Chapter Fifteen – Molecular Hydrogen as a Novel Antioxidant: Overview of the Advantages of Hydrogen for Medical Applications

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Abstract

Molecular hydrogen (H$_2$) was believed to be inert and nonfunctional in mammalian cells. We overturned this concept by demonstrating that H$_2$ reacts with highly reactive oxidants such as hydroxyl radical ("OH) and peroxynitrite (ONOO$^-$) inside cells. H$_2$ has several advantages exhibiting marked effects for medical applications: it is mild enough neither to disturb metabolic redox reactions nor to affect signaling by reactive oxygen species. Therefore, it should have no or little adverse effects. H$_2$ can be monitored with an H$_2$-specific electrode or by gas chromatography. H$_2$ rapidly diffuses into tissues and cells to exhibit efficient effects. Thus, we proposed the potential of H$_2$ for preventive and therapeutic applications. There are several methods to ingest or consume H$_2$: inhaling H$_2$ gas, drinking H$_2$-dissolved water (H$_2$-water), injecting H$_2$-dissolved saline (H$_2$-saline), taking an H$_2$ bath, or dropping H$_2$-saline onto the eyes. Recent publications revealed that, in addition to the direct neutralization of highly reactive oxidants, H$_2$ indirectly reduces oxidative stress by regulating the expression of various genes. Moreover, by regulating gene expression, H$_2$ functions as an anti-inflammatory, antiallergic, and antiapoptotic molecule, and stimulates energy metabolism. In addition to growing evidence obtained by model animal experiments, extensive clinical examinations were performed or are under way. Since most drugs specifically act on their specific targets, H$_2$ seems to differ from conventional pharmaceutical drugs. Owing to its great efficacy and lack of adverse effects, H$_2$ has potential for clinical applications for many diseases.

Keywords

Clinical examination; Hydroxyl radical; Inert gas; No adverse effect; Peroxynitrite; Rapid diffusion; Selective reduction

1. Introduction

Molecular hydrogen with the molecular formula H$_2$ is a colorless, odorless, tasteless, nonmetallic, and nontoxic gas at room temperature. Hydrogen gas is flammable and will burn in air at a very wide range of concentrations between 4% and 75% by volume. Its autoignition temperature, the temperature of spontaneous ignition in air, is about 500 °C (http://en.wikipedia.org/wiki/Hydrogen). These facts suggest that H$_2$ is not so dangerous
In daily life when its concentration is under 4%.

In terms of biological reactions in several microorganisms, H₂ is a product of certain types of anaerobic metabolism, usually via reactions catalyzed by iron- or nickel-containing enzymes called hydrogenases (Adams et al., 1980 and Fritsch et al., 2013). H₂ is also enzymatically metabolized as an energy source by providing electrons to the electron transport chain. These enzymes catalyze the reversible redox reaction between H₂ and its constituent two protons and two electrons (van Berkel-Arts et al., 1986).

On the other hand, in all photosynthetic organisms, the water-splitting reaction occurs in the light reactions, where water is decomposed into protons, electrons, and oxygen. Some organisms, including the alga *Chlamydomonas reinhardtii* and cyanobacteria, have evolved a second step in the dark reactions in which protons and electrons are reduced to form H₂ gas by specialized hydrogenases in cyanobacteria or chloroplast (Carrieri, Wawrousek, Eckert, Yu, & Maness, 2011). For industrial uses, extensive efforts have also been undertaken with alga in a bioreactor by genetically modifying cyanobacterial hydrogenases to synthesize H₂ gas efficiently (King, 2013 and van Berkel-Arts et al., 1986).

In contrast, H₂ was accepted to behave as an inert gas in mammalian cells because of the lack of no hydrogenase genes. Thus, it had been believed that H₂ is nonfunctional in our cells. In fact, H₂ seemed to react with no biological compounds, including oxygen (O₂), in the absence of catalysts at body temperature. Indeed, owing to its characteristics, H₂ gas was used for measuring local blood flow (Aukland, Bower, & Berliner, 1964).

We overturned this concept in a publication in 2007 describing that H₂ acts as a therapeutic and preventive antioxidant by selectively reducing highly active oxidants, such as hydroxyl radical (·OH) and peroxynitrite (ONOO⁻) in cultured cells, and that H₂ has cytoprotective effects against oxidative stress (Ohsawa et al., 2007). Since then, a large number of studies have explored therapeutic and preventive effects of H₂. These publications cover many biological effects against oxidative stress in almost all organs (Ohta, 2011 and Ohta, 2012). Moreover, it has been revealed that H₂ has more roles, including anti-inflammatory, antiapoptotic, and antiallergic effects, in most tissues of model animals, and that H₂ stimulates energy metabolism. In addition to publications on model animal experiments, more than 10 papers on clinical examinations have been published. As of 2013, the number of publications on its biologically or medically beneficial effects had surpassed 300 (Ohta, 2014).

2. Comparison of H₂ with Other Medical Gasses

Gas inhalation as disease therapy has recently received attention (Kajimura et al., 2010 and Szabó, 2007). In recent decades, there has been extraordinary and rapid growth in our knowledge of gaseous molecules, including hydrogen sulfide (H₂S), nitric oxide (NO•), and carbon monoxide (CO). H₂S, CO, and NO• are extremely toxic molecules; however, they play important roles as signaling molecules in biological systems (Kimura, 2010 and Motterlini and Otterbein, 2010).

In contrast, H₂ has advantages in terms of toxicity: it has no cytotoxicity even at high concentration (Abraini et al., 1994, Fontanari et al., 2000, Lillo and Parker, 2000 and Lillo et al., 1997). Furthermore, safety standards have been established for high concentrations of hydrogen gas for inhalation since high-pressure hydrogen gas was actually used in deep diving gas mixes to prevent decompression sickness and arterial gas thrombi (Fontanari et al., 2000). The safety of H₂ for humans is demonstrated by its application in Hydreliox, an exotic, breathing gas mixture of 49% H₂, 50% helium, and 1% O₂, which is used to prevent decompression sickness and nitrogen narcosis during very deep technical diving (Abraini et al., 1994, Fontanari et al., 2000, Lillo and Parker, 2000 and Lillo et al., 1997).

As the primary target of H₂S, CO, and NO•, heme-based proteins play central roles.
Integrated approaches revealed the physiological significance of H$_2$S, CO, and NO$^-$ on mitochondrial cytochrome c oxidase, a key target and central mediator of mitochondrial respiration (Kajimura et al., 2010). As far as briefly examined (Ohsawa et al., 2007), H$_2$ does not reduce the oxidized heme of cytochrome c. Thus, the primary target of H$_2$ seems to differ from that of the other medical gaseous molecules.

Moreover, the production of NO$^-$, H$_2$S, or CO is carried out by different enzymes, NO$^-$ synthases, cystathionine γ-lyase/cystathionine β-synthase, or hemeoxygenase-1 (HO-1), respectively (Kashfi & Olson, 2013). In contrast, as mentioned above, mammalian cells have no enzyme for producing intracellular H$_2$.

Regarding the interaction between H$_2$ and the other toxic medical gasses, combined therapy with H$_2$ and CO demonstrated additional therapeutic efficacy via both antioxidant and anti-inflammatory mechanisms, and may be a clinically feasible approach for preventing ischemia/reperfusion injury in the myocardium (Nakao et al., 2010). Breathing NO$^-$ plus H$_2$ during ischemia/reperfusion reduced the infarct size and maintained cardiac function, and reduced the generation of myocardial nitro-tyrosine associated with NO$^-$ inhalation (Shinbo et al., 2013). These findings suggest that the target of H$_2$ differs from those of CO and NO$^-$.

3. Oxidative Stress as Pathogenic Sources

First, the author would like to introduce how the biological function of H$_2$ was discovered regarding its contribution to reducing oxidative stress.

Reactive oxygen species (ROS) are generated inside the body during daily life as a by-product of energy metabolism by oxidative phosphorylation in every aerobic organism. Occasionally, excess ROS are produced, such as by smoking or air pollution, exposure to ultraviolet or irradiation rays, intense exercise, and physical or psychological stress (Agarwal, 2005, Grassi et al., 2010, Harma et al., 2006, Liu et al., 1996 and Tanriverdi et al., 2006). When ROS are produced excessively or endogenous antioxidant capacity is diminished, indiscriminate oxidation elicits harmful effects, resulting in “oxidative stress.”

Acute oxidative stress arises from various different situations: inflammation, ischemia/reperfusion in cardiac or cerebral infarction, organ transplantation, and cessation of operative bleeding, among others (Ferrari et al., 1991, Reuter et al., 2010 and Vaziri and Rodriguez-Iturbe, 2006). Under normal conditions, ROS induced by strenuous exercise result in muscle fatigue (Westerblad & Allen, 2011). Evidence has established strong links between chronic oxidative stress and a wide variety of pathologies, including malignant diseases, diabetes mellitus, atherosclerosis, and chronic inflammatory processes, as well as many neurodegenerative diseases and the aging process (Andersen, 2004, El Assar et al., 2013 and Kim and Byzova, 2014).

As a first step in generating ROS, superoxide anion radicals (O$_2^-$) are the primary ROS mostly generated by electron leakage from the mitochondrial electron transport chain (Andersen, 2004, Finkel and Holbrook, 2000, Lin and Beal, 2006 and Turrens, 2003). Other enzymes, including NADPH oxidases, cytochrome p450s, lipoxygenase, cyclooxygenase, and xanthine oxidase, also participate in ROS generation in the immune- or detoxifying system (Droge, 2002). Superoxide dismutase enzymatically converts O$_2^-$ to hydrogen peroxide (H$_2$O$_2$), which is metabolized to generate water (H$_2$O). Highly reactive *OH is generated from H$_2$O$_2$ or O$_2^-$ via the Fenton or Weiss reaction in the presence of catalytically active metals, such as Fe$^{2+}$ and Cu$^+$. Reaction of *O$_2^-$ with NO$^-$ generates ONOO$^-$, which is a very active nitrogen species (RNS) (Radi, 2013). *OH is the major cause of the oxidation and destruction of biomolecules by direct reaction or by triggering the chain reaction of free radicals (Lipinski, 2011). Ionizing radiation, including cosmic rays, also generates *OH as a damaging intermediate through the reaction with water, a process termed radiolysis (Schoenfeld et al., 2012 and Schoenfeld et al., 2011).
Although antioxidation therapy or prevention of various diseases is expected owing to the clinical importance of oxidative damage, many antioxidants have been of limited therapeutic success (Steinhubl, 2008). Antioxidant supplements have exhibited little effect on preventing cancer, myocardial infarction, and atherosclerosis, but conversely have increased mortality (Bjelakovic et al., 2007, Brambilla et al., 2008, Hackam, 2007, Hercberg et al., 2010 and Steinhubl, 2008).

4. Physiological Roles of H$_2$O$_2$

As mentioned above, ROS had historically been believed to cause cellular damage and to lack physiological functions; however, cellular redox homeostasis is a delicate balance between ROS production and the antioxidant system (Bashan et al., 2009 and Brewer et al., 2013). Some ROS are now appreciated to function as signaling molecules to regulate a wide variety of physiological process (Bell et al., 2007 and Liu et al., 2005). H$_2$O$_2$ was shown to be required for cytokine, insulin, growth factor, AP-1, c-Jun N-terminal kinase 1, p53, and nuclear factor kappa B signaling and to promote phosphatase inactivation by cysteine oxidation (Chandel, Trzyna, McClintock and Schumacker, 2000, Chandel, Vander Heiden, Thompson and Schumacker, 2000 and Finkel, 1998). These reactions provide a plausible biochemical mechanism by which ROS can impinge on signaling pathways (Collins et al., 2012).

Additionally, oxidative stress caused by H$_2$O$_2$ and NO$^•$ induces enzymes involved in antioxidation and tolerance to protect cells against oxidative stress (Endo et al., 2009 and Ristow and Zarse, 2010). For example, translocation of NF-E2-related factor 2 (Nrf2) into the nucleus leads to the regulation of gene expression involved in defense systems against oxidative stress (Jazwa & Cuadrado, 2010) and other toxic sources including heavy metals (Gan & Johnson, 2014). Moreover, H$_2$O$_2$ is a key factor to regulate cellular differentiation (Tormos et al., 2011 and Tsukagoshi et al., 2010), the immune system (West et al., 2011 and Zhou et al., 2011), autophagy (Garg et al., 2013 and Li et al., 2012), and apoptosis (Mates, Segura, Alonso, & Marquez, 2012). Thus, it is crucial for functional H$_2$O$_2$ not to be completely eliminated in order to maintain homeostasis; as such, it is very important to be aware of side effects when developing an effective antioxidant for the prevention of oxidative stress-related diseases.

Unexpectedly, recent notable studies have suggested that excessive antioxidants increased mortality and rates of cancer (Bjelakovic et al., 2007, Bjelakovic et al., 2008, Gray et al., 2008, Hackam, 2007, Hercberg et al., 2010 and Walker, 2008) probably because they may interfere with some essential defensive mechanisms (Bjelakovic and Gluud, 2007, Bjelakovic et al., 2008, Carriere et al., 2004, Chandel et al., 1998, Mandal et al., 2010, Miller et al., 2005 and Salganik, 2001). Against this background, an ideal antioxidant is expected to mitigate excess oxidative stress, but not disturb redox homeostasis. In other words, an ideal molecule should not reduce signaling molecules, such as H$_2$O$_2$ but should effectively reduce strong oxidants, such as ‘OH.

Since H$_2$ reduces ‘OH but does not react with ‘O$_2^−$, H$_2$O$_2$, and NO$^•$ that have physiological roles (Ohsawa et al., 2007), we propose that the adverse effects of H$_2$ are very small compared with those of other antioxidants. Thus, we have reached the conclusion that the ideal antioxidant could be H$_2$.

5. Measurement of H$_2$ Gas Concentration

H$_2$ gas concentration is measureable by gas chromatography. Additionally, H$_2$ concentration dissolved in a solution can be measured by this method. For example, H$_2$ in blood can be monitored by the following method: Venous or arterial blood (e.g., 5 ml) is collected in a closed aluminum bag with no dead space, followed by the addition of a defined volume of air (e.g., 30 ml) into the bag. After complete transfer of the H$_2$ gas from the blood to the air in the closed bag, H$_2$ can be measured by gas chromatography (Fig. 1). The inhalation of H$_2$ actually increased H$_2$ dissolved in arterial blood in a hydrogen gas concentration-dependent manner, and the H$_2$ levels in venous blood were lower than
in arterial blood; the different level between arterial and venous blood indicates the amount of H₂ incorporated into and consumed by tissues (Ohsawa et al., 2007). In a clinical examination, Ono et al. also showed a difference in H₂ concentrations between arterial and venous blood (Ono et al., 2012).

Figure 1. Incorporation of H₂ into blood by inhaling hydrogen gas. Rats inhaled a mixed gas of H₂ (1% or 2%) and O₂ (30%) under anesthetic N₂O and halothane for 1 h, and arterial (indicated by A) or venous blood (indicated by V) was collected into a closed aluminum bag from a three-way stopper (upper panel). After the transfer of H₂ into an accurate volume of air phase from the blood, amounts of H₂ were examined by gas chromatography. Lower panel shows profiles of gas chromatography. The vertical scale indicates the amounts of blood H₂ after calculations.

Adapted from after Ohsawa et al. (2007) modified version of Fig. 5A, with permission from Nature Publishing Group.

H₂ concentration can be measured using an H₂ electrode that specifically detects H₂; however, this sensor is also somewhat sensitive to H₂S. Thus, when H₂S is contaminated in a solution, one must take into consideration its effects.

H₂ can be measured in tissues using a needle-type H₂ sensor (Unisense, Aarhus, Denmark). The electrode current was measured with a picoammeter (Keithley, Cleveland, Ohio) attached to a recorder. The negative current obtained from the H₂ sensor was converted to regional H₂ concentration using a calibration curve generated from known levels of H₂-saturated saline.

6. Advantages of Hydrogen in Medical Applications

6.1. Selective reaction of H₂ with highly reactive ROS

H₂ dissolved in culture medium did not change the cellular levels of *O₂*⁻ and H₂O₂, as judged by the fluorescent signals of MitoSOX and dichlorofluorescein-diacetate (DCF-DA), respectively (Ohsawa et al., 2007). Additionally, H₂ did not decrease the cellular level of NO⁻. In contrast, H₂ treatment significantly decreased levels of *OH*, as judged by the decrease in the fluorescent signal of hydroxyphenyl fluorescein (HPF) (Setsukinai, Urano, Kakinuma, Majima, & Nagano, 2003).

In terms of the experimental protocol, culture media containing H₂ were prepared as follows: H₂ was dissolved beyond the saturated level into DMEM medium under 0.4 MPa pressure of hydrogen gas for 2 h, O₂ was also dissolved into another medium by bubbling, the third medium contained CO₂, and then fetal bovine serum was supplemented to 1% in all three media. The three media were combined at various ratios to obtain the desired concentration of H₂ and 8.5 mg/l of O₂ at 25 °C. For culture,
combined media were put into a culture flask and immediately examined for \( \text{H}_2 \) or \( \text{O}_2 \) concentration with an \( \text{H}_2 \) or \( \text{O}_2 \) electrode, and in turn gas composed of the desired ratio of \( \text{H}_2 \) and \( \text{N}_2 \) (\( \text{H}_2 + \text{N}_2 = 75\% \)), 20\% of \( \text{O}_2 \), and 5\% of \( \text{CO}_2 \) was filled into the culture flask; for example, when the medium contained 0.6 \( \text{mM} \) \( \text{H}_2 \), the \( \text{H}_2 \) gas was adjusted to 75\%. The mixed gas was obtained by regulating the flow rates of its constituents with connected flow meters. As a control, degassed medium lacking \( \text{H}_2 \) was prepared by stirring medium that had been saturated with \( \text{H}_2 \) in an open vessel for 4 h, and the concentration of \( \text{H}_2 \) was checked with an \( \text{H}_2 \) electrode.

Then, PC12 cells were incubated in medium with or without 0.6 \( \text{mM} \) \( \text{H}_2 \), and exposed to antimycin A or \( \text{L} \)-NAME (\( \text{N}^6 \)-nitro-\( \text{L} \)-arginine methyl ester) to induce \( \cdot \text{OH} \), \( \cdot \text{O}_2^- \), and \( \text{H}_2\text{O}_2 \). Fluorescent images of MitoSOX-, DCF-DA (2′,7′-dichlorodihydrofluorescein)-, HPF-, and DAF-2 DA (diaminofluorescein-2 diacetate)-treated cells were obtained by laser-scanning confocal microscopy (Olympus FV300) to estimate intracellular \( \cdot \text{O}_2^- \), \( \text{H}_2\text{O}_2 \), \( \cdot \text{OH} \), and \( \text{NO} \), respectively (Fig. 2).

![Figure 2](image_url)

Figure 2.
Selective reduction of reactive oxygen or nitrogen species by \( \text{H}_2 \) in cultured cells. PC12 cells were kept in medium with or without 0.6 \( \text{mM} \) \( \text{H}_2 \) (indicated by + \( \text{H}_2 \)) or without \( \text{H}_2 \) (indicated by − \( \text{H}_2 \)), and exposed to antimycin A or \( \text{L} \)-NAME (\( \text{N}^6 \)-nitro-\( \text{L} \)-arginine methyl ester) to induce \( \cdot \text{OH} \), \( \cdot \text{O}_2^- \), \( \text{H}_2\text{O}_2 \), and \( \text{NO} \). Each ROS or RNS was detected using flowing fluorescent dye; HPF, \( \text{H}_2\text{DCF} \) (2′,7′-dichlorodihydrofluorescein), MitoSOX, and DAF-2 DA (diaminofluorescein-2 diacetate) were used to detect \( \cdot \text{OH} \), \( \cdot \text{O}_2^- \), and \( \text{NO} \), respectively. These representative fluorescence images obtained by laser-scanning confocal microscopy demonstrate the selective reduction of \( \cdot \text{OH} \) by \( \text{H}_2 \).

Adapted from Ohsawa et al. (2007) modified version of Fig. 1A, B and supplementary Fig. 1A, C, with permission from Nature Publishing Group.

Alternatively, PC12 cells were exposed to intracellular \( \cdot \text{OH} \) produced by the Fenton reaction (\( \text{H}_2\text{O}_2 + \text{Cu}^+ \rightarrow \text{OH} + \text{OH}^- + \text{Cu}^{2+} \)), with or without 0.6 \( \text{mM} \) \( \text{H}_2 \). Cells were preincubated with 1 \( \text{mM} \) \( \text{CuSO}_4 \), washed, and exposed for 1 h to 0.1 \( \text{mM} \) ascorbate (Vit. C) in order to reduce intracellular \( \text{Cu}^{2+} \) to \( \text{Cu}^+ \). In this case, endogenous \( \text{H}_2\text{O}_2 \) would be sufficient to produce \( \cdot \text{OH} \). \( \text{H}_2 \) indeed protected the cells against \( \cdot \text{OH} \).

Moreover, the decrease in the cellular \( \cdot \text{OH} \) level by \( \text{H}_2 \) was confirmed by spin-trapping technology (Halliwell & Gutteridge, 1992). Standard electron spin resonance (ESR) signals of the DMPO - \( \cdot \text{OH} \) radical were obtained by trapping \( \cdot \text{OH} \) with a spin-trapping reagent (DMPO). PC12 cells were preincubated with 0.1 \( \text{M} \) DMPO and 2 \( \text{mM} \) \( \text{CuSO}_4 \) for 30 min at 37 °C with or without 0.6 \( \text{mM} \) \( \text{H}_2 \). After removal of this medium, the cells were treated with 0.2 \( \text{mM} \) ascorbate and 0.1 \( \text{mM} \) \( \text{H}_2\text{O}_2 \) for 5 min at 23 °C to produce \( \cdot \text{OH} \) by the Fenton reaction, and then scraped into a flat cuvette for ESR measurement. Alternatively, PC12 cells were incubated in PBS containing 0.1 \( \text{M} \) DMPO and 30 \( \text{g/ml} \) antimycin A for 7 min at 23 °C to produce excess \( \cdot \text{OH} \), with or without 0.6 \( \text{mM} \) \( \text{H}_2 \), and then scraped into a flat cuvette for ESR measurement.

The selective reduction of ROS can be explained by the marked oxidative strength of \( \cdot \text{OH} \). In other words, \( \cdot \text{OH} \) is strong enough to react with even inert \( \text{H}_2 \), but that \( \cdot \text{O}_2^- \),
H₂O₂, and NO• are insufficient to react with H₂ according to their activities. Namely, H₂ is mild enough neither to disturb metabolic redox reactions nor to affect ROS that function in cellular signaling (Fig. 3).

Figure 3.
Relative oxidative activities in each reactive oxygen and nitrogen species. "OH and ONOO− are highly reactive to damaged cells, whereas "O₂•, NO•, and H₂O₂ have physiological roles as signaling molecules. This graph is based on data from a previous publication (Setsukinai et al., 2003).

6.2. Rapid diffusion
Most hydrophilic antioxidants cannot penetrate biomembranes and most hydrophobic antioxidants remain on the membranes. In contrast, H₂ can be infused into lipids as well as aqueous solutions. It has favorable distribution characteristics having the physical ability to penetrate biomembranes and diffuse into the cytosol, as illustrated in Fig. 4.

Figure 4.
Illustration of gaseous diffusion of H₂ into a cell. Most hydrophilic compounds are retained at membranes and cannot reach the cytosol, whereas most hydrophobic ones cannot penetrate biomembranes in the absence of specific carriers. In contrast, H₂ can be rapidly distributed into cytosol and organelles. On the membrane, "OH triggers the initiation of a free radical chain reaction to generate lipid peroxides, which are converted to some oxidative stress markers, 4-hydroxyl-2-nonenal (4-HNE), and malondialdehyde (MDA). In the nucleus, "OH oxidizes DNA for modification to 8-OHdG (8-hydroxy-deoxyguanine).

Despite the clinical importance of overcoming oxidative damage, antioxidants had limited therapeutic success. This may be because most antioxidants do not reach specific regions (Murphy, 1997, Murphy and Smith, 2000 and Smith and Murphy, 2011). As H₂ effectively reaches the nucleus and mitochondria, the protection of nuclear DNA and mitochondria suggests preventive effects against lifestyle-related diseases, cancer, and the aging process (Ohsawa et al., 2007). Moreover, H₂ passes through the blood brain...
barrier, although most antioxidant compounds cannot; this is also an advantage of H₂.

The gaseous diffusion of H₂ can be monitored inside various tissues by detection with a specific H₂ electrode. For example, H₂ concentration has been monitored within the rat myocardium. The electrode was inserted into the "at-risk" area for infarction to estimate the diffusion of H₂ into the ischemic myocardium area after coronary artery occlusion. H₂ concentration was increased by its diffusion, even with coronary artery occlusion (Hayashida et al., 2008) (Fig. 5).

Moreover, we devised eye drops with dissolved H₂ to administer H₂ to the retina directly, and monitored the time course of changes in H₂ levels using the needle-shaped hydrogen sensor electrode inserted through the sclera to the vitreous body in rats. H₂ could reach the vitreous body by administering H₂ saturated in normal saline. When H₂ eye drops were administered continuously, approximately 70% H₂ was detected on the ocular surface (Oharazawa et al., 2010).

These experiments indicate that H₂ can rapidly diffuse into tissues even without blood flow.

7. Methods of Ingesting Molecular Hydrogen

7.1. Inhalation of hydrogen gas

Inhalation of H₂ gas is the most straightforward therapeutic method. H₂ gas can be inhaled through a ventilator circuit, facemask, or nasal cannula. Since inhaled H₂ gas acts rapidly, it may be suitable for defense against acute oxidative stress. In particular, inhalation of gas does not affect blood pressure (Ohsawa et al., 2007); on the other hand, drip infusion of drugs increases blood pressure and causes serious obstacles during the treatment of myocardial infarction. In particular, excess oxidative stress gives damages to tissues at the time of the initiation of reperfusion. Notably, most antioxidants cannot reach the at-risk area for infarction before initiating reperfusion. As pointed out above, H₂ can reach the region without blood flow by rapid diffusion (Fig. 5).

By a clinical examination, Ono et al. monitored H₂ and showed that inhalation of 3–4% H₂ gas did not affect any physiological parameters, suggesting no adverse effects (Ono et al., 2012).

7.2. Oral ingestion by drinking hydrogen water

Inhalation of H₂ gas is actually unsuitable or impractical for continuous H₂ consumption.
in daily life for preventive use. In contrast, solubilized H₂ (H₂-dissolved water; i.e., H₂-water) may be beneficial since it is a portable, easily administered, and safe way to ingest H₂ (Nagata et al., 2009 and Ohsawa et al., 2008). H₂ can be dissolved in water up to 0.8 mM (1.6 mg/l) under atmospheric pressure at room temperature without any change of pH.

H₂-water can be made by several methods: infusing H₂ gas into water under high pressure, electrolyzing water to produce H₂, and reacting magnesium metal or its hydride with water. These methods may be applicable not only to water but also to other solvents. H₂ penetrates glass and plastic walls of any vessel in a short time, while aluminum containers can retain H₂ for a long time.

In brief, for experimental treatments, H₂ was dissolved in water under high pressure (0.4 MPa) to a supersaturated level and the saturated H₂-water was stored under atmospheric pressure in an aluminum bag with no dead volume. Mice were given water freely using closed glass vessels equipped with an outlet line containing two ball bearings, which kept the water from being degassed. The vessel was refilled with fresh H₂-water at the same time (e.g., at 4:00 pm) every day.

When water saturated with H₂ was placed into the stomach of a rat, H₂ was detected at several micromoles in blood (Nagata et al., 2009 and Nakashima-Kamimura et al., 2009). In addition, a rat received H₂-water (0.8 mmol/l H₂ in water) orally by stomach gavage, for example, at 15 ml/kg. Hepatic H₂ was monitored with a needle-type hydrogen electrode (Kamimura, Nishimaki, Ohsawa, & Ohta, 2011) (Fig. 6).

Furthermore, after seven adult volunteers had drunk H₂-water, the H₂ content of their expired breath was measured by gas chromatography with a semiconductor (Shimouchi, Nose, Shirai, & Kondo, 2012). The ingestion of H₂-water rapidly increased breath H₂.
content to its maximal level 10 min after ingestion, which thereafter decreased to the baseline level within 60 min. H₂ lost from the water during the experimental procedures accounted for 3% or less of the total. The rate of H₂ release from the skin surface was estimated as approximately 0.1%. On the basis of the remaining H₂ mass balance, approximately 40% of H₂ that had been drunk was consumed inside the body. This report suggests that exogenous H₂ is at least partially trapped by oxygen radicals, such as •OH (Shimouchi et al., 2012).

7.3. Injection of hydrogen-saline

H₂ is intravenously or intraperitoneally injectable as H₂-saline (H₂-dissolved saline), which allows the delivery of H₂ with great efficacy in model animals (Cai et al., 2009, Li et al., 2013 and Sun et al., 2011).

Nagatani et al. performed an open-label, prospective, nonrandomized study of intravenous H₂ administration in 38 patients hospitalized for acute ischemic stroke. All patients received an H₂ intravenous solution immediately after the diagnosis of acute ischemic stroke. Data from this study indicated that an H₂ intravenous solution is safe for patients with acute cerebral infarction, including patients treated with tissue-plasminogen activator (Nagatani et al., 2013).

To rats, H₂-water, H₂-saline, and hydrogen gas were orally administered, intraperitoneally or intravenously injected, and inhaled, respectively. A method for determining the H₂ concentration was applied using high-quality sensor gas chromatography, after which the specimen was prepared via tissue homogenization in airtight tubes. The hydrogen concentration reached a peak at 5 min after oral and intraperitoneal administration, compared with 1 min after intravenous administration. These results indicate that H₂ can reach most organs or blood independently by the three methods (Liu et al., 2014).

7.4. Direct incorporation of molecular hydrogen by diffusion: Eye drops, bath, and cosmetics

Alternatively, H₂-loaded eye drops were prepared by dissolving H₂ in saline and directly administering them to the ocular surface (Kubota et al., 2011 and Oharazawa et al., 2010).

H₂ should easily penetrate the skin and is distributed throughout the body via blood flow. Thus, taking a warm water bath with dissolved H₂ is a method of incorporating H₂ into the body in daily life. It takes only 10 min for it to be distributed throughout the whole body, as judged by measuring H₂ gas in expiration (unpublished results). Indeed, powders that can be used to produce H₂ baths are commercially available in Japan.

H₂ delivery to cardiac grafts during cold preservation using a hydrogen-supplemented water bath efficiently ameliorated myocardial injury due to cold ischemia and reperfusion. This device to saturate organs with H₂ during cold storage merits further investigation for possible therapeutic and preventative use during transplantation (Noda et al., 2013).

7.5. Maternal intake of H₂

H₂ intake helps prevent the hippocampal impairment of offspring induced by ischemia/reperfusion during pregnancy (Mano et al., 2014). The effects of H₂ on rat fetal hippocampal damage caused by ischemia and reperfusion in pregnancy were examined with the transient occlusion of bilateral utero-ovarian arteries. Starting 2 days before the operation, the mothers were provided with H₂-saturated water ad libitum until vaginal delivery. A significant increase in the concentration of H₂ in the placenta was observed after the oral administration of H₂-saturated water to the mothers, with less placental oxidative damage after ischemia and reperfusion in the presence of H₂. Neonatal growth retardation was observed in the ischemia/reperfusion group, which was alleviated by H₂ administration. Maternal H₂ administration improved oxidative stress and the reference
memory of the offspring to the sham level after ischemia and reperfusion injury during pregnancy. Thus, this finding supports the idea that maternal H\textsubscript{2} intake helps prevent the impairment of offspring induced by oxidative stress.

8. Medical Effects of H\textsubscript{2}

8.1. Acute oxidative stress by ischemia/reperfusion

As a type of acute oxidative stress, ischemia/reperfusion induces serious oxidative stress, and its injuries should be considered in many clinical treatments. Inhalation of H\textsubscript{2} gas improved ischemia/reperfusion injuries in cerebral (Ohsawa et al., 2007) and myocardial infarction (Hayashida et al., 2008 and Yoshida et al., 2012). Hydrogen-saline protected against renal ischemia/reperfusion injury (Wang et al., 2011). All clinical manifestations related to postcardiac arrest (CA) syndrome are attributed to ischemia/reperfusion injury in various organs, including the brain and heart. H\textsubscript{2} gas inhalation yielded great improvement in survival and the neurological deficit score in post-CA syndrome in a rat model (Hayashida et al., 2012). H\textsubscript{2} also mitigated damage during the transplantation of various organs in the form of H\textsubscript{2} gas (Buchholz et al., 2008), H\textsubscript{2}-water (Cardinal et al., 2010), and H\textsubscript{2}-preservation solution (Noda et al., 2013). A clinical study showed a positive effect of H\textsubscript{2} on patients with acute brain stem infarction (Ono et al., 2011). These acute effects may be due to the direct reduction of oxidative stress by H\textsubscript{2} because no lag time was necessary.

8.2. Chronic oxidative stress loading to neurodegeneration

Chronic oxidative stress is accepted as one of the causes of neurodegeneration, including dementia and Parkinson's disease (PD) (Andersen, 2004 and Federico et al., 2012). Experimental oxidative stress in the brain can be induced by chronic physical restraint stress and can impair learning and memory (Abrous et al., 2005 and Liu et al., 1996). Drinking H\textsubscript{2}-water suppressed the increase in this oxidative stress and prevented this cognitive impairment (Nagata et al., 2009). In PD, mitochondrial dysfunction and associated oxidative stress are major causes of dopaminergic cell loss in the substantia nigra (Schapira, 2008 and Yoritaka et al., 1996). H\textsubscript{2} in drinking water was given before or after stereotactic surgery for 6-hydroxydopamine-induced nigrostriatal degeneration in a rat model of PD. H\textsubscript{2}-water prevented both the development and the progression of nigrostriatal degeneration in rats (Fu et al., 2009). Moreover, drinking H\textsubscript{2}-water also suppressed dopaminergic neuronal loss in another PD mouse model induced by MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) (Fujita et al., 2009). In a placebo-controlled, randomized, double-blind, parallel-group clinical pilot study, the efficacy of H\textsubscript{2}-water in patients with PD was assessed for 48 weeks. Total Unified Parkinson's Disease Rating Scale (UPDRS) scores in the H\textsubscript{2}-water group significantly improved, whereas UPDRS scores in the placebo group worsened (Yoritaka et al., 2013).

8.3. Stimulatory effects on energy metabolism

Obesity induces oxidative stress (Matsuda & Shimomura, 2013). H\textsubscript{2}-water significantly alleviated fatty liver in db/db mice, which are type 2 diabetes model mice with obesity, as well as high-fat diet-induced fatty liver in wild-type mice. Long-term H\textsubscript{2}-water drinking significantly decreased fat and body weights, despite no increase in the consumption of diet and water, in db/db mice, and decreased levels of plasma glucose, insulin, and triglyceride by stimulating energy metabolism (Kamimura et al., 2011). Analysis of gene expression revealed that a hepatic hormone, fibroblast growth factor 21 (FGF21), showed increased expression upon drinking H\textsubscript{2}-water (Kamimura et al., 2011). FGF21 functions to stimulate fatty acid and glucose expenditure. Thus, H\textsubscript{2}-water stimulates energy metabolism (Kamimura et al., 2011). Beneficial roles of H\textsubscript{2}-water in the prevention of potential metabolic syndrome were also reported by a clinical study (Song et al., 2013).

8.4. Anti-inflammatory effects
Inflammation is closely involved in oxidative stress. H$_2$-reduced inflammation in experimental model animals induced by concanavalin A and dextran sodium sulfate (Kajiya, Silva, Sato, Ouhara, & Kawai, 2009), lipopolysaccharide (Chen et al., 2013 and Xu et al., 2012), Zymosan, an inducer of generalized inflammation and polymicrobial sepsis (Li et al., 2013). H$_2$ gas, H$_2$-saline, and H$_2$-water decreased the levels of proinflammatory cytokines to suppress inflammation. Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by the destruction of bone and cartilage. The symptoms of RA were significantly improved with H$_2$-water (Ishibashi et al., 2012).

In terms of the current state of knowledge, H$_2$ exhibits not only antioxidative effects but also affects many phenotypes in various model animals. H$_2$ has many beneficial effects on animal models and patients besides its antioxidative effects: anti-inflammation, antiapoptosis, anti-allergy, and stimulation of energy metabolism (Ohta, 2011, Ohta, 2012 and Ohta, 2014). Their mutual relationships are not clear, but the reduction of oxidative stress may primarily lead to various subsequent effects. H$_2$ seems to exhibit a variety of phenotypic effects toward improving many pathogenic states by regulating the expression of various genes. The molecules encoding by these genes are, probably, not primary responders to H$_2$, but indirectly act to enable the various effects of H$_2$. The primary target of H$_2$ remains unknown.

9. Possible Molecular Mechanisms Underlying Various Effects of Molecular Hydrogen

9.1. Direct reduction of hydroxyl radicals with molecular hydrogen

H$_2$ was shown to reduce •OH in an experiment using cultured cells (Ohsawa et al., 2007). Later, it was shown that H$_2$ eye drops directly decreased •OH induced by ischemia/reperfusion in retinas (Oharazawa et al., 2010). Moreover, it has been demonstrated that, at the tissue level, H$_2$ neutralized •OH that had been induced by ionizing irradiation in testes, as judged by the decreased HFP signal, and exhibited a radioprotective role (Chuai et al., 2012).

Considering the reaction rate of •OH with H$_2$ in dilute aqueous solutions, this rate may be too slow to enable fully a decrease in •OH in order to exhibit its beneficial roles (Buxton, Greenstock, Helman, & Ross, 1988). Mammalian cells are, however, highly structured with complicated biomembranes and viscous solutions with multiple concentrated components. Since collision frequency is rate-limiting in a viscous environment, the marked diffusion rate of H$_2$ could be advantageous to overcome the slow reaction rate constant. •OH is known as a major trigger of the chain reaction of free radicals (Niki, 2009). Once this chain reaction occurs on biomembranes, it continues and expands causing serious damage to cells (Fig. 4). H$_2$ accumulates in the lipid phase more than in the aqueous phase, especially in unsaturated lipid regions, which are the major target of the initial chain reaction (unpublished results). Thus, H$_2$ may have an advantage to suppress the chain reaction, which produces lipid peroxide, and leads to the generation of oxidative stress markers, such as 4-hydroxyl-2-nonenal (4-HNE) and malondialdehyde (MDA) (Niki, 2014). Indeed, H$_2$ decreased these oxidative markers in many studies (Ning et al., 2013, Ohsawa et al., 2008 and Zhou et al., 2013). Additionally, •OH can modify deoxyguanine (dG) to 8-hydroxy-deoxyguanine (8-OHdG) (Delaney et al., 2012 and Kawai et al., 2012) (Fig. 4). H$_2$ decreased the level of 8-OHdG in most of the examined patients and animals (Ishibashi et al., 2012 and Kawai et al., 2012).

These experimental observations suggest that sufficient H$_2$ can efficiently mitigate tissue oxidation induced by •OH. However, when animals or humans drink H$_2$-water, it is not clear whether H$_2$-water provides a sufficient amount of H$_2$ to scavenge •OH efficiently (Fig. 7).
9.2. Direct reduction of peroxynitrite with molecular hydrogen to regulate gene expression

As another molecular mechanism, the scavenging of ONOO\(^-\) by H\(_2\) should be considered. ONOO\(^-\) is known to modify tyrosine of proteins to generate nitro-tyrosine (Radi, 2013). Several studies have shown that H\(_2\) efficiently decreases nitro-tyrosine in animal models regardless of whether H\(_2\)-water (Cardinal et al., 2010), H\(_2\) gas (Shinbo et al., 2013), or H\(_2\)-saline (Chen et al., 2010, Yu et al., 2011, Zhang et al., 2011 and Zhu et al., 2011) is used. Moreover, drinking H\(_2\)-water decreased nitro-tyrosine in patients with RA (Ishibashi et al., 2012). Thus, at least part of the effect of H\(_2\) can be attributed to the decreased production of nitro-tyrosine in proteins.

Many protein factors involved in transcriptional control are nitrolated (–O-NO\(_2\)) or nitrosolated (–S-NO\(_2\)). It is possible that the decrease in –O-NO\(_2\) or –S-NO\(_2\) may regulate the expression of various genes (Radi, 2013). However, major targets have not been identified and are under investigation.

9.3. Indirect reduction of oxidative stress by regulating gene expression

H\(_2\) reduces oxidative stress not only directly, but also indirectly, by inducing antioxidation systems, including HO-1 SOD (Zhai et al., 2013), catalase (Cai, Zhang, Yu, & Cai, 2013), and myeloperoxidase (Zhang et al., 2011). Nrf2 is known to function as a defense system against oxidative stress and various poisons by inducing various genes including HO-1. HO-1, a microsomal enzyme degrading heme to carbon monoxide, free iron, and biliverdin, participates in the cell defense against oxidative stress (Jazwa & Cuadrado, 2010).

In Nrf2-deficient mice, mitigating effects by the inhalation of H\(_2\) gas declined in hyperoxic lung injury accompanying by a decrease in HO-1, indicating that H\(_2\) gas can ameliorate hyperoxic lung injury in an Nrf2-dependent manner (Kawamura et al., 2013). Activation of Nrf2 is also required for the amelioration of cerebral ischemia–reperfusion injury in rats by H\(_2\) (Zhai et al., 2013).

H\(_2\) influences some signal transductions as an indirect modulator; however, it is unlikely that H\(_2\) could directly bind to some receptors involved in the signal transductions. The primary target molecule of H\(_2\) has not been identified in these signal transduction pathways. These regulatory molecules are, probably, not primary responders to H\(_2\), but
10. Unresolved Questions and Closing Remarks

H₂ can be incorporated or ingested into the body by various methods: inhalation of H₂ gas, drinking H₂-infused water (H₂-water), injection of H₂-infused saline, and incorporation through the skin. Drinking H₂-water was efficacious for various disease models and patients; however, H₂ can be infused up to only 0.8 mM under atmospheric pressure and drinking H₂-water provides a blood H₂ concentration up to only ~ 10 µM with short dwelling time in the body (Nagata et al., 2009 and Nakashima-Kamimura et al., 2009). Moreover, inhaling 1–4% (vol/vol) of H₂ gas was effective, by which H₂ should reach 8–32 µM in blood. Under these conditions, H₂ should be insufficient to scavengé ‘OH for fully exhibiting H₂ benefits because the direct reaction rate of ‘OH with H₂ in an aqueous solution may be too slow to decrease ‘OH (Buxton et al., 1988) as pointed out earlier (Wood & Gladwin, 2007). Thus, it remained elusive how such low levels of H₂ with a short dwelling time could effectively compete with the numerous cellular targets in chronic or acute pathogenesis. Unexpectedly, H₂ was shown to regulate the expression of many genes and the phosphorylation of factors involved in various types of signal transduction to exhibit various phenotypes. For example, drinking H₂-water reduces the gene expressions of proinflammatory cytokines to relieve inflammation, as mentioned above, FGF21 to stimulate energy metabolism (Kamimura et al., 2011), and Grelin for neuroprotection (Matsumoto et al., 2013). However, it essentially remains unsolved what the primary target of H₂ is.

Many other mysteries regarding H₂ therapy also remain unresolved. For initiating cellular signals by H₂, H₂ should be too inert to react with most molecules except highly reactive ones, such as ‘OH or ONOO⁻. To activate H₂ to react with other molecules, a sufficient level of a putative catalyst must be present; however, it is highly unlikely that such a putative catalyst would be abundant. Moreover, H₂ should be too small to bind a putative H₂-binding receptor because its intramolecular fluctuation should lead to the instability.

H₂ can be easily applied because of a lack of adverse effects and great efficacy for nearly all pathogenic statuses involved in oxidative stress and inflammation. Since most pharmacological drugs specifically act on their targets, H₂ seems to differ from conventional drugs or other medical gasses because of its extensive and varied effects. H₂ has great potential for preventive and therapeutic applications owing to its great efficacy and its “novel” concept.

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