Decompression sickness risk in rats by microbial removal of dissolved gas

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Kayar, Susan R., Terry L. Miller, Meyer J. Wolin, Eugenia O. Aukhert, Milton J. Axley, and Lutz A. Kiesow. Decompression sickness risk in rats by microbial removal of dissolved gas. Am. J. Physiol. 275 (Regulatory Integrative Comp. Physiol. 44): R677–R682, 1998.—We present a method for reducing the risk of decompression sickness (DCS) in rats exposed to high pressures of H2. Suspensions of the human colonic microbe Methanobrevibacter smithii were introduced via a colonic cannula into the large intestines of the rats. While the rats breathed H2 in a hyperbaric chamber, the microbe metabolized some of the H2, diffusing into the intestine, converting H2 and CO2 to methane and water. Rate of release of methane from the rats, which was monitored by gas chromatography, varied with chamber H2 pressure. This rate was higher during decompression than during compression, suggesting that during decompression the microbe was metabolizing H2 stored in the rats’ tissues. Rats treated with M. smithii had a 25% (5 of 20) incidence of DCS, which was significantly lower (P < 0.01) than the 56% (28 of 50) incidence of untreated controls, brought on by a standardized compression and decompression sequence. Thus using a microbe in the intestine to remove an estimated 5% of the body burden of H2 reduced DCS risk by more than one-half. This method of biochemical decompression may potentially facilitate human diving.

biochemical decompression; hydrogen diving; hydrogen metabolism; hyperbaria; methane; methanogens

LARGE VOLUMES OF GAS dissolve in the tissues of divers. The gases not metabolized by the diver, usually nitrogen or helium, are eliminated slowly during decompression. Gradual decompression lasting for days is needed following extended human dives to pressures of 20 atm or more, to avoid the excessive gas supersaturation and bubble formation that may lead to decompression sickness (DCS) (14). We describe a method for biochemically converting some of the dissolved gas to a nongaseous form. We call this method biochemical decompression.

Hydrogen has been used as the nonmetabolic portion of a breathing gas for deep hyperbaric exposures. Its lower density compared with helium reduces the effort of ventilation under high pressures (1, 2). Hydrogen is not metabolized by mammalian tissues (5, 12), but it can be readily converted to methane by microbes of the kingdom Archaea that can be found in the human colon (8). Methanobrevibacter smithii is the species responsible for the production of almost all human colonic methane; it uses H2 to reduce carbon dioxide

\[4 \text{H}_2 + \text{CO}_2 \rightarrow \text{CH}_4 + 2 \text{H}_2\text{O}\]

Under normal circumstances, the H2 is produced by colonic bacteria that ferment dietary plant polysaccharides (8). The magnitude of methane production in humans can be quite large. Some people produce up to three liters per day (15).

Utilizing the metabolism of M. smithii for our purposes is ideal: 4 mol of H2 are converted to 2 mol of an innocuous, nongaseous molecule (water) and only 1 mol of a gas (methane) (Eq. 1). CO2, the electron acceptor for this reaction, is abundant in mammalian tissues. Our approach was to place live suspensions of M. smithii into the proximal end of the large intestines of rats. While the rats breathed hyperbaric H2, the metabolism of H2 by the methanogen was demonstrated by monitoring the rate of methane release from the rats. With the use of a standardized compression and decompression sequence, efficacy at reducing DCS was confirmed by determining the incidence of DCS in rats with and without M. smithii treatments.

METHODS

Animals. Male Sprague-Dawley rats (Rattus norvegicus, n = 163, body mass range 215–324 g; Table 1) were used for all experiments. The rats were housed before experiments in an accredited animal care facility and had ad libitum access to rat chow (Prolab; Agway, Syracuse, NY) and water. All procedures were approved by an Animal Care and Use Committee. The experiments reported here were conducted according to the principles set forth in the Guide for the Care and Use of Laboratory Animals.

Analysis of variance was used to confirm that the various groups of control and treated animals did not differ from each other in body mass (P = 0.446). Maximum likelihood analysis was used to demonstrate that differences in body mass did not influence DCS outcome in these experiments.

Culturing of methanogen. M. smithii, strain PS, was obtained from the stock culture collection of the Wadsworth Center for Laboratories and Research, New York State Department of Health. This microbe was originally isolated from sewage. The microbe was cultured in 2-liter glass bottles modified with a serum bottle neck and side arm (9). The medium, which was based on rumen fluid (400 ml), was described elsewhere (10). Duplicate cultures were incubated at 37°C with stirring and 80% H2-20% CO2 at 1–2 atm. The gas within the culture bottles was replaced once daily. At 48 h, the cultures were centrifuged under H2-CO2 to concentrate the cells. The cells were resuspended in 12 ml O2-free sodium bicarbonate solution (0.5 g NaHCO3/100 ml) containing 0.005

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M dithiothretol (DTT) as a reducing agent. The suspension was stored under H2-CO2 at 4°C. In vitro methanogenic activity was measured in duplicate with 0.2 ml of suspension and 0.3 ml of the bicarbonate-DTT solution in serum bottles with ~8 ml headspace volume. The bottles were pressurized to 2 atm with H2-CO2 and incubated on a platform shaker (280 excursions/min) for 1 h at 37°C. Methane and H2 concentrations were determined by gas chromatography (11). The methane production rate per unit volume for the suspensions was retained in storage at 4°C for at least 8 days. In most experiments, the suspensions were used within 2 days of completion of culturing.

Surgical procedure to introduce methanogen. Sixty-three rats (Table 1) were anesthetized by inhalation of halothane (2-bromo-2-chloro-1,1,1-trifluoroethane). With the use of aseptic surgical techniques, an incision was made in the abdomen (2-bromo-2-chloro-1,1,1-trifluoroethane). With the use of aseptic surgical techniques, an incision was made in the abdomen to expose the cecum. A cannula flushed with the bicarbonate-DTT solution was introduced from the rectum. The cannula was slowly passed through the entire length of the large intestine until it could be seen through the wall of the cecum. When the anaerobic conditions for the injection of microbes were met due to improperly made DTT (n = 2), or 3) when animals experienced DCS immediately after their hyperbaric exposure for measuring methane (n = 10).

Dive protocol for measuring methane release rate. Four or five rats were placed together in a clear box (40-liter internal volume). Dry rat chow pellets and slices of apple were also put into the box to provide food and liquids. The box was placed inside a hyperbaric chamber (5.6 m3 internal volume, WSF Industries, Buffalo, NY) within a facility specially built for safe handling of high pressures of H2 (following NFPA Code 50A for H2 at consumer sites). Observation ports on the chamber allowed ad libitum viewing of the animals. A thermometer installed inside the rats’ box was monitored throughout the dive. Chamber temperature was thermostatically controlled to keep the box at a temperature that appeared comfortable for the rats: ~28°C at 11 atm and 30°C at 23.7 atm.

The chamber was pressurized with He to an absolute pressure of 11 atm for 1 h. A stream of chamber gas (12–18 l/min STP, depending on gas composition) passed continuously through the animals’ box and was sampled by a gas chromatograph (Hewlett-Packard 5890A, Series II, Wilmington, DE) every 12 min. This analysis of the ventilation of the box allowed regulation of chamber O2, which could be added independently of other gases, to maintain normoxia (0.2–0.4 atm O2) inside the box throughout the experiment. The He in the chamber was then replaced with H2 at constant total pressure to a final concentration of 89.4 ± 0.6% H2 (range 85.5–92.3%). This was accomplished primarily by opening chamber outlet and intake valves simultaneously and replacing the escaping chamber gases with H2 and O2 as needed to maintain constant pressure and normoxia. To speed the increase in H2 concentration after concentration exceeded 50%, the chamber was pressurized two or three times with pure H2 from 11 to 12 atm briefly and then returned to 11 atm. This exchange of H2 for He was always completed in ~2 h. The chamber was then further pressurized with H2 to a total pressure of 23.7 atm (95 ± 0.6% H2, range 90.7–98.1% H2). This maximum pressure was maintained for 2.5 h. The chamber was then depressurized to 11 atm absolute pressure at 0.2–0.45 atm/min, which was intended to be slow enough to be unlikely to cause DCS in the rats (6). After 45 min at 11 atm, the H2 in the chamber was replaced by He, maintaining a constant total pressure of 11 atm. In some cases, two or three excursions to 12 atm with pure He were used to speed the replacement of H2 with He. To reduce the H2 concentration to <4% typically required 2–2.5 h. The chamber was then further decompressed at 0.1–0.25 atm/min until the chamber had returned to 1 atm.

The animals were removed from the chamber, observed for overt signs of DCS, and left in cages with food and water overnight. Those animals with methanogen (n = 18; Table 1) that appeared in normal health after a 12-h interval in 1-atm air were then tested for their risk of DCS, as described below.

Dive protocol for testing of decompression sickness risk. A separate dive protocol, which has been described in detail previously (3), was followed to test animals for their risk of DCS (Table 1). Five rats per test were placed inside a chamber (140-liter internal volume) that was pressurized to 10 atm with pure He at a rate of 1–2 atm/min. The chamber was flushed at constant pressure with a mixture of 2% O2 in H2. The chamber was further pressurized to 23.7 atm, and a final gas composition of 2% O2, 4% He, 94% H2. The rats remained at this pressure for 20 min, which was sufficient time to saturate the animals with H2 (6). The animals were then decompressed within 36–40 s to a pressure of 10.8 atm and observed for signs of DCS over the next 30 min. While inside the chamber, rats were kept inside a drum mill made of wire mesh, rotating at 3.6 m/min, which obligates the rats to walk continuously throughout the experiment. The gait and position of rats on the mill was monitored through observation ports on the chamber. Signs of DCS were recorded as a limping or sideways gait, difficulty keeping up with the motion of the mill, falling, difficulty righting after falling, or rolling passively on the mill (3, 6). Two or three observers arrived at a consensus opinion to diagnose DCS, which required at least 1 min of signs.

### Table 1. Animal use

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Body Mass, g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animals injected with M. smithii</td>
<td>63</td>
<td>269 ± 4</td>
</tr>
<tr>
<td>Tested for methane release rate</td>
<td>34</td>
<td>245 ± 6</td>
</tr>
<tr>
<td>Tested for DCS risk</td>
<td>20</td>
<td>249 ± 3</td>
</tr>
<tr>
<td>Tested for retention of M. smithii at 1 atm</td>
<td>9</td>
<td>251 ± 10</td>
</tr>
<tr>
<td>Animals with surgical control procedure</td>
<td>40</td>
<td>274 ± 3</td>
</tr>
<tr>
<td>Tested for methane release rate</td>
<td>20</td>
<td>254 ± 3</td>
</tr>
<tr>
<td>Tested for DCS risk</td>
<td>18</td>
<td>252 ± 1</td>
</tr>
<tr>
<td>Untreated control animals</td>
<td>60</td>
<td>265 ± 3</td>
</tr>
<tr>
<td>Tested for methane release rate</td>
<td>10</td>
<td>250 ± 2</td>
</tr>
<tr>
<td>Tested for DCS risk</td>
<td>50</td>
<td>263 ± 3</td>
</tr>
</tbody>
</table>

Body mass values are means ± SE; n = no. of animals in each group. DCS, decompression sickness.
All animals were euthanized in the chamber at 11 atm by introducing 2 atm CO₂. This terminated any discomfort for those rats with DCS.

RESULTS

Release of methane. Figure 1 presents the results of one experiment in which each of the five rats was supplied with a 2-ml suspension of M. smithii with an in vitro metabolic activity of 50 µmol H₂ uptake/min. The rats released an average of 0.76 ± 0.05 µmol CH₄/min per animal (mean ± SE 4 gas chromatographic measurements) during the initial hour at 11 atm, while their breathing mixture contained He rather than H₂ (Fig. 1). We presume that most of this methane was released as flatus, but any methane released with the breath or transcutaneously would have been sampled as well.

Methane release rate increased, beginning within minutes of the appearance of H₂ in the chamber, reaching 2.21 ± 0.11 µmol CH₄/min per animal by the end of the H₂ exposure at 11 atm. As both chamber pressure and H₂ partial pressure increased, methane release rate increased again and reached 2.74 ± 0.12 µmol CH₄/min per animal by the end of the 2.5-h period at maximum pressure. Animals did not appear to be in any discomfort and often slept throughout large portions of the pressurization.

During chamber depressurization, methane release rate increased sharply, reaching a peak of 3.55 µmol CH₄/min per animal 45 min after depressurization was initiated (Fig. 1). Methane release rate then fell as both chamber pressure and H₂ partial pressure fell. There was no appearance of discomfort in the animals during depressurization; they were more wakeful and often groomed themselves.

This experimental procedure was executed a total of seven times, always using a 2-ml suspension of M. smithii per rat. In vitro activity for the suspensions ranged from 27 to 104 µmol H₂ uptake/min. Results from each procedure were qualitatively similar to those presented in Fig. 1, but there were quantitative differences in methane release rate that depended on the activity of methanogen delivered (Fig. 2). Animals supplied with doses of <50 µmol H₂ uptake/min activity (the dose for Fig. 1) released less methane throughout the experiment than illustrated in Fig. 1. However, animals supplied with >50 µmol H₂ uptake/min activity released approximately the same amounts of methane as shown in Fig. 1. Untreated control animals (n = 10; Table 1) and animals that had undergone the control surgical procedure (n = 20; Table 1) released no detectable methane while breathing either He or H₂ (limit of detection ~0.1 µmol CH₄/min).

DCS risk. Animals supplied with M. smithii at a mean activity of 51 ± 16 µmol H₂ uptake/min (range 32–101 µmol H₂ uptake/min) and tested immediately for DCS risk had a 25% (5 of 20) incidence of DCS, brought on by the standardized sequence of compression to 23.7 atm and rapid decompression to 11 atm (Fig. 3A). This was significantly lower than the incidence of DCS for untreated rats of 56% (28 of 50; P < 0.01, χ² two-sided test, Fig. 3A). Animals that underwent the control surgical procedure, followed immediately by the test for DCS risk, had a DCS incidence that was not significantly different from that of the untreated controls (65% of 20, P = 0.34, Fisher’s exact test; Fig. 3A), but was significantly higher than that of the animals treated with methanogen (P < 0.01, χ² one-sided test; Fig. 3A).
controls (Fig. 3), and being metabolized by methanogen. Hydrogen delivery within the intestine is governed by diffusional processes through the intestinal wall and also by transport of dissolved H₂ to the intestine via the blood. Because diffusional processes are proportional to pressure gradients, this nonproportionality in methane release rate versus H₂ partial pressure suggests a perfusion limitation to H₂ delivery to the methanogen.

The sigmoidal relationship between in vitro activity of methanogen supplied to animals and methane release rate at maximum pressure (Fig. 2) also suggests that H₂ delivery to the microbe became rate limiting to their metabolism at the higher levels of methanogenic activity tested. Thus there is apparently a limit to the amount of H₂ that can be removed per unit time by a microbe in the intestine. If so, biochemical decompression cannot be extended indefinitely to greater levels of effectiveness by adding greater quantities or activities of microbes. A limitation of the magnitude found in these experiments may, however, be only an artifact of our experimental design. If the microbe were dispersed through a larger volume of the contents of the large intestine, or if drugs to increase intestinal blood flow were administered, it might be possible to increase the amount of H₂ eliminated per unit time by supplying higher methanogenic activity than used here.

We had not expected the spike in methane release shortly after initiating decompression (Fig. 1). This spike may reflect an increase in discharge of methane produced earlier in the dive and released by the rats as flatus either as a startle response to the depressurization of the chamber or as a simple phenomenon of the expansion of intestinal gases. However, in several of the 7 replications of this experiment, there was a second, smaller increase in methane release rate associated with the chamber H₂ washout, when there was a change in H₂ partial pressure but not total chamber pressure. We speculate that these periods of high methane release rate during decompression reflected bubble formation of H₂ in the cecum due to H₂ supersaturation. Gaseous H₂ may have supported a higher methanogenesis than when the H₂ supply was in physical solution in intestinal and surrounding tissue fluids during compression or constant chamber pressure.

The rate of methane release during decompression was ~20%-40% higher than the rate during compression at the same H₂ partial pressures (Fig. 1). This probably indicates that during compression there is methanogenesis primarily of H₂ recently inhaled by the rats, but during decompression there is additional methanogenesis of H₂ stored in the tissues of the animals, as well as H₂ recently inhaled. This observation is particularly encouraging: feasibility of biochemical decompression depends on reduction of tissue stores of inert gas before onset of DCS symptoms.

The treatment of rats with M. smithii was responsible for significantly lowering their risk of DCS from a chosen compression and decompression sequence to less than one-half of the risk in untreated controls (Fig. 3). The results from the surgical control groups showed...
that this decreased risk was not an artifact of the surgical procedure, either a few hours after surgery (Fig. 3A) or on the following day (Fig. 3B). The negative outcome of the surgical controls was critical because it has been documented that some activations of the immune system before a dive can reduce DCS risk (3).

The protective effect against DCS of the treatments with M. smithii persisted for at least 24 h (Fig. 3B). However, M. smithii was probably not retained in a viable state for much longer than 24 h, due to loss of these alien microbes from the intestinal flora. Although fecal or cecal samples of some animals treated with M. smithii and left at 1 atm (Table 1) showed traces of methanogenic activity 24 h after the delivery of methanogen (7 of 8 animals sampled), no methanogenesis was observed after 2–8 days (0 of 3 animals sampled). It is significant that no health problems were observed that appeared to be caused by the methanogen itself. Animals kept at 1 atm after receiving methanogen treatment remained in good health and with feces normal in appearance during observation periods of days to weeks.

We are persuaded that there is a causal link between the methane released from rats treated with M. smithii (Figs. 1 and 2) and their reduced incidence of DCS (Fig. 3). As an approximation, assume that the average solubility of H₂ in the various tissues of a rat is 0.02 ml H₂·ml tissues⁻¹·atm⁻¹ (13). Assume also that H₂ production by bacteria in the gut flora is inhibited and falls to zero at elevated environmental H₂ pressures (8). A rat with a body mass of 250 g and a core body temperature of 38°C, breathing 91% H₂ at 23.7 atm total pressure (21.6 atm H₂ partial pressure) will contain 4.2 mmol H₂ when saturated. Saturation of a rat with H₂ is predicted to occur within 15 min (6). While the rats were breathing H₂ at this partial pressure, they released an average of 2.5 µmol CH₄/min when the in vitro activity of M. smithii placed in their intestines equaled or exceeded 50 µmol H₂ uptake/min (Fig. 2). We use the average methane release rate after 2 h at maximum pressure, rather than the methane release rate in the first 20 min, because we do not know the lag time between methane production in the intestine and our sampling time.) Assuming that 4 µmol H₂ were consumed for each micromole of methane we detected (Eq. 1), we estimate that M. smithii was eliminating ~10 µmol H₂/min from the rats while at this pressure. Over a 20-min period, the methanogen thus eliminated ~200 µmol H₂. The H₂ elimination by the methanogen would thus reduce the total H₂ load in the rat by nearly 5%. This is equivalent to saturating a rat with H₂ at a 5% lower pressure than its actual ambient pressure; i.e., at 22.5 atm vs. 23.7 atm total pressure. A study in which DCS risk was computed as a function of pressure for rats breathing 2% O₂ and 4% He in H₂ and using a compression and decompression sequence identical to ours (6) estimated that the risk at 23.7 atm is ~60% and that at 22.5 atm is ~25%. These risk values correspond closely to those we actually measured for untreated rats versus those treated with M. smithii (Fig. 3).

This calculation utilizes several simplifying assumptions. In addition to those already listed, it ignores the volume of H₂ that diffuses from the environment into a subsaturated rat as a consequence of the increased H₂ partial pressure gradient generated by the methanogenic elimination of H₂ inside the rat. It also ignores the elevated rate of H₂ removal by the methanogen at the critical time during and immediately following decompression (Fig. 1). This simple calculation is nevertheless illustrative that the fluxes of H₂ under consideration are within the appropriate range to explain our results.

We envision that packaging M. smithii in an ingestible, enteric-coated capsule that targets delivery to the large intestine may make biochemical decompression applicable to human divers breathing H₂. Hydrogen diving is, however, restricted in its use to great depths and limited professional applications. The gas mixture most commonly used in sport, professional, and military diving is air. Thus even more appealing is the idea of replicating this approach using nitrogen-metabolizing bacteria (4). Biochemical decompression following hyperbaric exposures to air would revolutionize diving.

Perspectives

The approaches to avoiding DCS that are currently in use are to allow enough time during decompression for the passive diffusion of inert gas out of the diver or to facilitate this diffusion by increasing the O₂ content of the breathing gas. Biochemical decompression is a fundamentally different approach: gases that are inert to the body, but metabolizable to some microbes, are actively scrubbed out within the intestine. Our research indicates that a significant gradient for H₂ through the body of a rat to its intestine can be created. This gradient eliminates a small but critical volume of H₂ and elicits a whole body response of lower DCS risk. Small incremental increases in the inert gas load in tissues add greatly to the risk of DCS; likewise, removing increments as small as the value of 5% of the total body burden we estimated for these rats has the startling effect of reducing DCS risk by more than one-half. Demonstrating the feasibility of H₂ biochemical decompression in a human-sized animal model and determining the feasibility of N₂ biochemical decompression are challenges that remain.

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