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Review

Hydrogen Therapy in Cardiovascular and Metabolic Diseases: from Bench to Bedside

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Abstract
Hydrogen (H_2) is colorless, odorless, and the lightest of gas molecules. Studies in the past ten years have indicated that H_2 is extremely important in regulating the homeostasis of the cardiovascular system and metabolic activity. Delivery of H_2 by various strategies improves cardiometabolic diseases, including atherosclerosis, vascular injury, ischemic or hypertrophic ventricular remodeling, intermittent hypoxia- or heart transplantation-induced heart injury, obesity and diabetes in animal models or in clinical trials. The purpose of this review is to summarize the physical and chemical properties of H_2, and then, the functions of H_2 with an emphasis on the therapeutic potential and molecular mechanisms involved in the diseases above. We hope this review will provide the future outlook of H_2-based therapies for cardiometabolic disease.

Hydrogen gas-background to it use

Hydrogen (H_2), produced by intestinal bacteria in mammals, is colorless, odorless, and the lightest of all gas molecules. The earth's atmosphere contains less than 1 part per million (ppm) of H_2 [1]. H_2 is a highly combustible diatomic gas when it is present with a specific catalyst or in the presence of heat [2]. H_2 is flammable only at temperatures higher than 527 °C. It will explode by a rapid chain reaction with O_2 only in the explosive range of H_2 concentration (4-75%, vol/vol) [1]. H_2 can be dissolved in approximately 0.8mM (1.6 ppm, wt/vol) of water at one atmospheric pressure [2].

Endogenous H_2 is catalyzed and produced by hydrogenases (H_2ases) in bacteria, such as *Escherichia coli, Bacteroidetes* and *Firmicutes* in colon [3-5]. The great majority of H_2ases contain iron-sulfur clusters and two metal atoms at their active center, a Ni and a Fe atom, the [NiFe]-H_2ases, or two Fe atoms, the [FeFe]-H_2ases [6]. Enzymes of these two classes catalyze

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the reversible oxidation of H_2 ($H_2 \rightleftharpoons 2\ H^+ + 2\ e^-$) and play a central role in microbial energy metabolism [6], for example, H_2 functions as an energy source for *Helicobacter pylori* [3], *Salmonella typhimurium* [7] *et al.* However, mammalian cells have no functional hydrogenase genes [8]. In mammalian cells, the endogenous or exogenous H_2 is qualified to cross the blood brain barrier, it has the ability to penetrate most membranes and diffuse into organelles, such as mitochondria and nucleus [1, 9]. In 2007, Ohsawa et al [10]. reported that H_2 is able to react with cytotoxic oxygen radicals by reacting with the hydroxyl radical (•OH), but not $•O_2^-$, H_2O_2 and NO in cultured cells. Due to its potential ability to inhibit oxidative stress, inflammation, and apoptosis, H_2 is emerging as a fourth gaseous signaling molecule (NO, carbon monoxide, hydrogen sulfide, and H_2) within the body [2].

During the past ten years, basic and clinical research has indicated that H_2 is an important pathophysiological regulatory factor with anti-oxidative, anti-inflammatory and anti-apoptotic effects on cells and organs [11]. Delivery of H_2 by inhalation or injection with H_2 [12, 13], injection with H_2-rich saline [14, 15], drinking H_2-rich water [16, 17], taking an H_2-rich bath [18], and increasing the production of intestinal H_2 by bacteria [19], has been shown to protect against cardiovascular and metabolic diseases, such as atherosclerosis, glucose and lipid metabolism disorder, myocardial ischemia/reperfusion (I/R) injury, myocardial transplantation injury, or cardiovascular hypertrophy. All of these will be discussed below.

Table 1. Effects of H_2 in vascular disease models

Animal Models	Mechanism of H_2 on vascular diseases improvement
Apolipoprotein E knockout ($ApoE^{-/-}$) mice	• iNOS↓ • 4-hydroxyl-2-nonenal (HNE)↓ • Macrophage (MOMA-2)↓ • Improves HDL function • Decreases athero-susceptibility
LDL receptor-knockout (LDLR-/-) mice	• Collagen↑ • Regulatory T cells↑ • Macrophages↓ • Dendritic cells(DCs)↓ • Lipid level↓ • Endoplasmic reticulum stress(ERS)↓ • NF-E2-related factor-2(Nrf2) antioxidant pathway↑
High-fat diet-fed hamsters	• Plasma LDL cholesterol and apo B levels↓ • Improves hyperlipidemia-injured HDL functions
Rat periodontitis model	• Serum ox-LDL↓ • Aortic oxidative stress↓ • Lipid deposition↓
Abdominal aortic coarctation (AAC)	• Oxidative stress↓ • MAPK activation↓ • Ezrin/Radixin/Moesin activation↓
Spontanerously hypertensive rats (SHR)	• Oxidative stress↓ • Restores antioxidant enzymes • NADPH oxidase↓ • Pro-inflammatory cytokines(IL-6, IL-1β)↓ • NF-κB activation↓ • Mitochondrial function impairments ↓ • eNOS expression↓ • Dimethylarginine dimethylaminohydrolase 2↑
Carotid balloon injury	• ROS↓ • TNF-α/NF-κB signaling pathway↓ • Ras-MEK1/2-ERK1/2 and Akt signaling pathway↓

The effects of H_2 in vascular diseases

The vasculature is an active, integrated organ primarily composed of endothelial cells (ECs) in tunica intima, vascular smooth muscle cells (VSMCs) in tunica media, and fibroblasts in adventitia, all of which interact in a complex autocrine-paracrine manner [20]. Besides fibroblasts, the adventitia includes many other cell types, such as nerves, microvascular endothelium, resident macrophages, dendritic cells, T cells, B cells, and mast cells [21]. The adventitia is essential for maintaining vessel wall homeostasis *via* regulating immune and inflammatory responses. Under various vascular stresses, such as high fat diet (HFD), disturbed flow with oscillatory and low shear stress, mechanical injury and hypertension, blood vessels will undergo structural alteration through inducing inflammatory responses and eNOS uncoupling in ECs, proliferation and migration of VSMCs, and fibroblasts activation [20]. H_2 has been reported to regulate these cellular events in vessel walls through their native antioxidant functions directly, or *via* lipid regulation, cell death and growth (Table 1).

Ikuroh Ohsawa et al. revealed that drinking H_2-rich water for 4 months reduced atherosclerotic lesion in apolipoprotein E knockout mice ($ApoE^{-/-}$ mice) [16]. H_2-rich water intake also prevents lipid deposition in the rat aorta induced by periodontitis by decreasing serum ox-LDL levels and aortic oxidative stress [22]. A series of studies from the *Qin Shucun* group indicated that the anti-atherosclerotic effect of H_2 is achieved by suppressing NF-κB activation and subsequently blocking cytokine-induced lectin-like oxidized LDL receptor-1 (LOX-1) gene expression in ECs [23]; decreasing plasma LDL cholesterol and apolipoprotein B100 and apo B48 levels in LDL, and improving HDL functions, including the capacity to enhance cellular cholesterol efflux and anti-oxidative properties [24-26]. More importantly, H_2 can enhance plaque stability in low-density lipoprotein receptor-knockout ($LDLR^{-/-}$) mice by increasing levels of collagen and numbers of regulatory T cells, reducing macrophages, dendritic cells numbers and lipid levels in plaques, as well as inhibiting endoplasmic reticulum stress and activating the NF-E2-related factor-2 (Nrf2) antioxidant pathway [27]. *In vitro* studies also support the antioxidant functions of H_2. H_2-rich medium has long-lasting antioxidant and anti-aging effects on ECs through the Nrf2 pathway, even after transient exposure to H_2 [28].

Our recent study indicates that intraperitoneal injection of H_2 (99.999%, 1 ml/100 g/day) prevents abdominal aortic coarctation (AAC)-induced vascular hypertrophy *in vivo* [29]. However, we find that H_2 had no effect on circulating angiotensin II (Ang II) levels, thereby the protective effect of H_2 on vascular hypertrophy is possibly by blocking circulating Ang II actions on vessels (especially targeting in VSMCs) rather than inhibiting its synthesis and secretion. Similarly, intraperitoneal injection of H_2-rich saline has been reported to ameliorate aortic hypertrophy and improve endothelium-dependent vascular relaxation and baroreflex function in spontaneously hypertensive rats (SHR) [30]. Drinking H_2-rich water reduced endothelial denudation, macrophage infiltration, and neointimal formation in vein grafts by reducing the activation of p38 MAPK inflammatory cascades, and decreasing the expression and activity of MMP-2 and MMP-9 [31]. H_2-rich saline also prevents neointimal hyperplasia induced by carotid balloon injury in rat by suppressing ROS and the TNF-α/NF-κB signaling pathway [32], and inactivating the Ras-MEK1/2 - extracellular signal-regulated kinase1/2 (ERK1/2) and Akt signaling pathways [33]. In addition, H_2-rich saline protects cerebral microvascular endothelial cells from apoptosis after hypoxia/reoxygenation *via* inhibiting PI3K/Akt/GSK3β signaling pathway [34].

Moreover, H_2 can also influence VSMCs proliferation and migration *in vitro*. H_2-rich medium inhibits PDGF-BB-induced VSMCs proliferation [32] and 10% FBS-induced VSMCs proliferation and migration, and blocks FBS-induced progression from the G0/G1 to the S-phase and increases the apoptosis of VSMCs [33]. H_2-rich medium inhibits Ang II-induced proliferation and migration of VSMCs *in vitro* by blocking ROS-dependent ERK1/2, p38 MAPK, c-Jun NH_2-terminal kinase (JNK) and ezrin/radixin/moesin signaling [29]. However, the *Atsunori Nakao* group [31] indicated that H_2-rich medium inhibits VSMCs migration with or without FBS, but has no effects on proliferation.

H_2 inhibits vascular remodeling by improving ECs and lipid function, suppressing VSMCs proliferation and migration, and attenuating inflammatory cell accumulation. Therefore, to design a kind of intravascular stent which can release H_2 might be a good strategy for suppressing restenosis.

Table 2. Effects of H_2 in heart disease models

Pathological stimuli	Mechanism of H_2 on heart diseases improvement
Ischemia/reperfusion (I/R)	• RNS (eg. Peroxynitrite) ↓ • Anti-oxidative stress • Anti-apoptosis • Anti-inflammatory response
Intermittent hypoxia (IH)	• Oxidative stress↓ • Dyslipidemia↓ • Expression of embryonic gene BNP, β-MHC, c-fos, c-jun ↓
Heart transplantation	• Oxidative stress↓ • Immune and inflammatory responses↓ • Mitochondria function ↑ • Energy metabolism↑
Isoproterenol (ISO)	• Anti-inflammatory response • NADPH oxidase expression↓ • Mitochondrial damage↓ • ROS ↓ • ERK1/2, p38, JNK signaling ↓ • Autophagy inhibition

The effects of H_2 in heart diseases

In response to pathophysiological stimuli, such as myocardial I/R, hypertension, or neurohumoral triggers, multiple molecular and cellular processes contribute to ventricular remodeling [35]. The increased production of endothelin-1 (ET-1), Ang II, catecholamines and pro-inflammatory cytokines activate their cognate receptors and downstream signaling events, which lead to cardiomyocytes necrosis, apoptosis, autophagy, or hypertrophy; and promote fibroblast activation to produce collagen and other proteins that cause fibrosis [35-37]. Recently, we and others have shown that H_2 can prevent various heart diseases through blocking parts of these molecular and cellular signaling events described above (Table 2).

Gut microbiota-derived H_2 slightly but significantly reduces myocardial infarct size [38]. The inhaled H_2 was rapidly transported to the ischemic myocardium before coronary blood flow was reestablished in the occluded region, and inhalation of 2% H_2 at the onset of ischemia and continued for 60 min after reperfusion reduces infarct size, lowers LV-end-diastolic pressure (LVEDP), and reduces pathological remodeling and improves cardiac function 30 days after myocardial I/R injury [12]. In swine, inhalation of 2% H_2 improves myocardial stunning, and inhalation of 4% but not 2% H_2 reduces myocardial infarct size [39]. Similar to H_2, nitric oxide (NO) also has the ability to decrease the infarct size in myocardial I/R injury [40]. However, NO has cytotoxicity by producing reactive nitrogen species (RNS), such as peroxynitrite, which can react with the tyrosine at the active site of vital enzymes (such as Tyr^6, Tyr^{32}, and Tyr^{78} in mouse GST-μ) and cellular components [38, 41]. These adverse effects can be reversed by H_2 inhalation. Breathing NO plus H_2 can reduce cardiac injury and augment recovery of the left ventricular function, by eliminating the adverse by-products of NO inhalation alone, nitrotyrosine [38]. Besides H_2 inhalation, *Sun xuejun* group indicated that intraperitoneal injection of H_2-rich saline attenuates myocardial I/R injury and improves cardiac function through anti-oxidative, anti-apoptotic and anti-inflammatory effects [14, 15]. Recently, *Yan fei* group have developed an ultrasound-visible H_2 delivery system by loading H_2 inside microbubbles (H_2-MBs) to prevent myocardial I/R injury [42]. Moreover, an *in vitro* study revealed that the cardioprotection by hypoxic postconditioning can be augmented by molecular H_2 infusion [43]. A clinical study has shown that H_2 inhalation (1.3% H_2) during primary percutaneous coronary intervention (PCI) is a feasible and safe treatment option for patients with ST-elevated myocardial infarction and may prevent adverse left ventricular remodeling after primary PCI [44].

Intermittent hypoxia, which is the major feature of sleep apnea syndrome, increases superoxide production and accelerates adverse left ventricular remodeling [45]. Inhalation of H_2 at low concentrations (1.3 vol/100 vol) reduces intermittent hypoxia-induced dyslipidemia, oxidative stress, and also prevents cardiomyocyte hypertrophy and perivascular fibrosis in left ventricular myocardium of C57BL/6J mice [46]. Inhalation of H_2 (3.05 vol/100 vol) by cardiomyopathic (CM) hamsters inhibits oxidative stress and decreases embryonic gene *BNP, β-MHC,* c-*fos* and c-*jun* expression, thus preserving cardiac function in CM hamsters [47].

Besides ischemic heart diseases and sleep apnea syndrome above, neurohumoral activation, such as β-adrenoceptor and Ang II stimulation, hypertension, will contribute to cardiac hypertrophy and heart failure [13]. Our recent study indicated that intraperitoneal injection of H_2 protects against isoproterenol (ISO, mice received H_2 for 7 days before ISO subcutaneous injection, and then received ISO with H_2 for another 7 days)-induced cardiac hypertrophy and dysfunction *in vivo,* and H_2-rich medium attenuates ISO-mediated cardiomyocyte hypertrophy *in vitro* [13]. H_2 exerts its protective effects by direct interruption of NADPH oxidase expression and alleviating mitochondrial damage, these lead to the inhibition of ROS accumulation, and subsequently block downstream ERK1/2, p38, and JNK signaling. However, our unpublished data indicate if ISO was given followed by H_2 (H_2 was given one hour before ISO injection) on the same day for the first time, H_2 fails to suppress ISO (5mg/kg, 10 days, intraperitoneal injection)-induced cardiac hypertrophy in Wistar rat. Our mice model also indicated that H_2 can suppress ISO-induced excessive autophagy in cardiomyocytes both *in vivo* and *in vitro* [48]. Similarly, inhalation of 2% H_2 attenuates myocardial I/R injury by attenuating cardiac endoplasmic reticulum stress and autophagy [49]. Moreover, H_2-rich saline protects high dose ISO-induced acute myocardial infarction in rat by anti-oxidative and anti-inflammatory activities [50]. The protective effects of H_2 on cardiac hypertrophy were also confirmed in SHR. H_2-rich saline attenuates left ventricular hypertrophy in SHR *via* suppressing inflammatory process, abating oxidative stress, preserving mitochondrial function, and inhibition of Ang II levels in left ventricles locally might also be involved [51].

Heart transplantation remains the surgical procedure of choice for eligible patients with severe advanced heart failure and inoperable congenital heart disease [52]. However, the cardiac transplant procedure obligates cold preservation and warm reperfusion of cardiac grafts and results in a certain degree of I/R injury in all grafts [18, 53]. The injury occurring during preservation or reperfusion can affect cardiac function after heart transplantation. Reducing injury is important for preserving cardiac function. Importantly, H_2-rich Histidine–Tryptophan–Ketoglutarate (HTK), H_2 inhalation, drinking H_2-rich water or H_2-rich water bath have the abilities to inhibit oxidative stress, suppress immune and inflammatory responses, improve mitochondria function and energy metabolism, enhance graft survival, and attenuate cardiac injury during preservation or reperfusion in heart transplantation [18, 53-55].

H_2 has comprehensive cardiac activities. H_2 administration protects against cardiac remodeling and improves cardiac function induced by I/R, intermittent hypoxia, neurohumoral activation, hypertension and transplantation injury. However, there is long way to develop H_2 into a clinical drug to treat heart failure.

The effects of H_2 in metabolic diseases

Metabolic syndrome (MS), which includes obesity, insulin resistance, hyperglycemia, hypertension, elevated VLDL triglycerides and low HDL cholesterol, is a primary risk factor for type 2 diabetes and cardiovascular diseases [56-58]. The pathophysiology of MS appears to be largely due to insulin resistance with excessive flux of fatty acids implicated, and a pro-inflammatory state probably also contributes to the syndrome [56, 58]. Moreover, inflammation, insulin resistance and hepatic steatosis influence one another to form a vicious circle [59-65]. Therefore, targeting inflammatory responses and lipid metabolism are important strategies to treat metabolic diseases. Interestingly, H_2 has the ability to regulate inflammation and lipid metabolism.

Long-term of drinking H_2-rich water markedly improves obesity, hyperglycemia, and the plasma triglycerides of diabetic *db/db* mice [66]. H_2 accumulates in the liver with glycogen after oral administration of H_2-rich water. H_2 markedly reduces hepatic oxidative stress levels and improves fatty liver in *db/db* as well as diet-induced obesity mice [66]. H_2 enhances the expression of hepatic hormone, fibroblast growth factor 21 (FGF21), which

functions to enhance fatty acid and glucose expenditure, and stimulates energy metabolism in *db/db* mice [66]. The beneficial effects of H_2-rich water are also identified in patients with potential metabolic syndrome. Drinking H_2-rich water decreases thiobarbituric acid reactive substances (TBARS) in urine and serum LDL-cholesterol levels, increases antioxidant enzyme superoxide dismutase (SOD) and HDL-cholesterol, and improves HDL function in patients with potential metabolic syndrome [26, 67]. Similarly, H_2-rich water activates ATP-Binding cassette transporter A1-dependent efflux *ex vivo* and improves HDL function in patients with hypercholesterolemia [68]. H_2-rich water also improves lipid and glucose metabolism in patients with type 2 diabetes or impaired glucose tolerance [17]. Moreover, a recent study indicates that subcutaneous injection of H_2 significantly improves T2DM and diabetic nephropathy related outcomes in a mouse model [69]. Thus, results from animals and clinical trials consistently indicate that drinking H_2-rich water shows beneficial effects in improving metabolic diseases.

Perspective

Current studies of H_2 focus on anti-oxidation, anti-inflammation, and anti-apoptosis. However, the effective target and the precise molecular mechanisms of H_2 are not clear. Recent studies have indicated that H_2 can regulate both innate and adaptive immune responses, such as inhibiting lipopolysaccharide/interferon γ-induced NO *via* blocking ASK-1 and its downstream signaling molecules, p38 and JNK, as well as IκBα in macrophages [70], restoring the L-arginine-induced $CD25^+Foxp3^+$ regulatory T cells loss in mice [71]. However, the functions of H_2 in regulating cardiovascular immune responses still need further investigation. Moreover, NO, CO, and H_2S are important signaling molecules in the cardiovascular system [72-80]. Breathing NO plus H_2 during I/R can reduce the generation of myocardial nitrotyrosine associated with NO inhalation [38]. Combination of H_2 and CO can elicit better results than either one alone for inhibiting inflammation and enhancing graft survival [55]. These indicate that H_2 can regulate the function of NO and CO. However, whether the effects of H_2S or other gas can be regulated by H_2 are not known. What is the relationship between endogenous H_2 and exogenous H_2 [81]? What's the role of higher density of H_2 in protoatmosphere during organic evolution, especially in the evolution and development of cardiovascular system [81, 82]? To date, there have been no reported side effects of H_2 therapy, however, long-term toxicology evaluation has not been performed. These are the interesting questions needing to be investigated in the near future.

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Disclosure Statement

No conflict of interests exists.

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