Free Radical Biology and Medicine 69 (2014) 324–330

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

Maternal molecular hydrogen administration ameliorates rat fetal hippocampal damage caused by in utero ischemia–reperfusion

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ARTICLE INFO

Article history:
Received 30 June 2013
Received in revised form
25 January 2014
Accepted 30 January 2014
Available online 6 February 2014

Keywords:
Ischemia–reperfusion
In utero
Neuronal damage
Molecular hydrogen
Free radicals

ABSTRACT

Molecular hydrogen (H_2) scavenges hydroxyl radicals. Recently, H_2 has been reported to prevent a variety of diseases associated with oxidative stress in model systems and in humans. Here, we studied the effects of H_2 on rat fetal hippocampal damage caused by ischemia and reperfusion (IR) on day 16 of pregnancy with the transient occlusion of the bilateral utero-ovarian arteries. Starting 2 days before the operation, we provided the mothers with hydrogen-saturated water ad libitum until vaginal delivery. We observed a significant increase in the concentration of H_2 in the placenta after the oral administration of hydrogen-saturated water to the mothers, with less placental oxidative damage after IR in the presence of H_2. Neonatal growth retardation was observed in the IR group, which was alleviated by the H_2 administration. We analyzed the neuronal cell damage in the CA1 and CA3 areas of the hippocampus at day 7 after birth by immunohistochemical analysis of the 8-oxo-7,8-dihydro-2'-deoxyguanosine- and 4-hydroxy-2-nonenal-modified proteins. Both oxidative stress markers were significantly increased in the IR group, which was again ameliorated by the H_2 intake. Last, 8-week-old rats were subjected to a Morris water maze test. Maternal H_2 administration improved the reference memory of the offspring to the sham level after IR injury during pregnancy. Overall, the present results support the idea that maternal H_2 intake helps prevent the hippocampal impairment of offspring induced by IR during pregnancy.

Cerebral hypoxia–ischemia (asphyxia) occurring in the fetus and newborn infant is a major cause of acute mortality and chronic disability in survivors [1]. Certain forms of newborn brain injury, such as stroke, have an incidence as high as 1 in 4000 live births [2]. More than 95% of infants who have a stroke survive to adulthood, and many have residual motor or cognitive disabilities. Stroke and other forms of brain damage cause considerable consequences to surviving babies, their families, and society [3]. Therefore, the development of preventive and therapeutic measures for fetal and neonatal brain injury is eagerly awaited.

Molecular hydrogen (H_2) can reduce hydroxyl radicals ($^{\bullet}OH$) and peroxynitrite but not superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), or nitric oxide ($^{\bullet}NO$), indicating that H_2 can antagonize damaging species without affecting physiologically important signaling molecules. Furthermore, to date, H_2 has no known side effects, including mutagenicity in rodents or humans [4]. Prompted by these unique characteristics, studies on H_2 for oxidative stress-associated diseases have flourished the past few years. H_2 in the form of gas reduces the cerebral infarction volume in rats [5,6], suppresses hepatic ischemia–reperfusion (IR)[1] injury in mice [7], reduces the infarct size of myocardial IR injury [8] and cardiac cold IR injury [9] in rats, and reduces apoptosis in neonatal hypoxic brain injury in rats [10]. It also mitigates small intestine transplantation-induced inflammation in rats [11] and decreases the hippocampal neuronal injury in mouse cerebral infarction model [12]. H_2 dissolved in drinking water or saline similarly prevents stress-induced learning impairment in mice [13], protects against 6-hydroxydopamine-induced nigrostriatal degeneration in a rat model of Parkinson disease [14], improves lipid and glucose metabolism in type 2 diabetes [15], and reduces oxidative stress, inflammatory cytokines, and apoptosis in a rabbit model of spinal cord ischemia–reperfusion injury [16].

Memory and learning are among the highest functions of the central nervous system, and the hippocampus, which is responsible

Abbreviations: HE, hematoxylin and eosin; HNE, 4-hydroxy-2-nonenal; HW, ~50% saturated hydrogen water; IR, ischemia and reperfusion; 8-oxodG, 8-oxo-7,8-dihydro-2'-deoxyguanosine

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http://dx.doi.org/10.1016/j.freeradbiomed.2014.01.037
0891-5849

for memory and learning [17], has been established as being highly sensitive to ischemia [18]. Accordingly, we focused on this area in the present preclinical study. To examine a neuroprotective effect of H_2 for neonatal cognitive and memory function, an intrauterine IR rat model was given free access to ~50%-saturated hydrogen water (HW) starting 2 days before the transient ligation of the bilateral utero-ovarian arteries [19]. Pathological and behavioral studies demonstrated that molecular oxygen efficiently prevents both the developmental failure of and the deterioration of the hippocampal function in rats after birth.

Materials and methods

Reagents

The following antibodies were used: mouse monoclonal antibody against 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) (N45.1) [20], monoclonal antibody against 4-hydroxy-2-nonenal (HNE)-modified proteins (HNEJ-2) [21,22] (Nikken Seil Co., Ltd., Shizuoka, Japan), mouse monoclonal antibody against NeuN [1B7] (Abcam, Tokyo, Japan). Approximately 50%-saturated hydrogen water prepared by dissolving H_2 gas in water under a pressure of 0.4 MPa as previously described [23] was a kind gift from Blue Mercury, Inc. (Tokyo, Japan). HW (more than 0.4 mM) was stored in an aluminum bag and aliquotted every 24 h to glass drinking bottles for rats with two ball bearings at the outlet, which prevents hydrogen degassing as well as air refill. With this glass bottle, the hydrogen concentration remained more than 0.2 mM after 24 h. In the IR + HW group, the rats drank HW ad libitum from day 14 of pregnancy to vaginal delivery (day 22 of pregnancy). The pregnant rats drank approximately 60 ml/kg of regular water or HW per day. Chemical agents used were of analytical quality.

Animal model

The animal experiment committee of Nagoya University Graduate School of Medicine approved this study. Pregnant Wistar/ST rats (Japan SLC, Shizuoka, Japan) were purchased and housed in plastic cages. They received a standard chow (F2) and water ad libitum and were maintained on a 12-h light/12-h dark cycle (lights on at 9:00 AM, off at 9:00 PM). The pregnant rats were assigned randomly to the following three groups of five dams each: sham group that underwent laparotomy on day 16 of pregnancy without IR procedure; IR group, in which IR operation was performed on day 16 of pregnancy; and IR + HW group, in which pregnant rats were given HW, starting from 2 days before operation and thereafter until vaginal birth with IR operation on day 16 of pregnancy. Each pregnant rat gave birth to 5–10 offspring without apparent sex difference ($n = 42$ for sham, $n = 40$ for IR group, and $n = 38$ for IR + HW).

Determination of hydrogen concentration

Previous studies reported that the hydrogen concentration in rat tissue peaked 5 to 15 min after oral HW administration and returned to the basal level after 25 to 30 min [15,24]. Cesarean section was performed under anesthesia 5 min after 4 ml of HW administration orally by gavage, and amniotic fluid, placenta, and fetuses were collected (HW group). The fetuses were decapitated immediately; the fetal head and body were examined separately. Pure air (100 ml) was equilibrated with either amniotic fluid or homogenized tissue in an aluminum bag, and the hydrogen concentration in the air was measured with a gas chromatograph connected to a semiconductor gas detector (EAGanalyzer GS-23, SensorTec Co. Ltd., Shiga, Japan). The pregnant rats without HW administration served as the control group ($n = 3$–5 fetuses for each group, consisting of six dams).

Ischemia–reperfusion operation

IR operation was performed as previously reported [25] with a minor modification. Briefly, the pregnant rats were operated on on day 16 of pregnancy after injection of ketamine (30 mg/kg, ip) and xylazine (10 mg/kg, ip). To induce fetal ischemia, we occluded bilateral utero-ovarian arteries for 30 min by using forceps covered with a soft polyvinyl tube. After 30-min occlusion, the clamp was released to restore circulation for 30 min and reperfusion was achieved. The same operation, but omitting the ischemia–reperfusion procedure, was considered the sham operation. Investigators involved in the following experiments were blinded to the operation procedure.

Neonatal growth evaluation and histological analysis of neonatal brain

After vaginal delivery, each pup was weighed on postnatal days 1, 3, 5, and 7. Then, each pup ($n = 10$ for each group with at least one pup from each dam) was decapitated under anesthesia with ice and each head portion was fixed in 10% phosphate-buffered formalin. Each specimen was embedded in paraffin, and frontal plane sections were cut at a 4-μm thickness. Neonatal hippocampal damage was observed with hematoxylin and eosin (HE) staining and quantified as the ratio of degenerated to total hippocampal pyramidal cell number under ×400 magnification in one field for each section. The average of total cell counts in one field was 78 cells for the CA1 and 45 cells for the CA3 region. The hippocampal pyramidal cells were confirmed as neuronal cells by immunohistochemistry of NeuN, a specific neuronal cell marker. The cells of which the nucleus showed a pyknotic change were defined as degenerated cells.

Immunohistochemistry

Immunohistochemistry was performed as previously described [26] with minor modifications. For antigen retrieval, deparaffinized sections were heated in an Immunosaver (Nisshin EM Corp., Tokyo, Japan) for 45 min at 98 °C. Immunohistochemical staining was performed employing the avidin–biotin immunoperoxidase technique using the Histofine SAB-PO kit (Nichirei, Tokyo, Japan)

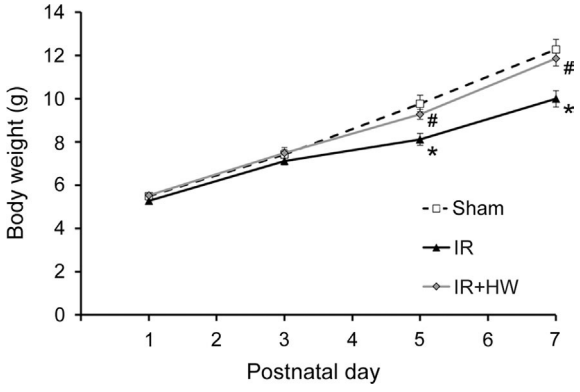

Fig. 1. Neonatal growth in body weight. Neonatal growth was significantly retarded in the IR group compared with the sham group at postnatal days 5 and 7. In the IR + HW group, maternal administration of hydrogen water restored neonatal growth to the control level ($n = 30$–42; means ± SEM; $^{*}P < 0.01$, IR group vs sham, and $^{\#}P < 0.01$, IR + HW group vs IR group, by both ANOVA according to the Tukey HSD test and Student's t test). HW, ~50% saturated hydrogen water; IR, ischemia–reperfusion. Refer to text for details.