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Molecular hydrogen as a preventive and therapeutic medical gas: initiation, development and potential of hydrogen medicine

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ABSTRACT

Molecular hydrogen (H_2) has been accepted to be an inert and nonfunctional molecule in our body. We have turned this concept by demonstrating that H_2 reacts with strong oxidants such as hydroxyl radical in cells, and proposed its potential for preventive and therapeutic applications. H_2 has a number of advantages exhibiting extensive effects: H_2 rapidly diffuses into tissues and cells, and it is mild enough neither to disturb metabolic redox reactions nor to affect signaling reactive oxygen species; therefore, there should be no or little adverse effects of H_2. 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 into the eyes. The numerous publications on its biological and medical benefits revealed that H_2 reduces oxidative stress not only by direct reactions with strong oxidants, but also indirectly by regulating various gene expressions. Moreover, by regulating the gene expressions, H_2 functions as an anti-inflammatory and anti-apoptotic, and stimulates energy metabolism. In addition to growing evidence obtained by model animal experiments, extensive clinical examinations were performed or are under investigation. Since most drugs specifically act to their targets, H_2 seems to differ from conventional pharmaceutical drugs. Owing to its great efficacy and lack of adverse effects, H_2 has promising potential for clinical use against many diseases.

Contents

1. Introduction

Molecular hydrogen (H_2, dihydrogen, or hydrogen gas) has been accepted to behave as an inert gas at body temperature in mammalian cells. In fact, H_2 seems to react with no biological compound, including oxygen gas in the absence of catalysts at body temperature. On the other hand, in some bacteria, H_2 is enzymatically catabolyzed as an energy source for providing electrons, or is a product of some types of anaerobic metabolism. These reactions are usually catalyzed by iron- or nickel-containing enzymes called hydrogenases. In contrast, mammals have no functional hydrogenase genes (Fritsch et al., 2013). Thus, it has been believed that H_2 is nonfunctional in our cells.

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We turned this concept in a publication in 2007 that H_2 acts as a therapeutic and preventive antioxidant by selectively reducing highly strong oxidants such as hydroxyl radical (•OH) and peroxynitrite ($ONOO^-$) in cells, and that H_2 exhibits 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 published papers cover many biological effects against oxidative stress in almost all organs (Ohta, 2011, 2012). Moreover, it has been revealed that H_2 has more functions, including anti-inflammatory, anti-apoptotic, and anti-allergic effects, and that H_2 stimulates energy metabolism, in most tissues of model animals. Until 2013, the number of publications on its biologically or medically beneficial effects had been increasing and had surpassed ~300 as shown in Fig. 1. Previous review articles mainly introduced various cellular and animal experiments (Ohta, 2011, 2012).

In addition to publications on model animal experiments, more than 10 papers on clinical examinations have been published. Thus, this article will review the results of recent clinical examinations toward actual applications. Moreover, this review article will look back the discovery process of the biological effects of H_2, and propose the possible mechanisms to explain the effects of H_2.

2. Oxidative stress as pathogenic sources and physiological roles

2.1. Oxidative stress as pathogenic sources

Reactive oxygen species (ROS) are generated inside the body throughout our daily lives as a side-product of energy metabolism by oxidative phosphorylation in every aerobic organism. Occasionally, excess ROS are produced, such as smoking or air pollution, exposure to ultraviolet or irradiation rays, hard exercise, physical or psychological stress, and so on (Liu et al., 1996; Agarwal, 2005; Harma et al., 2006; Tanriverdi et al., 2006; Grassi et al., 2010). 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, or others (Ferrari et al., 1991; Vaziri & Rodriguez-Iturbe, 2006; Reuter et al., 2010). Mounting 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; Bagul & Banerjee, 2013; El Assar et al., 2013; Kim & Byzova, 2014). Under normal conditions, ROS induced by strenuous exercise results in muscle fatigue (Westerblad & Allen, 2011).

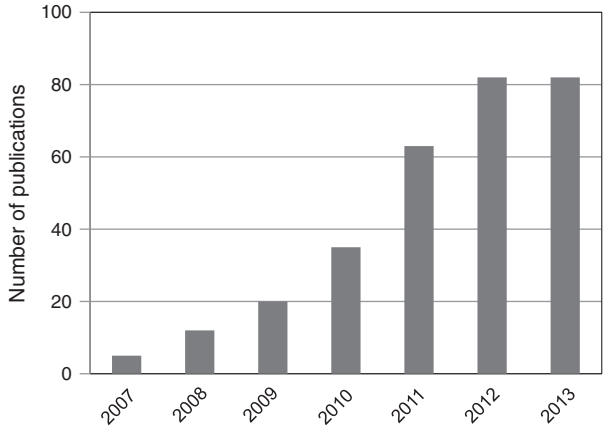

Fig. 1. The number of publications on biological effects of molecular hydrogen in each year.

2.2. The process of generation of oxygen species

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 (Finkel & Holbrook, 2000; Turrens, 2003; Andersen, 2004; Lin & Beal, 2006). Superoxide dismutase (SOD) enzymatically converts $O_2•^-$ to hydrogen peroxide (H_2O_2), which is metabolized to generate water (H_2O). Very reactive •OH is generated from H_2O_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 $O_2•^-$ with nitric oxide (•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 the 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 interaction with water, a process termed radiolysis (Schoenfeld et al., 2011, 2012).

Other enzymes, including NADPH oxidases, cytochrome p450s, lipoxygenase, cyclooxygenase, and xanthine oxidase, also participate in ROS generation in the immuno- or detoxifying-system (Droge, 2002).

2.3. Physiological roles of H_2O_2

As mentioned above, ROS had historically been believed to cause cellular damage and to lack physiological functions. Indeed, accumulation of oxidative damage by ROS has been linked to multiple pathologies, as mentioned above. Cellular redox homeostasis is, however, a delicate balance between ROS production and the antioxidant system (Bashan et al., 2009; Brewer et al., 2013). Oxidative stress is now appreciated to function as signaling molecules to regulate a wide variety of physiology (Liu et al., 2005; Bell et al., 2007a,b). H_2O_2 was shown to be required for cytokine, insulin, growth factor, AP-1, c-Jun N-terminal kinase 1 (JNK1), p53, and nuclear factor kappa B (NF-κB) signaling and to promote phosphatase-inactivation by cysteine oxidation (Finkel, 1998; Chandel et al., 2000a,b;). These reactions provide a plausible biochemical mechanism by which ROS can impinge on signaling pathways. There have been numerous reports highlighting the importance of ROS-dependent signaling in a variety of systems (Salganik, 2001; Sauer et al., 2001; Collins et al., 2012).

Additionally, oxidative stress caused by H_2O_2 and •NO induces enzymes involved in anti-oxidation 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, 2013). Moreover, H_2O_2 is a key factor to regulate cellular differentiation (Tsukagoshi et al., 2010 Tormos et al., 2011), the immune system (West et al., 2011; Zhou et al., 2011), autophagy (Li et al., 2012; Garg et al., 2013) and apoptosis (Mates et al., 2012). Thus, it is crucial not to completely eliminate H_2O_2 to maintain homeostasis.

2.4. Exploration of an ideal antioxidant

Although antioxidant therapy or prevention of various diseases is expected owing to the clinical importance of oxidative damage, antioxidants have been of limited therapeutic success (Steinhubl, 2008). Antioxidant supplements have exhibited little effect on preventing cancer, myocardial infarction and atherosclerosis, but rather conversely have increased mortality (Bjelakovic et al., 2007; Hackam, 2007; Brambilla et al., 2008; Steinhubl, 2008; Hercberg et al., 2010); thus, it is very important to be aware of side effects in developing an effective antioxidant for the prevention of oxidative stress-related diseases.

Under these situations, an ideal antioxidant molecule is expected to mitigate excess oxidative stress, but not disturb the redox homeostasis. In other words, the ideal molecule should not reduce signaling molecules

such as H_2O_2 but should effectively reduce strong oxidants such as •OH. After experiments, we have reached the current conclusion that the ideal antioxidant could be H_2.

3. Discovery of the biological effects of molecular hydrogen

During the process of searching for the ideal antioxidant, we infused H_2 into culture medium without changing pH, O_2 and CO_2 concentrations or other conditions. When cultured PC12 cells were exposed to oxidative stress by treatment with antimycin A, an inhibitor of the mitochondrial electron translocation chain, the cells apparently shrank and extended short fibers in response to oxidative stress (Fig. 2A). In contrast, when the cells were treated with the inhibitor in the presence of H_2, the cells did not change their shape (Fig. 2B). In H_2-degassed medium from H_2-medium, the cells again responded to oxidative stress. This finding indicates that H_2 affected no components in the original medium, but directly acted to the cells. By this finding of the first experiment, we foresaw that H_2 has great potential for actual clinical use.

After this experiment, we tried to identify the target of H_2 in cultured cells. H_2 dissolved in culture medium did not change the cellular levels of O_2•$^-$ and H_2O_2, as judged by the fluorescent signals of MitoSOX and dichlorofluorescein-diacetate (DCF-DA), respectively. Additionally, H_2 did not decrease the cellular level of •NO. In contrast, H_2 treatment significantly decreased levels of •OH, as judged by the decrease in the fluorescent signal of hydroxyphenyl fluorescein (HPF) (Setsukinai et al., 2003). Moreover, the decrease in the cellular •OH level by H_2 was confirmed by spin trapping technology (Halliwell & Gutteridge, 1992).

The selective reduction of ROS can be explained by the marked oxidative strength of •OH, as shown in Fig. 3, which was profiled according to published data (Setsukinai et al., 2003). This means that •OH is strong enough to react with even inert H_2, but that O_2•$^-$, H_2O_2, and •NO are insufficient to react with H_2 according to their activities. In other words, H_2 is mild enough neither to disturb metabolic redox reactions nor to affect ROS that function in cellular signaling.

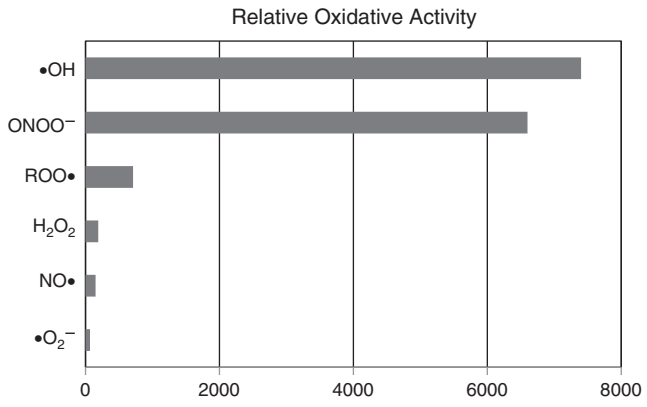

Fig. 3. Relative oxidative activities in each reactive oxygen and nitrogen species. This graph is illustrated based on data from a previous publication (Setsukinai et al., 2003).

4. Methods of how to ingest molecular hydrogen

4.1. Safety of molecular hydrogen

Previous review articles have summarized the nature of H_2 as a chemical element (Ohta, 2011, 2012). Here, the following point should be emphasized regarding the safety of H_2 for medical use: H_2 gas is flammable only at temperatures higher than 527 °C, and explodes by a rapid chain reaction with O_2 only in the explosive range of H_2 concentration (4-75%, vol/vol). Thus, H_2 can be used for medical applications without surplus worries by several ingestion methods because inhalation of 1-4% H_2 gas exhibits great efficacy (Ohsawa et al., 2007; Hayashida et al., 2008).

4.2. Inhalation of hydrogen gas

Inhalation of H_2 gas is a 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.

By a clinical examination, Ono et al. showed that inhalation of 3–4% H_2 gas reached a plateau at approximately 10–20 μM in the arterial and venous blood, respectively, in about 20 min and affected no physiological parameters, suggesting no adverse effects (Ono et al., 2012a).

4.3. Oral ingestion by drinking hydrogen water

Inhalation of H_2 gas may be unsuitable or impractical for continuous H_2 consumption in daily life for preventive use. In contrast, solubilized H_2 (H_2-dissolved water; namely, H_2-water) may be beneficial since it is a portable, easily administered and safe way to ingest H_2 (Nagata et al., 2009; Nakashima-Kamimura et al., 2009). H_2 can be dissolved in water up to 0.8 mM (1.6 mg/L) under atmospheric pressure at room temperature without changing pH. Although *ad libitum* drinking saturated H_2-water was more effective than diluted one, 80 μM of H_2-water was still effective for improving obesity (Kamimura et al., 2011).

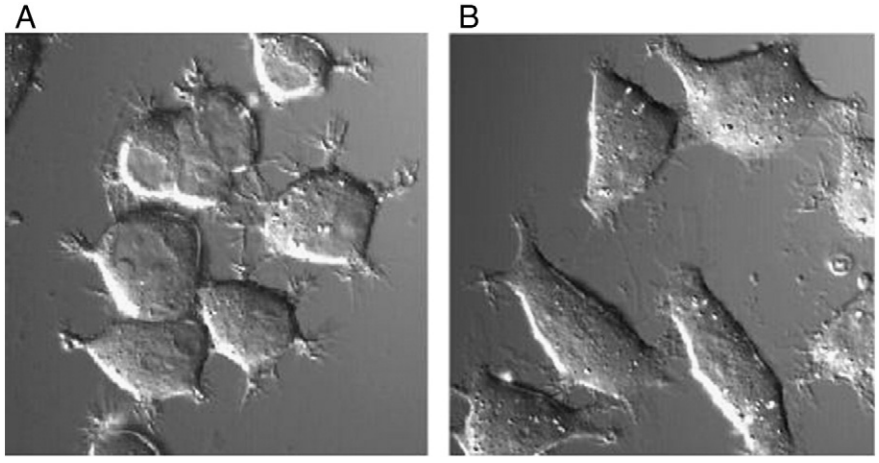

Fig. 2. Photographs of cultured PC12 exposed to oxidative stress by treatment with antimycin A without (A) or with (B) hydrogen in media.

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

4.4. 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; Sun et al., 2011a,b; G.M. Li et al., 2013).

Nagatani et al. performed an open-label, prospective, non-randomized study of intravenous H_2 administration in 38 patients hospitalized for acute ischemic stroke. All patients received an H_2 intravenous solution (200 mL twice a day) 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 (t-PA) (Nagatani et al., 2013).

4.5. Direct incorporation of molecular hydrogen by diffusion: Eye-drop, bath and cosmetics

Alternatively, H_2-loaded eye drops were prepared by dissolving H_2 in saline and directly administering to the ocular surface (Oharazawa et al., 2010; Kubota et al., 2011).

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).

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. Indeed, powders that produce H_2 baths are commercially available in Japan.

5. Monitoring the movement of molecular hydrogen

H_2 has a number of advantages as a potential antioxidant. It has favorable distributing characteristics with its own physical ability to penetrate biomembranes and diffuse into the cytosol. H_2 rapidly reaches the nucleus and mitochondria to protect nuclear DNA and mitochondria (Ohsawa et al., 2007). Moreover, H_2 passes through the blood-brain barrier although most antioxidant compounds cannot.

H_2 in blood can be monitored by the following method; venous or arterial blood is collected in a closed aluminum bag with no dead space, followed by the addition of a definite volume of air into the bag. H_2 in the air phase transferred from the blood can be measured by gas chromatography. The inhalation of H_2 actually increased H_2 dissolved in arterial blood in a H_2 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 and consumed into 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., 2012a).

The gaseous diffusion of H_2 can be monitored inside various tissues by detecting with a specific 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).

Moreover, we devised eye drops with dissolved H_2 to directly administer H_2 to the retina, and monitored the time course of changes in H_2 levels using a needle-shaped hydrogen sensor electrode inserted through the sclera to the vitreous body in rats. H_2 could reach the vitreous body by administering H_2 saturated in normal saline. When H_2 eye drops were administered continuously, approximately 70% H_2 was detected on the ocular surface (Oharazawa et al., 2010).

When water saturated with H_2 was placed into the stomach of a rat, H_2 was detected at several μM in blood (Nagata et al., 2009; Nakashima-Kamimura et al., 2009). Moreover, hepatic H_2 was monitored with a needle-type hydrogen electrode, and hepatic glycogen maintained H_2 after oral administration of H_2-water, partly explaining why consumption of even a small amount of H_2 over a short dwell time could efficiently improve various disease models (Kamimura et al., 2011).

When seven adult volunteers drank H_2-water, the H_2 content of their expired breath was measured by gas chromatography with a semiconductor (Shimouchi et al., 2012). The ingestion of H_2-water rapidly increased breath H_2 content to its maximal level 10 min after ingestion and thereafter decreased to the baseline level within 60 min. The loss of H_2 from the water during the experimental procedures accounted for 3% or less of the H_2. H_2 release from the skin surface was estimated as approximately 0.1%. Based on 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 •OH (Shimouchi et al., 2012).

6. Effects of molecular hydrogen and clinical examinations

6.1. Safety of molecular hydrogen for humans

H_2 has advantages from the aspect of toxicity: H_2 has no cytotoxicity even at high concentration (Abraini et al., 1994; Lillo et al., 1997; Fontanari et al., 2000; Lillo & Parker, 2000). Safety standards have been established for high concentrations of H_2 gas for inhalation since high-pressure H_2 gas is used in deep-diving gas mixes to prevent decompression sickness and arterial gas thrombi (Fontanari et al., 2000). Importantly for clinical use, the safety of H_2 for humans is demonstrated by its application of an extremely high concentration of H_2 gas 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; Lillo et al., 1997; Fontanari et al., 2000; Lillo & Parker, 2000). Considering that H_2 is an inert gas and nonfunctional in our body, its lack of toxic effects is easily understandable. As mentioned above, inhalation of 1–4% H_2 gas exhibits great efficacy (Ohsawa et al., 2007; Hayashida et al., 2008).

Many reports have supported that H_2 exhibited great efficacy in extensive disease models regardless of the ingestion methods of H_2 as summarized in Fig. 4. This review article focuses mainly on clinical examinations.

6.2. Protective effects against reperfusion injury

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 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 by H_2 gas (Buchholz et al., 2008), or H_2-water (Cardinal et al., 2010) and H_2-preservation solution (Noda et al., 2013).

Ono et al. intravenously administered H_2-saline with Edaravone, a clinically approved radical scavenger, in 8 patients with acute brain

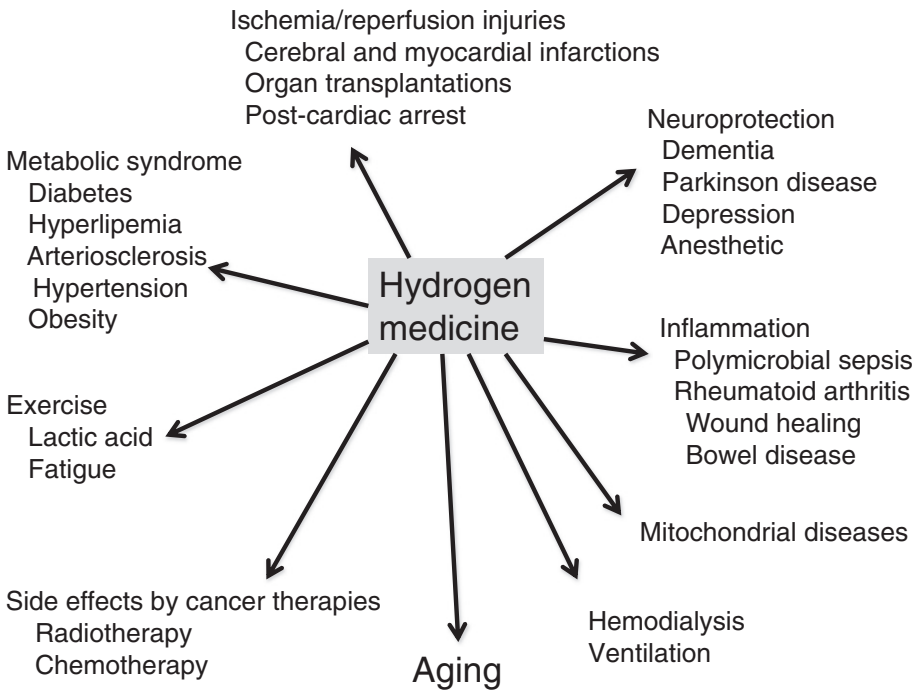

Fig. 4. Summary of potential of various preventive and therapeutic effects of H_2.

stem infarction and compared magnetic resonance imaging (MRI) indices of 26 patients who received Edaravone alone. The relative diffusion-weighted images (rDWIs), regional apparent diffusion coefficients (rADCs), and pseudo-normalization time of rDWI and rADC were all improved with the combined infusion of H_2 with Edaravone (Ono et al., 2011).

6.3. Protective effects against neurodegeneration

Chronic oxidative stress is widely accepted as one of the causes of neurodegeneration, including dementia and Parkinson 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 (Liu et al., 1996; Abrous et al., 2005). Additionally, neural proliferation in the dentate gyrus of the hippocampus is suppressed by the restraint stress (Abrous et al., 2005). Drinking H_2-water suppressed the increase in this oxidative stress and prevented this cognitive impairment (Nagata et al., 2009). Moreover, H_2-water restored neural proliferation in the dentate gyrus of the hippocampus in this model animal (Nagata et al., 2009). Since antidepressants increase adult neurogenesis (Becker & Wojtowicz, 2007; Sahay & Hen, 2007), H_2-water may be applicable for improving depression and some mental disorders.

In PD, mitochondrial dysfunction and the associated oxidative stress are major causes of dopaminergic cell loss in the substantia nigra (Yoritaka et al., 1996; Schapira, 2008). H_2 in drinking water was given before or after stereotactic surgery for 6-hydroxydopamine-induced nigrostrital degeneration in a rat model of PD. H_2-water prevented both the development and progression of nigrostriatal degeneration in rats (Fu et al., 2009). Moreover, drinking H_2-water also suppressed dopaminergic neuronal loss in another PD mice 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 levodopa-medicated PD was assessed (Yoritaka et al., 2013). Participants drank 1 L per day of H_2-water or pseudo water for 48 weeks. Total Unified Parkinson's Disease Rating Scale (UPDRS) scores in the H_2-water group ($n = 9$) improved, whereas UPDRS scores in the placebo group ($n = 8$) worsened. Despite the low number of patients and the short duration of the trial, the difference was significant ($P < .05$).

6.4. Preventive effects against metabolic syndrome

Drinking H_2-water stimulates energy metabolism (Kamimura et al., 2011). H_2-water significantly alleviated fatty liver in *db*/*db* mice, which is a 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. Moreover, drinking H_2-water decreased levels of plasma glucose, insulin, and triglyceride by stimulating energy metabolism (Kamimura et al., 2011).

Beneficial roles of H_2-water in the prevention of potential metabolic syndrome were reported by 3 independent clinical studies as follows.

Kajiyama et al. performed a randomized, double-blinded, placebo-controlled, crossover study in 30 patients with diabetes mellitus type II and 6 patients with impaired glucose tolerance. The patients consumed either 900 mL H_2-water or placebo water for 8 weeks, with a 12-week washout period. Statistical significance was observed in the improvement of electronegative charge-modified low-density lipoprotein (LDL)-cholesterol, small dense LDL, and urinary 8-isoprostanes. In four of six patients with impaired glucose tolerance, H_2 improved their indexes of the oral glucose tolerance test to a normal level (Kajiyama et al., 2008).

Nakao et al. performed an open-label trial in 20 subjects with potential metabolic syndrome (Nakao et al., 2010b). The participants drank H_2-water (1.5–2.0 L/day) for 8 weeks and showed an increase in urinary SOD; a decrease in urinary thiobarbituric acid reactive substances (TBARS), a marker of lipid peroxidation; an increase in high-density-lipoprotein (HDL)-cholesterol; and a decrease in total cholesterol.

Song et al. characterized the effects of H_2-water (0.9–1.0 L/day) on the content, composition, and biological activities of serum lipoproteins in 20 patients with potential metabolic syndrome. Serum analysis showed that drinking H_2-water for 10 weeks resulted in decreased serum total-cholesterol (TC) and LDL-cholesterol levels. In addition, H_2-water significantly improved i) protection against LDL oxidation, ii) inhibition of tumor necrosis factor-α (TNF-α)-induced monocyte

adhesion to endothelial cells, iii) stimulation of cholesterol efflux from macrophage foam cells, and iv) protection of endothelial cells from TNF-α-induced apoptosis (Song et al., 2013).

Taken together, these results strongly suggest the benefits of drinking H_2-water in patients with metabolic syndrome.

6.5. Suppressive effects on inflammation

H_2 reduced inflammation in experimental model animals induced by concanavalin A (Kajiya et al., 2009), dextran sodium sulfate (Kajiya et al., 2009), lipopolysaccharide (LPS) (Xu et al., 2012; Chen et al., 2013), Zymosan, an inducer of generalized inflammation (Xie et al., 2010b), and polymicrobial sepsis (G.M. Li et al., 2013). H_2 gas, H_2-saline and H_2-water decreased the levels of pro-inflammatory cytokines to suppress inflammation.

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by the destruction of bone and cartilage. Ishibashi et al. administered H_2-water containing 4–5 mg/L H_2 (high H_2-water) (0.5 L/day) for 4 weeks to 20 patients with RA. Urinary 8-hydroxy-deoxyguanine (8-OHdG) was significantly reduced by 14.3% ($P < 0.01$) on average. Disease activity (DAS28, using C-reactive protein levels also decreased ($P < 0.01$) during the same period. After the washout period, both the urinary 8-OHdG and the mean DAS28 had decreased compared to the end of the drinking period. During the second drinking period, the mean DAS28 was reduced ($P < 0.01$). All the 5 patients with early RA (duration < 12 months) who did not show antibodies against cyclic citrullinated peptides (ACPAs) achieved remission, and 4 of them became symptom-free at the end of the study. Thus, the symptoms of RA were significantly improved with H_2-water (Ishibashi et al., 2012; Ishibashi, 2013).

6.6. Mitigation of side effects in treatments of cancers

Inhalation of H_2 gas and drinking H_2-water improved mortality and body-weight loss caused by treatment with an anti-cancer drug, cisplatin, and alleviated nephrotoxicity in mice. Despite its protective effects against cisplatin-induced toxicity, H_2 did not affect the anti-tumor activity of cisplatin against cancer cell lines in vitro and tumor-bearing mice in vivo (Nakashima-Kamimura et al., 2009).

Kang et al. performed a randomized placebo-controlled clinical study of H_2-water (0.55–0.65 mM, 1.5–2.0 L/day) for 6 weeks in 49 patients receiving radiation therapy for malignant liver tumors. H_2 suppressed the elevation of total hydroperoxide levels, maintained serum antioxidant capacity, and improved the quality of life (QOL) scores. In particular, H_2-water efficiently prevented loss of appetite. There was no difference in tumor response to radiotherapy between the two groups (Kang et al., 2011).

6.7. Effects on dermatomyositis and mitochondrial diseases

Ito et al. performed an open-label trial of H_2-water (1.0 L/day) for 12 weeks in 14 patients with muscular diseases, including muscular dystrophies, polymyositis/dermatomyositis, and mitochondrial myopathies. In the open-label trial, significant improvements were observed in the lactate:pyruvate ratio, fasting blood glucose, serum matrix metalloproteinase-3 (MMP3), and triglycerides. In particular, the lactate:pyruvate ratio, which is a sensitive biomarker of a compromised mitochondrial electron transport system, was decreased by 28% in mitochondrial myopathies. In addition, MMP3, which represents the activity of inflammation, was decreased by 27% in dermatomyositis (Ito et al., 2011).

Then, they performed a randomized, double-blind, placebo-controlled, crossover trial of H_2-water or placebo-dehydrogenized water (0.5 L/day) for 8 weeks in 22 patients with dermatomyositis and mitochondrial myopathies. In the double-blind trial, a statistically significant improvement was observed only in serum lactate in mitochondrial myopathies by H_2-water consumption, but the lactate:pyruvate ratio in mitochondrial myopathies and MMP3 in dermatomyositis also tended to be decreased by H_2-water consumption (Ito et al., 2011).

6.8. Mitigating effects of hemodialysis

Nakayama et al. performed an open-label placebo-controlled crossover trial of 12 sessions of hemodialysis in eight patients (Nakayama et al., 2009, 2010) and an open-label trial of 78 sessions of hemodialysis in 21 patients (Nakayama et al., 2010). In both studies, continuous sessions of hemodialysis with H_2-dialysis solution decreased systolic blood pressure before and after dialysis. In the short-term study, plasma methylguanidine was significantly decreased. In the long-term study, plasma monocyte chemoattractant protein 1 and myeloperoxidase were significantly decreased.

6.9. Effects on acute erythematous skin diseases

Ono et al. treated 4 patients with acute erythematous skin diseases with fever and/or pain by intravenous administration of 0.5 L of H_2-fluid for 30 min for more than 3 days. Erythema of these 4 patients and associated symptoms improved significantly after H_2 treatment. In conclusion, an improvement in acute erythemtous skin diseases followed the administration of H_2-fluid without compromising safety (Ono et al., 2012b).

6.10. Effects on exercise

Aoki et al. recruited ten young male soccer players and subjected them to exercise tests and blood sampling. Each subject was examined twice in a crossover double-blind manner; they were given H_2-water or placebo water for one-week intervals. Subjects were requested to use a cycle ergometer at 75 % maximal oxygen uptake (VO_2) for 30 min, followed by measurement of peak torque and muscle activity throughout 100 repetitions of maximal isokinetic knee extension. Acute exercise resulted in an increase in blood lactate levels in the subjects given placebo water, whereas oral intake of H_2-water prevented the elevation of blood lactate during heavy exercise. Peak torque in the placebo water-group significantly decreased during maximal isokinetic knee extension, indicating muscle fatigue (Aoki et al., 2012).

7. Possible molecular mechanisms underlying various effects of molecular hydrogen

As mentioned, H_2 has various beneficial effects on animal models and patients; anti-oxidation, anti-inflammation, anti-apoptosis, anti-allergy and stimulation of energy metabolism (Ohta, 2011, 2012). Their mutual relationships are not clear, but the reduction of oxidative stress may primarily lead to various subsequent effects. There remain many unresolved questions regarding the molecular mechanism to fully explain the effects of H_2. In particular, although H_2 apparently regulates gene expressions and the protein phosphorylation involved in signal transduction, the primary target(s) of H_2 in these regulations has not been identified. Here, possible mechanisms are proposed as summarized in Fig. 5.

7.1. Direct reduction of hydroxyl radicals with molecular hydrogen

Considering the reaction rate of •OH with H_2 in dilute aqueous solutions, the reaction rate may be too slow to fully enable a decrease in •OH for exhibiting its beneficial roles (Buxton et al., 1988). Mammalian cells are, however, highly structured with complicated biomembranes and viscous solutions with concentrated multiple 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.

H_2 was shown to reduce •OH in an experiment using cultured cells (Ohsawa et al., 2007); however, the reduction of •OH to exhibit therapeutic and preventive effects had not been directly demonstrated at tissue level at this time. 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 a 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 of H_2 (Chuai et al., 2012).

•OH is known as a major trigger of the chain reaction of free radicals (Niki, 2009). Once the chain reaction occurs on biomembranes, it continues and expands causing serious damage to cells. H_2 may accumulate 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. 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 (Ohsawa et al., 2008; Ning et al., 2013; Zhou et al., 2013). Additionally, •OH can modify deoxy-guanine (dG) to 8-OHdG (Delaney et al., 2012; Kawai et al., 2012). H_2 decreased the level of 8-OHdG in most of the examined patients and animals (Ishibashi et al., 2012; Kawai et al., 2012).

Thus, these observations support that sufficient H_2 can efficiently mitigate tissue oxidation induced by •OH. However, when animals or humans drink H_2-water, the question remains whether H_2-water provides a sufficient amount of H_2 to efficiently scavenge •OH, which are continuously generated in normal and disease states.

7.2. Direct reduction of peroxynitrite with molecular hydrogen to regulate gene expressions

As another molecular mechanism, scavenging $ONOO^-$ 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 drinking H_2-water efficiently decreases nitro-tyrosine in animal models regardless of H_2-water (Cardinal et al., 2010), H_2 gas (Shinbo et al., 2013) or H_2-saline (C.H. Chen et al., 2010; Yu et al., 2011; Zhang et al., 2011; Zhu et al., 2011). Moreover, drinking H_2-water decreased nitrotyrosine in patients with RA (Ishibashi et al., 2012). Thus, at least a part of the effect of H_2 can be attributed to the decreased production of nitro-tyrosine of 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 various gene expressions (Radi, 2013). However, major targets have not been identified and are under investigation.

7.3. Indirect reduction of oxidative stress by regulating gene expression

H_2 reduces oxidative stress not only directly, but indirectly by inducing anti-oxidation systems, including hemeoxygenase-1 (HO-1) (Huang et al., 2010; Park et al., 2010), SOD (Zhai et al., 2013), catalase (Cai et al., 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 inhalation of H_2 gas declined in hyperoxic lung injury accompanying with 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 amelioration of cerebral ischemia-reperfusion injury in rats by H_2 (Zhai et al., 2013).

7.4. Regulation of gene expression of pro-inflammatory cytokines and hormones

H_2 seems to display various effects against various pathogenic situations indirectly by up- or down-regulation of gene expression.

In most inflammatory models, H_2 functions as anti-inflammation by decreasing the expressions of pro-inflammatory factors (Ohta, 2011). These pro-inflammatory factors include NF-κB (H. Chen et al., 2010), TNF-α, interleukin (IL)-1β, IL-6, IL-10, IL-12, CCL2 and interferon (INF)-γ, ICAM-1 (Buchholz et al., 2008), PGE2 and PGE2, (Kawasaki

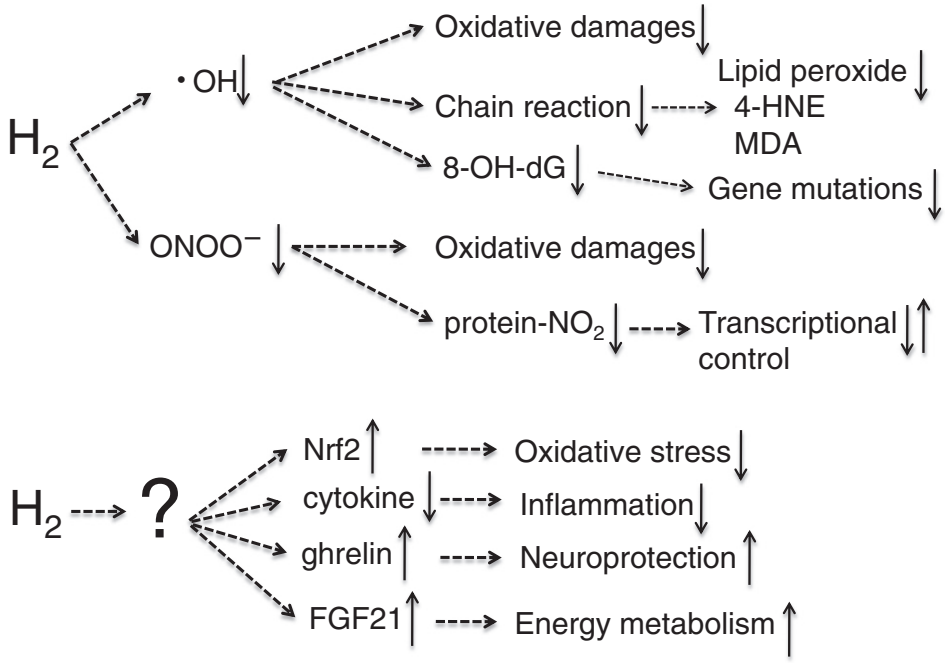

Fig. 5. Possible mechanisms of the marked efficacy by H_2 in various disease models and patients.

et al., 2010), and high mobility group box 1 (HMGB-1) (Xie et al., 2010a),

H_2-water improves obesity and metabolic parameters as mentioned above. Analysis of gene expression revealed that a hepatic hormone, fibroblast growth factor 21 (FGF21), showed increased expression by drinking H_2-water. FGF21 functions to stimulate fatty acid and glucose expenditure (Kamimura et al., 2011).

H_2-water supplementation increased the gastric expression of mRNA encoding ghrelin, a growth hormone secretagogue, and ghrelin secretion. Strikingly, the neuroprotective effect of H_2-water was abolished by administration of an antagonist to the ghrelin receptor. Thus, a neuroprotective effect of H_2 may be mediated by enhanced production of ghrelin (Matsumoto et al., 2013).

It is unlikely that H_2 could directly react with transcriptional factors to regulate the gene expressions as shown in Fig. 5.

7.5. Regulation of other genes

H_2 functions toward the suppression of apoptosis by up-regulation of anti-apoptotic factors, and/or by down-regulation of pro-apoptotic factors against various pathogenic statuses. Indeed, H_2 stimulated expressions of the anti-apoptoptic factors of Bcl-2, and Bcl-x_L (Kawamura et al., 2010) and suppressed the expressions of pro-apoptotic factors including casapase 3 (Cai et al., 2008; Sun et al., 2009), caspase 8 (Kawamura et al., 2010) and caspase 12 (J. Cai et al., 2008). In pro-apoptotic Bax, H_2 did not only down-regulate its gene expression (Kawamura et al., 2010; Huang et al., 2011), but also inhibited the translocation of Bax to mitochondria (Terasaki et al., 2011) by an unknown mechanism.

Expression profiling of rat liver demonstrated that H_2-water has a minimal effect on the expression levels of individual genes in normal rats (Nakai et al., 2011); however, more apparent up- or down-regulation of individual genes were observed in disease models and patients against the pathogenic stimuli.

H_2 seems to exhibits a variety of phenotypes toward improving many pathogenic states by regulating various gene expressions. Genes up- or down-regulated by H_2 are as follows; MMP2 and MMP9 (C.H. Chen et al., 2010); MMP3 and MMP13 (Hanaoka et al., 2011); brain natriuretic peptide; intercellular-adhesion-molecule-1 (ICAM-1) and myeloperoxidase; cyclooxygenase 2 (COX-2), neuronal nitric oxide synthase (nNOS), and connexins 30 and 43 (Hugyecz et al., 2011); collagen III (Terasaki et al., 2011); and ionized calcium binding adaptor molecule 1 (Iba1) (Sun et al., 2011b).

Additionally, recent reports indicated that ventilation with H_2 significantly increased expression of surfactant-related molecules, ATP synthases and stress-response molecules in lung grafts (Tanaka et al., 2012), and that H_2 reduced mRNA levels of osteoclast-specific markers, including tartrate resistant acid phosphatase, calcitonin receptor, cathepsin K, metalloproteinase-9, carbonic anhydrase type-II, and vacuolar-type H^+-ATPase (D.Z. Li et al., 2013).

These 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.

7.6. Modulation of signaling

H_2 attenuates the phosphorylation of FcεRI-associated Lyn and its downstream signaling molecules (Itoh et al., 2009). H_2 inhibits the phosphorylation of ASK1 and its downstream signaling molecules, p38 MAP kinase, JNK, and IκB without affecting ROS production derived from NADPH oxidase. The other study also indicated that H_2 inhibited the phosphorylations of some signal proteins, including MEK, p38, ERK, JNK (Cardinal et al., 2010). Pretreatment with H_2 reduced fatty acid uptake and lipid accumulation after palmitate overload in HepG2 cells, which was associated with inhibition of JNK activation (Iio et al., 2013). H_2 reduced the phosphorylation of p38, extracellular signal-regulated kinase, JNK, and protein kinases B stimulated with the receptor activator of NFκB ligand-induced osteoclast differentiation (D.M. Li et al., 2013). These studies suggest that 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.

8. Comparison with the other medical gasses

Several medical gasses are expected to provide more effective therapeutic interventions and preventive medicine. Hydrogen sulfide (H_2S), carbon monoxide (CO), and •NO are extremely toxic molecules; however, they play important roles as signaling molecules (Kimura, 2010; Motterlini & Otterbein, 2010). CO is poisonous even in low concentrations due to its ability to interfere with oxygen delivery, whereas endogenous CO is important in the physiological functioning of organs and possesses anti-inflammatory properties (Motterlini & Otterbein, 2010). In contrast, H_2 is advantageous to have no cytotoxicity.

In the past decade, there has been marked, rapid growth in the knowledge of gaseous molecules. Gas inhalation as disease therapy has received recent interest (Szabó, 2007; Kajimura et al., 2010). It has recently been revealed that heme-based proteins play central roles in their generation- and reception-mechanisms as the primary target of these gas molecules. An integrated approach to the interaction of these gasses revealed the physiological significance of H_2S, 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 did not reduce the oxidized heme of cytochrome c. Thus, the primary target of H_2 seems to differ from that of the other medical gasses.

Regarding the interaction between H_2 and the other toxic medical gasses, combined therapy with H_2 and CO demonstrated enhanced therapeutic efficacy via both anti-oxidant and anti-inflammatory mechanisms, and may be a clinically feasible approach for preventing ischemia/reperfusion injury in the myocardium (Nakao et al., 2010a).

Breathing NO plus H_2 during ischemia/reperfusion reduced the infarct size and maintained cardiac function, and reduced the generation of myocardial nitrotyrosine associated with •NO inhalation. Administration of •NO plus H_2 gasses for inhalation may be useful for planned coronary interventions or for the treatment of ischemia/reperfusion injury (Shinbo et al., 2013).

The production of •NO, H_2S or CO is catalyzed by •NO synthases, cystathionine γ-lyase and cystathionine β-synthase or HO-1, respectively (Kashfi & Olson, 2013). In contrast, mammalian cells have no enzyme for producing intracellular H_2.

9. Concluding remarks

This article reviewed the progress of hydrogen medicine from its initiation toward clinical applications. H_2 is easily applicable because it has no adverse effects and great efficacy on nearly all pathogenic states involved in oxidative stress and inflammation. Indeed, the clinical effects of H_2 were positive in patients with more than 10 various diseases. Since most pharmacological drugs specifically act on their targets, H_2 seems to differ from conventional drugs because of its extensive and various effects. H_2 has great potential for preventive and therapeutic applications in many diseases owing to its great efficacy and its novel concept.

Conflict of Interest Statement

The author is a Chief Scientic Officer of MitoGene, LLC (Little Rock, AR, USA).

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