CHAPTER FIFTEEN

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

Shigeo Ohta[1]

Department of Biochemistry and Cell Biology, Institute of Development and Aging Sciences, Graduate School of Medicine, Nippon Medical School, Kawasaki, Japan
[1]Corresponding author: e-mail address: ohta@nms.ac.jp

Contents

Methods in Enzymology, Volume 555
ISSN 0076-6879
http://dx.doi.org/10.1016/bs.mie.2014.11.038

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 ($^{\bullet}OH$) 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 anti-apoptotic 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.

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 turns of biological reactions in several microorganisms, H_2 is a product of certain types of anaerobic metabolism, usually via reactions catalyzed by iron- or nickel-containing enzymes called hydrogenases (Adams, Mortenson, & Chen, 1980; Fritsch, Lenz, & Friedrich, 2013). H_2 is also enzymatically metabolized as an energy source by providing electrons to the electron transport chain. These enzymes catalyze the reversible redox reaction between H_2 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 H_2 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 H_2 gas efficiently (King, 2013; van Berkel-Arts et al., 1986).

In contrast, H_2 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 H_2 is nonfunctional in our cells. In fact, H_2 seemed to react with no biological compounds, including oxygen (O_2), in the absence of catalysts at body temperature. Indeed, owing to its characteristics, H_2 gas was used for measuring local blood flow (Aukland, Bower, & Berliner, 1964).

We overturned this concept in a publication in 2007 describing that H_2 acts as a therapeutic and preventive antioxidant by selectively reducing highly active oxidants, such as hydroxyl radical ($^{\bullet}OH$) and peroxynitrite ($ONOO^-$) in cultured cells, and that H_2 has cytoprotective effects against oxidative stress (Ohsawa et al., 2007). Since then, a large number of studies have explored therapeutic and preventive effects of H_2. These publications cover many biological effects against oxidative stress in almost all organs (Ohta, 2011, 2012). Moreover, it has been revealed that H_2 has more roles, including anti-inflammatory, antiapoptotic, and antiallergic effects, in most tissues of model animals, and that H_2 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 H_2 WITH OTHER MEDICAL GASSES

Gas inhalation as disease therapy has recently received attention (Kajimura, Fukuda, Bateman, Yamamoto, & Suematsu, 2010; Szabó, 2007). In recent decades, there has been extraordinary and rapid growth in our knowledge of gaseous molecules, including hydrogen sulfide (H_2S), nitric oxide ($NO^{\bullet}$), and carbon monoxide (CO). H_2S, CO, and $NO^{\bullet}$ are extremely toxic molecules; however, they play important roles as signaling molecules in biological systems (Kimura, 2010; Motterlini & Otterbein, 2010).

In contrast, H_2 has advantages in terms of toxicity: it has no cytotoxicity even at high concentration (Abraini, Gardette-Chauffour, Martinez, Rostain, & Lemaire, 1994; Fontanari et al., 2000; Lillo & Parker, 2000; Lillo, Parker, & Porter, 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 H_2 for humans is demonstrated by its application in Hydreliox, an exotic, breathing gas mixture of 49% H_2, 50% helium, and 1% O_2, which is used to prevent decompression sickness and nitrogen narcosis during very deep technical diving (Abraini et al., 1994; Fontanari et al., 2000; Lillo & Parker, 2000; Lillo et al., 1997).

As the primary target of H_2S, CO, and $NO^{\bullet}$, heme-based proteins play central roles. Integrated approaches revealed the physiological significance of H_2S, CO, and $NO^{\bullet}$ 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 $NO^{\bullet}$, H_2S, or CO is carried out by different enzymes, $NO^{\bullet}$ 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 $NO^{\bullet}$ plus H_2 during ischemia/reperfusion reduced the infarct size and maintained cardiac function, and reduced the generation of myocardial nitro-tyrosine associated with $NO^{\bullet}$ inhalation (Shinbo et al., 2013). These findings suggest that the target of H_2 differs from those of CO and $NO^{\bullet}$.

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, Harma, & Erel, 2006; Liu et al., 1996; 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, Gupta, Chaturvedi, & Aggarwal, 2010; Vaziri & 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, Angulo, & Rodriguez-Manas, 2013; Kim & Byzova, 2014).

As a first step in generating ROS, superoxide anion radicals ($^{\bullet}O_2^-$) are the primary ROS mostly generated by electron leakage from the mitochondrial electron transport chain (Andersen, 2004; Finkel & Holbrook, 2000; Lin & Beal, 2006; 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 $^{\bullet}O_2^-$ to hydrogen peroxide (H_2O_2), which is metabolized to generate water (H_2O). Highly reactive $^{\bullet}OH$ is generated from H_2O_2 or $^{\bullet}O_2^-$ via the Fenton or Weiss reaction in the presence of catalytically active metals, such as Fe^{2+} and Cu^+ (Halliwell & Gutteridge, 1992). Reaction of $^{\bullet}O_2^-$ with $NO^{\bullet}$ generates $ONOO^-$, which is a very active nitrogen species (RNS) (Radi, 2013). $^{\bullet}OH$ 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 $^{\bullet}OH$ as a damaging intermediate through the reaction with water, a process termed radiolysis (Schoenfeld, Ansari, Nakao, & Wink, 2012; 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, Nikolova, Gluud, Simonetti, & Gluud, 2007; Brambilla et al., 2008; Hackam, 2007; Hercberg et al., 2010; Steinhubl, 2008).

4. PHYSIOLOGICAL ROLES OF H_2O_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, Kovsan, Kachko, Ovadia, & Rudich, 2009; Brewer, Mustafi, Murray, Rajasekaran, & Benjamin, 2013). Some ROS are now appreciated to function as signaling molecules to regulate a wide variety of physiological process (Bell, Klimova, Eisenbart, Schumacker, & Chandel, 2007; Liu, Colavitti, Rovira, & Finkel, 2005). H_2O_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, & Schumacker, 2000; Chandel, Vander Heiden, Thompson, & Schumacker, 2000; 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_2O_2 and $NO^{\bullet}$ induces enzymes involved in antioxidation and tolerance to protect cells against oxidative stress (Endo et al., 2009; Ristow & 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_2O_2 is a key factor to regulate cellular differentiation (Tormos et al., 2011; Tsukagoshi, Busch, & Benfey, 2010), the immune system (West et al., 2011; Zhou, Yazdi, Menu, & Tschopp, 2011), autophagy (Garg et al., 2013; Li, Ishdorj, & Gibson, 2012), and apoptosis (Mates, Segura, Alonso, & Marquez, 2012). Thus, it is crucial for functional H_2O_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, Nikolova, Gluud, Simonetti, & Gluud, 2008; Gray et al., 2008; Hackam, 2007; Hercberg et al., 2010; Walker, 2008) probably because they may interfere with some essential defensive mechanisms (Bjelakovic & Gluud, 2007; Bjelakovic et al., 2008; Carriere et al., 2004; Chandel et al., 1998; Mandal et al., 2010; Miller et al., 2005; 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_2O_2 but should effectively reduce strong oxidants, such as $^{\bullet}OH$.

Since H_2 reduces $^{\bullet}OH$ but does not react with $^{\bullet}O_2^-$, H_2O_2, and $NO^{\bullet}$ 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 H_2 incorporated into and consumed by tissues (Ohsawa et al., 2007). In a clinical examination, Ono et al. also showed a difference in H_2 concentrations between arterial and venous blood (Ono et al., 2012).

H_2 concentration can be measured using an H_2 electrode that specifically detects H_2; however, this sensor is also somewhat sensitive to H_2S. Thus, when H_2S is contaminated in a solution, one must take into consideration its effects.

H_2 can be measured in tissues using a needle-type H_2 sensor (Unisense, Aarhus, Denmark). The electrode current was measured with a picoammeter (Keithley, Cleveland, Ohio) attached to a recorder. The negative

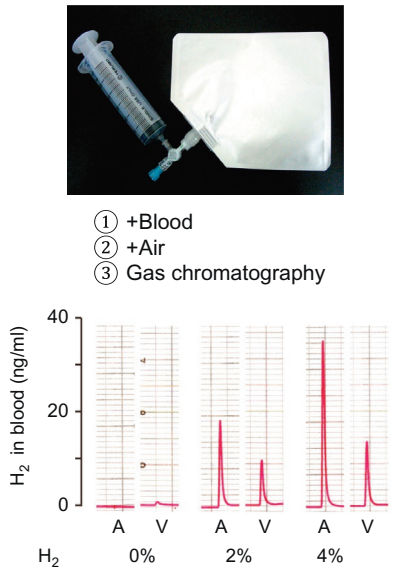

Figure 1 Incorporation of H_2 into blood by inhaling hydrogen gas. Rats inhaled a mixed gas of H_2 (1% or 2%) and O_2 (30%) under anesthetic N_2O and halothane for 1 h, and arterial (indicated by A) or venous blood (indicated by V) was collected into a closed aluminum bag from a three-way stopper (upper panel). After the transfer of H_2 into an accurate volume of air phase from the blood, amounts of H_2 were examined by gas chromatography. Lower panel shows profiles of gas chromatography. The vertical scale indicates the amounts of blood H_2 after calculations. *Adapted from after Ohsawa et al. (2007) modified version of Fig. 5A, with permission from Nature Publishing Group.*

current obtained from the H_2 sensor was converted to regional H_2 concentration using a calibration curve generated from known levels of H_2-saturated saline.

6. ADVANTAGES OF HYDROGEN IN MEDICAL APPLICATIONS

6.1. Selective reaction of H_2 with highly reactive ROS

H_2 dissolved in culture medium did not change the cellular levels of $^{\bullet}O_2^-$ and H_2O_2, as judged by the fluorescent signals of MitoSOX and dichlorofluorescein-diacetate (DCF-DA), respectively (Ohsawa et al., 2007). Additionally, H_2 did not decrease the cellular level of $NO^{\bullet}$. In contrast, H_2 treatment significantly decreased levels of $^{\bullet}OH$, 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 H_2 were prepared as follows: H_2 was dissolved beyond the saturated level into DMEM medium under 0.4 MPa pressure of hydrogen gas for 2 h, O_2 was also dissolved into another medium by bubbling, the third medium contained CO_2, 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 H_2 and 8.5 mg/l of O_2 at 25 °C. For culture, the combined media were put into a culture flask and immediately examined for H_2 or O_2 concentration with an H_2 or O_2 electrode, and in turn gas composed of the desired ratio of H_2 and N_2 ($H_2 + N_2 = 75\%$), 20% of O_2, and 5% of CO_2 was filled into the culture flask; for example, when the medium contained 0.6 m*M* H_2, the 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 H_2 was prepared by stirring medium that had been saturated with H_2 in an open vessel for 4 h, and the concentration of H_2 was checked with an H_2 electrode.

Then, PC12 cells were incubated in medium with or without 0.6 m*M* H_2, and exposed to antimycin A or L-NAME (N^G-nitro-L-arginine methyl ester) to induce $^{\bullet}O_2^-$, H_2O_2, and $^{\bullet}OH$ or $NO^{\bullet}$. 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 $^{\bullet}O_2^-$, H_2O_2, $^{\bullet}OH$, and NO, respectively (Fig. 2).

Alternatively, PC12 cells were exposed to intracellular $^{\bullet}OH$ produced by the Fenton reaction ($H_2O_2 + Cu^+ \rightarrow OH + OH^- + Cu^{2+}$), with or without 0.6 m*M* H_2. Cells were preincubated with 1 m*M* $CuSO_4$, washed, and exposed for 1 h to 0.1 m*M* ascorbate (Vit. C) in order to reduce intracellular Cu^{2+} to Cu^+. In this case, endogenous H_2O_2 would be sufficient to produce $^{\bullet}OH$. H_2 indeed protected the cells against $^{\bullet}OH$.

Moreover, the decrease in the cellular $^{\bullet}OH$ level by H_2 was confirmed by spin-trapping technology (Halliwell & Gutteridge, 1992). Standard electron spin resonance (ESR) signals of the DMPO-$OH^{\bullet}$ radical were obtained by trapping $^{\bullet}OH$ with a spin-trapping reagent (DMPO). PC12 cells were preincubated with 0.1 *M* DMPO and 2 m*M* $CuSO_4$ for 30 min at 37 °C with or without 0.6 m*M* H_2. After removal of this medium, the cells were treated with 0.2 m*M* ascorbate and 0.1 m*M* H_2O_2 for 5 min at 23 °C to produce $^{\bullet}OH$ by the Fenton reaction, and then scraped into a flat cuvette for ESR measurement. Alternatively, PC12 cells were incubated in PBS containing 0.1 *M* DMPO and 30 g/ml antimycin A for 7 min at 23 °C to produce

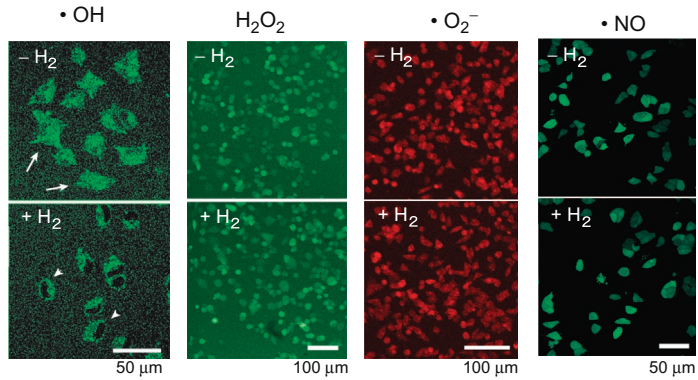

Figure 2 Selective reduction of reactive oxygen or nitrogen species by H_2 in cultured cells. PC12 cells were kept in medium with 0.6 m*M* H_2 (indicated by $+H_2$) or without H_2 (indicated by $-H_2$), and exposed to antimycin A or L-NAME (N^G-nitro-L-arginine methyl ester) to induce $^{\bullet}OH$, H_2O_2, and $^{\bullet}O_2^-$ (ROS) or $NO^{\bullet}$ (RNS) for 30 min. Each ROS or RNS was detected using flowing fluorescent dye; HPF, H_2DCF (2′,7′-dichlorodihydrofluorescein), MitoSOX, and DAF-2 DA (diaminofluorescein-2 diacetate) were used to detect $^{\bullet}OH$, H_2O_2, $^{\bullet}O_2^-$, and $NO^{\bullet}$, respectively. These representative fluorescence images obtained by laser-scanning confocal microscopy demonstrate the selective reduction of $^{\bullet}OH$ by 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.*

excess $^{\bullet}OH$, with or without 0.6 m*M* 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 $^{\bullet}OH$. In other words, $^{\bullet}OH$ is strong enough to react with even inert H_2, but that $^{\bullet}O_2^-$, H_2O_2, and $NO^{\bullet}$ are insufficient to react with H_2 according to their activities. Namely, H_2 is mild enough neither to disturb metabolic redox reactions nor to affect ROS that function in cellular signaling (Fig. 3).

6.2. Rapid diffusion

Most hydrophilic antioxidants cannot penetrate biomembranes and most hydrophobic antioxidants remain on the membranes. In contrast, H_2 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.

Despite the clinical importance of overcoming oxidative damage, antioxidants had limited therapeutic success. This may be because most

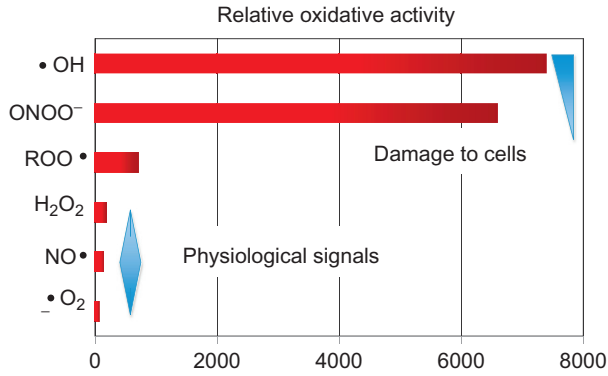

Figure 3 Relative oxidative activities in each reactive oxygen and nitrogen species. $^{\bullet}OH$ and $ONOO^{-}$ are highly reactive to damaged cells, whereas $^{\bullet}O_2^{-}$, $NO^{\bullet}$, and H_2O_2 have physiological roles as signaling molecules. *This graph is based on data from a previous publication (Setsukinai et al., 2003).*

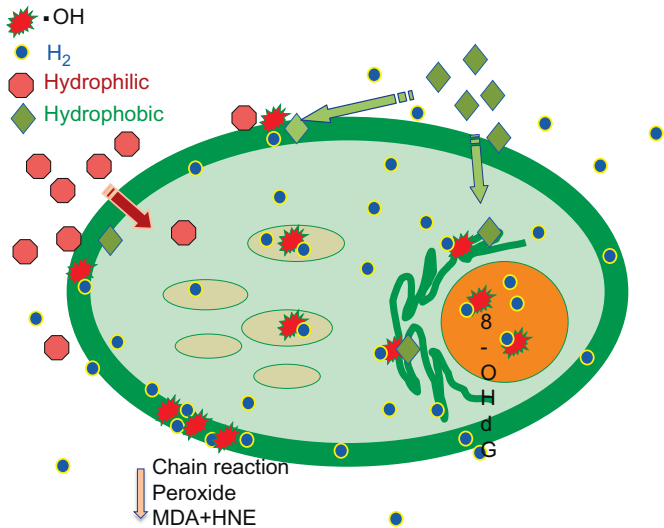

Figure 4 Illustration of gaseous diffusion of H_2 into a cell. Most hydrophilic compounds are retained at membranes and cannot reach the cytosol, whereas most hydrophobic ones cannot penetrate biomembranes in the absence of specific carriers. In contrast, H_2 can be rapidly distributed into cytosol and organelles. On the membrane, $^{\bullet}OH$ 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, $^{\bullet}OH$ oxidizes DNA for modification to 8-OHdG (8-hydroxy-deoxyguanine).

antioxidants do not reach specific regions (Murphy, 1997; Murphy & Smith, 2000; Smith & Murphy, 2011). As H_2 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, H_2 passes through the blood brain barrier, although most antioxidant compounds cannot; this is also an advantage of H_2.

The gaseous diffusion of H_2 can be monitored inside various tissues by detection with a specific H_2 electrode. For example, H_2 concentration has been monitored within the rat myocardium. The electrode was inserted into the "at-risk" area for infarction to estimate the diffusion of H_2 into the ischemic myocardium area after coronary artery occlusion. H_2 concentration was increased by its diffusion, even with coronary artery occlusion (Hayashida et al., 2008) (Fig. 5).

Moreover, we devised eye drops with dissolved H_2 to administer H_2 to the retina directly, and monitored the time course of changes in H_2 levels using the needle-shaped hydrogen sensor electrode inserted through the sclera to the vitreous body in rats. H_2 could reach the vitreous body by administering H_2 saturated in normal saline. When H_2 eye drops were

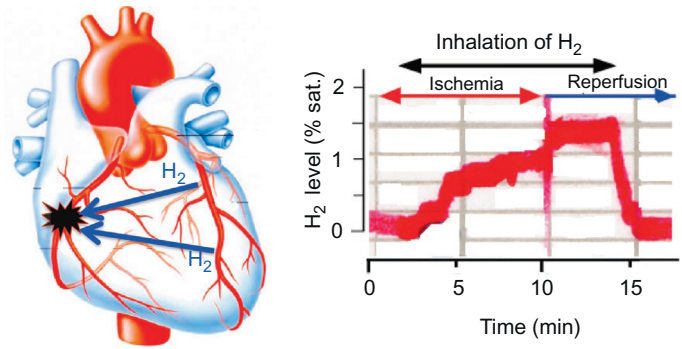

Figure 5 Inhalation of H_2 gas increases the intramyocardial H_2 by diffusion. Left panel illustrates that H_2 can reach an infarct area by diffusion even without blood flow. Right panel indicates an experimental result obtained as follows: Regional myocardial ischemia was induced by transient occlusion of the left anterior descending coronary artery of a rat. A needle-type hydrogen sensor (Unisense, Aarhus, Denmark) was inserted in the LV cavity (arterial blood) and H_2 gas at 2% was administered by respiration to intubated rats receiving mechanical ventilation and the concentration of H_2 in the "at-risk" area for infarction during ischemia and reperfusion was monitored with the needle-type H_2 sensor. *Adapted from Hayashida et al. (2008) modified version of Fig. 2C, with permission from Elsevier.*

administered continuously, approximately 70% H_2 was detected on the ocular surface (Oharazawa et al., 2010).

These experiments indicate that H_2 can rapidly diffuse into tissues even without blood flow.

7. METHODS OF INGESTING MOLECULAR HYDROGEN

7.1. Inhalation of hydrogen gas

Inhalation of H_2 gas is the most straightforward therapeutic method. H_2 gas can be inhaled through a ventilator circuit, facemask, or nasal cannula. Since inhaled H_2 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, H_2 can reach the region without blood flow by rapid diffusion (Fig. 5).

By a clinical examination, Ono et al. monitored H_2 and showed that inhalation of 3–4% H_2 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 H_2 gas is actually unsuitable or impractical for continuous H_2 consumption in daily life for preventive use. In contrast, solubilized H_2 (H_2-dissolved water; i.e., H_2-water) may be beneficial since it is a portable, easily administered, and safe way to ingest H_2 (Nagata, Nakashima-Kamimura, Mikami, Ohsawa, & Ohta, 2009; Ohsawa, Nishimaki, Yamagata, Ishikawa, & Ohta, 2008). H_2 can be dissolved in water up to 0.8 m*M* (1.6 mg/l) under atmospheric pressure at room temperature without any change of pH.

H_2-water can be made by several methods: infusing H_2 gas into water under high pressure, electrolyzing water to produce H_2, and reacting magnesium metal or its hydride with water. These methods may be applicable not only to water but also to other solvents. H_2 penetrates glass and plastic walls of any vessel in a short time, while aluminum containers can retain H_2 for a long time.

In brief, for experimental treatments, H_2 was dissolved in water under high pressure (0.4 MPa) to a supersaturated level and the saturated H_2-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 H_2-water at the same time (e.g., at 4:00 pm) every day.

When water saturated with H_2 was placed into the stomach of a rat, H_2 was detected at several micromoles in blood (Nagata et al., 2009; Nakashima-Kamimura, Mori, Ohsawa, Asoh, & Ohta, 2009). In addition, a rat received H_2-water (0.8 mmol/l H_2 in water) orally by stomach gavage, for example, at 15 ml/kg. Hepatic H_2 was monitored with a needle-type hydrogen electrode (Kamimura, Nishimaki, Ohsawa, & Ohta, 2011) (Fig. 6).

Furthermore, after seven adult volunteers had drunk H_2-water, the H_2 content of their expired breath was measured by gas chromatography with a semiconductor (Shimouchi, Nose, Shirai, & Kondo, 2012). The ingestion of H_2-water rapidly increased breath H_2 content to its maximal level 10 min after ingestion, which thereafter decreased to the baseline level within 60 min. H_2 lost from the water during the experimental procedures accounted for 3% or less of the total. The rate of H_2 release from the skin surface was estimated as approximately 0.1%. On the basis of the remaining H_2 mass balance, approximately 40% of H_2 that had been drunk was consumed inside the body. This report suggests that exogenous H_2 is at least partially trapped by oxygen radicals, such as $^{\bullet}OH$ (Shimouchi et al., 2012).

7.3. Injection of hydrogen-saline

H_2 is intravenously or intraperitoneally injectable as H_2-saline (H_2-dissolved saline), which allows the delivery of H_2 with great efficacy in model animals (Cai et al., 2009; Li et al., 2013; Sun et al., 2011).

Nagatani et al. performed an open-label, prospective, nonrandomized study of intravenous H_2 administration in 38 patients hospitalized for acute ischemic stroke. All patients received an H_2 intravenous solution immediately after the diagnosis of acute ischemic stroke. Data from this study indicated that an H_2 intravenous solution is safe for patients with acute cerebral infarction, including patients treated with tissue-plasminogen activator (Nagatani et al., 2013).

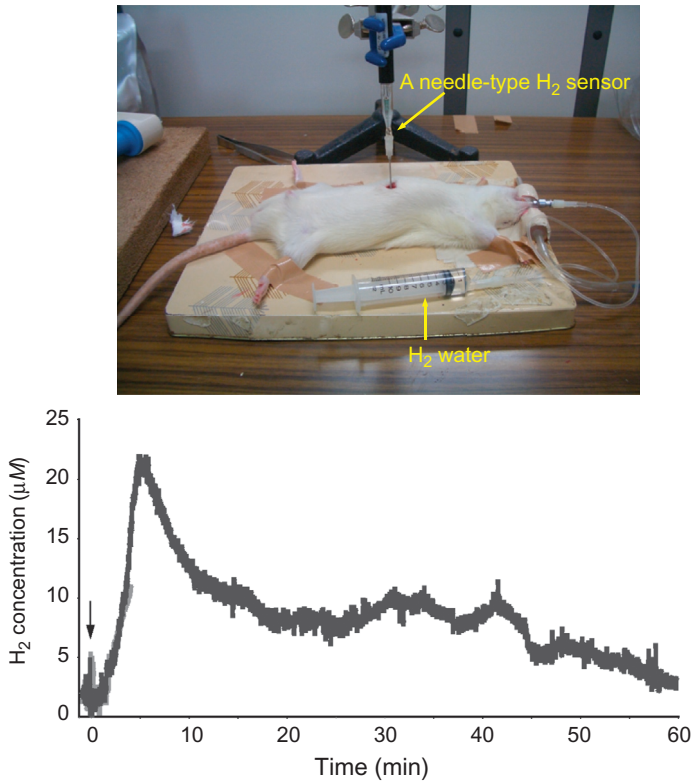

Figure 6 Incorporation of H_2 into the liver from the stomach. H_2 was dissolved in water under high pressure (0.4 MPa) to a supersaturated level. Upper panel: A needle-type hydrogen sensor (Unisense, Aarhus, Denmark) was inserted into rat liver and the rat received saturated H_2-water orally by stomach gavage at 15 ml/kg (upper panel). Lower panel: H_2 concentration was monitored with a picoammeter (Keithley, Cleveland, Ohio). Arrow indicates the time point when rat was administered H_2-water. *Adapted from Kamimura et al. (2011), with permission from Wiley Online Library.*

To rats, H_2-water, H_2-saline, and hydrogen gas were orally administered, intraperitoneally or intravenously injected, and inhaled, respectively. A method for determining the H_2 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 H_2 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, H_2-loaded eye drops were prepared by dissolving H_2 in saline and directly administering them to the ocular surface (Kubota et al., 2011; Oharazawa et al., 2010).

H_2 should easily penetrate the skin and is distributed throughout the body via blood flow. Thus, taking a warm water bath with dissolved H_2 is a method of incorporating H_2 into the body in daily life. It takes only 10 min for it to be distributed throughout the whole body, as judged by measuring H_2 gas in expiration (unpublished results). Indeed, powders that can be used to produce H_2 baths are commercially available in Japan.

H_2 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 H_2 during cold storage merits further investigation for possible therapeutic and preventative use during transplantation (Noda et al., 2013).

7.5. Maternal intake of H_2

H_2 intake helps prevent the hippocampal impairment of offspring induced by ischemia/reperfusion during pregnancy (Mano et al., 2014). The effects of H_2 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 H_2-saturated water *ad libitum* until vaginal delivery. A significant increase in the concentration of H_2 in the placenta was observed after the oral administration of H_2-saturated water to the mothers, with less placental oxidative damage after ischemia and reperfusion in the presence of H_2. Neonatal growth retardation was observed in the ischemia/reperfusion group, which was alleviated by H_2 administration. Maternal H_2 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_2 intake helps prevent the impairment of offspring induced by oxidative stress.

8. MEDICAL EFFECTS OF H_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_2 gas improved ischemia/reperfusion injuries in cerebral (Ohsawa et al., 2007) and myocardial infarction (Hayashida et al., 2008; Yoshida et al., 2012). Hydrogen-saline protected against renal ischemia/reperfusion injury (Wang et al., 2011). All clinical manifestations related to post-cardiac arrest (CA) syndrome are attributed to ischemia/reperfusion injury in various organs, including the brain and heart. H_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_2 also mitigated damage during the transplantation of various organs in the form of H_2 gas (Buchholz et al., 2008), H_2-water (Cardinal et al., 2010), and H_2-preservation solution (Noda et al., 2013). A clinical study showed a positive effect of H_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_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; 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, Koehl, & Le Moal, 2005; Liu et al., 1996). Drinking H_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; Yoritaka et al., 1996). H_2 in drinking water was given before or after stereotactic surgery for 6-hydroxydopamine-induced nigrostriotal degeneration in a rat model of PD. H_2-water prevented both the development and the progression of nigrostriatal degeneration in rats (Fu et al., 2009). Moreover, drinking H_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_2-water in patients with PD was assessed for 48 weeks. Total Unified Parkinson's Disease Rating Scale (UPDRS) scores in the H_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_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_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_2-water (Kamimura et al., 2011). FGF21 functions to stimulate fatty acid and glucose expenditure. Thus, H_2-water stimulates energy metabolism (Kamimura et al., 2011). Beneficial roles of H_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; 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, antiallergy, and stimulation of energy metabolism (Ohta, 2011, 2012, 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 $^{\bullet}OH$ in an experiment using cultured cells (Ohsawa et al., 2007). Later, it was shown that H_2 eye drops directly decreased $^{\bullet}OH$

induced by ischemia/reperfusion in retinas (Oharazawa et al., 2010). Moreover, it has been demonstrated that, at the tissue level, H_2 neutralized $^{\bullet}OH$ 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 $^{\bullet}OH$ with H_2 in dilute aqueous solutions, this rate may be too slow to enable fully a decrease in $^{\bullet}OH$ 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. $^{\bullet}OH$ 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; Zhou et al., 2013). Additionally, $^{\bullet}OH$ can modify deoxyguanine (dG) to 8-hydroxy-deoxyguanine (8-OHdG) (Delaney, Jarem, Volle, & Yennie, 2012; 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; Kawai et al., 2012).

These experimental observations suggest that sufficient H_2 can efficiently mitigate tissue oxidation induced by $^{\bullet}OH$. 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 $^{\bullet}OH$ 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

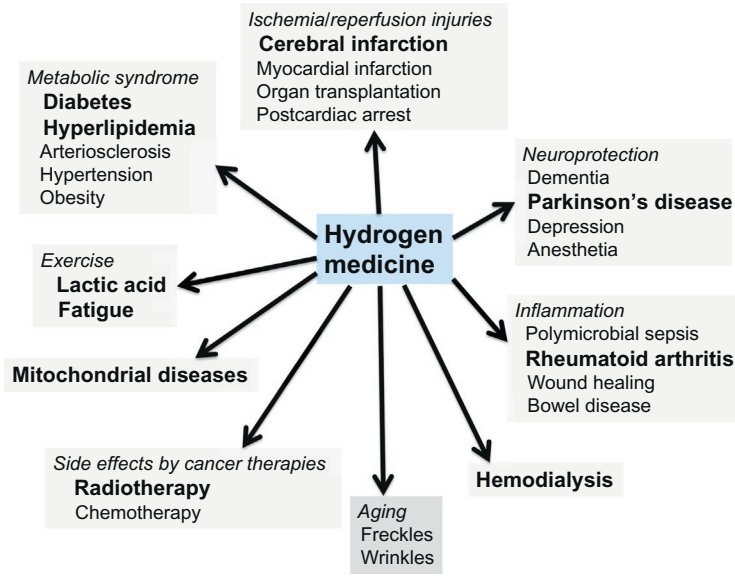

Figure 7 Summary of potential of various preventive and therapeutic effects of H_2. Bold letters indicate published results for clinical examinations (Ohta, 2014). Positive effects obtained by animal experiments for disease models are shown by normal text.

(Chen et al., 2010; Yu et al., 2011; Zhang et al., 2011; 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 indirectly act to enable the various effects of H_2. The primary target of H_2 remains unknown.

10. UNRESOLVED QUESTIONS AND CLOSING REMARKS

H_2 can be incorporated or ingested into the body by various methods: inhalation of H_2 gas, drinking H_2-infused water (H_2-water), injection of H_2-infused saline, and incorporation through the skin. Drinking H_2-water was efficacious for various disease models and patients; however, H_2 can be infused up to only 0.8 m*M* under atmospheric pressure and drinking H_2-water provides a blood H_2 concentration up to only ~10 μ*M* with short dwelling time in the body (Nagata et al., 2009; Nakashima-Kamimura et al., 2009). Moreover, inhaling 1–4% (vol/vol) of H_2 gas was effective, by which H_2 should reach 8–32 μ*M* in blood. Under these conditions, H_2 should be insufficient to scavenge $^{\bullet}OH$ for fully exhibiting H_2 benefits because the direct reaction rate of $^{\bullet}OH$ with H_2 in an aqueous solution may be too slow to decrease $^{\bullet}OH$ (Buxton et al., 1988) as pointed out earlier (Wood & Gladwin, 2007). Thus, it remained elusive how such low levels of H_2 with a short dwelling time could effectively compete with the numerous cellular targets in chronic or acute pathogenesis. Unexpectedly, H_2 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 H_2-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 H_2 is.

Many other mysteries regarding H_2 therapy also remain unresolved. For initiating cellular signals by H_2, H_2 should be too inert to react with most molecules except highly reactive ones, such as $^{\bullet}OH$ or $ONOO^{-}$. To activate H_2 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, H_2 should be too small to bind a putative H_2-binding receptor because its intramolecular fluctuation should lead to the instability.

H_2 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, H_2 seems to differ from conventional drugs or other medical gasses because of its extensive and varied effects. H_2 has great potential for preventive and therapeutic applications owing to its great efficacy and its "novel" concept.

REFERENCES

Abraini, J. H., Gardette-Chauffour, M. C., Martinez, E., Rostain, J. C., & Lemaire, C. (1994). Psychophysiological reactions in humans during an open sea dive to 500 m with a hydrogen–helium–oxygen mixture. *Journal of Applied Physiology*, *76*(3), 1113–1118.

Abrous, D. N., Koehl, M., & Le Moal, M. (2005). Adult neurogenesis: From precursors to network and physiology. *Physiological Reviews*, *85*(2), 523–569.

Adams, M. W., Mortenson, L. E., & Chen, J. S. (1980). Hydrogenase. *Biochimica et Biophysica Acta*, *594*(2–3), 105–176.

Agarwal, R. (2005). Smoking, oxidative stress and inflammation: Impact on resting energy expenditure in diabetic nephropathy. *BMC Nephrology*, *6*, 13.

Andersen, J. K. (2004). Oxidative stress in neurodegeneration: Cause or consequence? *Nature Medicine*, *10*(Suppl.), S18–S25.

Aukland, K., Bower, B. F., & Berliner, R. W. (1964). Measurement of local blood flow with hydrogen gas. *Circulation Research*, *14*, 164–187.

Bashan, N., Kovsan, J., Kachko, I., Ovadia, H., & Rudich, A. (2009). Positive and negative regulation of insulin signaling by reactive oxygen and nitrogen species. *Physiological Reviews*, *89*(1), 27–71.

Bell, E. L., Klimova, T. A., Eisenbart, J., Schumacker, P. T., & Chandel, N. S. (2007). Mitochondrial reactive oxygen species trigger hypoxia-inducible factor-dependent extension of the replicative life span during hypoxia. *Molecular and Cellular Biology*, *27*, 5737–5745.

Bjelakovic, G., & Gluud, C. (2007). Surviving antioxidant supplements. *Journal of the National Cancer Institute*, *99*(10), 742–743.

Bjelakovic, G., Nikolova, D., Gluud, L. L., Simonetti, R. G., & Gluud, C. (2007). Mortality in randomized trials of antioxidant supplements for primary and secondary prevention: Systematic review and meta-analysis. *JAMA*, *297*(8), 842–857.

Bjelakovic, G., Nikolova, D., Gluud, L. L., Simonetti, R. G., & Gluud, C. (2008). Antioxidant supplements for prevention of mortality in healthy participants and patients with various diseases. *The Cochrane Database of Systematic Reviews*, *2*, CD007176.

Brambilla, D., Mancuso, C., Scuderi, M. R., Bosco, P., Cantarella, G., Lempereur, L., et al. (2008). The role of antioxidant supplement in immune system, neoplastic, and neurodegenerative disorders: A point of view for an assessment of the risk/benefit profile. *Nutrition Journal*, *7*, 29.

Brewer, A. C., Mustafi, S. B., Murray, T. V., Rajasekaran, N. S., & Benjamin, I. J. (2013). Reductive stress linked to small HSPs, G6PD, and Nrf2 pathways in heart disease. *Antioxidants & Redox Signaling*, *18*(9), 1114–1127.

Buchholz, B. M., Kaczorowski, D. J., Sugimoto, R., Yang, R., Wang, Y., Billiar, T. R., et al. (2008). Hydrogen inhalation ameliorates oxidative stress in transplantation induced intestinal graft injury. *American Journal of Transplantation*, *8*(10), 2015–2024.

Buxton, G. V., Greenstock, C. L., Helman, W. P., & Ross, A. B. (1988). Critical view of rate constants for reactions of hydrated electrons, hydrogen atoms and hydroxyl radicals ($^{\bullet}OH/^{\bullet}OH^{-}$) in aqueous solution. *Journal of Physical and Chemical Reference Data*, *17*, 513–886.

Cai, J., Kang, Z., Liu, K., Liu, W., Li, R., Zhang, J. H., et al. (2009). Neuroprotective effects of hydrogen saline in neonatal hypoxia-ischemia rat model. *Brain Research*, *1256*, 129–137.

Cai, W. W., Zhang, M. H., Yu, Y. S., & Cai, J. H. (2013). Treatment with hydrogen molecule alleviates TNFalpha-induced cell injury in osteoblast. *Molecular and Cellular Biochemistry*, *373*(1–2), 1–9.

Cardinal, J. S., Zhan, J., Wang, Y., Sugimoto, R., Tsung, A., McCurry, K. R., et al. (2010). Oral hydrogen water prevents chronic allograft nephropathy in rats. *Kidney International*, *77*(2), 101–109.

Carriere, A., Carmona, M. C., Fernandez, Y., Rigoulet, M., Wenger, R. H., Penicaud, L., et al. (2004). Mitochondrial reactive oxygen species control the transcription factor CHOP-10/GADD153 and adipocyte differentiation: A mechanism for hypoxia-dependent effect. *The Journal of Biological Chemistry*, *279*(39), 40462–40469.

Carrieri, D., Wawrousek, K., Eckert, C., Yu, J., & Maness, P. C. (2011). The role of the bidirectional hydrogenase in cyanobacteria. *Bioresource Technology*, *102*(18), 8368–8377.

Chandel, N. S., Maltepe, E., Goldwasser, E., Mathieu, C. E., Simon, M. C., & Schumacker, P. T. (1998). Mitochondrial reactive oxygen species trigger hypoxia-induced transcription. *Proceedings of the National Academy of Sciences of the United States of America*, *95*(20), 11715–11720.

Chandel, N. S., Trzyna, W. C., McClintock, D. S., & Schumacker, P. T. (2000). Role of oxidants in NF-kappa B activation and TNF-alpha gene transcription induced by hypoxia and endotoxin. *Journal of Immunology*, *165*(2), 1013–1021.

Chandel, N. S., Vander Heiden, M. G., Thompson, C. B., & Schumacker, P. T. (2000). Redox regulation of p53 during hypoxia. *Oncogene*, *19*(34), 3840–3848.

Chen, C. H., Manaenko, A., Zhan, Y., Liu, W. W., Ostrowki, R. P., Tang, J., et al. (2010). Hydrogen gas reduced acute hyperglycemia-enhanced hemorrhagic transformation in a focal ischemia rat model. *Neuroscience*, *169*(1), 402–414.

Chen, H. G., Xie, K. L., Han, H. Z., Wang, W. N., Liu, D. Q., Wang, G. L., et al. (2013). Heme oxygenase-1 mediates the anti-inflammatory effect of molecular hydrogen in LPS-stimulated RAW 264.7 macrophages. *International Journal of Surgery*, *11*(10), 1060–1066.

Chuai, Y., Gao, F., Li, B., Zhao, L., Qian, L., Cao, F., et al. (2012). Hydrogen-rich saline attenuates radiation-induced male germ cell loss in mice through reducing hydroxyl radicals. *The Biochemical Journal*, *442*(1), 49–56.

Collins, Y., Chouchani, E. T., James, A. M., Menger, K. E., Cocheme, H. M., & Murphy, M. P. (2012). Mitochondrial redox signalling at a glance. *Journal of Cell Science*, *125*(Pt. 4), 801–806.

Delaney, S., Jarem, D. A., Volle, C. B., & Yennie, C. J. (2012). Chemical and biological consequences of oxidatively damaged guanine in DNA. *Free Radical Research*, *46*(4), 420–441.

Droge, W. (2002). Free radicals in the physiological control of cell function. *Physiological Reviews*, *82*(1), 47–95.

El Assar, M., Angulo, J., & Rodriguez-Manas, L. (2013). Oxidative stress and vascular inflammation in aging. *Free Radical Biology & Medicine*, *65*, 380–401.

Endo, J., Sano, M., Katayama, T., Hishiki, T., Shinmura, K., Morizane, S., et al. (2009). Metabolic remodeling induced by mitochondrial aldehyde stress stimulates tolerance to oxidative stress in the heart. *Circulation Research*, *105*, 1118–1127.

Federico, A., Cardaioli, E., Da Pozzo, P., Formichi, P., Gallus, G. N., & Radi, E. (2012). Mitochondria, oxidative stress and neurodegeneration. *Journal of the Neurological Sciences*, *322*(1–2), 254–262.

Ferrari, R., Ceconi, C., Curello, S., Cargnoni, A., Pasini, E., & Visioli, O. (1991). The occurrence of oxidative stress during reperfusion in experimental animals and men. *Cardiovascular Drugs and Therapy*, *5*(Suppl. 2), 277–287.

Finkel, T. (1998). Oxygen radicals and signaling. *Current Opinion in Cell Biology*, *10*(2), 248–253, S0955-0674(98)80147-6 [pii].

Finkel, T., & Holbrook, N. J. (2000). Oxidants, oxidative stress and the biology of ageing. *Nature*, *408*(6809), 239–247.

Fontanari, P., Badier, M., Guillot, C., Tomei, C., Burnet, H., Gardette, B., et al. (2000). Changes in maximal performance of inspiratory and skeletal muscles during and after the 7.1-MPa Hydra 10 record human dive. *European Journal of Applied Physiology*, *81*(4), 325–328.

Fritsch, J., Lenz, O., & Friedrich, B. (2013). Structure, function and biosynthesis of O(2)-tolerant hydrogenases. *Nature Reviews Microbiology*, *11*(2), 106–114.

Fu, Y., Ito, M., Fujita, Y., Ichihara, M., Masuda, A., Suzuki, Y., et al. (2009). Molecular hydrogen is protective against 6-hydroxydopamine-induced nigrostriatal degeneration in a rat model of Parkinson's disease. *Neuroscience Letters*, *453*(2), 81–85.

Fujita, K., Seike, T., Yutsudo, N., Ohno, M., Yamada, H., Yamaguchi, H., et al. (2009). Hydrogen in drinking water reduces dopaminergic neuronal loss in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson's disease. *PLoS One*, *4*(9), e7247.

Gan, L., & Johnson, J. A. (2014). Oxidative damage and the Nrf2-ARE pathway in neurodegenerative diseases. *Biochimica et Biophysica Acta*, *1842*(8), 1208–1218.

Garg, A. D., Dudek, A. M., Ferreira, G. B., Verfaillie, T., Vandenabeele, P., Krysko, D. V., et al. (2013). ROS-induced autophagy in cancer cells assists in evasion from determinants of immunogenic cell death. *Autophagy*, *9*(9), 1292–1307.

Grassi, D., Desideri, G., Ferri, L., Aggio, A., Tiberti, S., & Ferri, C. (2010). Oxidative stress and endothelial dysfunction: Say no to cigarette smoking! *Current Pharmaceutical Design*, *16*(23), 2539–2550.

Gray, S. L., Anderson, M. L., Crane, P. K., Breitner, J. C., McCormick, W., Bowen, J. D., et al. (2008). Antioxidant vitamin supplement use and risk of dementia or Alzheimer's disease in older adults. *Journal of the American Geriatrics Society*, *56*(2), 291–295.

Hackam, D. G. (2007). Review: Antioxidant supplements for primary and secondary prevention do not decrease mortality. *ACP Journal Club*, *147*(1), 4.

Halliwell, B., & Gutteridge, J. M. (1992). Biologically relevant metal ion-dependent hydroxyl radical generation. An update. *FEBS Letters*, *307*(1), 108–112.

Harma, M. I., Harma, M., & Erel, O. (2006). Measuring plasma oxidative stress biomarkers in sport medicine. *European Journal of Applied Physiology*, *97*(4), 505, author reply 506–508.

Hayashida, K., Sano, M., Kamimura, N., Yokota, T., Suzuki, M., Maekawa, Y., et al. (2012). H(2) gas improves functional outcome after cardiac arrest to an extent comparable to

therapeutic hypothermia in a rat model. *Journal of the American Heart Association*, *1*(5), e003459.
Hayashida, K., Sano, M., Ohsawa, I., Shinmura, K., Tamaki, K., Kimura, K., et al. (2008). Inhalation of hydrogen gas reduces infarct size in the rat model of myocardial ischemia-reperfusion injury. *Biochemical and Biophysical Research Communications*, *373*(1), 30–35.
Hercberg, S., Kesse-Guyot, E., Druesne-Pecollo, N., Touvier, M., Favier, A., Latino-Martel, P., et al. (2010). Incidence of cancers, ischemic cardiovascular diseases and mortality during 5-year follow-up after stopping antioxidant vitamins and minerals supplements: A postintervention follow-up in the SU.VI.MAX Study. *International Journal of Cancer*, *127*(8), 1875–1881.
Ishibashi, T., Sato, B., Rikitake, M., Seo, T., Kurokawa, R., Hara, Y., et al. (2012). Consumption of water containing a high concentration of molecular hydrogen reduces oxidative stress and disease activity in patients with rheumatoid arthritis: An open-label pilot study. *Medical Gas Research*, *2*(1), 27.
Jazwa, A., & Cuadrado, A. (2010). Targeting heme oxygenase-1 for neuroprotection and neuroinflammation in neurodegenerative diseases. *Current Drug Targets*, *11*(12), 1517–1531.
Kajimura, M., Fukuda, R., Bateman, R. M., Yamamoto, T., & Suematsu, M. (2010). Interactions of multiple gas-transducing systems: Hallmarks and uncertainties of CO, NO, and H2S gas biology. *Antioxidants & Redox Signaling*, *13*(2), 157–192.
Kajiya, M., Silva, M. J., Sato, K., Ouhara, K., & Kawai, T. (2009). Hydrogen mediates suppression of colon inflammation induced by dextran sodium sulfate. *Biochemical and Biophysical Research Communications*, *386*(1), 11–15.
Kamimura, N., Nishimaki, K., Ohsawa, I., & Ohta, S. (2011). Molecular hydrogen improves obesity and diabetes by inducing hepatic FGF21 and stimulating energy metabolism in db/db mice. *Obesity*, *19*(7), 1396–1403.
Kashfi, K., & Olson, K. R. (2013). Biology and therapeutic potential of hydrogen sulfide and hydrogen sulfide-releasing chimeras. *Biochemical Pharmacology*, *85*, 689–703.
Kawai, D., Takaki, A., Nakatsuka, A., Wada, J., Tamaki, N., Yasunaka, T., et al. (2012). Hydrogen-rich water prevents progression of nonalcoholic steatohepatitis and accompanying hepatocarcinogenesis in mice. *Hepatology*, *56*(3), 912–921.
Kawamura, T., Wakabayashi, N., Shigemura, N., Huang, C. S., Masutani, K., Tanaka, Y., et al. (2013). Hydrogen gas reduces hyperoxic lung injury via the Nrf2 pathway in vivo. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, *304*(10), L646–L656.
Kim, Y. W., & Byzova, T. V. (2014). Oxidative stress in angiogenesis and vascular disease. *Blood*, *123*(5), 625–631.
Kimura, H. (2010). Hydrogen sulfide: From brain to gut. *Antioxidants & Redox Signaling*, *12*(9), 1111–1123.
King, P. W. (2013). Designing interfaces of hydrogenase-nanomaterial hybrids for efficient solar conversion. *Biochimica et Biophysica Acta*, *1827*(8–9), 949–957.
Kubota, M., Shimmura, S., Kubota, S., Miyashita, H., Kato, N., Noda, K., et al. (2011). Hydrogen and N-acetyl-L-cysteine rescue oxidative stress-induced angiogenesis in a mouse corneal alkali-burn model. *Investigative Ophthalmology & Visual Science*, *52*(1), 427–433.
Li, L., Ishdorj, G., & Gibson, S. B. (2012). Reactive oxygen species regulation of autophagy in cancer: Implications for cancer treatment. *Free Radical Biology & Medicine*, *53*(7), 1399–1410.
Li, G. M., Ji, M. H., Sun, X. J., Zeng, Q. T., Tian, M., Fan, Y. X., et al. (2013). Effects of hydrogen-rich saline treatment on polymicrobial sepsis. *The Journal of Surgical Research*, *181*(2), 279–286.
Lillo, R. S., & Parker, E. C. (2000). Mixed-gas model for predicting decompression sickness in rats. *Journal of Applied Physiology*, *89*(6), 2107–2116.

Lillo, R. S., Parker, E. C., & Porter, W. R. (1997). Decompression comparison of helium and hydrogen in rats. *Journal of Applied Physiology*, *82*(3), 892–901.
Lin, M. T., & Beal, M. F. (2006). Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. *Nature*, *443*(7113), 787–795.
Lipinski, B. (2011). Hydroxyl radical and its scavengers in health and disease. *Oxidative Medicine and Cellular Longevity*, *2011*, 809696.
Liu, H., Colavitti, R., Rovira, I. I., & Finkel, T. (2005). Redox-dependent transcriptional regulation. *Circulation Research*, *97*, 967–974.
Liu, C., Kurokawa, R., Fujino, M., Hirano, S., Sato, B., & Li, X. K. (2014). Estimation of the hydrogen concentration in rat tissue using an airtight tube following the administration of hydrogen via various routes. *Scientific Reports*, *4*, 5485.
Liu, J., Wang, X., Shigenaga, M. K., Yeo, H. C., Mori, A., & Ames, B. N. (1996). Immobilization stress causes oxidative damage to lipid, protein, and DNA in the brain of rats. *The FASEB Journal*, *10*(13), 1532–1538.
Mandal, C. C., Ganapathy, S., Gorin, Y., Mahadev, K., Block, K., Abboud, H. E., et al. (2010). Reactive oxygen species derived from Nox4 mediate BMP2 gene transcription and osteoblast differentiation. *The Biochemical Journal*, *433*(2), 393–402.
Mano, Y., Kotani, T., Ito, M., Nagai, T., Ichinohashi, Y., Yamada, K., et al. (2014). Maternal molecular hydrogen administration ameliorates rat fetal hippocampal damage caused by in utero ischemia-reperfusion. *Free Radical Biology & Medicine*, *69*, 324–330.
Mates, J. M., Segura, J. A., Alonso, F. J., & Marquez, J. (2012). Oxidative stress in apoptosis and cancer: An update. *Archives of Toxicology*, *86*(11), 1649–1665.
Matsuda, M., & Shimomura, I. (2013). Increased oxidative stress in obesity: Implications for metabolic syndrome, diabetes, hypertension, dyslipidemia, atherosclerosis, and cancer. *Obesity Research & Clinical Practice*, *7*(5), e330–e341.
Matsumoto, A., Yamafuji, M., Tachibana, T., Nakabeppu, Y., Noda, M., & Nakaya, H. (2013). Oral 'hydrogen water' induces neuroprotective ghrelin secretion in mice. *Scientific Reports*, *3*, 3273.
Miller, E. R., 3rd, Pastor-Barriuso, R., Dalal, D., Riemersma, R. A., Appel, L. J., & Guallar, E. (2005). Meta-analysis: High-dosage vitamin E supplementation may increase all-cause mortality. *Annals of Internal Medicine*, *142*(1), 37–46.
Motterlini, R., & Otterbein, L. E. (2010). The therapeutic potential of carbon monoxide. *Nature Reviews Drug Discovery*, *9*(9), 728–743.
Murphy, M. P. (1997). Selective targeting of bioactive compounds to mitochondria. *Trends in Biotechnology*, *15*(8), 326–330.
Murphy, M. P., & Smith, R. A. (2000). Drug delivery to mitochondria: The key to mitochondrial medicine. *Advanced Drug Delivery Reviews*, *41*(2), 235–250.
Nagata, K., Nakashima-Kamimura, N., Mikami, T., Ohsawa, I., & Ohta, S. (2009). Consumption of molecular hydrogen prevents the stress-induced impairments in hippocampus-dependent learning tasks during chronic physical restraint in mice. *Neuropsychopharmacology*, *34*(2), 501–508.
Nagatani, K., Nawashiro, H., Takeuchi, S., Tomura, S., Otani, N., Osada, H., et al. (2013). Safety of intravenous administration of hydrogen-enriched fluid in patients with acute cerebral ischemia: Initial clinical studies. *Medical Gas Research*, *3*(1), 13.
Nakao, A., Kaczorowski, D. J., Wang, Y., Cardinal, J. S., Buchholz, B. M., Sugimoto, R., et al. (2010). Amelioration of rat cardiac cold ischemia/reperfusion injury with inhaled hydrogen or carbon monoxide, or both. *The Journal of Heart and Lung Transplantation*, *29*, 544–553.
Nakashima-Kamimura, N., Mori, T., Ohsawa, I., Asoh, S., & Ohta, S. (2009). Molecular hydrogen alleviates nephrotoxicity induced by an anti-cancer drug cisplatin without compromising anti-tumor activity in mice. *Cancer Chemotherapy and Pharmacology*, *64*(4), 753–761.

Niki, E. (2009). Lipid peroxidation: Physiological levels and dual biological effects. *Free Radical Biology & Medicine*, *47*(5), 469–484.
Niki, E. (2014). Biomarkers of lipid peroxidation in clinical material. *Biochimica et Biophysica Acta*, *1840*(2), 809–817.
Ning, Y., Shang, Y., Huang, H., Zhang, J., Dong, Y., Xu, W., et al. (2013). Attenuation of cigarette smoke-induced airway mucus production by hydrogen-rich saline in rats. *PLoS One*, *8*(12), e83429.
Noda, K., Shigemura, N., Tanaka, Y., Kawamura, T., Hyun Lim, S., Kokubo, K., et al. (2013). A novel method of preserving cardiac grafts using a hydrogen-rich water bath. *The Journal of Heart and Lung Transplantation*, *32*(2), 241–250.
Oharazawa, H., Igarashi, T., Yokota, T., Fujii, H., Suzuki, H., Machide, M., et al. (2010). Protection of the retina by rapid diffusion of hydrogen: Administration of hydrogen-loaded eye drops in retinal ischemia-reperfusion injury. *Investigative Ophthalmology & Visual Science*, *51*(1), 487–492.
Ohsawa, I., Ishikawa, M., Takahashi, K., Watanabe, M., Nishimaki, K., Yamagata, K., et al. (2007). Hydrogen acts as a therapeutic antioxidant by selectively reducing cytotoxic oxygen radicals. *Nature Medicine*, *13*(6), 688–694.
Ohsawa, I., Nishimaki, K., Yamagata, K., Ishikawa, M., & Ohta, S. (2008). Consumption of hydrogen water prevents atherosclerosis in apolipoprotein E knockout mice. *Biochemical and Biophysical Research Communications*, *377*(4), 1195–1198.
Ohta, S. (2011). Recent progress toward hydrogen medicine: Potential of molecular hydrogen for preventive and therapeutic applications. *Current Pharmaceutical Design*, *17*(22), 2241–2252.
Ohta, S. (2012). Molecular hydrogen is a novel antioxidant to efficiently reduce oxidative stress with potential for the improvement of mitochondrial diseases. *Biochimica et Biophysica Acta*, *1820*(5), 586–594.
Ohta, S. (2014). Molecular hydrogen as a preventive and therapeutic medical gas: Initiation, development and potential of hydrogen medicine. *Pharmacology & Therapeutics*, *144*, 1–11. http://dx.doi.org/10.1016/j.pharmthera.2014.04.006.
Ono, H., Nishijima, Y., Adachi, N., Sakamoto, M., Kudo, Y., Kaneko, K., et al. (2012). A basic study on molecular hydrogen (H2) inhalation in acute cerebral ischemia patients for safety check with physiological parameters and measurement of blood H2 level. *Medical Gas Research*, *2*(1), 21.
Ono, H., Nishijima, Y., Adachi, N., Tachibana, S., Chitoku, S., Mukaihara, S., et al. (2011). Improved brain MRI indices in the acute brain stem infarct sites treated with hydroxyl radical scavengers, Edaravone and hydrogen, as compared to Edaravone alone. A non-controlled study. *Medical Gas Research*, *1*(1), 12.
Radi, R. (2013). Peroxynitrite, a stealthy biological oxidant. *The Journal of Biological Chemistry*, *288*(37), 26464–26472.
Reuter, S., Gupta, S. C., Chaturvedi, M. M., & Aggarwal, B. B. (2010). Oxidative stress, inflammation, and cancer: How are they linked? *Free Radical Biology & Medicine*, *49*(11), 1603–1616.
Ristow, M., & Zarse, K. (2010). How increased oxidative stress promotes longevity and metabolic health: The concept of mitochondrial hormesis (mitohormesis). *Experimental Gerontology*, *45*(6), 410–418.
Salganik, R. I. (2001). The benefits and hazards of antioxidants: Controlling apoptosis and other protective mechanisms in cancer patients and the human population. *Journal of the American College of Nutrition*, *20*(5 Suppl.), 464S–472S, discussion 473S–475S.
Schapira, A. H. (2008). Mitochondria in the aetiology and pathogenesis of Parkinson's disease. *Lancet Neurology*, *7*(1), 97–109.
Schoenfeld, M. P., Ansari, R. R., Nakao, A., & Wink, D. (2012). A hypothesis on biological protection from space radiation through the use of new therapeutic gases as medical counter measures. *Medical Gas Research*, *2*, 8.

Schoenfeld, M. P., Ansari, R. R., Zakrajsek, J. F., Billiar, T. R., Toyoda, Y., Wink, D. A., et al. (2011). Hydrogen therapy may reduce the risks related to radiation-induced oxidative stress in space flight. *Medical Hypotheses*, *76*(1), 117–118.

Setsukinai, K., Urano, Y., Kakinuma, K., Majima, H. J., & Nagano, T. (2003). Development of novel fluorescence probes that can reliably detect reactive oxygen species and distinguish specific species. *The Journal of Biological Chemistry*, *278*(5), 3170–3175.

Shimouchi, A., Nose, K., Shirai, M., & Kondo, T. (2012). Estimation of molecular hydrogen consumption in the human whole body after the ingestion of hydrogen-rich water. *Advances in Experimental Medicine and Biology*, *737*, 245–250.

Shinbo, T., Kokubo, K., Sato, Y., Hagiri, S., Hataishi, R., Hirose, M., et al. (2013). Breathing nitric oxide plus hydrogen gas reduces ischemia-reperfusion injury and nitrotyrosine production in murine heart. *American Journal of Physiology. Heart and Circulatory Physiology*, *305*(4), H542–H550.

Smith, R. A., & Murphy, M. P. (2011). Mitochondria-targeted antioxidants as therapies. *Discovery Medicine*, *11*(57), 106–114.

Song, G., Li, M., Sang, H., Zhang, L., Li, X., Yao, S., et al. (2013). Hydrogen-rich water decreases serum LDL-cholesterol levels and improves HDL function in patients with potential metabolic syndrome. *Journal of Lipid Research*, *54*(7), 1884–1893.

Steinhubl, S. R. (2008). Why have antioxidants failed in clinical trials? *The American Journal of Cardiology*, *101*(10A), 14D–19D.

Sun, Q., Cai, J., Liu, S., Liu, Y., Xu, W., Tao, H., et al. (2011). Hydrogen-rich saline provides protection against hyperoxic lung injury. *The Journal of Surgical Research*, *165*(1), e43–e49.

Szabó, C. (2007). Hydrogen sulphide and its therapeutic potential. *Nature Reviews Drug Discovery*, *6*, 917–935.

Tanriverdi, H., Evrengul, H., Kuru, O., Tanriverdi, S., Seleci, D., Enli, Y., et al. (2006). Cigarette smoking induced oxidative stress may impair endothelial function and coronary blood flow in angiographically normal coronary arteries. *Circulation Journal*, *70*(5), 593–599.

Tormos, K. V., Anso, E., Hamanaka, R. B., Eisenbart, J., Joseph, J., Kalyanaraman, B., et al. (2011). Mitochondrial complex III ROS regulate adipocyte differentiation. *Cell Metabolism*, *14*(4), 537–544.

Tsukagoshi, H., Busch, W., & Benfey, P. N. (2010). Transcriptional regulation of ROS controls transition from proliferation to differentiation in the root. *Cell*, *143*(4), 606–616.

Turrens, J. F. (2003). Mitochondrial formation of reactive oxygen species. *The Journal of Physiology*, *552*(Pt. 2), 335–344.

van Berkel-Arts, A., Dekker, M., van Dijk, C., Grande, H. J., Hagen, W. R., Hilhorst, R., et al. (1986). Application of hydrogenase in biotechnological conversions. *Biochimie*, *68*(1), 201–209.

Vaziri, N. D., & Rodriguez-Iturbe, B. (2006). Mechanisms of disease: Oxidative stress and inflammation in the pathogenesis of hypertension. *Nature Clinical Practice Nephrology*, *2*(10), 582–593.

Walker, C. (2008). Antioxidant supplements do not improve mortality and may cause harm. *American Family Physician*, *78*(9), 1079–1080.

Wang, F., Yu, G., Liu, S. Y., Li, J. B., Wang, J. F., Bo, L. L., et al. (2011). Hydrogen-rich saline protects against renal ischemia/reperfusion injury in rats. *The Journal of Surgical Research*, *167*(2), e339–e344.

West, A. P., Brodsky, I. E., Rahner, C., Woo, D. K., Erdjument-Bromage, H., Tempst, P., et al. (2011). TLR signalling augments macrophage bactericidal activity through mitochondrial ROS. *Nature*, *472*(7344), 476–480.

Westerblad, H., & Allen, D. G. (2011). Emerging roles of ROS/RNS in muscle function and fatigue. *Antioxidants & Redox Signaling*, *15*(9), 2487–2499.

Wood, K. C., & Gladwin, M. T. (2007). The hydrogen highway to reperfusion therapy. *Nature Medicine*, *13*(6), 673–674.

Xu, Z., Zhou, J., Cai, J., Zhu, Z., Sun, X., & Jiang, C. (2012). Anti-inflammation effects of hydrogen saline in LPS activated macrophages and carrageenan induced paw oedema. *Journal of Inflammation*, *9*, 2.

Yoritaka, A., Hattori, N., Uchida, K., Tanaka, M., Stadtman, E. R., & Mizuno, Y. (1996). Immunohistochemical detection of 4-hydroxynonenal protein adducts in Parkinson disease. *Proceedings of the National Academy of Sciences of the United States of America*, *93*(7), 2696–2701.

Yoritaka, A., Takanashi, M., Hirayama, M., Nakahara, T., Ohta, S., & Hattori, N. (2013). Pilot study of H(2) therapy in Parkinson's disease: A randomized double-blind placebo-controlled trial. *Movement Disorders*, *28*(6), 836–839.

Yoshida, A., Asanuma, H., Sasaki, H., Sanada, S., Yamazaki, S., Asano, Y., et al. (2012). H(2) mediates cardioprotection via involvements of K(ATP) channels and permeability transition pores of mitochondria in dogs. *Cardiovascular Drugs and Therapy*, *26*(3), 217–226.

Yu, P., Wang, Z., Sun, X., Chen, X., Zeng, S., Chen, L., et al. (2011). Hydrogen-rich medium protects human skin fibroblasts from high glucose or mannitol induced oxidative damage. *Biochemical and Biophysical Research Communications*, *409*(2), 350–355.

Zhai, X., Chen, X., Shi, J., Shi, D., Ye, Z., Liu, W., et al. (2013). Lactulose ameliorates cerebral ischemia-reperfusion injury in ratsby inducing hydrogen by activating Nrf2 expression. *Free Radical Biology & Medicine*, *65*, 731–741.

Zhang, Y., Sun, Q., He, B., Xiao, J., Wang, Z., & Sun, X. (2011). Anti-inflammatory effect of hydrogen-rich saline in a rat model of regional myocardial ischemia and reperfusion. *International Journal of Cardiology*, *148*(1), 91–95.

Zhou, H., Fu, Z., Wei, Y., Liu, J., Cui, X., Yang, W., et al. (2013). Hydrogen inhalation decreases lung graft injury in brain-dead donor rats. *The Journal of Heart and Lung Transplantation*, *32*(2), 251–258.

Zhou, R., Yazdi, A. S., Menu, P., & Tschopp, J. (2011). A role for mitochondria in NLRP3 inflammasome activation. *Nature*, *469*(7329), 221–225.

Zhu, W. J., Nakayama, M., Mori, T., Nakayama, K., Katoh, J., Murata, Y., et al. (2011). Intake of water with high levels of dissolved hydrogen (H2) suppresses ischemia-induced cardio-renal injury in Dahl salt-sensitive rats. *Nephrology, Dialysis, Transplantation*, *26*(7), 2112–2118.