Hydroxyl-radical scavenging activity of hydrogen does not significantly contribute to its biological function

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Abstract

Since Ohsawa et al. reported a biological antioxidant function of hydrogen in 2007, researchers have now shown it to exert protective effects in a wide range of human and animal disease models. Clinical observations and scientific arguments suggest that a selective scavenging property of H_2 cannot adequately explain the beneficial effects of hydrogen. However, there is no experiment challenging the original published data, which suggested that molecular hydrogen dissolved in solution reacts with hydroxyl radicals in cell-free systems. Here we report that a hydrogen-saturated solution (0.6 mM) did not significantly reduce hydroxyl radicals in the Fenton system using 1 mM H_2O_2. We replicated the same condition as Ohsawa's study (i.e. 5 μM H_2O_2), and observed a decrease in $^{\bullet}OH$ radicals in both the H_2-rich and N_2-rich solutions, which may be caused by a decreased dissolved oxygen concentration. Finally, we determined the effect of hydrogen on a high-valence iron enzyme, horseradish peroxidase (HRP), and found that hydrogen could directly increase HRP activity in a dose-dependent manner. Overall, these results indicate that although H_2 and $^{\bullet}OH$ can react, the reaction rate is too low to have physiological function. The target of hydrogen is more complex, and its interaction with enzymes or other macro-molecules deserve more attention and in-depth study.

Introduction

In 2007, Ohsawa et al. [1] reported neuroprotective effects of molecular hydrogen in a rat model of cerebral infarction. They further demonstrated that H_2 could act as a therapeutic antioxidant by selectively reducing hydroxyl radicals without disturbing important physiological reactive oxygen species (ROS). Data generated since have demonstrated potential benefits of H_2 in over 170 different human and animal disease models [2]. For example, H_2 has beneficial effects in both type 1 [3] and type 2 diabetes, insulin resistance [4,5], experimental liver injury [6], acute oxidative stress in focal brain ischemia/reperfusion injury [1], acute and chronic stress [7], and organophosphorus pesticide-induced neurotoxicity [8]. H_2 was also shown to have some anti-cancer effects [9,10], and alleviate nephrotoxicity induced by anti-tumor drugs [11]. Several clinical trials, such as in patients with potential metabolic syndrome [12,13], Parkinson's disease [14], rheumatoid arthritis [15], mild cognitive impairments [16], and others, as reviewed previously [2,17], further demonstrate hydrogen's potential as a medical gas.

These therapeutic effects are associated with beneficial changes in several transcription factors (e.g. Nf-$\kappa\beta$, Nrf2, NFAT, etc.) and subsequent regulation of anti-oxidant and anti-inflammatory mediators (e.g. glutathione, superoxide dismutase, cytokines, TNF-α, etc.) [17]. However, the underlying mechanism(s) and primary target(s) remain elusive, as many of these molecules are simply passenger molecules that are changed secondarily by modulation of their upstream regulators [2].

To date, there are only four reported primary actions of molecular hydrogen that may help explain some, but not all of its ubiquitous biological effects. The first, and most widely cited, is direct $^{\bullet}OH$ radical scavenging by H_2 (i.e. $H_2 + {}^{\bullet}OH \rightarrow H_2O$) [1]. However, this hypothesis does not adequately explain the many effects of H_2. In fact, a companion paper published in the same issue of the *2007-Nature Medicine* has questioned this hypothesis theoretically [18], indicating that there was no conclusion on this issue at that time. Although more than 1000 original articles have been published in the field of molecular hydrogen from 2007, and the specific hydroxyl radical scavenging effect of hydrogen has been repeatedly proposed in these articles, only a few articles have questioned this hypothesis again [19], and no conclusive experimental evidence has been presented to confirm or refute this hypothesis so far. A second hypothesis, showing the oxidation of deuterium gas *in vitro*, suggests an iron species-dihydrogen interaction with the iron sulfur clusters within

mitochondrial complex I [20]. A third in vitro experiment, suggests an interaction between hydrogen and certain enzymes, such as increasing the activity of acetylcholine esterase (AChE) [8]. The fourth hypothesis is of an *in vitro* experiment where it was demonstrated that H_2 gas could suppress the auto-oxidation of linoleic acid, and subsequently modify lipid mediators thus affecting Ca^{2+} signaling and gene expression [21]. However, these proposed mechanisms require further investigation to falsify and/or reproduce their results, and to determine how much, and to what extent they can address the ubiquitous therapeutic effects of only small amounts of molecular hydrogen used *in vivo*.

In this study, we directly investigated the first hypothesis to determine if H_2 significantly reacts with $^{\bullet}OH$ radicals or if it could be explained by other study confounds (e.g. decreased dissolved O_2). We also investigated another potential target of molecular hydrogen, and report the preliminary positive *in vitro* results, which may further elucidate the mechanisms by which H_2 exerts its biological benefits.

Materials and Methods

Reagents

Hydrogen peroxide (30%), ferrous (II) perchlorate, $K_3Fe(CN)_6$, were purchased from Sigma, 2-[6-(4'-hydroxy) phenoxy-3H-xanthen-3-on-9-yl] benzoate (HPF) from Molecular Probes® and Horse radish peroxidase (HRP) was purchased from Aladdin®. Solutions were prepared with deionized water, which was purified using a Milli-Q water purification system for laboratory tests.

Hydroxyl radical producing methods

The composition of $^{\bullet}OH$ -radical generating system is shown in Table 1. H_2 or N_2 gas was bubbled in ultrapure water beyond the saturated level under 0.4 MPa of pure hydrogen pressure for 30 min, and the resultant H_2 or N_2-rich water was then used to prepare $^{\bullet}OH$-producing system under atmospheric pressure. We determined the H_2 concentration with a hydrogen electrode (Unisense A/S, Aarhus, Denmark) in each experiment. The concentration of dissolved oxygen in H_2-rich water (3.65 mg/L), N_2-rich water (3.50 mg/L) and normal water without treatment (Air) (8.75 mg/L) was determined using an oxygen electrode (Thermo Fisher Scientific, USA).

114 Table 1. The composition of the $^{\bullet}OH$-generation reaction system

Reaction System (50 μL)	Composition with H_2 (0.8 mM) or N_2-rich water	
Fenton reaction	H_2O_2 (5 μM or 1 mM)	Ferrous (II) perchlorate (0.1 mM)

115

116 **Detection of $^{\bullet}OH$ formation by hydroxyphenyl fluorescein**

117 To detect the reaction of H_2 and $^{\bullet}OH$, the Fenton reaction system as
118 described above was used in the presence of HPF (0.4 μM). The fluorescence
119 intensity of HPF was measured at 535 nm with excitation at 485 nm by using a
120 Victor 1420 Multilabel Counter (Wallac, Perkin-Elmer, Wellesley, MA, USA).

121 **Horseradish peroxidase (HRP) enzyme activity test**

122 We incubated solutions containing 0.4 μM HPF, 10 μM H_2O_2 with H_2 or
123 N_2-rich water at different concentrations (85%, 50% and 25%). The reaction
124 was initiated by adding 0.2 μg/mL HRP. The fluorescence of HPF was
125 measured at 535 nm with excitation at 485 nm by using a Victor 1420
126 Multilabel Counter (Wallac, Perkin-Elmer, Wellesley, MA, USA).

127 **Reduction of Fe(III) by H_2**

128 The fluorescent intensity of HPF was also used to test the possible reduction
129 of ferric iron (Fe^{3+}) by H_2, $FeCl_3$ (0.1 mM), H_2O_2 (5 μM), HPF (0.4 μM) and
130 either H_2 or N_2-rich water were mixed together. The fluorescent intensity of
131 HPF was measured at 535 nm with excitation at 485 nm by using a Victor 1420
132 Multilabel Counter (Wallac, Perkin-Elmer, Wellesley, MA, USA). For $K_3Fe(CN)_6$
133 reaction, 2 mM $K_3Fe(CN)_6$, 0.4 μM HPF and H_2 or N_2 rich water was mixed,
134 followed by HPF fluorescence measurement.

135 **Statistical analysis**

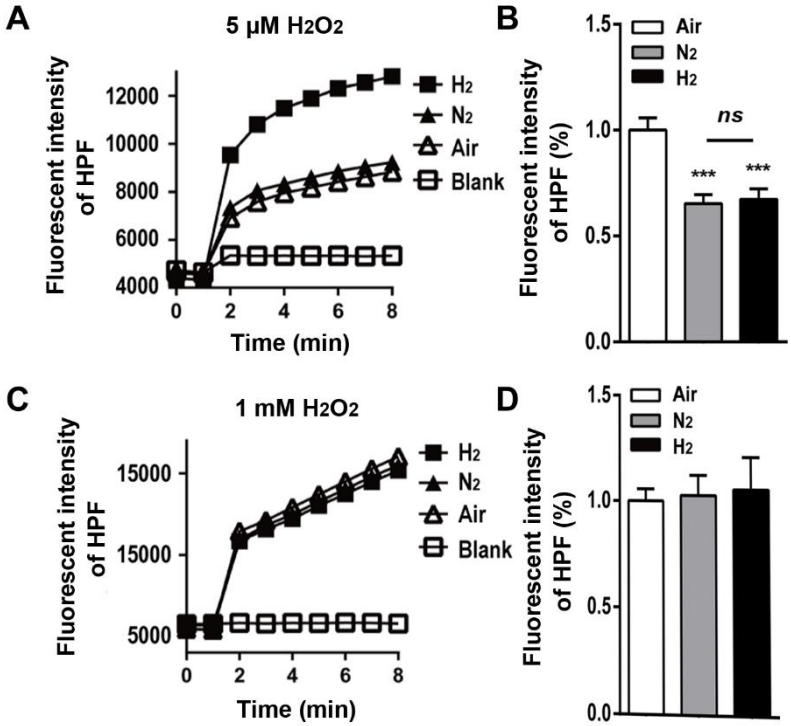
136 Results are expressed as means ± S.E.M. for each experiment. For single
137 comparisons, we used an unpaired two-tailed Student's *t* test; for multiple
138 comparisons, we used ANOVA. $p < 0.05$ was considered to be statistically
139 significant.

140

141 **Results**

142 **The effect of hydrogen on the $^{\bullet}OH$ production in cell-free *in vitro* system**

143 In Ohsawa's study, they produced •OH radicals by the Fenton reaction and
144 semi-quantified the levels of •OH by HPF fluorescence [1]. A previous study
145 showed that both the presence of oxygen and the $[Fe^{2+}]/H_2O_2$ ratio may have
146 an impact on •OH production in the Fenton reaction [22]. To determine whether
147 the H_2 induced •OH decrease is caused by the reduced oxygen concentration,
148 N_2 was used to induce similar anaerobic conditions and the •OH formation was
149 detected. To further determine whether a different $[Fe^{2+}]/H_2O_2$ ratio could affect
150 •OH production in the Fenton reaction, we used a H_2O_2 concentration of either
151 5 μM (the same as Ohsawa's study) or 1 mM. These concentrations represent
152 a high (20:1) and low (1:10) $[Fe^{2+}]/H_2O_2$ ratio, respectively. The •OH formation
153 in Fenton reaction was assessed via HPF fluorescence detection. As expected,
154 both H_2 and N_2 decreased the HPF fluorescent signal of •OH radicals in the
155 presence of 5 μM H_2O_2 (Fig. 1A and B). However, in the presence of 1 mM
156 H_2O_2, there was neither a decrease nor an increase in •OH formation in either
157 the H_2 or N_2 group (Fig. 1C and D).



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159 **Fig. 1. The effect of hydrogen on •OH formation in Fenton reaction assessed by HPF**
160 **fluorescence.** (A) and (C) are the time course of •OH formation in the presence of 5 μM (A)
161 or 1 mM H_2O_2 (C). (B) and (D) are the quantification of the HPF fluorescence intensity (5 μM
162 H_2O_2 (B), n = 8; 1 mM H_2O_2 (D), n = 10). ***$p < 0.001$, *ns* $p > 0.05$.

163

Effect of hydrogen on horseradish peroxidase activity

We tested the activity of HRP, based on the HPF fluorescence intensity, in the presence of 10 μM H_2O_2 in water with or without H_2 or N_2. The results showed that both hydrogen and nitrogen treatment greatly increased HPF signals in a dose-dependent manner (Fig. 2A), and the HPF signals in each dilution in H_2 group were markedly higher than that in the N_2 group (Fig. 2B). These results indicated that hydrogen and nitrogen treatment may enhance the HRP activity, an affect which may not be attributed to the removal of oxygen.

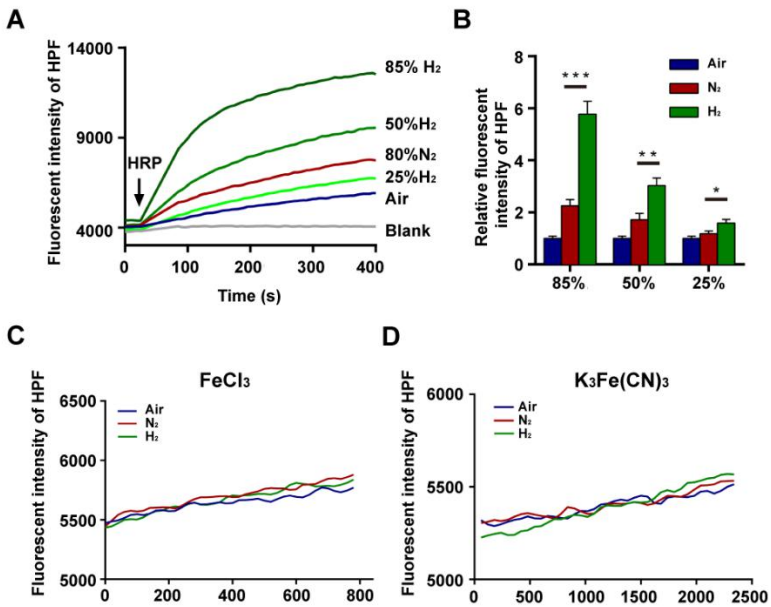

Fig. 2. The possible effects of hydrogen on Fe(III) and on HRP activity were determined by HPF florescence. (A) is the representative time course of HPF fluorescence at different concentration of H_2 and 85%N_2. Baseline showed HPF fluorescence in absence of HRP. (B) is the quantification of HPF fluorescence at 300 seconds after adding HRP. (C) and (D) is the representative time course of HPF fluorescence in the presence of $FeCl_3$ and $K_3Fe(CN)_6$, respectively. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Effect of hydrogen on the redox state of Ferric iron (Fe^{3+})

To further confirm whether H_2 could possibly reduce Fe(III), the HPF fluorescence based method was used. The results showed no observable reduction in either $FeCl_3$ (Fig. 2C) or $K_3Fe(CN)_6$ (Fig. 2D).

Discussion

Since Ohsawa et al. published the first paper in *Nature Medicine*, the effects of hydrogen have been extensively reported using more than 170

188 different human and animal diseases models [2]. The idea that H_2 acts as a
189 therapeutic antioxidant via directly interacting with $^{\bullet}OH$ radicals was first
190 proposed in Ohsawa's study [1]. They evaluated the $^{\bullet}OH$ scavenging activity of
191 H_2 in an *in vitro* cell-free Fenton reaction by HPF fluorescence based method,
192 and found that H_2 reduced the HPF fluorescent signal of $^{\bullet}OH$ radicals in a
193 dose-dependent manner. One of the widely interpretations of this study, is that
194 hydrogen can selectively scavenge $^{\bullet}OH$, which account of its therapeutic and
195 antioxidant-like effects. This perspective has also been questioned [18,19],
196 due to two main concerns. The first is the cellular concentration of H_2 only
197 reaches the micromolar range, but in order to be an effective biological $^{\bullet}OH$
198 scavenger, the required concentration should be in the millimolar range, and in
199 some organs (e.g. brain) the H_2 level may never even increase after drinking
200 H_2-rich water [23]. The second is that the observed rate constant for the H_2 and
201 $^{\bullet}OH$ reaction (i.e. 4.2×10^{7} M^{-1} s^{-1}), is likely too low to effectively compete with
202 the other reactions between $^{\bullet}OH$ and numerous cellular targets such as
203 proteins or lipids, whose rate constants are on the order of 10^{9} or 10^{10} M^{-1} s^{-1}
204 [19].

205 In the present study, to determine whether H_2 significantly reacts with $^{\bullet}OH$
206 radicals, we evaluated the $^{\bullet}OH$ scavenging activity of H_2 in an *in vitro* cell-free
207 Fenton reaction by using HPF fluorescence based method. The fluorescent
208 intensity of HPF was used as the indicator of the levels of $^{\bullet}OH$ radicals. In the
209 presence of 1 mM H_2O_2 concentration, there was no reduction of $^{\bullet}OH$ radicals
210 in neither the H_2 nor the N_2 group. We note that in Ohsawa's study, they used
211 a very low H_2O_2 concentration (5 μM), which is close to the *in vivo*
212 physiological level, but started the reaction with 0.1 mM Fe^{2+}. This leads to an
213 excess of Fe^{2+} in conditions with 5 μM H_2O_2 which is oxidized by the oxygen in
214 the air-saturated media but not in the H_2 purged solutions where oxygen is
215 removed or diminished. In our study, the same concentration of H_2O_2 in the
216 Fenton reaction was also used. In contrast to the higher level of H_2O_2, the
217 results showed that the levels of $^{\bullet}OH$ radicals were significantly decreased in
218 H_2 group compared to the control, which was consistent with Ohsawa's study.
219 However, this marked reduction in $^{\bullet}OH$ radical formation was also observed in
220 the N_2 group. It was reported that besides changes in O_2 concentration, the
221 $[Fe^{2+}]/H_2O_2$ ratio also impacts $^{\bullet}OH$ production in the Fenton reaction [22].
222 When the $[Fe^{2+}]/H_2O_2$ ratio is high (> 1.5), the $^{\bullet}OH$ production in anaerobic
223 conditions is markedly lower than in aerobic conditions [22]. In our study, in the
224 presence of 5 μM H_2O_2, the $[Fe^{2+}]/H_2O_2$ ratio reached to 20:1. We speculate
225 that in the high $[Fe^{2+}]/H_2O_2$ ratio condition, the decrease in $^{\bullet}OH$ formation may

226 be attributed to the reduced oxygen concentration. A previous study also
227 showed that in a low $[Fe^{2+}]/H_2O_2$ ratio condition (< 1), the decrease in oxygen
228 concentration had no significant effect on the $^{\bullet}OH$ production [22]. This may
229 explain why in the presence of 1 mM H_2O_2 ($[Fe^{2+}]/H_2O_2$ ratio: 1:10), there was
230 neither a decrease nor an increase in $^{\bullet}OH$ formation in either the H_2 or the N_2
231 group. Our results demonstrate that under these conditions with infeasible
232 supraphysiological concentrations of H_2O_2 (1 mM) and H_2 (≈ 0.6 mM) there
233 was no reduction in $^{\bullet}OH$ radicals in the H_2 group. Thus, we propose that
234 although H_2 can react with $^{\bullet}OH$ radicals at an observed rate constant of
235 4.2×10^{7} M^{-1} s^{-1} [19], hydroxyl radical scavenging of hydrogen does not
236 significantly contribute to its biological function.

237 Next, we hypothesized that, because direct $^{\bullet}OH$-radical scavenging activity
238 of H_2 likely does not have biological significance, H_2 must interact with other
239 biomolecules. Besides $^{\bullet}OH$ radicals, HPF can also be oxidized by various
240 enzymes containing high-valence iron, such as horseradish peroxidase (HRP)
241 [24]. HRP, a related heme enzyme of cytochrome c peroxidase and
242 cytochrome P450, has an analogous high-valence iron intermediate, but does
243 not release hydroxyl radicals [25]. To determine whether hydrogen could
244 directly modulate the activity of an enzyme containing high-valence iron, we
245 tested the activity of HRP based on the HPF fluorescence intensity with or
246 without H_2 or N_2 in a cell-free *in vitro* system. Our study clearly showed that
247 hydrogen treatment greatly increased HPF signals in a dose-dependent
248 manner, which cannot be fully explained by removal of oxygen, as is
249 evidenced by significantly higher level of HPF signals in the H_2 group
250 compared to the N_2 group. These results suggest that high-valence iron
251 containing enzyme(s) could be one of the potential targets of hydrogen.

252 Our previous study was the first to report an interaction between molecular
253 hydrogen and biological macromolecules [8]. We found that molecular
254 hydrogen could directly increase the activity of acetylcholinesterase (AChE). In
255 combination with the evidence provided in the present study, we propose that
256 the biological macromolecules, especially the enzymes, might be a potential
257 target of hydrogen involved in its biological functions.

258 Lastly, a previous study has also investigated the notion that H_2 may exert
259 its beneficial effects by reducing Fe(III) centers, which are oxidized during
260 oxidative stress. However, they reported that neither hemes nor iron–sulfur
261 clusters were reduced in cytochrome P450cam, myoglobin, and putidaredoxin
262 [19]. Liu *et al*. showed that in addition to hydroxyl radicals, HPF can also be

oxidized by the high-valence iron and $K_3Fe(CN)_6$ [24]. In this study, we determined the possible reductive effect of hydrogen on $K_3Fe(CN)_6$ and $FeCl_3$ by measuring the intensity of HPF fluorescence. Consistently, no significant effect was observed both in the presence of either H_2 or N_2, which indicated that molecular hydrogen does not reduce Fe(III) centers.

In conclusion, in this study we provided evidence that the selective-scavenging activity of hydrogen for $^{\bullet}OH$ radicals may not be the principal factor that contributes to its biological functions. Instead, other mechanisms/targets such as biological macromolecules, especially enzymes, might be responsible for its therapeutic effects.

Conflict of interest statement

We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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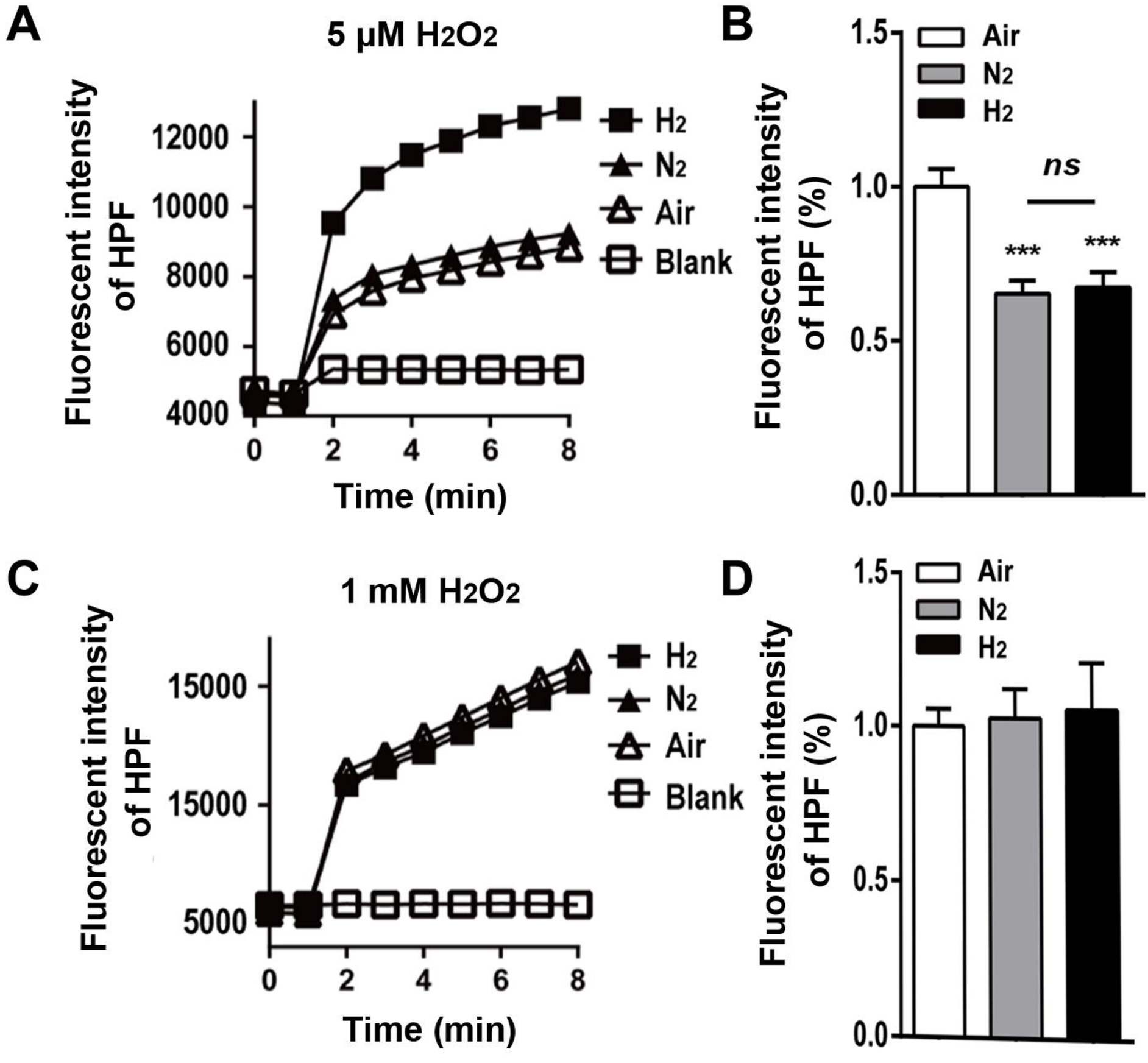
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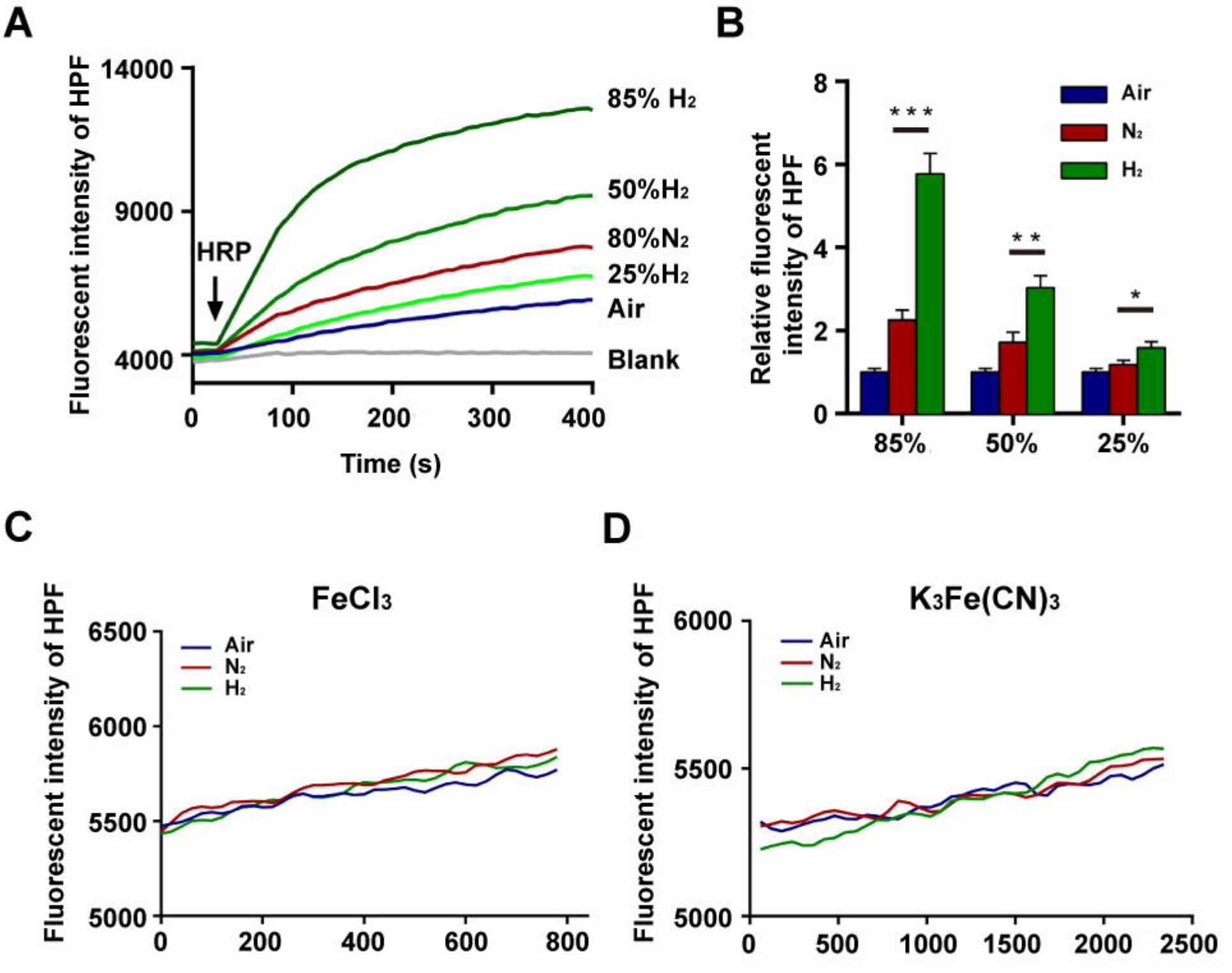
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