2 gas in the human body occurs via the fermentation of undigested carbohydrates by resident enterobacterial flora [56]. H_2 is transferred to the portal circulation and excreted through the breath in significant amounts [57]. For this reason, measurement of H_2 levels in expired air is used to detect carbohydrate malabsorption [47]; however, there have been few studies on the physiological function of gastrointestinal tract-derived H_2 gas as an antioxidant.

α-Glucosidase inhibitors are pharmacological agents that specifically reduce postprandial hyperglycemia through retardation of disaccharide digestion, thereby reducing glucose absorption. A large scale epidemiologic trial has demonstrated that the treatment of patients with impaired glucose tolerance with an α-glucosidase inhibitor was associated with a 25% reduction in the risk of progression to diabetes, a 34% reduction in the risk of developing de novo hypertension, and a 49% risk reduction for cardiovascular events [58]. Furthermore, meta-analysis of seven long-term studies suggested that acarbose reduced the risk of myocardial infarction for patients with type 2 diabetes [59]. Such risk reduction for coronary heart disease events in patients with type 2 diabetes was not observed by the improved glycemic control achieved with intensified treatment with insulin and glibenclamid [60]. Actually, acarbose, which is an α-glucosidase inhibitor, markedly increased H_2 production in volunteers. Thus, we propose that H_2 produced by intestinal bacteria acts as a unique antioxidant and prevents cardiovascular events [61].

7. Biological benefits of molecular hydrogen

As mentioned, more than 70 publications have demonstrated the biological benefits of hydrogen as of now. Here, we would like to focus on our studies and introduce some papers that significantly contribute to this field.

7.1. Scavenging effects on hydroxyl radicals in cultured cells

Cultured cells were treated with a mitochondrial respiratory complex III inhibitor, antimycin A, to induce excess $\bullet O_2^-$ production. Following such treatment, $\bullet O_2^-$ is rapidly converted into H_2O_2 and then •OH. The addition of antimycin A actually increased levels of $\bullet O_2^-$ and H_2O_2; however, H_2 dissolved in culture medium did not change their levels. Additionally, H_2 did not decrease the steady-state level of NO. In contrast, H_2 treatment significantly decreased levels of •OH. Notably, H_2 decreased •OH levels even in the nuclear region [10].

After antimycin A treatment, H_2 prevented the decline of the mitochondrial membrane potential. This suggested that H_2 protected mitochondria from •OH. Along with this protective effect, H_2 also prevented a decrease in the cellular level of ATP synthesized in mitochondria. The fact that H_2 protected mitochondria and nuclear DNA provided evidence that H_2 penetrated most membranes and diffused into organelles. Consequently, H_2 protected cultured cells against oxidative stress [10].

7.2. Protective effects on ischemia–reperfusion model by rat cerebral infarction

H_2 gas was applied to a rat model of ischemia–reperfusion as an acute model [62]. We produced focal ischemia in rats by occlusion of the middle cerebral artery (MCA) for 90 min with subsequent reperfusion for 30 min. One day after MCA occlusion, infarct volumes decreased in an H_2-dependent manner by 2–4%. One week after MCA occlusion, the difference in infarct volume between non-treated and H_2-treated rats increased. H_2-treated rats also showed improvements in body weight and temperature vs. untreated rats. Moreover, movement defects were improved by the inhalation of H_2 during ischemia reperfusion. Thus, H_2 suppressed not only the initial brain injury, but also the progression of injury. H_2 markedly decreased several oxidative stress markers. In this experiment, H_2 was, for the first time, demonstrated to have the potential to markedly decrease oxidative stress and suppress brain injury [10] (Fig. 5).

7.3. Protective effects on hepatic ischemia reperfusion injury

Next, inhalation of H_2 gas was also applied to a hepatic ischemia reperfusion injury model [63]. Inhalation of H_2 clearly attenuated the degeneration induced by hepatic ischemia reperfusion and the protective effect was in a concentration-dependent manner. Since helium gas (He) exhibited no effect, any gaseous small molecules have no protective effect, but H_2 clearly has a protective effect [64].

7.4. Protective effects on myocardial ischemia–reperfusion injury model

The degree of cardioprotection against ischemia–reperfusion injury was evaluated by measuring oxidative damage and infarct size 30 min after left anterior descending coronary artery occlusion and reperfusion. Inhalation of an incombustible level of H_2 gas (2%) before reperfusion significantly reduced oxidative stress-induced myocardial injury and infarct size without affecting hemodynamic parameters, and thereby prevented deleterious left ventricle remodeling [39].

7.5. Improvement of glaucoma model

In acute glaucoma of the eyes, transient elevation of intraocular pressure causes significant reductions in the thickness of the retina by ischemia–reperfusion injury mediated through the generation of reactive oxygen species. The direct application of H_2 eye drops ameliorated ischemia-reperfusion injury of the retina in a rat model. When H_2 eye drops were continuously administered, H_2 concentration increased in the vitreous body, and •OH level decreased in the during retinal ischemia-reperfusion. H_2 eye drops reduced the number of apoptotic and oxidative stress marker-positive cells 1 day after ischemia–reperfusion injury, and reduced retinal thinning with accompanying activation of Müller glia, astrocytes and microglia at 7 days after ischemia–reperfusion injury. They improved the recovery of inner retinal layer thickness to >70%.

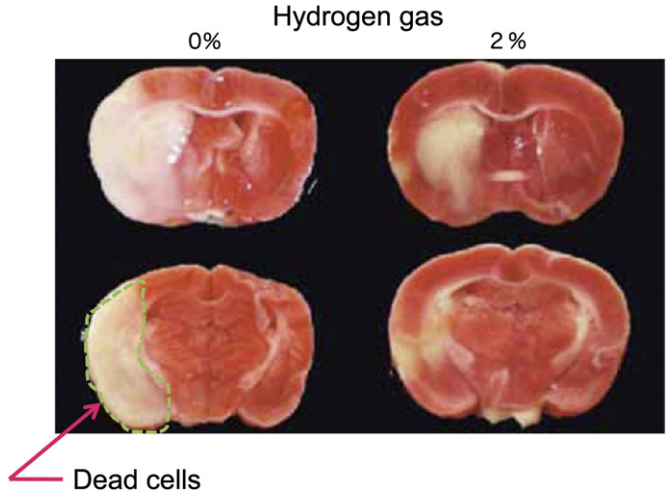

Fig. 5. Protective effects of inhaled hydrogen on ischemia–reperfusion induced by rat middle cerebral artery occlusion. During the 120-min ischemia–reperfusion procedure, hydrogen gas (2%) was inhaled. One day after occlusion, the forebrain was sliced into six coronal sequential sections and stained with the mitochondrial respiratory substrate TTC.

7.6. Prevention of cognitive decline

Chronic physical restraint stress on mice enhanced levels of oxidative stress in the brain, and impaired learning and memory [65,66]. Consumption of H_2 water ad libitum suppressed the increase in oxidative stress, and prevented cognitive impairment. Neural proliferation in the dentate gyrus of the hippocampus was suppressed by restraint stress [66]. The consumption of H_2 water ameliorated the reduced proliferation although a mechanistic link between hydrogen-dependent changes in neurogenesis and cognitive impairments remains unclear. Thus, continuous consumption of H_2 water reduces oxidative stress in the brain, and prevents the stress-induced decline in learning and memory caused by chronic physical restraint [51].

7.7. Preventive and therapeutic effects on Parkinson disease model

In Parkinson's disease, mitochondrial dysfunction and the associated oxidative stress are major causes of dopaminergic cell loss in the substantia nigra [67]. Molecular hydrogen in drinking water was given before or after stereotactic surgery for 6-hydroxydopamine-induced nigrostrital degeneration in a rat model of Parkinson's disease. Hydrogen prevents both the development and progression of nigros-triatal degeneration. Tyrosine hydroxylase staining of the substantia nigra and the striatum also demonstrated that pre- and post-treatment with hydrogen suppressed the dopaminergic cell loss. Hydrogen water likely retards the development and progression of Parkinson's disease [68]. Drinking H_2 water improved another model of Parkinson's disease induced by MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) [69].

7.8. Prevention of atherosclerosis model

Oxidative stress is accepted to be involved in atherosclerosis [70,71]; however, most clinical trials of dietary antioxidants failed to show marked success in preventing atherosclerotic diseases [9,72,73]. Drinking H_2-dissolved water ad libitum decreased the oxidative stress level in the aorta and prevented arteriosclerosis in an apolipoprotein E knockout mouse [74]. Thus, consumption of H_2-dissolved water has the potential to prevent arteriosclerosis [75] (Fig. 6).

7.9. Improvement of metabolic syndrome

Increased oxidative stress in obesity affects metabolic syndrome [76]. Long-term drinking of H_2 water significantly controlled fat and body weights, despite no increase in the consumption of food and water. Moreover, drinking H_2-water decreased levels of plasma glucose, insulin and triglyceride, the effect of which on hyperglycemia was similar to diet restriction [77]. A mechanistic study revealed that the gene expression of a hepatic hormone, fibroblast growth factor 21 (FGF21) was enhanced, which should function to enhance fatty acid and glucose expenditure. Indeed, H_2 stimulated energy metabolism, as measured by oxygen consumption. These results suggest the potential benefit of H_2 in improving obesity, diabetes and metabolic syndrome [77].

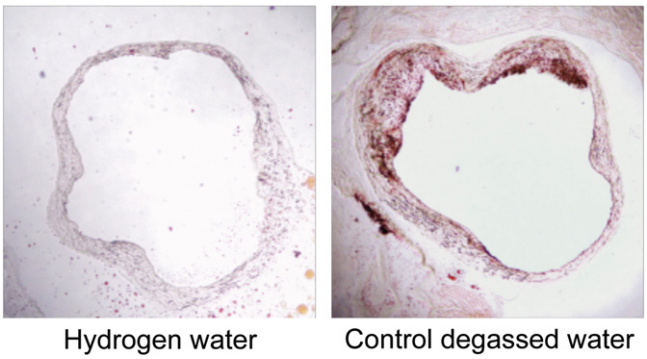

Fig. 6. Preventive effects of drinking hydrogen water on an atherosclerosis model. Drinking H_2 water decreased atherosclerotic lesion. ApoE knockout mice drank H_2 water (left) or degassed control water (right) for 6 months from the age of 2 months old. Representative microscopic images of horizontal sections of the proximal aorta attached to the heart.

7.10. Prevention of adverse effects by an anti-tumor drug

Cisplatin is a widely used anti-cancer drug in the treatment of a wide range of tumors; however, its application is limited by causing nephrotoxicity, which may be mediated by oxidative stress [78]. Inhalation of hydrogen gas (1% H_2 in air) or drinking a saturated level of H_2 water improved mortality and body-weight loss caused by cisplatin, and alleviated nephrotoxicity. Consumption of H_2 water improved the metamorphosis accompanying decreased apoptosis in the kidney. Despite its protective effects against cisplatin-induced toxicity, hydrogen did not impair the anti-tumor activity of cisplatin against cancer cell lines in vitro and in tumor-bearing mice in vivo. Thus, hydrogen, whether H_2 gas or H_2 water, has the potential to improve the quality-of-life of patients during chemotherapy [52].

7.11. Anti-inflammatory effects

It has been reported that H_2 acts as an anti-inflammatory and anti-allergic regulator by inducing inflammatory cytokines, such as TNF-α, IL-6 and some phosphorylating signal factors [79–82].

Some intestinal bacteria, such as *Escherichia coli*, can produce a considerable amount of H_2 by catalyzing with hydrogenase. Kawai et al. examined whether H_2 released from intestinally colonized bacteria could affect Concanavalin A-induced mouse hepatitis. Reconstitution of intestinal flora with H_2-producing *E. coli*, but not hydrogenase-deficient mutant *E. coli*, down-regulated Concanavalin A-induced liver inflammation. These results indicate that H_2 released from intestinal bacteria can suppress inflammation [82].

Sepsis/multiple organ dysfunction syndrome is the leading cause of death in critically ill patients [83]. Hydrogen gas inhalation significantly improved the survival rate and organ damage of septic mice with moderate or severe cecal ligation and puncture by reducing levels of early and late proinflammatory cytokines in serum and tissues, suggesting the potential use of H_2 as a therapeutic agent in the therapy of conditions associated with inflammation-related multiple organ dysfunction syndrome [84].

7.12. Anti-allergic reactions

Itoh et al. demonstrated using a mouse model that drinking H_2 water could attenuate an immediate-type allergic reaction by suppressing the phosphorylation of FcεRI-associated Lyn and its downstream signaling molecules, which subsequently inhibited NADPH oxidase activity and reduced the generation of hydrogen peroxide [81]. These findings imply that the beneficial effects of hydrogen are not only imparted by its radical scavenging activity, but also by modulating a specific signaling pathway.

7.13. Protective effects after organ transplantation

ROS contribute to the development of interstitial fibrosis and tubular atrophy seen in chronic allograft nephropathy. Nakao's group tested the effect of treatment with H_2 water in a model of kidney transplantation, in which allografts from Lewis rats were orthotopically transplanted into Brown Norway recipients that had undergone bilateral nephrectomy. Drinking H_2 water improved allograft function, slowed the progression of chronic allograft nephropathy, reduced oxidant injury and inflammatory mediator production, and improved overall survival. Inflammatory signaling pathways, such as mitogen-activated protein

kinases, were less activated in renal allografts from H_2 water-treated rats as compared with normal water-treated rats. Thus, oral H_2 water is an effective antioxidant and anti-inflammatory agent that reduced chronic allograft nephropathy, improved the survival of rat renal allografts, and may be of therapeutic value in the setting of transplantation [80]. Previously, Nakao's group has demonstrated the marked effects of inhaling hydrogen gas on intestinal graft and lung transplantation [79,85].

7.14. Clinical tests

Clinical tests have revealed that drinking H_2-water reduced oxidative stress markers in patients with type 2 diabetes [86] or subjects with potential metabolic syndrome [87], and influenced glucose [86] and cholesterol metabolism [87]. Hemodialysis using dialysis solution with H_2 significantly decreased the levels of plasma monocyte chemoattractant protein 1 and myeloperoxidase [88].

Mitochondrial disorders seem to induce oxidative stress due to damage, and in turn, oxidative stress enhances mitochondrial damages, worsening the pathogenesis in a vicious circle. Thus, mitochondrial disorders are candidate conditions for application of H_2 water [11].

Dr. Mikio Hirayama (Department of Neurology, Kasugai City Hospital) and his colleagues presented their clinical report of treating a patient with mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episode (MELAS). He reported that a 33-year-old female patient was successfully treated by drinking H_2 water for 18 months, which reduced the frequency of episodic cerebral ischemia. Blood lactate and pyruvate concentrations decreased depending upon the period of drinking H_2 water. HbA1c level and urinary proteins decreased. The patient clinically appeared improved by drinking H_2 water [11].

Dr. Tohru Ibi (Department of Neurology, Aichi Medical School) conducted an open label trial on 4 MELAS patients, one CPEO (chronic progressive external ophthalmoplegia) patient with mitochondrial disorders, and 5 patients with inflammatory myopathies, and demonstrated a marked reduction of several serum markers, including lactate. The patients drank 1 l of H_2 water per day for 12 weeks. He also conducted a double-blind crossover trial; however, the trial showed no significant effects, except on blood lactate concentration. These insignificant results may be likely due to the half-volume of H_2 water and to the limited observation period [11].

Both reports suggest that oral administration of H_2 water seems to be effective for mitochondrial diseases.

8. Advantage of molecular hydrogen

Despite their cytotoxic effects, low concentrations of ROS, such as $\bullet O_2^-$ and H_2O_2 function as signaling molecules and regulate apoptosis, cell proliferation, and differentiation [13,14]. Recent studies have suggested that excessive antioxidants increase mortality and rates of cancer, because they may interfere with some essential defensive mechanisms [12,89,90]. At higher concentrations, H_2O_2 is converted into hypochlorous acid by myeloperoxidase to defend against bacterial invasion [91]. Additionally, nitric oxide (NO•) functions as a neurotransmitter and is essential for the dilation of blood vessels [92]. Thus, cytotoxic radicals such as •OH must be neutralized without compromising the essential biological activities of other ROS including NO•.

Since hydrogen reduces •OH selectively but does not affect $\bullet O_2^-$ and H_2O_2 having physiological roles [10], we suggest that the side effects of H_2 should be small compared to other antioxidants.

9. Issues to be dissolved in future

Although biological benefits have been confirmed by the publication of more than 70 original articles, there remain many issues to be resolved.

The primary molecular target of hydrogen remains unknown. In our first report published in 2007 [10], we indicated that cells cultured in H_2-rich medium were protected against oxidative stress by the •OH-scavenging activity of H_2; however, recent evidence shows that the scavenging property is not the only explanation for the potent beneficial effects of hydrogen. For example, the amount of orally administered H_2 may not be enough to scavenge •OH. In addition, it is likely that the dwell time of H_2 in the body is too short to scavenge the large amount of •OH that are generated continuously.

Several reports have demonstrated an effect on the regulation of gene expressions and protein-phosphorylation; however, the transcriptional factors and kinases involved in the effects afforded by H_2 have not been identified. It also remains unknown whether the regulations are performed directly by H_2. The amount of administered H_2 seems to be, in many cases, independent of the magnitude of effects. Intestinal bacteria produce more than 1 l H_2 gas per day, whereas the amount of H_2 originating from drinking H_2 water is less than 50 ml. Nevertheless, additional H_2 from drinking H_2 water is unambiguously effective. The molecular mechanisms underlying the marked effects of a very small amount of H_2 remain elusive.

10. Evolutionary aspect: Hydrogen was closely involved with the ancestors of mitochondria

Before closing this review article, we would like to briefly refer to the relationship of hydrogen with mitochondria. According to the hydrogenosome hypothesis [93,94], ancestor mitochondria should have produced H_2 using a hydrogenase as some current anaerobic bacteria are doing. Hydrogen would have been provided to methane-producing bacteria, a type of archaea, as an energy source for producing methane in symbiosis ($CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$). Although current mitochondria of higher eukaryotes maintain no hydrogenase for producing or conversely for using H_2, a hypothetic system in mitochondria might have been maintained for using H_2 with essential roles throughout evolution. Future studies might reveal the relationship between hydrogen and mitochondria.

Disclosure

The author declares no conflicts of interest.

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