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Original Contribution

ONOOH does not react with H_2: Potential beneficial effects of H_2 as an antioxidant by selective reaction with hydroxyl radicals and peroxynitrite

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ABSTRACT

H_2 has been suggested to act as an antioxidant when administered just before the reperfusion phase of induced oxidative stress. These effects have been reported, for example, for the heart, brain, and liver. It is hypothesized that this beneficial effect may be due to selective scavenging of $HO^\bullet$ and ONOOH by H_2. The reaction of H_2 with $HO^\bullet$ has been studied by pulse radiolysis in the past and is too slow to be physiologically relevant, not to mention that the reaction yields the reactive $H^\bullet$ radical. We therefore investigated whether H_2 reacts with ONOOH and whether the presence of H_2 influences the yield of nitration of tyrosine by ONOOH. With only negative results, we entertained the notion that H_2 may possibly exert its beneficial effects by reducing Fe(III) centers, oxidized during oxidative stress. However, neither hemes nor iron–sulfur clusters were reduced.

In recent years much research has been conducted on the potential beneficial effects of hydrogen (H_2) and its use as a therapeutic medical gas. In 2007, Ohsawa et al. [1] reported that H_2 could possibly selectively reduce the oxidizing species $HO^\bullet$ and $ONOO^-$ in cultured cells. According to this view, H_2 would scavenge these species directly, but not interfere with physiological oxidation and reduction reactions or react with physiologically important species as $NO^\bullet$, needed for neurotransmission, or $O_2^{\bullet-}$ and H_2O_2, which are thought to act as signaling molecules in processes such as apoptosis, cell proliferation, and differentiation. As with the claim that H_2 scavenges $HO^\bullet$ and ONOOH, no experimental evidence was proffered for the nonreactions [1]. These authors also found that H_2 gas protected neurons from ischemia/reperfusion injury when administered before the reperfusion phase, even in concentrations as low as 1–3% in the air to which the animals were exposed [2]. No direct mechanism for these beneficial effects has been put forward.

Indeed, K.C. Wood and M.T. Gladwin [3] questioned the effectiveness of H_2 as a direct scavenger. It would have to compete with numerous other cellular targets of $HO^\bullet$ (such as thiols or lipids), which react faster and are in far greater abundance. Furthermore the rate constant of the reaction of H_2 and $HO^\bullet$ to form H_2O and $H^\bullet$ is only $4.2 \times 10^7\ M^{-1}\ s^{-1}$ [4,5], which is very low compared to the $10^9\ M^{-1}\ s^{-1}$ for most radical reactions. In addition, the solubility of H_2 is low, at 0.100 MPa ($\approx$ 1 atm) it is 0.78 and 0.74 mM at 25 and 37 °C, respectively. These are maximum values, as no correction for ionic strength—estimated to lower these concentrations by 5%—was made.

Later research has built on the work of Ohsawa et al. [1] and found other promising applications for H_2, for example, reduction of hepatic ischemia/reperfusion injury (I/R) [2] or myocardial I/R injury [6] and the use of H_2 as a radioprotective agent [7,8]. Again, the aforementioned reactions of H_2 with $HO^\bullet$ and ONOOH are put forward as possible or even likely mechanisms, but these reactions have not been examined.

In a 2010 review article by C.S. Huang et al. [9], it is stated that the direct scavenging properties of H_2 are unlikely to be the only explanation for the beneficial effects. H_2 could also act as a signaling molecule and influence certain (enzymatic) pathways. This view is also mentioned in the 2012 review by J.Y. Zhang et al. [10].

Our goal is to demonstrate that H_2 can or cannot act as a direct scavenger for $HO^\bullet$ and ONOOH by determining and/or evaluating the respective rate constants. Nitration of tyrosine by ONOOH was selected, as formation of nitrotyrosine would prevent phosphorylation of that residue, a reaction detrimental to the function of a tyrosine kinase. Furthermore additional UV–visible analyses are performed to test whether H_2 reduces Fe(III)-containing proteins, such as cytochrome P450cam, myoglobin, and putidaredoxin.

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Experimental methods

Materials

All chemicals were of the highest quality available and were used without further purification. Water was purified in a Millipore Milli-Q unit. Peroxynitrite was used as prepared via the Bohle method as adapted by us [11,12] and had been stored at −80 °C. *N*-acetyl-3-nitrotyrosine ethyl ester, *N*-acetyl-L-tyrosine methyl ester, and *N*-acetyl-L-tyrosine were used as received by Sigma (St. Louis, MO, USA). Cytochrome P450cam was prepared from a single colony of *Escherichia coli* Bl-21 cells and kindly provided by Ms. H. Erdogan. Myoglobin (from horse heart) was used as received by Sigma and putidaredoxin was kindly provided by Professor D. Hilvert (ETH Zürich).

Stopped-flow spectrophotometry

An Applied Photophysics SX 18-MV stopped-flow spectrophotometer was used at ambient pressure and at 25.1 ± 0.1 °C. The mixing time was less than 2 ms. Ten kinetic traces were averaged to extract a pseudo-first-order rate constant from a single exponential fit.

ONOOH

A 0.20 mM solution of $ONOO^-$ was prepared by adding an Eppendorf vial of ~0.8 ml of 25 mM $ONOO^-$ solution in 10 mM NaOH (frozen) to a 100-ml 10 mM KOH solution cooled with ice to < 5 °C. The solution was kept on ice and shielded from light to prevent decay of peroxynitrite. The concentration of the $ONOO^-$ solution was determined by UV–visible spectroscopy (Analytic Jena Specord 200), at 300 nm, with $\varepsilon(ONOO^-) = 1705\ M^{-1}\ cm^{-1}$ [13]. Solutions of $ONOO^-$ and phosphate buffers were freshly prepared before the stopped-flow experiments. One syringe of the stopped-flow apparatus contained an ~0.20 mM $ONOO^-$ solution and the other syringe a phosphate buffer at pH 3 that contained or did not contain H_2.

Three series of 10 mixings each were performed and the exponential decay of peroxynitrite was observed with and without H_2.

Ion chromatography analysis of the decay products of peroxynitrite

With a simple flow-reactor setup, the decay of ONOOH to nitrate or nitrite was quantitatively evaluated with and without a H_2-saturated phosphate buffer. The flow reactor was set to a specific speed, so that the time from mixing to outlet was 10 s, the same as in the stopped-flow. Two milliliters of $ONOO^-$ solution (~0.2 mM in 10 mM KOH) and 2 ml of 40 mM phosphate buffer, pH 2.3, were added at a rate of 97 µl/min to a 1-ml 60 mM NaOH solution, so that the pH of the end solution would be around 7. To this 5-ml solution, 8.8 ml (1.75 equivalents) of saturated $Ca(OH)_2$ was added and the solution was stirred for 5 min to precipitate all phosphate. The solution was left to settle for 30 min. The supernatant was injected through a PALL Acrodisc 32-mm syringe filter (0.2-µm Supor membrane) into the ion chromatograph. A Metrohm 709 IC pump, 828 IC dual suppressor, and 732 IC detector were used, with resting absolute conductivity 2.1 µS/cm, eluent 30 mM KOH, 0.5 ml/min. With the use of a calibration curve (1–100 µM NO_2^-/NO_3^-) the absolute concentrations of NO_3^- and NO_2^- were determined.

Nitration of tyrosine by ONOOH

Solutions of 0.1 and 1 mM *N*-Ac-L-tyrosine methyl ester and *N*-Ac-L-3-nitrotyrosine ethyl ester were prepared, buffered at pH 3, 7.1, and 12. These solutions were mixed with 20 mM $ONOO^-$ in the stopped-flow spectrophotometer. Concentrations of tyrosine and nitrotyrosine were determined by UV spectroscopy. A solution of 1 mM *N*-Ac-L-tyrosine in 40 mM phosphate buffer at pH 3 was mixed with a 0.20 mM peroxynitrite solution in a stopped-flow spectrophotometer as described above. The experiment was repeated with a H_2- and a He-saturated, buffered, *N*-Ac-L-tyrosine solution.

Reduction of iron(III) in proteins by H_2

A 6 µM solution of cytochrome P450 Fe(III) in a 10 mM physiological phosphate buffer (pH 7.4) with 10 µM camphor was prepared in an air-tight UV–Vis cuvette under a nitrogen atmosphere. The solution was examined with a UV–Vis spectrophotometer (UVIKON 810, Kontron Analytical) from 360 to 720 nm before and after saturation of the solution with H_2. Spectra were recorded after 10, 20, and 90 min. Then the solution was diluted by a factor of 2 and again saturated with H_2; spectra were recorded after 10 and 20 min.

A 6 µM solution of Fe(III) myoglobin in a 40 mM phosphate buffer (pH 7.4) was prepared and analyzed in a manner similar to that for cytochrome P450. Spectra were recorded after 0, 10, and 20 min. A second (longer) saturation was performed and spectra were recorded after 0 and 10 min. The solution was then exposed to air for 1 h to ensure again full oxidation of the iron center and another spectrum was recorded. To obtain the spectrum of the completely reduced protein, a few grains of $Na_2S_2O_3$ were added to the solution.

For the putidaredoxin solution, a buffer was prepared as described by V. Reipa et al. [14] from 50 mM Tris–HCl, 200 mM KCl, 20 mM $MgCl_2$ and brought to pH 7.4 with 1 M NaOH. One hundred microliters of the 137 µM protein solution was added to the UV–Vis cuvette with 500 µl of buffer solution, which resulted in a protein concentration of 23 µM. UV–Vis spectra were taken from 200 to 800 nm (Analytik Jena Specord), to determine the spectrum of the oxidized form. The solution was then put under a H_2 atmosphere with continuous flow for 20 min and another spectrum was recorded. The spectrum of the fully reduced protein was similarly obtained by addition $Na_2S_2O_3$.

Results

Direct scavenging of peroxynitrite and hydroxyl radicals by H_2

Any scavenging of peroxynitrite by H_2 (Reaction (3)) competes with the acid-induced decay of ONOOH according to Reactions (1) and (2):

$$ONOO^- + H^+ \rightarrow ONOOH, \quad (1)$$

$$ONOOH \rightarrow NO_3^- + H^+, \quad (2)$$

$$ONOOH + H_2 \rightarrow NO_2^- + H_2O + H^+. \quad (3)$$

The reported rate constant for Reaction (2) is 1.11 ± 0.01 s^{-1} [15]. Rate constants of 1.13 s^{-1} were measured in the presence and absence of H_2 (Fig. 1). The presence of H_2 did not change the rate of decay of ONOOH.

ONOOH may oxidize H_2 in a reaction that is zero order in H_2. Such a reaction would kinetically not be different from the isomerization to NO_3^-, but result in the formation of NO_2^-. Thus, ion chromatography was used to quantitatively determine the