
REVIEW

Preventive and Therapeutic Application of Molecular Hydrogen in Situations With Excessive Production of Free Radicals

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Summary

Excessive production of oxygen free radicals has been regarded as a causative common denominator of many pathological processes in the animal kingdom. Hydroxyl and nitrosyl radicals represent the major cause of the destruction of biomolecules either by a direct reaction or by triggering a chain reaction of free radicals. Scavenging of free radicals may act preventively or therapeutically. A number of substances that preferentially react with free radicals can serve as scavengers, thus increasing the internal capacity/activity of endogenous antioxidants and protecting cells and tissues against oxidative damage. Molecular hydrogen (H₂) reacts with strong oxidants, such as hydroxyl and nitrosyl radicals, in the cells, that enables utilization of its potential for preventive and therapeutic applications. H₂ rapidly diffuses into tissues and cells without affecting metabolic redox reactions and signaling reactive species. H₂ reduces oxidative stress also by regulating gene expression, and functions as an anti-inflammatory and anti-apoptotic agent. There is a growing body of evidence based on the results of animal experiments and clinical observations that H₂ may represent an effective antioxidant for the prevention of oxidative stress-related diseases. Application of molecular hydrogen in situations with excessive production of free radicals, in particular, hydroxyl and nitrosyl radicals is relatively simple and effective, therefore, it deserves special attention.

Key words

Oxygen and nitrogen free radicals • Cardiovascular system • Oxidative stress • Inflammation • Apoptosis • Gene expression • Molecular hydrogen • Protection

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Introduction

Excessive production of reactive oxygen species (ROS) has been regarded as a common denominator of many pathological processes. Hydroxyl and nitrosyl radicals are the major cause of the destruction of biomolecules either by a direct action or by triggering the chain reaction of free radicals. Scavenging of excessive levels of ROS may result in preventive or therapeutic effects. A number of substances can serve as scavengers that increase the capacity/activity of endogenous antioxidants thereby augmenting cells and tissues protection against oxidative damage.

Targeted application of molecular hydrogen (H₂) as one of the potent scavengers may be used in the prevention and treatment of ROS-related diseases. H₂ reacts with strong oxidants, such as hydroxyl and/or nitrosyl radicals that makes it possible to utilize its potential for preventive and therapeutic applications. H₂ rapidly diffuses into tissues and cells, without affecting metabolic redox reactions and signaling ROS. H₂ is able to reduce oxidative stress also by regulating gene expression, and acts as an anti-inflammatory and anti-apoptotic agent.

There is a growing body of evidence based on animal experiments and clinical observations that H₂ may represent an effective antioxidant used for the prevention of oxidative stress-related pathologies.

States with increased production of free radicals

Regulation of cellular redox balance is important for the maintenance of human body integrity. Many important diseases, such as ischemia, cancer, inflammation, diabetes and others and also ageing are known to be associated with increased ROS production (Majzunova *et al.* 2013).

Oxidative stress occurs due to the imbalance between the production of reactive oxygen and nitrogen species and the capability of innate biological systems to eliminate reactive intermediates. Numerous studies (Halliwell and Gutteridge 1992, Citrin *et al.* 2010, Slezak *et al.* 2015, Patel *et al.* 2016) have reported beneficial effects of a therapy with different antioxidant agents acting through different mechanisms and thus preventing cell damage.

The process of excessive ROS generation causes cellular damage and loss of physiological functions of cells and tissues linked with multiple pathologies. On the other hand, short-lasting or moderate oxidative stress is desirable to mediate signaling mechanisms regulating a variety of physiological processes (Sies 1997, Bell *et al.* 2007, Klandorf and van Dyke 2012).

The primary ROS are mostly generated by electron leakage from the mitochondrial electron transport chain (Lin and Beal 2006). Cellular redox homeostasis is a delicate balance between ROS production and the antioxidant innate system (Brewer *et al.* 2013).

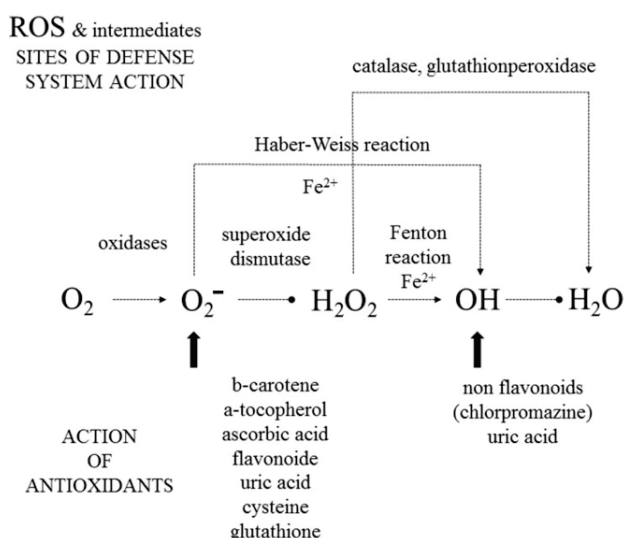


Fig. 1. ROS and intermediates sites of defense system action.

Superoxide dismutase (SOD) enzymatically converts $\bullet O_2^-$ to hydrogen peroxide (H_2O_2), which is metabolized to generate water (H_2O). Very reactive $\bullet OH$ is generated from H_2O_2 via the Fenton or Haber-Weiss reaction in the presence of catalytically active metals, such as Fe^{2+} and Cu^{2+} (Fig. 1) (Halliwell and Gutteridge 1992) or other redox-cycling compounds, which can cause damage to cells or tissues. The extent of the antioxidant depression is the key factor of cell viability where small antioxidant perturbations are readily corrected by the cell, but a large-scale depletion of antioxidants can cause cell death. DNA is a main target of this damage and its origin could be either somatic or mitochondrial. Mitochondrial DNA repair is less complete than chromosomal DNA repair. Damage to mitochondrial DNA is important for producing energy in the form of ATP, therefore, it can limit energy production leading to apoptosis and necrosis (Klandorf and van Dyke 2012).

$\bullet OH$ is the major cause of the oxidation and destruction of biomolecules by the direct reaction or by triggering the chain reaction of free radicals. Ionizing radiation including cosmic rays also generates $\bullet OH$ as a damaging intermediate through the interaction with water, a process termed radiolysis (Schoenfeld *et al.* 2012). $\bullet OH$ is strong enough to react with H_2 , on the other hand, $\bullet O_2^-$, H_2O_2 , and $\bullet NO$ are insufficient to react with H_2 (Ohta 2014).

In situation of the oxidative stress under physiological conditions, cells are able to increase the activities of antioxidant enzymes and other defense mechanisms to counteract effects of ROS. These include manganese-dependent superoxide dismutase (Mn-SOD), copper/zinc superoxide dismutase (Cu/Zn-SOD), glutathione peroxidase, glutathione reductase and catalase (CAT). Mn-SOD and Cu/Zn-SOD convert $\bullet O_2^-$ to hydrogen peroxide, which is then transformed to water by glutathione peroxidase or catalase (Halliwell and Gutteridge 1992).

General antioxidant defense is also provided by low molecular weight antioxidants, which are hydrogen atom-donating reducing agents such as ascorbic acid, tocopherols, polyphenols, and thiols such as glutathione. In such situation, the oxidants are neutralized by hydrogen atom donation, resulting in formation of a less reactive or nonreactive product from the original oxidant and a radical product from the antioxidant, which no longer can exert detrimental effects (Citrin *et al.* 2010, Slezak *et al.* 2015).

Acute oxidative stress occurs in different situations, such as inflammation, cardiac or cerebral ischemia, organ transplantation and others (Reuter *et al.* 2010, Vaziri and Rodriguez-Iturbe 2006). On the other hand, evidence has established strong links between chronic oxidative stress and numerous pathologies including oncological diseases, diabetes mellitus, atherosclerosis, and chronic inflammatory processes, as well as many neurodegenerative diseases and the process of aging (Ohta 2014).

Non-free radical species

Oxidative stress is implicated in the pathophysiology of multiple conditions including cardiovascular diseases (CVD). Although the harmful cellular effects of free radicals *in vitro* remain undisputed, observational evidence along with clinical trials of free radical scavengers has been disappointing (Steinhubl 2008, Bjelakovic *et al.* 2007, Pechanova and Simko 2009, Patel *et al.* 2016).

This has promoted the concept that free radicals may not constitute clinically important sources of oxidants and that non-free radical species may be of equal or greater importance (Jones 2008, Go and Jones 2013, Jones and Liang 2009).

Cysteine represents the major aminothiols pool extracellularly that reacts easily with oxidants to form oxidized disulphide cystine. Intracellularly, glutathione is a major antioxidant that helps to eliminate peroxides and maintain cellular redox state, and its oxidized form is glutathione disulfide. Increased oxidative stress, measured as higher levels of cystine, lower levels of glutathione, or altered ratios of oxidized to reduced aminothiols, is associated with cellular dysfunction, aging and risk factors of cardiovascular diseases (Patel *et al.* 2016).

Physiological role of H₂O₂ and signaling

Oxidative stress and/or nitrosative stress caused by H₂O₂ and •NO induces enzymes involved in anti-oxidation and tolerance to protect cells against oxidative damage (Endo *et al.* 2009, Jazwa and Cuadrado 2010). There have been numerous reports highlighting the importance of ROS-dependent signaling in a variety of systems (Collins *et al.* 2012).

H₂O₂ 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 (Matés *et al.* 2012). Thus, it is crucial not to completely eliminate H₂O₂ to maintain homeostasis (Ohta *et al.* 2014).

Selected situations with established excessive production of ROS

Ischemia and reperfusion

Ischemia and reperfusion (I/R) is a major cause for inducing acute oxidative stress. In ischemic myocardium, accelerated generation of ROS through incomplete reduction of oxygen upon reperfusion is a potential mediator of reperfusion injury (Bolli *et al.* 1988, 1989, Zweier 1988, Vanden Hoek *et al.* 2000). ROS are produced by different cellular mechanisms, and their accumulation in the myocardium is generally considered to result in deleterious effects. These oxygen species are highly reactive and can quickly overwhelm the cell's endogenous free radical scavenging system. This, in turn, triggers cellular injury by reactions with lipids, proteins, and nucleic acids. The enzyme, xanthine oxidase, has been particularly implicated as a generator of free radicals in the reperfused heart, as its substrates (xanthine and hypoxanthine) accumulate during ischemia (Xia and Zweier 1995). The central role of ROS in reperfusion injury was demonstrated in studies showing that inhibitors of mitochondrial respiratory complexes I and III prevented reperfusion ROS generation and improved cellular viability.

ROS mediate myocardial injury by inducing mitochondrial permeability transition pore opening, causing a loss of mitochondrial membrane potential, and leading to mitochondrial swelling with membrane rupture (Halestrap *et al.* 2007). Targeting mitochondria with the effective antioxidant could prevent mitochondrial permeability transition pore opening when ROS are produced during early phase of heart reperfusion, however, this therapy failed to abrogate pathological cascade of oxidative damage after reperfusion injury (Zhang *et al.* 2012).

The pathogenesis of myocardial I/R injury reflects the confluence of multiple pathways including ion channels, reactive oxygen species, inflammation, and endothelial dysfunction. Despite its complexity it is necessary to look for the possibility to mitigate this form of injury because many patients annually undergo either spontaneous or iatrogenic I/R events (Turer and Hill 2010).

Many attempts have been made to inhibit ROS production to limit the extent of reperfusion injury. The

administration of ROS scavengers at the time of reperfusion has brought conflicting results that can be partially explained by the dual role of ROS in I/R (Flaherty *et al.* 1994), since ROS play an important role as signaling molecules as well.

The majority of detrimental effects associated with lethal reperfusion injury are attributed to $\bullet\text{OH}$. By comparison, $\bullet\text{O}_2^-$ and H_2O_2 have less oxidative energy and, paradoxically, are implicated as crucial signaling components in the establishment of tolerance to oxidative stress (Ristow and Zarse 2010). Thus, cytotoxic radicals such as $\bullet\text{OH}$ must be neutralized without compromising the essential biological activities of other ROS and/or nitrosyl radicals, including $\bullet\text{NO}$ (Penna *et al.* 2006, Downey and Cohen 2006).

Increased production of ROS and failure of the antioxidant defense system are considered to play a central role in the pathogenesis of cardiovascular disease. The transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is a key master switch controlling the expression of antioxidant and protective enzymes, and was proposed to participate in protection of vascular and cardiac function. The central role of Nrf2 in maintenance of tissue redox balance, and Nrf2-dependent biochemical pathways may play an important role in preservation of cardiac and vascular function (Erkens *et al.* 2015).

Preconditioning and postconditioning

Strategy of myocardial protection based on the exploitation of the heart's own intrinsic protective mechanisms is known as preconditioning. Apart from the short-lasting protection afforded by classical ischemic preconditioning (IP) or its delayed ("second window") phase, adaptation to longer-lasting physiological stimuli or pathological processes is also known to increase myocardial resistance to ischemic injury. Although molecular mechanisms of cardiac adaptation conferring a higher ischemic tolerance still remain not sufficiently elucidated, multiple cascades of intracellular signalization are suggested to be involved in this process (Ravingerová 2007, 2012). Suppression of ROS production during IP procedure by antioxidant N-acetylcysteine and by mitochondrial K_{ATP} channel (mito K_{ATP}) blocker 5-hydroxydecanoate blunted cardioprotective effect of IP indicating an important role of ROS in preconditioning signaling cascades linked with mito K_{ATP} opening (Matejíková *et al.* 2009).

Myocardial ischemic postconditioning afforded by brief episodes of I/R applied immediately after the

ischemic insult enhances resistance to lethal I/R injury as well. Cardioprotection is conveyed by parallel pro-survival signaling pathways converging on mitochondria to prevent mitochondria permeability transition pore opening. Research indicated that postconditioning is associated with free radicals generation including nitric oxide ($\bullet\text{NO}$) and superoxide ($\bullet\text{O}_2^-$), and that cardioprotection is blunted by antioxidants. Since $\bullet\text{NO}$ and $\bullet\text{O}_2^-$ react to form peroxynitrite, it was hypothesized that postconditioning might trigger the formation of peroxynitrite to promote cardioprotection. Peroxynitrite generated in the myocardium during postconditioning activated cardioprotective mechanisms improving both structural and functional integrity of the left ventricle exposed to ischemia and reperfusion *in vivo* (Li *et al.* 2013).

Radiation

Mechanisms by which radiation causes tissue injury of both malignant and normal tissues involves induction of apoptosis due to free radical-mediated DNA damage, and the sequence of overlapping events that include activation of the coagulation system, inflammation, and tissue remodeling. This complex process is orchestrated by a large number of interacting molecular signals including cytokines, chemokines, and growth factors. Radiation affects tissues by direct and indirect effects.

Direct action. DNA is the principle target of biological effects of radiation. Radiation may damage the DNA directly, causing ionization of the atoms in the DNA molecule. Increased expression of pro-inflammatory and other genes has been demonstrated within hours following irradiation (Hong *et al.* 1995, Kyrkanides *et al.* 2002). These include genes of transcription factors such as nuclear factor-kappa B (NF- κ B), cytokines, such as tumor necrosis factor- α (TNF α), interleukin-1 β (IL-1 β), and basic fibroblast growth factor (bFGF) involved in inflammatory processes (Slezak *et al.* 2015). Radiation treatment causes also direct damage to blood vessels by the generation of ROS that disrupt DNA strands and leading to an inflammatory cascade (Hatoum *et al.* 2006, Slezak *et al.* 2015).

Indirect action via production of free oxygen radicals. Radiation interacts with non-critical target atoms or molecules, usually water. This results in the production of free radicals. Free radicals can then attack critical targets such as the DNA, because they are able to penetrate the cell. The initial ionization event does not have to occur so

close to the DNA in order to cause damage.

The 'law of Bergonié and Tribondeau' formulated in 1906 declares that X-rays are more effective in the cells which have a greater reproductive activity than any cells that are immature. Undifferentiated and actively dividing cells are more radiosensitive than mature non-dividing cells. A radiosensitive cell would have a greater chance of death after exposure to ionizing radiation than a radioresistant cell. Experiments have shown that the effects of ionizing radiation on a cell also depend on the total dose and exposure rate. A large dose given within a short time interval is more damaging than the same dose given during a longer period of time (Muller 1927).

Radiation induces tissue injury at the cellular level. Most susceptible tissues are those with a higher percentage of dividing cells (Brown and Rzucidlo 2011). To protect healthy tissue surrounding tumor, molecular hydrogen (H₂) may afford reasonable protection since it neutralizes detrimental ROS, especially the hydroxyl radical (Qian *et al.* 2010).

Inflammation

Oxidative and nitrosative stress is usually tightly connected with inflammatory response and metabolic processes accompanied by the generation of highly reactive free radicals. ROS and reactive nitrogen species (RNS), such as superoxide, nitric oxide, peroxynitrite and hydrogen peroxide and other ROS/RNS-related compounds can react with other substrates. Under physiological conditions, these are counterbalanced by an array of defense mechanisms. In situations where defense is compromised, ROS and RNS may react with fatty acids, proteins and DNA and damage them. Antioxidants (like coenzyme Q10, vitamin C and E, and glutathione) and innate antioxidant enzymes (e.g. superoxide dismutase, glutathione peroxidase and catalase) regulate oxidative and nitrosative reactions. Some proteins function as antioxidants by binding ROS and RNS, e.g. acute phase proteins such as albumin, transferrin, haptoglobin and ceruloplasmin. Oxidative and nitrosative stress occurs as a result of lower activity of tissue antioxidant enzymes and cause oxidative damage to membrane lipids (lipid peroxidation) and DNA, as well as nitrosative damage to proteins (Maes 2009a,b). Activation of inflammatory reaction and oxidative and nitrosative stress pathways is a key pathophysiological factor of a number of diseases including inflammation and depression (Maes 2008).

Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic inflammation associated with an increased risk of atherosclerosis, which can result in cardiovascular disease and increased mortality. While the mechanisms responsible for the onset of RA remain unclear, ROS play a significant role in the pathogenesis of RA (Mirshafiey and Mohsenzadegan 2008, Gelderman *et al.* 2007).

ROS play a central role both upstream and downstream of NF- κ B and TNF α pathways, which are considered to play a central role in the inflammatory response. Among ROS, the hydroxyl radical is the most harmful. Molecular hydrogen has been proposed as a selective ROS scavenger. Recently, it has been shown that H₂ is beneficial when administered along with the conventional therapy of RA and is able to reduce oxidative stress in the patients. Especially in the early stage of RA, H₂ showed significant therapeutic potential, which also seemed to determine diagnostic and treatment strategy (Ishibashi 2013).

Endothelium injury: role and impact of ROS

The endothelium plays an essential role in maintaining of cardiovascular function, and early changes in endothelial function are indicators of cardiovascular morbidity and mortality (Okruhlicova *et al.* 2012, Triggle *et al.* 2012). Important functional role of endothelium is represented by the control of blood flow, angiogenesis, inflammation, platelet aggregation, and vascular remodeling, as well as by control of metabolism (Stewart *et al.* 2010, Slezak *et al.* 2015).

I/R injury is dependent not only on the events within cardiomyocytes, but the endothelium is an active participant as well. The endothelium is the major source of the ephemeral molecule of nitric oxide (NO). Under normal conditions, NO generation elicits vasodilation, which has beneficial, protective effects during I/R, likely by influencing oxygen consumption, platelet aggregation, (Radomski *et al.* 1987) leukocyte adhesion, and free radical scavenging. Paradoxically, in high concentrations, NO may potentiate ROS-mediated toxicity by promoting the formation of highly reactive species, such as peroxynitrite (Wang and Zweier 1996, Turer and Hill 2010).

The equilibrium of the nitroso/redox balance between RNS and ROS is crucial for the function of cardiomyocytes. The key role of nitric oxide synthases (NOSs) in the maintenance of the nitroso/redox balance is obvious. Uncoupled endothelial NOS has been suggested

to shift the balance from nitric oxide to ROS production, contributing to increased oxidative stress within the myocardium (Zhang *et al.* 2012).

Endothelium is not only an important source of NO, but also of numerous other signaling molecules including the putative endothelium-derived hyperpolarizing factor, prostacyclin, and hydrogen peroxide, which have both vasodilating and vasoconstricting properties. It modulates flow-mediated vasodilatation as well as influences mitogenic activity, platelet aggregation, and neutrophil adhesion. These early effects are followed by endothelial cells proliferation and obstruction of myocardial capillary lumen (Gyenes 1998).

It has been postulated that damage to the microvascular components begins with the injury of endothelial cells within blood capillaries of the heart. Endothelial damage leads to an acute inflammatory reaction and to activation of the coagulation mechanisms with consequent fibrin deposition. The activation of macrophages and monocytes during the inflammatory process (Slezak *et al.* 2015) results in the continuous secretion of cytokines and growth factors, including tumor necrosis factor (TNF), interleukin IL-1, IL-6, IL-18, monocytes chemotactic factor. Besides induction of adhesion molecules, up-regulation of some cytokines (namely IL-6 and IL-8) has been observed after endothelial cell irradiation in a time- and dose-related manner (Burger *et al.* 1998, Van der Meeren *et al.* 1999).

The redox imbalance between nitric oxide and superoxide generated in the endothelium is thought to play a pivotal role in the development of endothelial dysfunction. H₂O₂ is known to exert both beneficial and detrimental effects on the vasculature. Nonetheless, the influence of the hydroxyl radical, a by-product of H₂O₂ decay, is unclear, and there is no direct evidence that the hydroxyl radicals impair endothelial function in conduit arteries (Sakai *et al.* 2014). Molecular hydrogen may protect the vasculature from shear stress-derived detrimental ROS, such as hydroxyl radical, by maintaining the NO-mediated vasomotor response (Sakai *et al.* 2014).

Protective effect of molecular hydrogen as a scavenger of hydroxyl radical

Acute oxidative stress causes serious damage to tissues, and persistent oxidative stress is one of the causes of many diseases including cancer, cardiovascular pathologies and the aging process. In fact, molecular

hydrogen has a potential as an effective antioxidant for many medical applications (Ohsawa *et al.* 2007, Ohta 2012, 2014). It was also demonstrated that hydrogen-rich saline significantly improved post-ischemic functional recovery of rat hearts (Chang and Toledo-Pereyra 2013).

To attenuate the harmful effects of oxidative stress and ROS, one can directly eliminate them by medical gases such as carbon monoxide (CO), hydrogen sulphide (H₂S), and H₂, or one can induce ROS-resistant proteins and antioxidant enzymes to antagonize oxidative stress (Noda *et al.* 2011).

Molecular hydrogen is the smallest gas molecule consisting of two protons and two electrons. Hydrogen is combustible when the concentration is 4-75 %. On the other hand, H₂ is a stable gas that can react only with oxide radical ion ($\bullet\text{O}^-$) and hydroxyl radical ($\bullet\text{OH}$) in water with low reaction rate constants (Buxton *et al.* 1988). Molecular hydrogen neutralizes detrimental ROS, especially the hydroxyl radical (Ohta 2014).

Molecular hydrogen was originally thought to be an inert gas in mammalian cells, however, previous studies showed that this is not always the case. It neutralizes the hydroxyl radical and peroxyxynitrite inside the cells and acts as an antioxidant to protect the cells against oxidative stress (Ishibashi 2013, Ohta 2014). Subsequently, H₂ was shown to exhibit multiple functions including anti-inflammatory, anti-apoptotic, anti-allergic, and antioxidant properties, as well as regulation of cell differentiation and involvement in energy metabolism. Molecular hydrogen also regulates various signal transduction pathways and the expression of many genes (Hanaoka *et al.* 2011, Ohta 2011).

Numerous studies have suggested that H₂ has the potential for a variety of therapeutic and preventive applications (Ohta 2014). In addition to extensive animal experiments, several clinical studies examining the efficacy of H₂ have been reported including double-blinded clinical studies in patients with Parkinson's disease and rheumatism (Yoritaka *et al.* 2013, Ishibashi *et al.* 2014).

As mentioned before, H₂ reacts with strong oxidants such as hydroxyl radical in cells, and is potentially proposed for preventive and therapeutic applications. Molecular hydrogen has a number of advantages exhibiting extensive effects: H₂ rapidly diffuses into tissues and cells, and it is mild enough neither to affect metabolic redox reactions nor to affect signaling reactive oxygen species. Therefore, no adverse effects of H₂ should be expected. In addition to a growing

number of results obtained from animal experiments, extensive clinical examinations have been reported or are currently running. Since most drugs act specifically to reach their targets, H₂ seems to differ from conventional pharmaceutical drugs. Due to its great efficacy and lack of adverse effects, H₂ has a promising potential for a clinical use to treat many diseases (Ohta 2014).

A major breakthrough in hydrogen research occurred after Ohsawa *et al.* (2007) reported a prominent effect of molecular hydrogen on a rat model of cerebral infarction. Rats were subjected to left middle cerebral artery occlusion and placed in 2-4 % hydrogen gas chamber. Results showed significantly smaller infarction volumes compared to controls. The authors attributed the hydrogen effect to the specific activity of scavenging hydroxyl radical (•OH). They also demonstrated that hydrogen scavenges peroxynitrite but to a lesser extent (Oshawa *et al.* 2007).

Ways of H₂ applications

There are several methods how to apply H₂. Inhalation of H₂ gas, ingestion by drinking H₂-rich water (H₂-water), injecting H₂-rich saline (H₂-saline), taking H₂ bath, or dropping H₂-saline into the eyes. The numerous studies of its biological and medical benefits revealed that H₂ reduces oxidative stress not only by direct reactions with strong oxidants, but also indirectly by regulating various gene expressions (Ohta 2014).

H₂ can be dissolved in water up to 0.8 mM (1.6 ppm, wt/vol) under higher pressure. H₂ rapidly disappears, it penetrates the glass and plastic walls of any vessel. However, aluminum containers are able to retain hydrogen gas at atmospheric pressure at refrigerator temperature for relatively long time (Ohta 2014).

More recent studies have also shown that hydrogen is able to reduce ROS *in vitro*, as a result of its proposed ability to scavenge the hydroxyl radical, “the most potent oxidant known to mankind” (Dole *et al.* 1975, Xie *et al.* 2010). Electrolyzed-reduced water scavenges active oxygen species and protects DNA from oxidative damage (Shirahata *et al.* 1997).

Inhalation of 1-4 % (v/v) H₂ gas is effective for the treatment of ischemia/reperfusion injury (Hayashida *et al.* 2008). Recently, inhalation of 1.3 % H₂ gas from a premixed gas was shown to protect neurons in a cardiac arrest model. However, the mechanism of how such a low concentration of H₂ exerts the positive effects is not known.

Oral administration is safe and convenient, H₂ in

water tends to escape over time and some H₂ is lost in the stomach or intestine, making it difficult to control the concentration of H₂ administered. Administration of H₂ *via* an injectable hydrogen saline (H₂-rich saline) vehicle may allow the delivery of more accurate concentrations of H₂ (Schoenfeld *et al.* 2011, Kajimura *et al.* 2010).

Medical gasses like CO, NO and H₂S are generated by endogenous enzymatic systems. Pharmaceutical research has taken advantage of these systems to design exogenous molecules to simulate those generated endogenously; however, mammals lack their own enzyme to produce H₂ (Kajimura *et al.* 2010).

Other findings indicate that H₂ released from intestinal bacteria can suppress inflammation. H₂ also mediates the suppression of colon inflammation induced by dextran sodium sulfate (Kajiya *et al.* 2009a).

Hydrogenized saline can also be administered either by intraperitoneal injection or drip infusion. Hydrogen water is mostly given *ad libitum*. Hydrogen gas is usually given by inhaling 1-4 % hydrogen gas, which is below the explosion level (4 %). Continuous exposure to hydrogen gas and *ad libitum per os* administration of hydrogen water modulated signaling pathways and gene expressions in different manners in mice (Shirahata *et al.* 1997).

The number of clinical trials is increasing every year. In most diseases, the effect of hydrogen has been reported with hydrogen water or hydrogen gas, which was followed by confirmation of the effect with hydrogen-rich saline or Krebs-Henseleit solution. Recently, Zálešák *et al.* (2016) performed pilot experiments demonstrating that hydrogen-rich Krebs-Henseleit solution administered in a mode of postconditioning improved post-ischemic recovery in isolated rat hearts. The improvement in functional recovery was coupled with a significant reduction in infarct size. Histological analysis revealed a substantial decrease in hemorrhage and necrosis within the ischemic zone. Application of hydrogen during early reperfusion appears to be cardioprotective and to facilitate anti-infarct potential of postconditioning without additional anti-stunning protection (Zálešák *et al.* 2016).

Possible molecular mechanisms of H₂ action

Iuchi *et al.* (2016) elucidated one of the molecular mechanisms by which H₂ mediates signal transduction and gene expression. The results suggested that low concentrations of H₂ modulated Ca²⁺ signal transduction and regulated gene expression by modifying

the production of oxidized phospholipid species (Iuchi *et al.* 2016).

Among genes involved in signaling pathways, the proportion of those involved in Ca^{2+} signaling was lower in the selected genes than in those in the entire genome indicating that H_2 regulates fewer components of the Ca^{2+} signaling pathways. This was consistent with the finding that H_2OxPAPC decreased Ca^{2+} signaling. In contrast, the proportion of genes belonging to the mitogen-activated protein kinase (MAPK) signaling was higher indicating that H_2 regulates more components of MAPK signal transduction pathways (Iuchi *et al.* 2016).

Low concentrations of H_2 contribute to various signal transduction pathways *via* oxidized phospholipid species. These data are consistent with the previous studies showing the Ca^{2+} -dependent regulation by transcription factors: cAMP response element-binding protein (CREB) is activated *via* phosphorylation by a calmodulin-dependent kinase (CaMK) in a Ca^{2+} -dependent manner, and nuclear factor of activated T-cells (NFAT) is dephosphorylated by calcineurin in a Ca^{2+} -dependent manner, translocates to the nucleus, and then acts as a transcription factor with its partner proteins, e.g. activator protein 1 (AP-1), CREB, or nuclear factor-kappa B (NF- κ B). Thus, H_2 -dependent oxidized mediators or putative antagonists could be associated with transcriptional regulation *via* Ca^{2+} signaling (Iuchi *et al.* 2016, Christl *et al.* 1992). Low concentrations of H_2 modulate Ca^{2+} signal transduction and regulate gene expression by modifying the production of oxidized phospholipid species. Data provide important insights into one of the molecular mechanisms by which H_2 mediates gene expression (Iuchi *et al.* 2016, Christl *et al.* 1992, Dixon *et al.* 2013, Ohno *et al.* 2012).

Understanding the exact molecular mechanisms of the effects of hydrogen will elucidate and clarify the pros and cons of hydrogen therapy. It will also lead to the development of another therapeutic modality to modulate the master regulator(s). Hydrogen exhibits statistically significant effects in patients but they are usually not as conspicuous as those observed in animal models. It might be caused by the difference in species, and to a certain extent by a technical difficulty in preparing a high concentration of hydrogen-rich water every day (Fujita *et al.* 2009). A similar dose-response effect is also observed in inhaled hydrogen gas (Ohsawa *et al.* 2007, Hayashida *et al.* 2008, Fukuda *et al.* 2007). Hydrogen-rich water generally shows a more prominent effect than hydrogen gas, although the amount of hydrogen taken up by

hydrogen water is ~ 100 times less than that given by hydrogen gas (Ito *et al.* 2012). Gastric secretion of ghrelin may partly account for that difference (Ichihara *et al.* 2015).

Effects of hydrogen in various situations have been attributed to four major molecular mechanisms: a specific scavenging activity to neutralize hydroxyl radical, a scavenging activity to neutralize peroxynitrite, modulation of altered gene expressions, and signal-modulating activities. The four mechanisms are not mutually exclusive and some of them may be causally associated with other mechanisms.

The first molecular mechanism identified for hydrogen appears to be its specific scavenging activity (Ohsawa *et al.* 2007). As was shown, oxidative stress markers like 8-OHdG, 4-hydroxyl-2-nonenal (4-HNE), malondialdehyde (MDA), and thiobarbituric acid reactive substances (TBARSs) are decreased in all examined patients and animals, as hydrogen can easily dissipate by exhalation. Hydrogen in drinking water is able to stay in human and rodent body for less than 10 min. Hydrogen, however, can bind to glycogen, and its stay is prolonged in rat liver after food intake (Kamimura *et al.* 2011).

Peroxynitrite and H_2 effect

Reaction of $\bullet\text{O}_2^-$ with $\bullet\text{NO}$ generates peroxynitrite, which is a very active nitrogen species (Radi 2013). Therefore, another molecular mechanism of hydrogen effect is its peroxynitrite scavenging activity. Although hydrogen cannot eliminate peroxynitrite as efficiently as hydroxyl radical *in vitro* (Ohsawa *et al.* 2007), hydrogen can markedly reduce NO-induced production of nitrotyrosine in rodents (Chen *et al.* 2010, Yu *et al.* 2011, Zhang *et al.* 2011, Zhu *et al.* 2011, Hanaoka *et al.* 2011). NO is a gaseous molecule that also exerts therapeutic effects including relaxation of blood vessels and inhibition of platelet aggregation (Thomas *et al.* 2008). However, NO is also toxic at higher concentrations because it leads to production of nitrotyrosine, which compromises protein functions. A part of hydrogen effects may thus be attributed to the reduced production of nitrotyrosine (Zhang *et al.* 2011, Zhu *et al.* 2011).

Specific scavenging activities of hydroxyl radical and peroxynitrite, however, cannot fully explain the anti-inflammatory and anti-apoptotic effects, which should involve a number of fine-tuned signaling pathways. It was shown that hydrogen suppresses signaling pathways in allergies (Itoh *et al.* 2009) and

inflammation (Itoh *et al.* 2011) without directly scavenging reactive oxygen/nitrogen species. Signaling molecules that are modulated by hydrogen include RAS (Chen *et al.* 2013).

Despite of positive action of H₂ as a ROS scavenger there remain some unanswered questions. First, no dose-response effect of hydrogen has been observed. Hydrogen has been administered to animals and humans in the forms of hydrogen gas, hydrogen-rich water, hydrogen-rich saline, instillation, and dialysis solution. As hydrogen mostly disappears within about 10 min by dissipation *via* exhalation, an individual is exposed to 2.8 % hydrogen only for about 10 min (Ohno *et al.* 2012).

The second unanswered question concerns intestinal production of hydrogen gas in rodents and humans. Although no mammalian cells can produce hydrogen endogenously, it is produced by intestinal bacteria carrying hydrogenase in both animals and humans. Humans are able to produce a maximum of 12 liters of hydrogen in the intestines (Christl *et al.* 1992, Strocchi and Levitt 1992). Specific pathogen-free animals are different from aseptic animals and carry intestinal bacteria that produce hydrogen. The amount of hydrogen taken by water or gas is much less than that produced by intestinal bacteria, but the exogenously administered hydrogen demonstrates a prominent effect. Kajiya *et al.* (2009b) reported that drinking hydrogen-rich water was more effective than the restitution of hydrogenase-positive bacteria in intestine. If intestinal hydrogen is as effective as the other hydrogen administration methods, we can easily increase hydrogen concentrations in our bodies by an alfa-glucosidase inhibitor, acarbose, an ingredient of curry, turmeric, or a nonabsorbable synthetic disaccharide, lactulose (Shimouchi *et al.* 2009, Corazza *et al.* 1992, Chen *et al.* 2011). Therefore, it is necessary to elucidate in more details molecular basis of hydrogen effects in more details.

Administration of H₂ serves as an antioxidant treatment, and it has also been shown to have anti-inflammatory, cytoprotective and signal modulatory effects.

Anti-inflammatory effect. Hydrogen has been described to possess anti-inflammatory properties. Gharib *et al.* (2001) observed these anti-inflammatory effects in a mouse model of chronic liver inflammation induced by the parasite *Schistosoma mansoni*. Hyperbaric hydrogen treatment was able to improve liver hemodynamics and reduce portal hypertension, as well as to reduce liver

fibrosis by attenuating inflammatory cytokines (Gharib *et al.* 2001).

Administration of H₂ revealed that oxidoreduction-related genes were upregulated in many pathological models. Administration of hydrogen downregulated pro-inflammatory cytokines including tumor necrosis-factor-(TNF-), interleukin-(IL-) 1 β , IL-6, IL-12, interferon-(IFN-), and high mobility group box 1 (HMGB1) (Buchholz *et al.* 2008, 2011, Kajiya *et al.* 2009a,b, Mao *et al.* 2009, Zheng *et al.* 2009, Nakao *et al.* 2010, Liu *et al.* 2010, Hayashi *et al.* 2011, Yoon *et al.* 2011, Song *et al.* 2011, Huang *et al.* 2011, Sun *et al.* 2009, 2011, Wang *et al.* 2011, Ji *et al.* 2011, Liu *et al.* 2011, Shen *et al.* 2011, Yang *et al.* 2011).

Hydrogen also downregulated nuclear factors including nuclear factor kappa B, JNK, and proliferation cell nuclear antigen and caspases (Itoh *et al.* 2009, Sun *et al.* 2009). Other important molecules including vascular endothelial growth factor (Ye *et al.* 2008), MMP2 and MMP9 (Chen *et al.* 2010), brain natriuretic peptide, intercellular-adhesion-molecule-1 and myeloperoxidase, B-cell lymphoma 2 and Bcl-2-associated X protein, MMP3 and MMP13, cyclooxygenase 2, neuronal nitric oxide synthase, connexins (Hugyecz *et al.* 2011, Ohno *et al.* 2012), and others were studied as well (Sun *et al.* 2011, Kamimura *et al.* 2011). However, most molecules are probably passengers that are secondarily changed by hydrogen administration, and some are potentially direct targets of hydrogen effects.

Cytoprotective effect of H₂. Although hydrogen has been strongly implicated in reduction of oxidative stress, it has been proposed that hydrogen affects signaling mechanisms and can also induce cytoprotection (Itoh *et al.* 2011, Sun *et al.* 2009). The exact mechanism when and how hydrogen is able to provide cytoprotection is unknown. It can potentiate other antioxidant enzymes such as superoxide dismutase and catalase (Xie *et al.* 2010). It is also proposed that hydrogen can confer cytoprotection by preventing the activation of caspase-3, which can subsequently reduce apoptosis (Sun *et al.* 2009). Shi *et al.* (2012) also proposed that molecular hydrogen may be able to affect signal transduction by interacting with metalloproteins, since metal ions can be a possible binding site for hydrogen.

It was also confirmed that hydrogen was able to modulate signal transduction, however, further studies need to be conducted to determine exactly under what circumstances hydrogen can be a signal modulator (Itoh *et al.* 2011). Recently, it has been reported that hydrogen

may be able to block signaling pathways as a result of its ability to reduce the levels of ROS. It has also been shown that molecular hydrogen is able to inhibit the TNF- α /NF- κ B pathway as well as the Ras-ERK1/2-MEK1/2 and Akt pathways (Ito *et al.* 2012, Chen *et al.* 2013). Possible gene regulatory effects were also reported (Qin *et al.* 2012, Chen *et al.* 2013). The suppression of these pathways by hydrogen was demonstrated in neointimal hyperplasia models in rats. Further investigation of these mechanisms is needed since these pathways are involved in inflammatory responses, gene regulation, and apoptosis. The possibilities to regulate these pathways are quite substantial (Chen *et al.* 2013, Ohta 2011).

Signal modulatory effect. It still remains to confirm that hydrogen exerts its beneficial effect as a signal modulator. Hydrogen modulates miRNA expression (Liu *et al.* 2013, Wei *et al.* 2015), such as expression of miR-9, miR-21, and miR-199, and possibly many others (Kura *et al.* 2016). Analysis of miRNA

profiles of hippocampal neurons during I/R injury revealed that hydrogen inhibits I/R-induced expression of the miR-200 family by reducing ROS production, which has led to suppression of cell death (Wei *et al.* 2015). However, modulation of miRNA expression cannot solely explain all the biological effects mediated by hydrogen. Mechanisms underlying modulated miRNA expression remain to be elucidated.

As reported by Ichihara *et al.* (2015) the effects of H₂ have been observed in practically all organs covering 31 disease categories that can be subdivided into 166 disease models, human diseases, treatment-associated pathologies, and pathophysiological conditions of plants with a predominance of oxidative stress-mediated diseases and inflammatory diseases (Ichihara *et al.* 2015). Administration of H₂ to astronauts by either inhalation or drinking hydrogen water may potentially be a novel preventative/therapeutic strategy to prevent radiation-induced adverse events (Schoenfeld *et al.* 2011, Kajiya *et al.* 2009b, Ohta 2011) (Fig. 2).

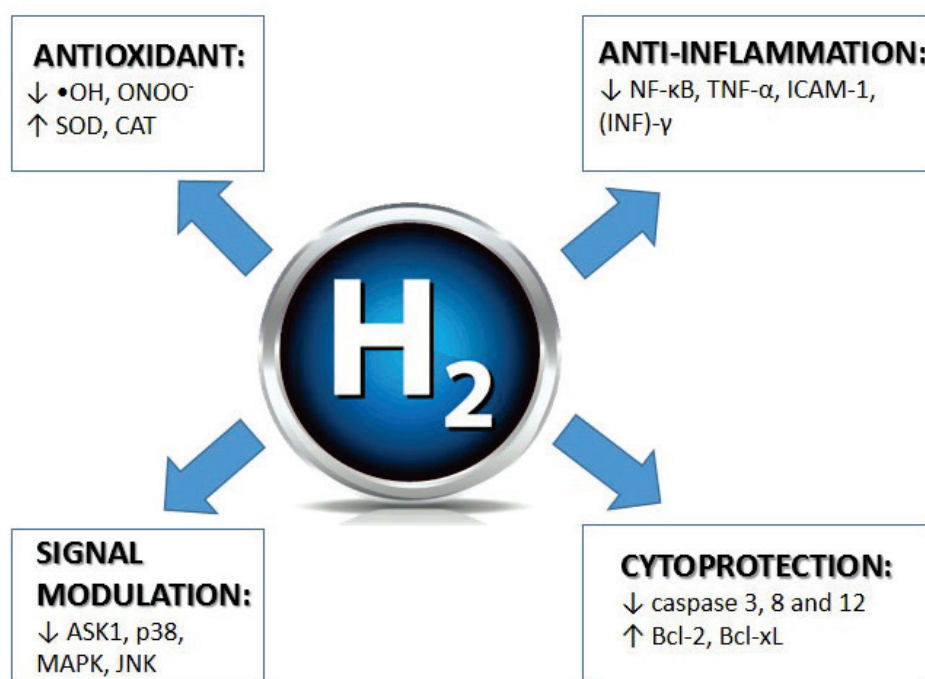


Fig. 2. Antioxidative, anti-inflammatory, cytoprotective and signal modulatory effect of H₂ administration.

Conclusion

Review aims to summarize our present knowledge on strategy how to control oxidative stress by molecular hydrogen. Despite some positive results in discovering the effective antioxidant that would be able to mitigate the adverse effect of excessive production of

reactive oxygen species, search for the proper compounds is still ongoing. Recently it was shown by numerous animal and clinical studies that hydrogen is an important physiological regulatory factor with antioxidant, anti-inflammatory, anti-apoptotic and signal modulatory protective effects on cells and organs. Application of molecular hydrogen in situations with excessive

production of free radicals and, in particular, hydroxyl radicals is relatively simple and effective, therefore, it deserves special attention.

Therapeutic application of hydrogen has been performed by different delivery methods including inhalation, drinking hydrogen rich water and/or infusion hydrogen-saturated solutions. This review summarizes currently available data regarding the protective role of hydrogen and provides an outline of recent advances in research of the use of hydrogen as a therapeutic modality in diverse models of pathologies associated with oxidative stress.

The exact mechanisms of how molecular hydrogen operates still need to be explored. The missing

links of how hydrogen is involved in cell signaling and activation or suppression of transduction pathways, and how hydrogen can reduce inflammation are all important. Moreover, the efficiency of specific concentrations in various disease models needs to be determined.

Conflict of Interest

There is no conflict of interest.

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