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Anti-inflammatory properties of molecular hydrogen: investigation on parasite-induced liver inflammation

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Abstract – Molecular hydrogen reacts with the hydroxyl radical, a highly cytotoxic species produced in inflamed tissues. It has been suggested therefore to use gaseous hydrogen in a new anti-inflammatory strategy. We tested this idea, with the aid of the equipment and skills of COMEX SA in Marseille, a group who experiments with oxygen–hydrogen breathing mixtures for professional deep-sea diving. The model used was schistosomiasis-associated chronic liver inflammation. Infected animals stayed 2 weeks in an hyperbaric chamber in a normal atmosphere supplemented with 0.7 MPa hydrogen. The treatment had significant protective effects towards liver injury, namely decreased fibrosis, improvement of hemodynamics, increased NOSII activity, increased antioxidant enzyme activity, decreased lipid peroxide levels and decreased circulating TNF-α levels. Under the same conditions, helium exerted also some protective effects, indicating that hydroxyl radical scavenging is not the only protective mechanism. These findings indicate that the proposed anti-inflammatory strategy deserves further attention.

inflammation / molecular hydrogen / hydroxyl radical / hyperbaric medicine

Résumé – Première mise en évidence des propriétés anti-inflammatoires potentielles de l'hydrogène moléculaire. Vis-à-vis de l'organisme, l'hydrogène moléculaire présente la particularité d'être inerte dans les conditions physiologiques, tout en étant susceptible d'agir comme antioxydant dans des situations pathologiques. En particulier, il peut réagir avec le radical hydroxyle, espèce hautement cytotoxique produite au cours de l'inflammation. Aussi a-t-il été suggéré de l'utiliser dans une thérapeutique anti-inflammatoire. Nous avons été en mesure, pour la première fois, de tester cette hypothèse, grâce à la société Comex, qui a développé un mélange respiratoire à base d'hydrogène pour la plongée professionnelle en profondeur. Le modèle pathologique utilisé est l'inflammation hépatique chronique due au ver parasite *Schistosoma mansoni*. Les souris infestées ont été placées pendant 15 jours dans une enceinte hyperbare, sous atmosphère normale plus 0.7 MPa d'hydrogène. Le traitement

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atténue significativement l'atteinte hépatique, ainsi qu'une réduction des taux sanguins de TNF-α. L'hélium, utilisé dans les mêmes conditions, exerce également un effet protecteur, mais à un degré moindre. Cela signifie que l'élimination du radical hydroxyle par l'hydrogène ne peut rendre compte de tous les effets observés. En tout état de cause, notre travail démontre l'intérêt thérapeutique potentiel de l'hydrogène dont l'avantage serait, a priori, de n'entraîner aucun effet secondaire.

inflammation / hydrogène moléculaire / radical hydroxyle / médecine hyperbare

. Version abrégée

L'hydrogène moléculaire réagit avec le radical hydroxyle:

$$H_2 + HO^{\cdot} \rightarrow H_2O + H^{\cdot}$$

$H^{\cdot}$ est converti en électron hydraté:

$$H^{\cdot} + OH^{-} \rightarrow H_2O + e^{-}_{aq}$$

$HO^{\cdot}$ est donc éliminé de la solution, avec l'avantage qu'aucune autre espèce moléculaire n'est formée. En outre, e^{-}_{aq} est un réducteur.

Le radical hydroxyle est une des principales espèces pathogènes produites au cours de l'inflammation. Il est en particulier responsable de l'initiation de la peroxydation des lipides membranaires et de l'oxydation des bases de l'ADN. Pour ces raisons, il a été suggéré d'utiliser l'hydrogène dans une thérapeutique anti-inflammatoire. Nous avons été en mesure de tester cette hypothèse, grâce à la société Comex SA, qui possède les compétences et les installations appropriées. Cette société a amplement démontré l'innocuité de l'hydrogène dans le cadre d'un programme visant la plongée professionnelle en profondeur. Le modèle pathologique utilisé est l'inflammation chronique du foie due au parasite *Schistosoma mansoni*. La pathologie est due à l'exacerbation de la réaction inflammatoire, en réponse au dépôt des œufs dans le foie. Il s'en suit un stress oxydatif résultant d'une part de la production de radicaux oxygène au contact même des œufs et d'autre part de la diminution des défenses anti-oxydantes du tissu hépatique. Des souris infestées par ce parasite ont été placées 15 jours dans un caisson hyberbare, sous atmosphère normale plus 0.7 MPa d'hydrogène. Les animaux témoins ont été infestés en même temps, dans les mêmes conditions. Deux expériences indépendantes ont été réalisées et le tout a été répété en utilisant l'hélium à la place de l'hydrogène. Au terme du traitement à l'hydrogène, les animaux étaient en meilleure condition que les témoins, ce que l'autopsie a confirmé. Nous avons observé une diminution significative de la fibrose hépatique. En particulier, l'analyse d'image a montré une diminution du dépôt de collagène périvasculaire, ce qui se traduit par une altération moindre de la microcirculation sanguine et une hypertension portale moindre, attestée par une splénomégalie atténuée. L'activité NO synthase induite (NOSII) est accrue. Il en est de même de l'activité des enzymes anti-oxydantes, catalase et glutathion peroxydase avec, corrélativement, une diminution de la concentration des peroxylipides hépatiques. On note également une diminution des taux sanguins de TNF-α, cytokine pro-inflammatoire. Ces effets de l'hydrogène hyperbare sont à rapprocher de ceux de la cytokine anti-inflammatoire IL-12, qui atténue de façon comparable l'inflammation hépatique due à ce parasite. Bien que le rôle de NO dans cette pathologie ne soit pas pleinement élucidé, il est frappant de constater que, d'une part les effets de l'IL-12 sont médiés par NOSII et, d'autre part, le traitement par l'hydrogène hyperbare se traduit par un accroissement de l'activité de cette enzyme. Notons enfin qu'une réduction significative de la fibrose hépatique et une augmentation de l'activité NOSII sont observés également après traitement à l'hélium hyperbare. Cela sous-entend que l'élimination du radical hydroxyle par l'hydrogène n'est pas le seul mécanisme responsable des effets observés. En tout état de cause, notre travail démontre l'intérêt thérapeutique potentiel de l'hydrogène dont l'avantage serait, a priori, de n'entraîner aucun effet secondaire.

1. Introduction

Molecular hydrogen reacts with the hydroxyl radical:

$$H_2 + HO^{\cdot} \rightarrow H_2O + H^{\cdot} \quad (1)$$

H atoms are converted to hydrated electrons:

$$H^{\cdot} + OH^{-} \rightarrow H_2O + e^{-}_{aq} \quad (2)$$

The rate constants are $6 \times 10^7\ M^{-1}\cdot s^{-1}$ for reaction (1) [1] and $2 \times 10^7\ M^{-1}\cdot s^{-1}$ for reaction (2) [2]. $HO^{\cdot}$ is thus removed from the solution by hydrogen, with the advantage that no species other than those in reactions (1) and (2) are formed. Moreover, the hydrated electron e^{-}_{aq} is a reducing species, especially in neutral and alkaline conditions.

The hydroxyl radical and its derivatives such as peroxyl are generated during inflammation [3]. They are highly cytotoxic, through processes such as the initiation of the peroxidation of cell membrane lipids [4] and DNA strand breakage [5]. It has been suggested therefore to use hydrogen in an anti-inflammatory strategy [6]. Hydrogen diffuses freely within the body, without any side effects. Its harmlessness has been demonstrated through 17-year long studies on cells, mice, monkeys and deep-sea divers (COMEX HYDRA program, Marseille).

Making use of the unique facilities of COMEX SA we tested the potential anti-inflammatory properties of gaseous hydrogen, under safe conditions. The biological model used was the chronic liver inflammation induced by the parasite *Schistosoma mansoni* in mice. Parasite eggs are trapped in the liver, leading to a delayed hypersensitivity response [7]. The ensuing pathology is the consequence of an exacerbated inflammatory response, as shown by the protective effects of IL-12 [8], a cytokine which down-regulates parasite egg-induced inflammation and fibrosis [9]. The host reaction gives rise to oxidative stress in the liver resulting from a combination of oxygen radical production by inflammatory cells and the alteration of liver antioxidant defenses. On the one hand, H_20_2 is released in the immediate vicinity of the eggs and it is associated with peroxidase activity [10]. On the other hand, the activities of the H_20_2 scavenging enzyme catalase and glutathione peroxidase decreased drastically and the reduced glutathione stores are depleted [11]. *Schistosoma*-associated oxidative stress occurs also in man [12] and is associated with increased cancer at the site of inflammation [13].

In the present experiments, infected mice stayed in a hyperbaric chamber in a normal atmosphere supplemented with 0.7 MPa hydrogen for 2 weeks. The treatment was applied at the time of the onset of parasite egg deposition, i.e. at 5 weeks of post-infection. Control mice were infected at the same time under the same conditions. At the end of the treatment, experimental and control mice were sacrificed and the parameters associated with liver inflammation were assessed. The experiment was done twice, and the entire protocol was repeated also twice using helium instead of hydrogen.

Hyperbaric treatment with hydrogen reduced significantly liver injury and oxidative stress. Helium also exerted protective effects, albeit to a lower extent.

2. Materials and methods

2.1. Animals and parasites

Eight-week-old female CBA/J mice (IFFA CREDO, France) were used. They had free access to water and to a complete rodent diet, including in the hyperbaric chamber. Animals were infected percutaneously with 150 cercariae of the Puerto Rican strain of *S. mansoni*, maintained in our laboratory by passage through *Biomphalaria glabrata* snails. Parasites circulate in the vascular system as schistosomula larvae. They settle in the mesenteric veins as they reach sexual maturity, 5 weeks after infection. All animals received humane care, according to the rules of « Décret n° 87-848 du 19/10/87, Paris ».

2.2. Hyperbaric treatment

For each experiment, a group of 14 mice were infected at the same time and were raised under the same conditions for 5 weeks. Then 7 of the animals were selected randomly and were introduced into a 50 L hyperbaric chamber in a normal atmosphere supplemented with 0.7 MPa H_2 (absolute pressure: 8 atm). Hydrogen pressure was set in order to avoid an explosive reaction of H_2 and O_2. Two weeks later, all animals were sacrificed and the pathology was evaluated.

2.3. Determination of the pathological parameters

The following parameters relevant to schistosomiasis-associated inflammation were determined in the liver: i) fibrosis, ii) NOSII activity, iii) catalase and glutathione peroxidase activities, iv) lipid peroxide concentrations. Spleen enlargement, which reflects portal hypertension, and circulating TNF-α levels were also evaluated. The surface of collagen deposits was determined by image analysis (Biocom) of histological sections (liver samples collected from identical sites within the organ). Slides were read blindly and collagen deposition was expressed as percent of the section area. Collagen deposition surronding blood microvessels, which was induced by antigens released in the circulation by the parasite eggs [14] was expressed as percent of the vessel cross section. Liver fibrosis was also quantified by the determination of hydroxyproline levels according to Bergman et al. [15]. NOSII activity was determined as the rate of arginine to citrulline conversion, using [^{3}H]-arginine (Amersham) as tracer, according to Rees et al. [16]. Catalase and glutathione peroxidase activities, and lipid peroxide concentrations were determined according to Gharib et al. [11]. Circulating TNF-α levels were quantified using a R&D Systems kit. Since schistosomiasis is associated with a decrease of protein concentration in the serum [17], TNF-α levels were expressed as pmol/mg protein. The improvement of hepatic hemodynamics was estimated by the reduction of spleen enlargement.

2.4. Statistical analysis

Values from the two experiments employing hydrogen and the two with helium, respectively, were pooled. Mean values in the experimental group were compared to those in the corresponding control group using Student's *t*-test.

3. Results and discussion

Mice treated with hyperbaric hydrogen or helium were clearly in better shape than the infected controls; bio

Table I. Effects of hyperbaric hydrogen and helium on parasite-induced liver inflammation in the mouse.

	Treatment			
	Hydrogen		Helium	
	Control group	Hyperbaric group	Control group	Hyperbaric group
Total collagen deposition (% of liver section area)	38.4 ± 6.0 *n* = 13	16.2 ± 7.4 # *n* = 14	41.9 ± 7.6 *n* = 14	22.1 ± 6.2 # *n* = 14
Perivascular collagen (% of vascular section area)	18.2 ± 2.26 *n* = 13	11.2 ± 4.9 # *n* = 14	16.5 ± 4.1 *n* = 14	10.9 ± 2.2 # *n* = 14
Hydroxyproline levels (µg/g wt)	1050 ± 166 *n* = 6	670 ± 94 # *n* = 7	960 ± 145 *n* = 7	693 ± 96 # *n* = 7
NOSII activity (pmol/min/mg protein)	43.3 ± 16.2 *n* = 13	70.7 ± 12.0 #§ *n* = 14	43.5 ± 2.9 *n* = 14	63.0 ± 7.8 # *n* = 14
Spleen weight (g)	0.44 ± 0.11 *n* = 13	0.34 ± 0.12 #§ *n* = 14	0.45 ± 0.09 *n* = 14	0.48 ± 0.08 *n* = 14
TNF-α levels (pg/mg protein)	1.32 ± 0.45 *n* = 13	0.63 ± 0.18 #§ *n* = 14	1.24 ± 0.29 *n* = 14	1.12 ± 0.48 *n* = 14
Catalase activity (U/mg protein)	53.7 ± 16.1 *n* = 7	102.3 ± 19.1 #§ *n* = 7	42.4 ± 24.5 *n* = 7	50.3 ± 9.6 *n* = 7
Glutathione peroxidase Activity (mU/mg protein)	455 ± 111 *n* = 7	718 ± 168 #§ *n* = 7	538 ± 109 *n* = 7	461 ± 107 *n* = 7
Lipid peroxides (nmol/g wt)	42.8 ± 10.7 *n* = 13	24.1 ± 5.6 #§ *n* = 14	50.1 ± 12.7 *n* = 14	37.6 ± 5.5 *n* = 14

The values are means ± SD; (*n*), number of animals used; (#), values significantly different from the cognate control group and (§), values significantly different from the hyperbaric helium group, according to Student *t*-test ($p < 0.05$).

chemical analyses confirmed the improvement. *Table I* shows that all the parameters evaluated changed significantly after hyperbaric hydrogen in the sense of a reduced pathology. Reduction of liver fibrosis was shown by a 60 % decrease of the relative surface of collagen deposits and by a 35 % decrease of hydroxyproline levels. In particular, there was less collagen deposition around blood microvessels, indicating a reduction of microvessel clogging. This event was associated with the improvement of liver hemodynamics and reduction of portal hypertension, as shown by the reduction of spleen enlargement. The activities of the H_2O_2 scavenging enzyme catalase and glutathione peroxidase were better preserved, with a decrease of lipid peroxide concentrations in the liver. We observed also that NOSII activity increased and that circulating levels of the inflammatory cytokine TNF-α decreased after the treatment. A significant reduction of liver fibrosis and increase in NOSII activity was also observed after hyperbaric treatment with helium. Comparison of the two hyperbaric treatments shows that animals treated with hydrogen differed from those treated with helium for most of the parameters examined; there was a significant reduction of spleen enlargement as well as decreased TNF-α and lipid peroxide levels; there was also a significant increase of antioxidant enzyme activities. These changes translate a reduction of the pathology under hydrogen treatment, as compared to helium.

Therefore hyperbaric hydrogen treatment reduced the parasite-induced liver pathology. In particular, the reduction of perivascular and periovular fibrosis indicates an attenuated inflammatory response to antigens released by parasitic worms and eggs. The effects of the treatment can be compared to those of IL-12 which down-regulates *S. mansoni* egg-induced inflammation and fibrosis in mice [9]. Even though the role of NO in liver inflammation is unclear [18], it is striking that on the one hand the anti-inflammatory effects of IL-12 are mediated by NOS II [8] and, on the other hand, the hyperbaric treatment resulted in the increase in NOSII activity.

This is the first report of the effect of hyperbaric treatment on an inflammatory disease. The experiments were unique in the sense that oxygen pressure was normal (0.02 MPa), whereas the hyperbaric conditions routinely experienced either in therapeutic use (hyperbaric oxygen) or in sea-diving, for example, result in hyperoxic conditions. Hydrogen presumably protects against inflammation at least in part by hydroxyl radical scavenging. However helium, i.e. pressure as such, exerted protective effects, through mechanisms which remain to be elucidated. It has be assumed that hyperbaric inert gases enhance superoxide radical production in the presence of iron [19]. However, our own experiments performed under the conditions described by the authors, failed to confirm such observations (data not shown). In conclusion, even though our findings are far from being fully understood, the proposed anti-inflammatory strategy deserves further attention.

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Appendix 1. Effects of hyperbaric hydrogen and helium on parasite-induced liver inflammation in the mouse : detailed experiments.

	Hyberbaric hydrogen			
	Experiment I		Experiment II	
	Control group	Hyperbaric group	Control group	Hyperbaric group
Total collagen deposition (% of liver section area)	35.4 ± 3.1 *n* = 6	15.9 ± 6.6 # *n* = 7	41.0 ± 6.1 *n* = 7	16.5 ± 7.6 # *n* = 7
Perivascular collagen (% of vascular section area)	17.1 ± 2.0 *n* = 6	12.7 ± 5.6 *n* = 7	19.1 ± 3.4 *n* = 7	9.7 ± 3.7 # *n* = 7
Hydroxyproline levels (µg/g wt)	1050 ± 166 *n* = 6	670 ± 94 # *n* = 7	nd	nd
NOSII activity (pmol/min/mg protein)	35.8 ± 10.1 *n* = 6	76.4 ± 8.1 # *n* = 7	49.9 ± 17.5 *n* = 7	65.0 ±10.5 *n* = 7
Spleen weight (g)	0.39 ± 0.07 *n* = 6	0.28 ± 0.04 # *n* = 7	0.49 ± 0.12 *n* = 7	0.40 ± 0.14 *n* = 7
TNF-α levels (pg/mg protein)	1.28 ± 0.41 *n* = 6	0.70 ± 0.15 # *n* = 7	1.38 ± 0.39 *n* = 7	0.56 ± 0.18 # *n* = 7
Catalase activity (U/mg protein)	nd	nd	53.7 ± 16.1 *n* = 7	102.3 ± 19.1 # *n* = 7
Glutathione peroxidase activity (mU/mg protein)	nd	nd	455 ± 111 *n* = 7	718 ± 168 # *n* = 7
Lipid peroxides (nmol/g wt)	39.3 ± 10.0 *n* = 6	21.4 ± 6.0 # *n* = 7	45.8 ± 11.1 *n* = 7	28.1 ± 5.6 # *n* = 7

Appendix 2. The values are means ± SD; (*n*), number of animals used; (#), values significantly different from the control group according to Student *t*-test ($p < 0.05$). (nd), not determined (not all the assays could be performed on a single set of liver samples).

	Hyberbaric helium			
	Experiment I		Experiment II	
	Control group	Hyperbaric group	Control group	Hyperbaric group
Total collagen deposition (% of liver section area)	37.9 ± 7.8 *n* = 7	20.7 ± 5.6 # *n* = 7	45.9 ± 7.8 *n* = 7	24.2 ± 4.6 # *n* = 7
Perivascular collagen (% of vascular section area)	17.7 ± 4.9 *n* = 7	9.7 ± 1.8 # *n* = 7	15.3 ± 2.7 *n* = 7	12.1 ± 2.0 # *n* = 7
Hydroxyproline levels (µg/g wt)	960 ± 145 *n* = 7	693 ± 96 # *n* = 7	nd	nd
NOSII activity (pmol/min/mg protein)	40.4 ± 4.4 *n* = 7	58.3 ± 8.7 # *n* = 7	46.6 ± 5.8 *n* = 7	67.8 ± 6.3 # *n* = 7
Spleen weight (g)	0.41 ± 0.06 *n* = 7	0.45 ± 0.04 *n* = 7	0.49 ± 0.06 *n* = 7	0.51 ± 0.10 *n* = 7
TNF-α levels (pg/mg protein)	1.17 ± 0.27 *n* = 7	1.01 ± 0.22 *n* = 7	1.31 ± 0.46 *n* = 7	1.23 ± 0.62 *n* = 7
Catalase activity (U/mg protein)	nd	nd	42.4 ± 24.5 *n* = 7	50.3 ± 9.6 *n* = 7
Glutathione peroxidase activity (mU/mg protein)	nd	nd	538 ± 109 *n* = 7	461 ± 107 *n* = 7
Lipid peroxides (nmol/g wt)	45.3 ± 14.3 *n* = 7	34.9 ± 6.0 *n* = 7	55.0 ± 8.1 *n* = 7	40.3 ± 4.6 # *n* = 7

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