Hydrogen gas, ROS metabolism and cell signaling: Are hydrogen spin states important?

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Short title: Hydrogen gas and magnetism

Abstract

It is becoming accepted that treatment with hydrogen gas (H_2) has profound and often beneficial effects on cells from both animals and plants. Future uses which have been suggested include for cancer treatment, for alleviating symptoms of Parkinson's disease and ischemia and for improving crops in agriculture. However, besides a direct interaction with hydroxyl radicals there is little resolution of how H_2 is having biological effects. Dihydrogen is known to exist in two spin states, ortho and para, and to have paramagnetic properties. The interconversion of hydrogen spin states has been reported in the presence of signaling molecules such as nitric oxide, and in the vicinity of transition metals and organometallic compounds. Therefore, it is proposed here that the relationship between the effects of hydrogen gas and paramagnetism are investigated as a possible mechanism which could account for the alterations of cell function reported following H_2 treatment.

Introduction

Cell signaling mechanisms, which control the functioning of cells from the moment of an organism's conception to the moment that the organism dies, is extremely complex but it is now well recognized that these systems include the involvement of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Compounds such as superoxide anions ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), nitric oxide ($NO^{\cdot}$) and peroxynitrite ($ONOO^-$) are all thought to mediate cell signaling events [1,2].

Several other reactive compounds also need to be considered to be involved in the control of cellular function [1]. Amongst these is hydrogen gas (H_2) [3], a compound gaining prominence in the literature. However to be considered as part of a suite of signaling molecules, the production, movement, perception, roles and removal of the signaling compound need to be considered [2], and this would also apply to H_2 if it is to be accepted as a regulatory component in cells.

H_2 can be made by organisms, for example, through the action of hydrogenase enzymes [4-6]. For example, *Chlamydomonas reinhardtii* has two [Fe]-hydrogenases, HydA1 and HydA2 [5]. Such enzymes are reliant on the presence of Fe-S centers. In plants H_2 generation can be increased by presence of hormones such as abscisic acid, ethylene, jasmonate, but also stress such as salt and drought. This suggests that H_2 is important in stress signaling [7]. Auxin has also been shown to cause an increase in H_2 generation [8]. In humans it appears that H_2 is not endogenously produced. However, mammals can be exposed to H_2 through the action of colonic bacteria [9,10].

In treating organisms with H_2 there are various methods which can be used [3]. Although H_2 can be used in the gaseous form more often a saturated solution is created and added to the biological system. Therefore H_2 is administered as hydrogen-rich

water (HRW) or hydrogen-rich saline (HRS), although the physiological validity of this methodology needs to be considered [11].

Being relatively inert means that H_2 can freely diffuse through cells. Although it is also quite insoluble in water the hydrophobic environment of a cell membrane or organellular membrane should not be inhibiting to its movement, and may enhance it, so in that regard H_2 should be able to move through cells and between cells, which would facilitate its role as a signaling molecule.

The most important aspect of any compound's involvement in cell signaling is the effect that might result. One of the major effects of H_2 is through the modulation of antioxidant levels [11]. For example, in plants exposed to cadmium stress HRW was seen to have effects on the levels of antioxidants in the tissues [12]. In a similar manner HRW was given to swimming mice and some of the anti-fatigue effects appeared to be mediated by antioxidant levels [13]. There was lower nitric oxide (NO) in the serum and increased levels of glutathione peroxidase (GPx) in serum and the liver. The paper concludes that HRW is altering the immune-redox balance, giving the effects on the animals seen. In a study on UVB-induced responses in HaCat cells hydrogen decreased the accumulation of ROS and increased the expression of several genes including heme-oxygenase-1 [14]. Here the PI3K/Akt signaling pathway was thought to be involved too.

As with the swimming mice study [13], H_2 effects are often the amelioration of metabolic events which can alleviate stress responses. HRS could be seen to protect against ischemia-reperfusion injury of the liver, for example, mediated by the inhibition of endoplasmic reticulum stress [15]. Molecular hydrogen is thought to be able to protect against radiation damage, a process which is likely to involve the presence of ROS [10]. In plants, hydrogen gas has been shown to increase the shelf life of kiwifruit, an effect mediated by the reduction of ethylene biosynthesis [16]. Previously it has been

shown that HRW delays postharvest ripening of kiwifruit, partly mediated by increased levels of superoxide dismutase (SOD) activity, decreased lipid peroxidation and higher mitochondrial inner membrane integrity [17].

Having provided evidence that organisms can make or be exposed to H_2, that H_2 can move around cells and importantly that H_2 can have effects of cells – all good measures of the involvement of H_2 in cell signaling events [3] – to truly be able to be involved in biological systems H_2 would need to be perceived. One mechanism, that has been studied which may account for some of the effects of molecular hydrogen is the heme oxygenase (HO) system. HRW upregulated HO-1 expression in mice [4,18] and in cucumber [19]. Here the HRW effects were sensitive to a HO-1 inhibitor zinc protoporphyrin IX (ZnPP).

Hydrogen treatment of organisms has been mooted as a cancer therapy [20], to alleviate the symptoms of inflammatory bowel disease [18] and to help with the symptoms of Parkinson's disease [21]. In plants it has been suggested to be excellent for many aspects of agriculture [22]. Therefore its impact on cell signaling events needs to be fully understood.

Physical properties of molecular hydrogen and possible effects

An issue with the involvement of hydrogen gas in cell signaling is how it might be perceived. H_2 is not very soluble in water and relatively inert. It is, however, known to react with hydroxyl radicals but not with other ROS [23]. However, this cannot account for all its actions. There are reports of HO-1 being important in mice [14,18,19] but there is no receptor for H_2 and so it must be exerting effects in other ways. It is hard to conceive how H_2 can be recognized by a receptor protein, being so small and inert. A parallel can be drawn here with the nitric oxide receptor. Here there is no classical receptor protein mooted to be involved, but rather NO has its effects through the action

on a heme group in guanylyl cyclase [24], or is involved in direct chemistry with thiol groups [25]. For H_2 the latter is not likely because of its lack of reactivity but the former, that is, a direct effect on a heme group, may need to be considered.

A property of hydrogen which may be important is that of its nuclear spin states [26]. Hydrogen can exist of two states: ortho- (nuclear triplet state) and parahydrogen (nuclear singlet state). In the former the two proton spins are aligned but in the parahydrogen state they are antiparallel. At room temperature approximately 25% would exist in a parahydrogen form, while 75% would be in the singlet state. Rychlewski's [26] treatise explores the magnetic effects of the lowest triplet state of hydrogen and suggested that it is the simplest molecular system which shows purely repulsive interactions. For further exploration of the magnetic properties of H_2 see also Rychlewski's earlier paper [27].

Of importance here is that interconversions between para- and ortho- forms of H_2 can be catalyzed by paramagnetic collisions. Steiner and Ulrich wrote a long review on magnetic field effects [28] which included a section on biological systems and a short discussion of hydrogen interconversions. Some of these interactions involve compounds involved in ROS metabolism and signaling, that is, molecular oxygen (O_2), NO and NO_2 [28]. NO is an immensely important signaling molecule that is known to exist is different states and have diverse effects: NO can exist in the NO^-, $NO^{\cdot}$, and NO^+ forms. Therefore, an effect on NO chemistry can have an important consequence. Although not carried out in biological systems, it has been suggested that magnetic interactions with paramagnetic centers and hydrogen spins are possible, such as with unpaired electrons [29]. Extrapolating to relevant systems here, this would include free radicals such as $NO^{\cdot}$, hydroxyl radicals and superoxide anions which are all relevant to ROS metabolism and signaling.

Of particular relevance to ROS and NO metabolism is that in the liquid phase such para- and ortho- interconversions of H_2 can be catalyzed by transition metal ions [28] and organometallic compounds [30]. An explanation of the mechanism involves the magnetic field of the paramagnetic center influencing the proton spins [cited in 28]. Bunkowsky [29] cites a series of papers that discuss the effects of dihydrogen in the coordination sphere of transition metals. Of importance here is that many of the enzymes involved in ROS metabolism and signaling contain metal centers that are instrumental in their enzymatic activities (Table 1). Such metal prosthetic groups would be easily accessible to a small diffusible molecule such as H_2: it is smaller and less likely to steric hindrance than either the substrates or products in all cases.

An immensely important enzyme for ROS metabolism and the impact of signaling is superoxide dismutase (SOD). It removes superoxide anions produced by electron leakage and has been studied for many years as a potential therapeutic target [31]. SOD exists in different isoforms but they all contain transition metal centers, either Cu and Zn, Fe , Mn or Ni (Table 1). It has been suggested that hydrogen para- and ortho- interconversions can be catalyzed by copper atoms and surfaces [29]. Perhaps of relevance here too is the report that SOD activity is also influenced by the presence of a magnetic field [32], showing that physical rather than chemical influences can alter the enzyme's activity.

Catalase is instrumental in the removal of H_2O_2 in many cells and organelles. This would reduce H_2O_2 signaling as well as alleviate oxidative stress. Catalase has a Fe-heme prosthetic group which acts as the active site. Again this would be easily accessible to H_2. The rate of H_2O_2 decomposition and the evolution of oxygen by catalase is increased by approximately 20% in a 0.8T magnetic field [33].

Similarly, on the other hand, generation of ROS [34] and NO [35] may be affected by the presence of magnetic fields. Enzymes which generate ROS and RNS often contain transition metal prosthetic groups: NADPH oxidase [36] contains Fe-heme as does nitric oxide synthase (NOS) [37], while nitrate reductase also contains a molybdenum group [38].

It can be seen therefore that many enzymes, and cellular activities in which such enzymes are involved, some which are listed in Table 1, can be affected by magnetic fields and it is therefore possible that such enzymes are influenced by the physical nature of dihydrogen molecules. Furthermore, due to their inherent function the prosthetic groups of such enzymes will be available to the enzymes environment, allowing H_2 intimate access. Substrates and interacting proteins must have close access to these functional groups and therefore they will be readily accessible to a small diffusible molecule such as H_2.

Other cell signaling effects of magnetic fields

Although dihydrogen is not magnetic as such, it is suggested here that some of the effects seen in biological systems are due to its paramagnetic properties. Therefore, it seems pertinent to have a short discussion of magnetic effects on cells. This is not a new field, but it is one which has not been given much prominence in the literature. Steiner and Ulrich wrote a long review on magnetic field effects [28] which included a section on biological systems. Here the main focus was on effects on the photosynthetic reaction centers, interestingly which also contain transition metal prosthetic groups and can release ROS through electron leakage [39]. Static magnetic fields have been shown to enhance the growth of *Chlorella kessleri*, which was partly mediated by an increase in net photosynthetic capacity and increased respiratory rate.

Of relevance here there was an increase in oxidative stress and a decrease in the antioxidant capacity [40].

In animals endothelial cells have been shown to be sensitive to magnetic fields. Reducing the magnetic field to low levels inhibited proliferation, while the addition of SOD decreased the increased proliferation caused by 120μT fields. It was concluded that endothelial cells were affected by static magnetic fields through a free radical mediated mechanism [41]. Others have used magnetic hydroxyapatite scaffolds and shown an increase in cell proliferation [42]. These effects appeared to be mediated by an activation of mitogen-activated protein kinase (MAPK) pathways, most notably involving MEK1/2 and ERK1/2, but interestingly neither of these enzymes contain a prosthetic group, suggesting that there may be an upstream effect that is yet to be identified.

On the flip side of an increase in proliferation is the report of the increase in cell death signaling by magnetism. Using magnetic nanoparticles a promotion of apoptosis in animal cells was seen [43]. However, contrary to this is the report that magnetic fields increase cell survival by reducing apoptosis [44]. This was because the magnetic field increased Ca^{2+} influx from the outside of the cells and it was suggested that the rescue of damaged cells by magnetism may increase mutation and tumor frequencies. How the magnetic field is having effects in this system seems unclear at the moment.

Untangling the direct effects of magnetism on intracellular events in cells has been the focus of some studies. Zhang et al. [45] studied the effects of extremely low frequency magnetic fields (ELF-MF: 50Hz, 8mT, 4 hours per day) on a myriad of cell signaling components in mice hippocampus. An increase in levels of G_i protein, inositol 1,4,5-trisphosphate, diacylglycerol, protein kinase A, protein kinase C (PKC) beta and calcium signaling were seen. Again, few of these enzymes and signaling components (with the exception of calcium) involve metals, although PKC has its activity modulated

by zinc ions [46]. Of more relevance here are the reports of magnetic field effect on antioxidants. In mice fibroblasts and using permanent magnets of 0.1 T to 0.7 T it was reported that there was a decrease in the activity of SOD and GPx, although the authors then concluded that the magnets did not cause oxidative stress but rather showed a slight antioxidising activity [47]. Supporting this was a study on the magnetic effects on restraint stressed rats. Using a static magnetic field of 0.8mT over 5 days (exposure being 30, 60 or 240 min/day) there was a decrease in nitric oxide, malondialdehyde, advanced oxidation proteins products and glycation end products, suggesting a decrease in oxidative stress. This was supported by the rise in reduced glutathione (GSH). Interestingly SOD levels also rose. The authors suggest that treatment with a static magnetic field may be a therapy to attenuate oxidative stress [48], an effect also seen with hydrogen gas [14,49].

Conclusions and perspectives

Molecular hydrogen (H_2) whether supplied as a gas or in solution (as HRW or HRS) has been mooted as a beneficial treatment in both in medicine [11] and agriculture [22]. Hydrogen has been shown to relieve stress in cells, ameliorating responses to stress challenge in plants [12] and disease in animals [15]. Although some direct interactions with ROS have been proposed, such as with hydroxyl ions [23] such chemistry would not account for all the effects reported for H_2. Chemically H_2 is relatively inert so a physical property may need to be considered to account for its actions. H_2 can exist in two spin states (para- and ortho-) while such spin states of hydrogen can be altered by direct interaction with some signaling molecules [28] such as NO, and organometallic compounds [30]. Some enzymes involved in ROS metabolism and signaling have been shown to be affected by magnetic fields, such as catalase [33], and such enzymes will have prosthetics groups which will be accessible

to close interaction with H_2. Therefore, it is proposed here that the relationship between the effects of hydrogen and magnetic fields is worth exploring. The direct effects of molecular hydrogen with a number of ROS metabolizing enzymes such as SOD and catalase is worth investigating in the presence and absence of static magnetic fields.

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Acknowledgements. This work was funded by the University of the West of England, Bristol, who financed the authors' time and literature sourcing for the preparation of this manuscript.

Competing interests statement. The authors declare that they have no competing financial interests.

390 Table 1: Some proteins involved in ROS/NO metabolism and signaling which contain

391 metal prosthetic groups.

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Protein	Function	Metal prosthetic group
Superoxide dismutase	Removes superoxide anions to produce H_2O_2	Cu and Zn
Superoxide dismutase	Removes superoxide anions to produce H_2O_2	Fe
Superoxide dismutase	Removes superoxide anions to produce H_2O_2	Mn
Superoxide dismutase	Removes superoxide anions to produce H_2O_2	Ni
Catalase	Removes H_2O_2 to make O_2	Fe/Heme
NADPH oxidase	Produces superoxide	Fe/Heme
Guanylyl cyclase	Produces cGMP	Fe/Heme
Nitric oxide synthase	Produces NO	Fe/Heme
Nitrate reductase	Can generate NO	Mo
Xanthine oxidoreductase	Can generate H_2O_2 and NO	Mo & [2Fe-2S] clusters
Cytochrome *c*	May trigger apoptosis	Fe/Heme
Myeloperoxidase	Removes H_2O_2 and produces hypochlorous acid	Fe/Heme

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