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Review

Theoretical evaluation of the biological activity of hydrogen

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Abstract

Hydrogen (H_2), the simplest and most ubiquitous molecule in the universe, has garnered significant scientific interest over the past two decades because of its potential as an effective antioxidant and anti-inflammatory agent. Traditionally considered inert, H_2 is now being re-evaluated for its unique bioactive properties. H_2 selectively neutralizes reactive oxygen and nitrogen species, mitigating oxidative stress without disrupting essential cellular functions. This review therefore aims to provide a theoretical evaluation of the biological activity of H_2, focusing on its pharmacokinetics, including absorption, distribution, and retention within biological systems. The pharmacokinetic profile of H_2 is crucial for understanding its potential therapeutic applications. The interaction of H_2 with protein pockets is of particular interest, as these sites may serve as reservoirs or active sites for H_2, influencing its biological activity and retention time. Additionally, the impact of H_2 on cellular signaling pathways, including those regulating glucose metabolism and oxidative stress responses, will be explored, offering insights into its potential as a modulator of metabolic and redox homeostasis. Finally, interactions with ferromagnetic molecules within biological environments, as well as effects on cellular signaling mechanisms, add another layer of complexity to the biological role of H_2. By synthesizing the current research, this review seeks to elucidate the underlying mechanisms by which H_2 may exert therapeutic effects while also identifying critical areas for further investigation. Understanding these aspects is essential for fully characterizing the pharmacodynamic profile of H_2 and assessing its clinical potential in the treatment of oxidative stress–related disorders.

Key Words: anti-inflammatory; antioxidant; cellular signalling; H_2; hydrogen; oxidative stress; pharmacokinetics; protein pockets; reactive oxygen species

Introduction

The exploration of hydrogen (H_2) as a therapeutic molecule has roots that stretch back several decades, but its significance has only been recognized relatively recently. Initially, H_2 was considered biologically inert, with no substantial role in physiological or therapeutic contexts. This perception began to change in 1975, when Dole et al.[1] published a landmark study demonstrating that hyperbaric H_2 therapy could have beneficial effects in treating squamous cell carcinoma in mice. Despite these promising findings, this research did not immediately attract widespread interest, and the therapeutic potential of H_2 has remained largely overlooked for several decades. However, interest in H_2 as a therapeutic agent was reignited when Ohsawa et al.[2] in 2007 published a pivotal study showing that inhalation of H_2 gas could selectively reduce cytotoxic oxygen radicals in a rodent model of cerebral ischemia–reperfusion injury. This study marked a significant turning point, demonstrating that H_2 could act as a selective antioxidant capable of neutralizing highly reactive hydroxyl radicals ($^{\bullet}OH$) without affecting other essential reactive species involved in normal cellular signaling (e.g., hydrogen peroxide (H_2O_2)).

Since 2007, the therapeutic applications of H_2 have expanded rapidly, with research exploring its effects in various forms, including H_2-enriched water (HRW),[3,4] inhalation,[5,6] and intravenous administration of H_2-saturated saline (HRS).[7,8] The versatility of H_2 and its ability to diffuse into tissues and cells without accumulating to toxic levels has made it a promising candidate for therapeutic intervention across a range of diseases. H_2 is now recognized for its potential therapeutic properties, particularly as an antioxidant.

With increasing momentum, international research institutions and commercial enterprises are devoting time and resources to understanding how H_2 affects both cellular and wider, somatic physiology. By incorporating current research and exploring the molecular interactions of H_2 within biological systems, such analyses aim to provide a comprehensive understanding of how H_2 can be harnessed to improve health outcomes.

H_2 is formed by the amalgamation of two hydrogen (H) atoms with an H–H bonding energy of 107 kcal/mol (4.64 eV)[9] and a redox potential ($2H + 2e^- \rightarrow H_2$) of –0.421 eV (pH 7) relative to the standard hydrogen electrode.[10] Although the primary mode of action in biological systems has yet to be illuminated,

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one favored postulation is that H_2 is able to reduce excessive reactive oxygen and reactive nitrogen species (ROS/RNS, respectively) through direct interactions with radical and ionic species (e.g., hydroxyl radicals (•OH) and peroxynitrite ($ONOO^-$). However, although the clinical advantages of H_2 therapeutics are now well documented,[11-16] there are still many questions surrounding the distribution and precise molecular mechanisms related to the biological activities of H_2. These include, how does H_2 reach target tissues? What are the primary physiological targets of H_2 interactions? And how does H_2 maintain its influence over time?

The purpose of this review is to explore the potential mechanisms of the distribution, retention and activity of H_2 and to critically examine the emerging role of H_2 as a therapeutic molecule. This exploration not only highlights the potential of H_2 as a medical gas but also identifies areas for further investigation to fully elucidate its role in human physiology. By synthesizing current research on the molecular mechanisms of H_2 and identifying key areas for future investigations, this review addresses critical questions. By exploring these areas, this review aims to enhance our understanding of the potential of H_2 to improve health outcomes.

Methodology

The retrieval strategy involved a comprehensive search of the following scientific databases: Scopus, Google Scholar, and PubMed. The following keywords were used in various combinations: "hydrogen," "molecular hydrogen," "H_2," "HRW (hydrogen-rich water)," "distribution," "calcium," "glucose/glycogen," "phosphorylation," "modality," "mechanisms," and "signaling." Studies were selected on the basis of their relevance to hydrogen distribution, molecular mechanisms, clinical outcomes, and the use of hydrogen in disease treatment. The inclusion criteria required that studies be freely accessible as full-text, peer-reviewed, available in English, and typically published within the last two decades to ensure that the most current findings were considered.

Distribution and Retention of Hydrogen

Pharmacokinetics

Pharmacokinetics explores how the body responds to a drug after administration and is crucial for determining the dosage, frequency, and duration of treatment, ensuring that the drug reaches its target in the body at the right concentration and for the right amount of time to be effective while minimizing side effects.[17]

Pharmacokinetic analysis of H_2 distribution in porcine models suggests that, owing to the increased half-life of H_2 in venous blood, this diatomic gas likely travels in the plasma and simply diffuses into tissue structures.[18] The physical and chemical characteristics of H_2 (e.g., low molecular weight, nonpolar nature, and electrochemical neutrality) allow the molecule to diffuse through biological fluids, the extracellular matrix, cellular membranes and cytosolic compartments. However, it is undetermined whether this mechanism of dispersal is able to explain the effects observed in distal organs. For example, when HRS is administered via intraperitoneal delivery, substantial amounts of H_2 are recorded in proximal sites such as the pancreas and spleen, whereas stepwise reductions, progressing radially, are shown for the distal organs.[19]

One explanation for H_2 distribution is that after imbibition, inhalation or injection, H_2 molecules diffuse into the bloodstream entering the circulation and allowing for dissemination into all the corporal systems. Supporting this theory is early research in 2014 conducted by Liu et al.[19] In rodent models, where HRW containing 1.25 ppm, 2.5 ppm and 5 ppm/H_2 (0.62, 1.25 and 2.5 mM, respectively) delivered by i) intraperitoneal and intravenous injection of HRS, and ii) inhalation of H_2 at various concentrations (1%, 2% and 4%) was studied. H_2 concentrations are increased in the blood, brain, intestines, kidney, liver, pancreas, skeletal muscle and spleen following HRW, intravenous HRS, and H_2 inhalation (**Table 1**).

Although this research revealed that intraperitoneal injection of HRS resulted in H_2 elevation in the majority of the tissues analyzed, this method was not effective at delivering large amounts of H_2 into the bloodstream or the wider cardiovascular system but was highly effective in delivering H_2 to proximal organs, including the intestines, liver, pancreas and spleen (**Table 1**).[19] Interestingly, when H_2 is delivered via inhalation, although the primary beneficiary organs are typically in close proximity (e.g., the brain, respiratory system and heart), significant increases in H_2 concentrations are also observed in the spleen and skeletal muscle 30 minutes after H_2 application.[19] These results suggest a preference for distribution by simple diffusion; however, this still does not explain the elevated levels of H_2 in distal organs such as the brain when elevated H_2 levels in the blood are not detected (**Table 2**).

Table 1 | Demographic and clinical data of all subjects

Tissue	Oral (5 ppm HRW) – (5 min)	Intraperitoneal (5 ppm HRS) (5 min)	Intravenous (5 ppm HRS) (1 min)	Inhalation (4%) (30 min)
Blood	5	5	5	5
Liver	170	170	15	25
Kidney	50	75	25	50
Heart	50	0	30	30
Spleen	500	300	40	50
Pancreas	400	250	40	45
Intestine	400	150	20	20
Muscle	20	20	20	140
Brain	15	15	20	15

Data extracted from Liu et al.[19] H_2: Hydrogen; HRS: H_2-saturated saline; HRW: H_2-enriched water.

Table 2 | The timing (min) of peak concentrations and retention of H_2

Tissue	Oral (5 ppm HRW)		Intraperitoneal (5 ppm HRS)		Intravenous (5 ppm HRS)		Inhalation (4%)	
	Peak	Return to baseline	Peak	Return to baseline	Peak	Return to baseline	Peak	Return to baseline
Blood	5	> 60	5	15	1	3	30	> 60
Liver	5	60	5	15	1	5	30	> 60
Kidney	5	30	5	15	1	5	30	> 60
Heart	5	15	5	15	1	5	–	–
Spleen	5	30	5	15	1	> 5	60	> 60
Pancreas	5	60	5	15	1	5	–	–
Intestine	5	60	5	15	1	3	60	> 60
Muscle	5	30	5	15	1	> 5	30	> 60
Brain	5	30	5	15	1	> 5	30	> 60

Data extracted from Liu et al.[19] H_2: Hydrogen; HRS: H_2-saturated saline; HRW: H_2-enriched water.

An investigation into the effects of 100% H_2 insufflation (the act of blowing something into a body cavity) revealed that H_2 likely remains in the plasma.[20] However, for this to be the case, the saturation levels would have to be vastly increased for such wide-ranging effects and distribution patterns to be seen. As physiological pressures are only ~120 mmHg (16 kPa) higher than atmospheric pressure (760 mmHg, 101.32 kPa), elevating the saturation levels of H_2 as a result of increased pressure is unlikely. Furthermore, simple diffusion through the plasma cannot explain the gradual increase in and retention of H_2 in skeletal muscle or why H_2 is not only effective in the organs proximal to the delivery site. One reason for this could be that in the experimental protocol, only H_2 dissolved in the plasma was recorded. The saturation point of plasma is in the range of 1.6 mg/L/H_2; therefore, inhalation likely provides more H_2 than can be dissolved in the bloodstream. The following question then arises: how does enough H_2 travel through the blood to still be present at detectable levels in the venous bloodstream and distal organs for up to 1 hour after treatment? This factor could be explained if H_2 transitorily resides within micropores or pockets formed within the hemoglobin of red blood cells[21-23] or if H_2 could be temporarily retained by molecules suspended in the serum, such as carbohydrates or inorganic ions, glycogen and calcium, as discussed below.

Glucose and glycogen

Glucose is a six-carbon monosaccharide with the chemical composition $C_6H_{12}O_6$, which is ubiquitously utilized as an energy source in animals, bacteria, fungi, plants and protozoa, where glucose is the key substrate for both aerobic and anaerobic metabolism.[24] Glycogen, on the other hand, is a large branched-chain polysaccharide storage complex formed of numerous glucose moieties that are connected by two glycosidic bonds: i) an α-1,4-glycosidic bond and ii) an α-1,6-glycosidic bond. In plants, a similar structure, referred to as starch, provides carbohydrate storage capacity. Unlike the branching-chain structure of glycogen, starch is formed by two polymers, i) amylose, which forms linear and coiled chains, and ii) amylopectin, which forms branched chains.[25] Although glycogen and starch structures vary in form, they are chemically identical, with both functioning as energy stores in their respective organisms.[26] Perhaps due to this chemical similarity, both glycogen and starch are likely to have similar H_2-retention capabilities.

Glycogen complexes have been identified as effective H_2-retaining molecules, as demonstrated in both the liver and skeletal muscle of rodents,[19,27] whereas consuming HRW was demonstrated to reduce glycogen utilization in the liver in laboratory models of endurance exercise,[28] suggesting that H_2 may either inhibit glycogen catalysis or promote glucose transportation into the cells, which would then negate the requirement for glucose release.

In aqueous solutions such as blood plasma or serum, glucose molecules, owing to their high hydrogen bond content, become glutinous (i.e., sticky)[29]; therefore, H_2 may temporarily bind with carbohydrate moieties of glycogen or glycoproteins (**Figure 1**), perhaps also on the surface of red blood cells (**Figure 2**). It can be assumed that weak noncovalent attachments to the glucose components of glycogen (or glucose moieties of proteins) can occur, entrapping H_2 molecules within the structure, which could account for the elevated levels of H_2 at distal sites and in both the hepatic and skeletal muscle systems.[25] Furthermore, if glutinisation is a mechanism by which H_2 can adhere, perhaps transiently, to carbohydrate molecules, it would be feasible to assume that in plants, the same effects would be seen with starch (**Figure 1**). If glucose moieties can retain H_2, it would also be reasonable to surmise that H_2 could bond with free glucose or glycoproteins (e.g., glycophorins A–D on the surface of red blood cells), perhaps preventing glycosylation or other posttranslational modifications. Considering dissociation, such weak chemical interactions could be expunged by the velocity of blood flow (**Figure 2**), releasing H_2 in the vicinity of distal organs. However, such speculation will need to be empirically assessed if the above theory is to be verified.

Protein pockets and cavities

Proteins are dynamic macromolecules with intricate three-dimensional structures that include pockets and cavities, which are often involved in binding small molecules, ligands, and ions. These pockets are crucial for various biological processes, including enzyme catalysis, ligand storage, signal transduction, and molecular recognition.[30,31] The structural properties of these cavities, such as their size, shape, and electrostatic environment, determine their binding affinity and specificity for different molecules.[32]

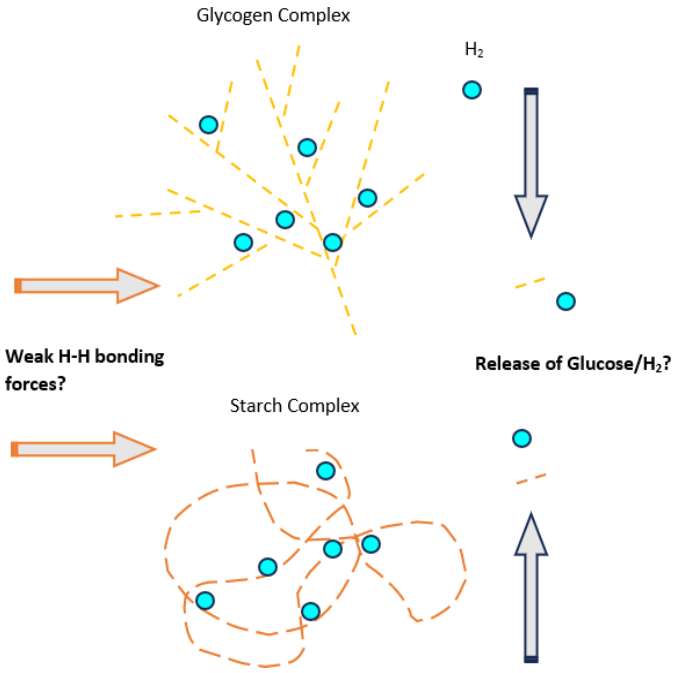

Figure 1 | A theorized mechanism behind the retention and release of H_2 in glycogen and starch complexes.
Schematic representation of how H_2 may be trapped and released by glucose storage molecules. Above: Glycogens (animals, bacteria, fungi). Below: Starch (plants). Created with Microsoft Word (Version 2409). H_2: Hydrogen.

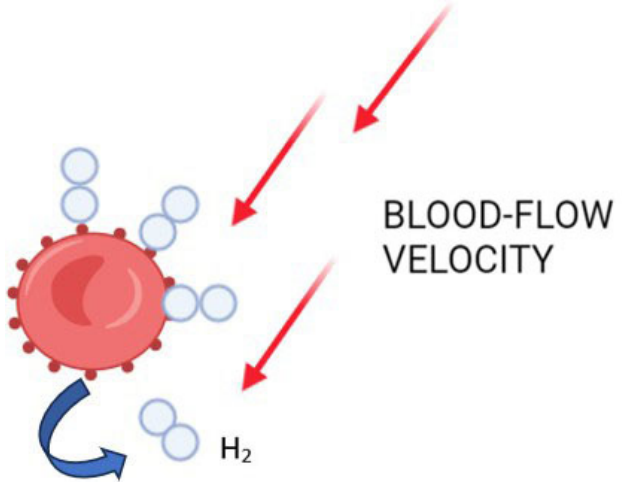

Figure 2 | A theorized mechanism for H_2 distribution via transient glucose binding.
Schematic representation of how H_2 may be trapped and released by glycolic moieties of glycolipids/proteins on red blood cells. Created with BioRender.com. H_2: Hydrogen.

Noble gases such as xenon (Xe) have unique interactions with protein pockets, primarily due to their inert nature and ability to dissolve in both hydrophobic and hydrophilic environments. Xe, in particular, has been extensively studied for its anesthetic and neuroprotective properties.[33] Additionally, Xe has been demonstrated to bind within the hydrophobic cavities of proteins, altering their conformational states and influencing their biological activity.[34-36] For example, research has shown that Xe exerts its effects by binding to specific protein sites, such as the N-methyl-D-aspartate receptor, stabilizing the receptor's closed state, and preventing excitotoxicity.[37] This binding is typically weak and reversible, which is advantageous for therapeutic applications where precise modulation of protein function is needed without long-term disruption.[38] The possibility of H_2 interacting with these protein pockets and cavities is an intriguing hypothesis, given the small size and nonpolar nature of H_2. Such interactions could facilitate the targeted distribution of H_2 throughout the body, enhancing its therapeutic efficacy. Moreover, protein-bound H_2 molecules might participate in modulating protein functions, either by stabilizing protein conformations or by influencing protein–protein interactions involved in cell signaling pathways. For example, H_2 may influence calcium ion (Ca^{2+})/calmodulin signaling, a critical process in various cellular activities, by interacting with Ca^{2+}-binding proteins that contain suitable pockets or cavities. By stabilizing these proteins or altering their conformation, H_2 could indirectly modulate calcium fluxes and related signaling cascades, thereby affecting processes such as muscle contraction, neurotransmission, and cell proliferation.[39] Similarly, H_2 may interact with kinases involved in phosphorylation signaling pathways, such as the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) pathways,[40-42] which are crucial for cell survival, proliferation, and apoptosis. By affecting specific protein sites, particularly those rich in histidine, serine, threonine or tyrosine,[43] H_2 could modulate kinase activity, leading to alterations in phosphorylation events that are central to cell signaling and stress responses.

Supporting the notion that H_2 may reside at least temporarily in proteinaceous pockets, it is insightful that for industrial storage purposes, research into H_2-retaining materials has tended to focus on the structural adsorption of H_2 molecules. In fuel cells, carbon nanotubes,[44,45] fullerene nanocages[46,47] and palladium nanolattice particles[48,49] are commonly used because of their superior and reversible H_2-retention properties. Metallic ion-containing organic crystalline structures are noted to have a high capacity for storing H_2. Metalloorganic frameworks are produced using biologically compatible metallic ions (e.g., Ca^{2+}, Cu^{2+}, Mn^{2+}, Zn^{2+}), which are connected by organic ligands, forming a microporous structure that is able to retain H_2 in relatively large quantities.[50,51]

The use of such microporous composites is intriguing from a biological stance as the architecture often provides micropockets that are similar to the structure of folded proteins. It is therefore feasible that, in accordance with the Xe pocket theory of H_2 activity and distribution,[22,23] H_2 could reside in such cavities, perhaps through metal–metal interactions. If such a process is indeed incorporated physiologically, then it can be extrapolated that a similar process may occur in other protein units. Furthermore, should this phenomenon occur in proteins other than hemoglobin (e.g., proteins involved in biomolecule synthesis, DNA repair and replication, and/or metabolism and energy production) (**Table 3**),[52-59] it could explain the pleiotropic effects of H_2 and account for the movement of H_2 within tissues. The protein pocket mechanism of H_2 activity may hold some validity.

Table 3 | Downstream effects of metalloproteins that may be influenced by H_2, identifying the immediate role and downstream effects of metalloproteins that may be influenced by H_2

Metalloprotein	Example	Immediate roles	Downstream effects	Reference
Dehydrogenases	Complex I (NADH dehydrogenase)	O_2 metabolism	Cellular energy dynamics	52
Hydratase/dehydratase	Succinate dehydrogenase/fumarate hydratase	Removal of H_2O groups (H + HO)	Cellular energy dynamics	53
Dioxygenases	Indoleamine 2,3-dioxygenase 1	Addition of oxygen	Amino acid metabolism	54
Dismutases	Superoxide dismutase	Converts oxygen radicals ($2O_2^{\bullet-} \rightarrow H_2O_2 + O_2$)	Redox status	55
Globins	Hemoglobin	O_2 transportation	Acid/base balance, oxidation status	56
Oxidases (oxidoreductase)	NADH oxidase	O_2 reduction	Redox status, signaling molecules	57
Proteases	A-disintegrin and metalloprotease 17	Posttranslational modification of proteins	Ligand binding, cell signaling	58
Synthases	Porphobilinogen synthase	Synthesis of proteins and small molecules	Regulation of cell function	59

H_2: Hydrogen.

Theories on the Action of H_2

Intracellular signaling

H_2 is a nonpolar, diatomic molecule, a factor that reduces solubility in biological fluids[60] but enhances the ability of molecules to traverse biological membranes and affect intracellular processes such as signal transduction, gene expression and perhaps protein activity and/or conformation.[61] When considering cell signaling events, arguably two of the most prominent messengers responsible for regulating cellular activities are the positively charged Ca^{2+} and the negatively charged phosphate ion (PO_3^{2-}).[62,63] Ca^{2+} regulates a wide range of cellular events, including cell contractility and motility, energy metabolism, and neuronal activity, whereas PO_3^{2-} is responsible for the reversible posttranslational modifications of proteins (catalyzed by kinases for the addition of PO_3^{2-} and phosphatases for the removal of PO_3^{2-}), an action known to alter the polarity of proteins from hydrophobic (apolar) to hydrophilic (polar), enabling protein–protein interactions.[62]

Ca^{2+} signaling is integral for optimal cellular function and can have wide-ranging influences within the cytoplasm, organelles, and the extracellular milieu. Ca^{2+} signaling is fundamental to canonical stress responses in many cell types, including endothelial and epithelial cells, which rely, directly and indirectly, on Ca^{2+} influx through membrane-bound ion channels.[64] Cellular responses to Ca^{2+} signaling are largely dependent on the concentration, spatial orientation and physicochemical characteristics of Ca^{2+}. Ca^{2+} molecules are highly mobile traveling through saline solutions rapidly at 40 nm/ms.[65] Therefore, Ca^{2+} signaling is tightly regulated to ensure highly spatially restricted signaling, with cells chelating, compartmentalizing, or extruding Ca^{2+} using an array of protein antiporters, ion channels and pumps controlling localized concentrations,[66] any, or all, of which may be influenced by H_2.

Interestingly, the industrial production of H_2-retaining nanoparticles can involve coating the preformed structures with a layer of Ca^{2+} to augment and reinforce H_2 adsorption,[67,68] indicating that H_2 may directly interact with such ions. *Ab initio* calculations demonstrate that H_2 can bind with the Ca^{2+} component of calcium oxide through weak electron-donation forces, wherein electron transfer occurs from the occupied H_2 σ-orbital to the unoccupied 3δ-orbital of Ca^{2+}.[69] If such binding were to occur under physiological conditions, it would be feasible to assume that a strong affinity between Ca^{2+} and H_2 exists, with H_2 possibly affecting the Ca^{2+}-calmodulin binding potential, an event that would have significant downstream effects on gene transcription, immune responses and muscle contraction. Calmodulin is a highly conserved sensor of Ca^{2+} that has a fundamental role in cellular signaling.[70] The double-lobed calmodulin molecule can bind up to four Ca^{2+} molecules, with each binding region asserting individual effects on protein conformation, thus affecting further Ca^{2+} binding and target recognition.[71]

Although evidence is sparse regarding H_2 interactions with Ca^{2+} and Ca^{2+}-derived signaling cascades, Iuchi et al.[72] in 2016 demonstrated that in a pure chemical system, H_2 can prevent autooxidation of unsaturated fatty acids, a factor that reduces Ca^{2+} signal transduction and downstream Ca^{2+}-regulated gene expression. The team theorized that the effects on Ca^{2+} signaling were likely a result of a decrease in agonist inducers or antagonist inhibitors, although they did not speculate as to whether H_2 could directly interact with the calcium ion. Direct H_2/Ca^{2+} interactions could also affect the production and activity of ROS, as Ca^{2+} overload can negatively impact the function of Kreb's cycle enzymes provoking the activation of ROS-generating enzymes (e.g., α-ketoglutarate dehydrogenase), and via indirect activation of nitric oxide synthases, a factor that can effectively inhibit the function of cytochrome c oxidase (complex IV), increase electron leakage, and ultimately promote mitochondrial dysfunction.[73] Therefore, if H_2 directly interacts with Ca^{2+}, it would likely influence both ROS production and cellular signaling.

Alternatively, H_2 may have an indirect influence on calcium signaling via upstream effects. For example, H_2 could influence ion channel function. If so, would the characteristics of H_2 activity be analogous to the proposed mechanism discussed for metalloproteins and enzymes? For example, Xe, which utilizes protein pockets, is noted to inhibit calcium signaling;[33] therefore, could H_2 utilize similar, or disparate, mechanisms to regulate Ca^{2+} signaling?

To address this conundrum, Itoh et al.[74] proposed that H_2 treatment suppressed lipopolysaccharide-induced phosphorylation of apoptosis signal-regulating kinase 1 and its downstream effector proteins (e.g., c-Jun N-terminal kinases, p38) in macrophages without affecting nicotinamide adenine dinucleotide oxidase-1 production of ROS. Therefore,

potential target(s) for H_2 interactions could be found either at the receptor proteins or immediately downstream of them. These findings are intriguing, as they suggest that i) Ca^{2+}, either directly or indirectly, could be a target for H_2, with cellular levels being directly affected by the configuration of transmembrane ion channels and antiporters and/or the size, shape and electrophilic status of Ca^{2+}; and ii) the primary mechanism of H_2 action may not be related to the direct reducing potential of H_2 but through the regulation of Ca^{2+} influx or activity. Therefore, the question "Does H_2 inhibit ROS/RNS generation by regulating Ca^{2+} influx?" should be considered.

Protein phosphorylation

Protein phosphorylation is another cardinal mechanism by which intracellular information is communicated in eukaryotic cells. Phosphorylation involves the reversible, posttranslational modification of an amino acid (serine, threonine, tyrosine or histidine) through the addition or removal of a PO_3^{2-} phosphoryl group.[43] The addition of the PO_3^{2-} molecule to protein residues alters the conformational structure, and therefore the function, of the modified protein.

Kinases are known to form cascades wherein phosphorylation of an antecedent protein leads to sequential phosphorylation of proteins downstream. A good example is the MAPK/extracellular signal-regulated kinase cascades that mediate between extracellular and intracellular responses to a range of stimuli, including cytokines, growth factors, mitogenic substances such as pathogen-associated compounds (e.g., lipopolysaccharides) and oxidative stress.[74] MAPK/ERK is a term for a supergroup of proteins related to signal transduction that are highly conserved throughout eukaryotic species. MAPK pathways utilize numerous, characteristically distinct proteins (e.g., extracellular signal-regulated kinase 1/2, c-Jun N-terminal kinase, p38) to relay a signal through the cell to inducible (e.g., nuclear factor erythroid 2-related factor 2 (Nrf2)) or transcriptional elements (e.g., antioxidant response elements), which further influence cellular activity. For example, the activation of serine/threonine MAPK3s through extracellular stimuli or guanosine triphosphate-binding proteins induces the phosphorylation and subsequent activation of MAPK2s. MAPK2s then participate in dual phosphorylation of the conserved Thr-X-Tyr motif within the activation loop of MAPK.[75] MAPKs phosphorylate target substrates on amino acid residues that are upstream of proline (e.g., Thr-X-Tyr-X-Pro). This mechanism confers specificity to signal transduction, an important factor when considering more than 13,000 human proteins are known to be altered through phosphorylation/dephosphorylation events.[76] A pro-oxidative cellular environment is known to initiate MAPK pathway signaling, which drives the cell toward protection and preservation responses through activating cellular differentiation, motility, proliferation, and survival pathways.[75] Therefore, if H_2 directly affects the phosphorylation/dephosphorylation of proteins, it could have significant downstream effects.

p38, a highly conserved evolutionary MAPK protein, is particularly associated with heightened inflammatory responses. For example, increased p38 activity is associated with a higher risk of malignancy, whereas MAPK/p38 inhibitors reduce inflammation, a hallmark of oncological disease.[77] H_2 has been demonstrated to inhibit MAPK/ERK/c-Jun N-terminal kinase/p38 signaling *in vitro*, utilizing both somatic cell cultures[78] and gametes.[79] Although whether the observed effects of H_2 application are due to the increased activity of phosphatases, a reduction in kinase activity as a result of reduced NFκB/TNF-α activity, or another yet-to-be-defined mechanism is largely unknown.

If H_2 is able to inhibit phosphorylation cascades, through either accumulating in protein pockets or acting directly on phosphoryl groups or targets of phosphorylation (e.g., serine, threonine, etc.), perhaps via quantum forces (e.g., London dispersion), it could provide a unifying theory to the pleiotropic effects noted in contemporary literature.[80-85]

Heme

Heme is an iron-containing prosthetic group found in cytochromes and red blood cells. The hemoglobin protein has a major role in the transportation and exchange of biological gases (such as oxygen (O_2), nitric oxide (NO), carbon monoxide (CO), and carbon dioxide (CO_2)) and achieves these functions through direct interactions with heme prosthetic groups and through covalent modifications of thiols and amine moieties.[22] Hemoglobin is composed of four individual subunits, each capable of binding a single molecule of oxygen. In both hemoglobin and myoglobin, the iron ion (Fe^{2+}) is coordinated by five nitrogen atoms within the porphyrin ring, with the sixth coordination position reserved for binding oxygen (**Figure 3**).

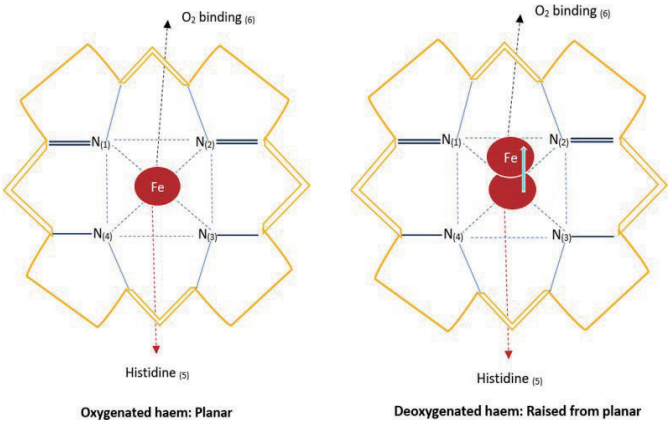

Figure 3 | The heme-containing porphyrin ring.
Left: Oxygenated heme (relaxed). Right: Heme in the deoxygenated (tense) form as the distance of Fe from the porphyrin ring increases by 0.06 nm. Brackets indicate binding positions. His represents the $Fe^{2+/3+}$-binding histidine residue of the protein (e.g., hemoglobin). Created with Microsoft Word (Version 2409).

Heme is versatile in that it can be present in numerous states, with the levels of each form influencing physiological processes. For example, ferrous iron (Fe^{2+}) is present in deoxygenated (deoxy-) heme and oxygenated (oxy-) heme, whereas ferric iron (Fe^{3+}) is present in (met-) heme, a non-oxygen-binding configuration. Additionally, there are significant differences in the conformation of hemoglobin

related to the ionic status of Fe. For example, deoxygenated hemoglobin is referred to as 'tense', as the globin is shifted 0.06 nm distally from the porphyrin ring.[85] Furthermore, oxygenated hemoglobin is defined as being relaxed, with the globin molecule sitting closer to the porphyrin ring (**Figure 3**). The change in oxidation to Fe^{3+} (met-heme) causes a change in the geometry of the heme pocket, favoring the relaxed position such that oxygen can no longer bind.

The ferromagnetism of heme may also influence the binding characteristics of gases. *In silico* analysis of the spin states of ferrous iron identifies that the Fe–N bond distance increases from 2.01 Å in the low-spin state to 2.1 Å in the high-spin state.[86] Therefore, as the high-spin state Fe^{2+} may extend further from the porphyrin ring, high spin state Fe^{2+} may be more effective in attracting O_2 (and H_2) to the heme prosthetic than low-spin Fe^{2+}. Structural characterization further identified high- and low-spin Fe-porphyrin derivatives, with research showing that ferric (Fe^{3+}) iron can also be found in the two spin states.[87] Moreover, the bond of high-spin Fe^{3+} was found to be increased by 0.4–0.5 Å, initiating a bond length of 2.04 Å away from the nitrogen moieties of porphyrin, whereas low-spin Fe^{3+} has a bond length of 1.99 Å.[88,89] Therefore, the question of whether H_2 interactions favor high-spin state $Fe^{2+/3+}$ arises.

Whether H_2 exerts its effects by extending the length of the $Fe^{2+/3+}$ bonds, optimizing O_2 adsorption by stabilizing the protein structure through direct interactions with the $Fe^{2+/3+}$ prosthetic moiety, or interactions with histidine 93 of human hemoglobin (and corresponding moieties in other proteins) has yet to be elucidated. H_2 may also impede O_2 dissociation from $Fe^{2+,}$ thereby improving O_2 saturation, as reported by Ostojic et al.[90] and Gaboreau et al.[91] When investigating the effect H_2 may have on heme utilization, daily administration of HRW (8 mM/day) was noted to improve O_2 saturation in middle-aged women and in patients recovering from COVID-19. To offer reasoned thought as to how this effect may occur and accounting for the relaxed and tense states of the heme prosthetic group, there are three ways in which H_2 could affect the oxygenation of haem. First, H_2 may protect Fe^{2+} from becoming oxidized via bilateral Kubas (involving the simultaneous donation of electrons from both hydrogen atoms to the iron center), or second, via asymmetrical bonding, where $Fe^{2+}/H^{\bullet}$ arrangements may directly reduce $^{\bullet}OH$ radicals and/or other reactive species (e.g., $Fe/H^{\bullet} + {}^{\bullet}OH \rightarrow H_2O$)[9] (**Figure 4**). Third, if the high-spin state of Fe^{2+} can increase the Fe^{2+}-N bond length, then H_2 may improve oxygen attachment to the prosthetic heme of low-spin ferrous iron through elongation of the 2.0 Å Fe^{2+}-N bond.

Magnetic electrochemistry

Research into biomagnetism, the study of magnetic fields generated by living organisms, is well established, with magnetic forces influencing electron spin dynamics.[92,93] This discussion in this section addresses whether quantum forces associated with ferromagnetic prosthetic groups, such as those in hemoglobin ($Fe^{2+/3+}$), superoxide dismutase (Mg^{2+}, Cu^{2+}), or calcium (Ca^{2+}), could impact spin states and thereby modulate the biological activity of H_2.

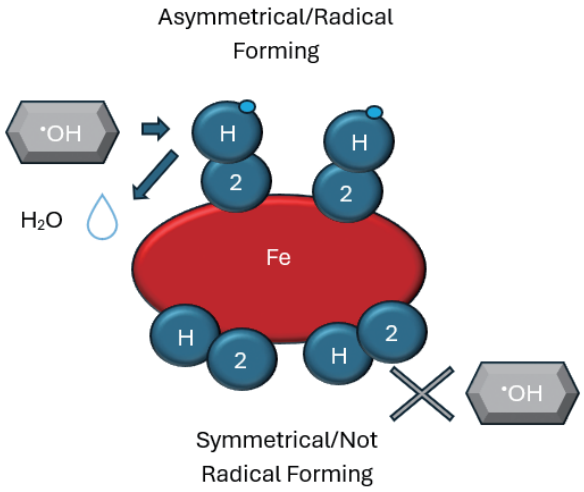

Figure 4 | Bonding of H_2 to Fe and $^{\bullet}OH$ reduction.
Schematic showing the possible mechanism(s) for the direct reduction of the hydroxyl radical ($^{\bullet}OH$) and the prevention of Fe oxidation. Created with Microsoft Word (Version 2409). H_2: Hydrogen.

Electromagnetism

Recent studies indicate that the thermal polarization of H_2 tends to favor the triplet electron state, with most H_2 molecules at room temperature existing in the ortho, or triplet, state (~75% ortho/25% para spin)[94] (**Figure 5**). Given that ortho-spin H_2 exhibits weak diamagnetic properties, which produce a repelling force,[95] it is improbable that ortho-spin H_2 directly reduces ROS, as the repulsive forces likely counteract such interactions. However, the ortho-spin state of H_2 might still influence cellular processes such as gene expression, protein conformation, or signal transduction. Although speculative, a detailed exploration of the quantum forces influencing H_2 interactions within biological systems could yield valuable insights.

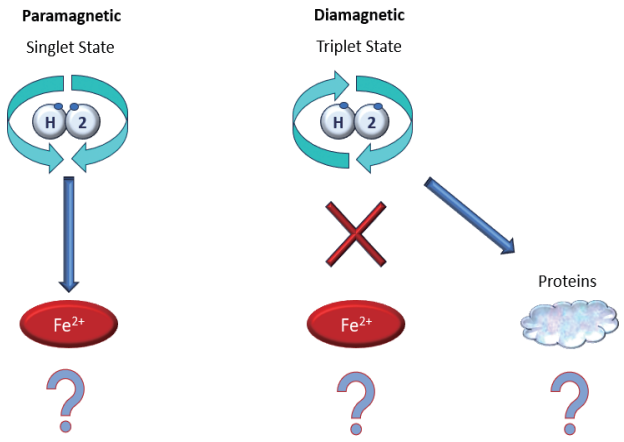

Figure 5 | How can electron spin affect H_2 biological activity?
Left: Singlet-state electron spin, electrons spin in opposing directions, creating a magnetic force (attracted to Fe), which may provide direct antioxidant activity. Right: Triplet-state electron spin, electrons spin in the same direction, creating a diamagnetic force (not attracted to Fe), which may have indirect antioxidant activity (via stabilization of protein structures). Created with Microsoft Word (Version 2409). H_2: Hydrogen.

Despite the predominance of diamagnetic H_2 under physiological conditions, the para or singlet state of H_2 (which comprises about 25% of physiological H_2) may also be significant. The para-spin state results in weak paramagnetic properties, potentially allowing for magnetic–electrochemical interactions with metalloproteins and prosthetic groups. For example, the inhibition of electron translocation, as observed in mitochondrial complex I, can lead to radical formation and subsequent ROS production.[96] The role of electron spin in ROS formation is exemplified by the reaction of a flavin-superoxide radical pair ($FH^{\bullet} + O_2^{\bullet-}$), where the singlet state leads to the formation of $F + H_2O_2$, whereas the triplet state results in two free radicals, $FH^{\bullet}$ and $O_2^{\bullet-}$.[97] Therefore, if H_2 in the para-spin state interacts with transition metals in proteins, as proposed by Hancock et al.,[22] it is conceivable that these metal groups could facilitate the reduction of radicals by lowering the dissociation energy of free H_2 (~4.64 eV to ~2.35 eV) and forming an acceptor/$H^{\bullet}$ complex. This interaction could potentially enable $H^{\bullet}$ radicals to neutralize oxidants such as $^{\bullet}OH$, although this would be contingent on the spatial and temporal availability of H_2 and the corresponding radicals. Supporting the assumption that para-spin H_2 may interact with Fe, Najera and Fout[98] reported that low-spin Fe^{2+} was capable of reacting with parahydrogen, as established through nuclear magnetic resonance and infrared spectroscopy, along with single-crystal X-ray diffraction.

Of further consideration for the magnetic electrochemical effects of H_2 are the metal catalytic centers of antioxidant enzymes such as catalase and superoxide dismutase, which are reported to have enhanced activity after exposure to H_2.[99] It can be surmised that para-spin H_2 may also improve the function of such enzymes by providing additional $H^{\bullet}$ and preserving the reductive capacity of the catalytic metal elements, although this has yet to be empirically verified.

In silico analysis of the effect of H_2 is predicted to occur within heme proteins, illustrating the feasibility of antioxidant activity via interactions between H_2 and protoheme. The authors reported that H_2 molecules can bind to the iron (Fe^{2+}) contained within heme asymmetrically via a dihydrogen bond and symmetrically through bilateral electron transfer, known as Kubas bonding (**Figure 4**).[9] Asymmetric binding is noted to be more favorable under physiological conditions due to a moderately lower activation energy (2.04 and 2.14 eV, respectively). In reciprocation, Fe^{2+} should reduce the dissociation energy of the H–H bond within the H_2 diatom, forming a $Fe^{2+}/H^{\bullet}$ complex, wherein $H^{\bullet}$ would be able to reduce highly reactive ions and radicals. The $Fe^{2+}/H^{\bullet}$ complex was calculated to have a relatively low dissociation energy of 2.78 eV, which would allow the bound $H^{\bullet}$ radical to neutralize another, coterminous, reactive nitrosative or oxidative species.[9] Accordingly, the authors state that this proposed activity may account for the direct reduction of $^{\bullet}OH$ in biological systems. It is therefore plausible that due to the weak magnetic forces at play, the para-spin H_2 molecule may have antioxidant effects, whereas the ortho-spin state H_2 molecule may influence cellular activities such as cell signaling, gene regulation and metabolic activity.

Future Directions

Many aspects need to be considered before H_2 can be considered a prescriptible therapy by global health agencies. Unidentified factors of H_2 biochemistry, such as diffusion and reactivity rates, interactions with metal-containing groups such as heme, and the magnetic spin state, may have effects. There is also a need to identify the primary target, or targets, of H_2 interactions so that the molecular mechanisms can be identified. Such work would support clinical data and could indicate whether there are likely to be any long-term or detrimental effects to H_2 therapies.

To advance the understanding of H_2 biochemistry, a variety of analytical methods should be employed across different eukaryotic cells. For example, electron paramagnetic resonance spectroscopy, combined with protein-specific spin labels, can detect radical or ionic adducts on traps that react with specific ROS or RNS.[100] When paired with chemiluminescence analysis, this approach would be applicable to both *ex vivo* and *in vitro* studies, allowing for a comparative evaluation of H_2 scavenging activity against $^{\bullet}OH$. Halliwell et al.[101] also developed a cost-effective deoxyribose assay to determine the reaction rate constants of antioxidants with $^{\bullet}OH$. By adding $^{\bullet}OH$-reductive antioxidants to this assay, they compete with deoxyribose (a primary target for $^{\bullet}OH$ oxidation) and inhibit chromogen formation. Spectrophotometric analysis can then deduce the reaction rate constant of the antioxidant with hydroxyl radicals.

Considering the hypothesis that H_2 may reside in protein pockets or influence heme dynamics, further research using X-ray crystallography, *in silico* modeling (e.g., X-PLOR),[102] and nuclear magnetic resonance spectroscopy could help define protein regions with surface pockets or intraprotein channels where H_2 might interact. A bioinformatics approach to compare Xe-binding pockets with potential H_2-binding sites, particularly in heme-containing proteins, could reveal whether these structures are conserved and whether H_2 has a significant role in maintaining the cellular and organismal redox status. Techniques, including infrared spectroscopy, protein film electrochemistry, and whole-cell biochemical assays, could also be used.[103] Additionally, advanced methods such as gene editing/mutation, transcriptomic profiling, and mass spectrometry could identify molecular regions targeted by H_2, offering valuable insights into the functional effects of H_2.

To explore the potential effects of H_2 on calcium (Ca^{2+}) signaling, techniques such as Ca^{2+}-specific fluorescent probes, such as green fluorescent protein-based genetically encoded calcium indicator sensors,[104] could be employed. To investigate the impact of H_2 on phosphorylation events, enzyme-linked immunosorbent assays could provide semiquantitative analyses of phosphorylated proteins by detecting antibody conjugation at phosphorylation sites. Alternatively, the Phos-Tag™/sodium dodecyl sulfate–polyacrylamide gel electrophoresis assay, which binds phosphoryl groups, could be used.[105] Additionally, radiolabeling, mass spectrometry, and western blot analyses, along with various kinase activity assays, could help determine whether H_2 interacts with signaling pathways upstream of protein phosphorylation.

Applying such analyses would contribute to a deeper understanding of whether H_2 directly influences these signaling cascades.

Conclusion

In conclusion, H_2, once distributed through the bloodstream, may be selectively retained by glucose moieties or within molecular pockets, which could explain the high concentrations found in skeletal muscle and distal tissues, as well as retention in organs such as the liver. Once H_2 is distributed through the bloodstream, it may be selectively retained by glucose or within protein pockets or cavities. It is also possible that H_2, through the influence of electron spin states, may target ferromagnetic ions such as Ca^{2+}, Fe^{2+} and others, affecting cellular signaling cascades and ROS levels.

Considering the direct antioxidant potential of H_2, ferromagnetism may attract singlet-state H_2, with heme potentially playing a pivotal role in its antioxidant capacity by modulating the production and reduction of ROS, whereas triplet-state H_2 could be integral to cell signaling and protein stabilization.

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