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Original Contribution

Neuroprotective potential of molecular hydrogen against perinatal brain injury via suppression of activated microglia

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ABSTRACT

Exposure to inflammation *in utero* is related to perinatal brain injury, which is itself associated with high rates of long-term morbidity and mortality in children. Novel therapeutic interventions during the perinatal period are required to prevent inflammation, but its pathogenesis is incompletely understood. Activated microglia are known to play a central role in brain injury by producing a variety of pro-inflammatory cytokines and releasing oxidative products. The study is aimed to investigate the preventative potential of molecular hydrogen (H_2), which is an antioxidant and anti-inflammatory agent without mutagenicity. Pregnant ICR mice were injected with lipopolysaccharide (LPS) intraperitoneally on embryonic day 17 to create a model of perinatal brain injury caused by prenatal inflammation. In this model, the effect of maternal administration of hydrogen water (HW) on pups was also evaluated. The levels of pro-inflammatory cytokines, oxidative damage and activation of microglia were determined in the fetal brains. H_2 reduced the LPS-induced expression of pro-inflammatory cytokines, oxidative damage and microglial activation in the fetal brains. Next, we investigated how H_2 contributes to neuroprotection, focusing on microglia, using primary cultured microglia and neurons. H_2 prevented LPS- or cytokine-induced generation of reactive oxidative species by microglia and reduced LPS-induced microglial neurotoxicity. Finally, we identified several molecules influenced by H_2, involved in the process of activating microglia. These results suggested that H_2 holds promise for the prevention of inflammation related to perinatal brain injury.

1. Introduction

Perinatal brain injury is a severe problem even in developed countries on the socioeconomic aspect, because it can cause lifelong neurologic disability [1]. Intrauterine infection/inflammation is known to be a principal risk factor for perinatal cerebral injury in both term and preterm newborns [2–6]. Infection-induced maternal immune activation leads to exaggerated secretion of cytokines, which are cytotoxic to the fetus and induce fetal inflammatory response syndrome (FIRS) [2]. FIRS has been reported to be involved in both short-term complications that include multi-organ dysfunction, sepsis, and fetal death and in long-term sequelae that include cerebral palsy [7–9]. A recent report has also implied that fetal infection/inflammation may be associated with other neurodevelopmental disorders, such as schizophrenia [10]. High levels of pro-inflammatory cytokines in cord blood, especially interleukin (IL)-6, are considered to be a marker of FIRS [7].

The pathophysiologic mechanisms of perinatal brain injury due to maternal or fetal infections are incompletely understood; however, oxidative stress and pro-inflammatory cytokines are

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reported to cause apoptotic or necrotic neuronal death and to disrupt the differentiation of neurons [11–13]. Pro-inflammatory cytokines can also induce damage by activating microglia, which are resident macrophages in the central nervous system [3]. Activated microglia are known to play a central role in brain injury by producing a variety of pro-inflammatory cytokines and releasing oxidative and nitrosative products [14–16]. Thus, it is well recognized that prenatal administration of agents that downregulate the exaggerated inflammatory response and/or activation of microglia will be a novel therapeutic approach to improve its prognosis [1,17–19].

Now, molecular hydrogen (H_2) has been widely used in medical applications as a safe and effective treatment without any known side effects, including mutagenicity [20,21]. The first article on the effects of H_2 was reported on cerebral infarction in 2007 [22]. H_2 is known to be a characteristic antioxidant, because it selectively reduces peroxynitrate and hydroxyl radicals. In addition, although most antioxidants are not able to reach neurons because of the blood–brain barrier, H_2 can penetrate cell membranes and can easily target organelles, including mitochondria and nuclei. There are papers reporting that H_2 gas reduced the cerebral infarction volume in adult rats [22] and suppressed apoptosis in a neonatal rat model of hypoxic brain injury [23]. Additionally, H_2 dissolved in drinking water or saline has been used to allow for ease of clinical administration. Moreover, our previous work has indicated that maternal intake of hydrogen water (HW) increased the H_2 concentration in the fetal head and relieved fetal hippocampal damage in an *in utero* ischemia–reperfusion rat model [24].

Based on previous findings, we investigated whether maternal oral intake of H_2 offers protection against fetal brain injury induced by inflammation or oxidative stress in a mouse model, and the effects of H_2 on microglial activation and neurotoxicity using primary cultured microglia *in vitro*.

2. Materials and methods

2.1. Reagents

Lipopolysaccharide (LPS, serotype O55:B5) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Tumor necrosis factor (TNF)-α and IL-1β (R&D, Minneapolis, MN, USA), IL-6 (Peprotech, Rocky Hill, NJ, USA), 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) (Invitrogen, Carlsbad, CA), and Diogenes chemiluminescence superoxide-detection kit (National Diagnostics, Atlanta, GA) were purchased from the indicated manufacturers.

2.2. Preparation of HW and hydrogen medium

HW was prepared by dissolving H_2 gas in water under a pressure of 0.4 MPa, as described previously [25], and hydrogen medium (HM), prepared in the same manner, were kind gifts from Blue Mercury, Inc. (Tokyo, Japan). Both HW and HM, stored in aluminum bags, contained a concentration of at least 0.4 mM H_2. In the *in vivo* pregnant mouse study, HW was aliquotted into glass drinking bottles with two ball bearings at the outlet, which prevented H_2 degassing as well as air refilling. With this glass bottle, the H_2 concentration of HW remained higher than 0.2 mM after 24 h. To maintain the H_2 concentration, HW from a new aluminum bag was placed into the bottle every 24 h. In the *in vitro* microglia study, H_2 treatment was conducted by replacing microglial media with HM, and dehydrogenated HM was used as control medium.

2.3. Animals and treatments

All protocols for the animal experiments were approved by the Animal Experiment Committee of Nagoya University (approval no. 25096). Pregnant ICR (CD-1) mice (7–8 weeks, Charles River Japan, Kanagawa, Japan) were purchased. The animals were allowed free access to food and water at all times and were maintained on a 12-h light/12-h dark cycle (lights on at 9:00 AM, off at 9:00 PM). Pregnant ICR mice were divided randomly into 3 groups of 5 dams. All pregnant mice, except for those in the Control group, received an intraperitoneal injection (i.p.) of 75 μg LPS dissolved in Phosphate buffered saline (PBS) on embryonic day 17 (E17). In the Control group, the pregnant mice were injected with an equal volume of sterile PBS. In the HW+LPS group, the pregnant mice drank HW beginning 24 h before LPS administration until they were sacrificed. The pregnant mice drank approximately 200 ml/kg of regular water or HW per day. In each group, the mice were anesthetized 6 or 8 h after the PBS or LPS i.p. to collect samples. For quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) and the immunohistochemistry analysis, brains from random 4–5 pups from every litter were harvested and analyzed individually. For measurement of the pro-inflammatory cytokine levels, blood from all decapitated pups from every litter was collected and pooled before being analyzed.

2.4. Cell culture

Primary microglia were isolated from primary mixed glial cell cultures prepared from newborn ICR mice on day 14 using the 'shaking off' method as described previously [26]. The purity of the cultures (> 99%) was determined by anti-CD11b immunostaining (BD Biosciences, Franklin Lakes, NJ, USA). The cultures were maintained in Dulbecco's modified Eagle's minimum essential medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Equitech-Bio, Kerrville, TX, USA), 5 mg/ml bovine insulin (Sigma-Aldrich) and 0.2% glucose. Primary neuronal cultures were prepared from the cortices of embryos on E17 of C57BL/6 mouse (Japan SLC, Hamamatsu, Japan) as described previously [27]. Briefly, cortical fragments were dissociated into single cells in dissociation solution (Sumitomo Bakelite, Akita, Japan) and were resuspended in nerve culture medium (Sumitomo Bakelite). The purity of the culture was more than 95% as determined by NeuN-specific immunostaining (Merck Millipore, Billerica, MA, USA).

2.5. qRT-PCR

Total RNA was isolated from the cortices of pups and microglial cultures using the RNeasy Mini Kit (Qiagen Inc.) following the manufacturer's directions. The RT reaction with 100 ng of total RNA was carried out with a first strand cDNA synthesis kit (ReverTra Ace; Toyobo Co., Ltd, Osaka, Japan). Thereafter, qRT-PCR was performed in 96-well PCR plates using the Thermal Cycler Dice (Takara Bio Inc., Tokyo, Japan) and SYBR‖ Premix Ex Taq (Takara Bio Inc.) reagents. Primers set for qRT-PCR are listed in Table 1. In the *in vitro* microglia study, replacement of microglial media with HM or control media was performed 3 h before the time of LPS or PBS addition, and at the time of LPS or PBS addition.

2.6. Immunostaining and quantification of data

The pups were fixed in formalin and embedded in paraffin, then serial sections were cut through whole heads in sagittal planes. Immunostaining was performed as described previously [28,29]. Images were collected using a BZ-8000 microscope. (Keyence Corporation, Osaka). The sections were stained using a mouse polyclonal anti-8-Hydroxy-2′-deoxyguanosine (8OHdG)

Table 1
List of primers for qRT-PCR.

Genes	Primer sequences	
	Forward	Reverse
TNF-α	5′-GTAGCCCACGTCGTAGCAAAC-3′	5′-CTGGCACCACTAGTTGGTTGTC-3′
IL-6	5′-ACAACCACGGCCTTCCCTAC-3′	5′-TCCACGATTTCCCAGAGAACA-3′
IL-1β	5′-CATCCAGCTTCAAATCTCGCAG-3′	5′-CACACACCAGCAGGTTATCATC-3′
iNOS	5′-CATGCTACTGGAGGTGGGTG -3′	5′-CATTGATCTCCGTGACAGCC-3′
SOD1	5′-GGGATTGCGCAGTAAACATTC-3′	5′-AATGGTTTGAGGGTAGCAGATGA-3′
SOD2	5′-CACATTAACGCGCAGATCATG-3′	5′-CCAGAGCCTCGTGGTACTTCTC-3′
Gpx1	5′-GCGGCCCTGGCATTG-3′	5′-GGACCAGCGCCCATCTG-3′
Gpx4	5′-GCATGCCCGATATGCTGAGT-3′	5′-CCTGCCTCCCAAACTGGTT-3′
Cat	5′-CCTTTGGCTACTTTGAGGTCACACA-3′	5′-GAACCCGATTCTCCAGCAACAGT-3′
Cacna1c	5′-CAAGCCCTCACAAAGGAATGC-3′	5′-AAAGTTGCCCCTGCTGTCACTC-3′
Gja5	5′-GAGGCCCACGGAGAAGAATG-3′	5′-TGGTAGAGTTCAGCCAGGCT-3′
Gja8	5′-GCATCCTGCCCCTCTATCG-3′	5′-TCTCAGTAGGCCGGGATACAA-3′
Fgf1	5′-GAAGCATGCGGAGAAGAACTG-3′	5′-CGAGGACCGCGCTTACA-3′
Fgfr2	5′-GAGAAGGAGATCACGGCTTC-3′	5′-AAGTCTGGCTTCTTGGTCGT-3′
Lepr	5′-CTTTGGAAGCCCCTGACGAA-3′	5′-CGTACCTCCTCACACTACACAGT-3′
β-actin	5′-CGTGGGCCGCCCTAGGCACCA-3	5′-ACACGCAGCTCATTGTA-3

antibody (1:20; JICA, Shizuoka, Japan), mouse polyclonal anti-4-hydroxy-2-nonenal (4HNE) antibody (1:5; JICA, Shizuoka, Japan) and goat polyclonal anti-ionized calcium binding adapter molecular 1 (Iba1) antibody (1:250; Abcam, Tokyo, Japan). The staining of the cortex for 8OHdG and Iba1 was quantified by manually counting the positively stained cells. The 4HNE staining of cortex was scored as 0 ($<0.05\%$), 1 (0.05–15%), 2 (15–30%) or 3 ($>30\%$), indicating the percentage of the area that was positively stained. Three microscopic images of the cortex were randomly captured in each stained section, and the mean value was used to represent one single data point. The quantification of 4HNE staining was performed using the BZ Image Measurement Software program (Keyence).

2.7. Intracellular ROS (iROS) assay and extracellular superoxide assay

Primary microglia enriched cultures were plated at 5×10^4 cells/well in 200 μl of culture medium in 96-well plates, and were incubated for 24 h at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. The microglial media were replaced with HM or control media 3 h before the addition of LPS or each cytokine. The production of iROS in the microglia was measured by the oxidation of CM-H_2DCFDA [30,31], a ROS-specific indicator, as previously reported [32]. Before the addition of LPS or each cytokine, the cells were exposed to 5 μM CM-H_2DCFDA for 0.5 h. After washing the cells with PBS, 5 μg/ml of LPS,10 ng/ml of TNF-α, 100 ng/ml of IL-6, or 100 ng/ml of IL-1β were added to each well. After the treatment, the cultures were incubated for 0.5 h at 37 °C with 5% CO_2 and 95% air. After incubation, the fluorescence of the wells was measured with a Wallac 1420 ARVOMX instrument (PerkinElmer Japan Co., Ltd., Yokohama, Japan). Extracellular superoxide production from the microglia was measured using DIOGENES, a superoxide-specific chemiluminescence reagent. After washing with PBS, the cells were exposed to 100 μl of DIOGENES. Then, 5 μg/ml of LPS was added to each well. After the treatment, the cultures were incubated for 2 h at 37 °C with 5% CO_2 and 95% air. After incubation, the measurements were performed with a Mithras Multimode Microplate Reader LB 940 (Berthold Technologies, Bad Wildbad, Germany).

2.8. Evaluation of the neurotoxic effects induced by microglia

To assess neurotoxic effects of microglia, we conducted immunocytochemical studies and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assays using the CellTiter 96 Aqueous One-solution assay (Promega, Madison, WI) as described previously [27]. Replacement of microglial media with HM or control media was started 3 h before the addition of LPS, and was performed twice during the experiments. Then, the microglia were treated with or without LPS for 10 h and the media were collected. These collected media were immediately added to cortical neuronal cultures for a further 24 h before the evaluation of microglial neurotoxicity was performed.

In the immunocytochemical studies, cortical neuronal cells, seeded onto 12 mm polyethyleneimine (PEI)-coated glass cover slips (Asahi Techno Glass Corp, Chiba, Japan) at a density of 5×10^4 cells/well in 24-well plates, were fixed with 4% paraformaldehyde for 10 min. The cells were then permeabilized with 0.05% Triton X-100 for 5 min and blocked with 5% bovine serum albumin for 1 h, followed by incubation with a rabbit polyclonal anti-microtubule-associated protein 2 (MAP2) antibody (1:1000; Chemicon, Temecula, CA) overnight at 4 °C. The cells were then incubated with Alexa 488-conjugated secondary antibodies (1:500; Invitrogen) for 1 h before the cells were examined with a BZ-8000 microscope. Neuronal survival was assessed by MAP2-specific immunofluorescence staining [33–35]. Viable neurons were stained strongly with the anti-MAP2 antibody, whereas damaged neurons showed weaker staining. In each experiment, 9 randomly selected fields in each well were evaluated by a scorer who was blind to the experimental conditions. In the MTS assay, cortical neuronal cells were seeded at a density of 3×10^4 cells/well in 96-well plates. The absorbance at 490 nm was measured using a multiple plate reader according to the manufacturer's protocol (Labsystems, Thermo BioAnalysis, Tokyo, Japan).

2.9. DNA microarray

Three hours after LPS addition, total RNA was prepared from the microglial cultures with or without HM using the RNeasy Mini Kit (Qiagen Inc.). Microarray experiments were performed using an Agilent Expression Array Whole Mouse Genome oligo DNA microarray (Agilent, Santa Clara, CA) at Takara-bio Corporation (Ootu, Japan) with the microarray service certified by Agilent.

2.10. ELISA

Commercial ELISA kit (eBioscience, San Diego, CA, USA) was used to determine the levels of TNF-α and IL-6 in the pup's serum.

The absorbance at 490 nm was measured using a multiple plate reader according to the manufacturer's protocol (ELx808, BioTek Instruments, VT, USA).

2.11. Determination of the H_2 concentration

Measurement of the H_2 concentration was performed as previously described, with minor modifications [24,36]. Briefly, cesarean sections were performed under anesthesia on the pregnant mice of each group 5 min after 1 ml of HW or regular water was administered orally via gavage; the pups were delivered and 1 ml of maternal blood was collected. The pups were decapitated immediately; the heads of 3 pups from every litter were collected in an aluminum bag and examined as one single data point. Pure air (50 ml) was equilibrated with either the homogenized fetal head or maternal blood in an aluminum bag, and the H_2 concentration in the air was measured with a gas chromatograph (EAGanalyzerGS-23, SensorTec Co., Ltd. Shiga, Japan). In the HM, the time-dependent changes in H_2 concentration for up to 180 min ($n=3$) were measured.

2.12. Statistical analyses

The data are presented as the means ± standard error of the mean (SEM). To analyze the pro-inflammatory cytokine expression in the cortices of the pups, a non-parametric Kruskal–Wallis ANOVA was used, followed by Dunn's test. Mortality was analyzed with the χ^2-test. All other data were analyzed by Student's *t*-test or Mann–Whitney *U*-test or a one-way ANOVA followed by Tukey's tests. The statistical analyses were performed using the Prism 5 for Windows software program (GraphPad Software, San Diego, CA). Values of $p<0.05$ were considered to be significant.

3. Results

3.1. H_2 treatment attenuated the LPS-induced pro-inflammatory cytokines, oxidative damage and microglial activation in the cortices of pups

In this mouse model, elevation of the fetal serum IL-6 level, which is the determination of FIRS [7], was confirmed (Control, 0.168 ± 0.007 ng/ml vs. LPS, 3.986 ± 1.080 ng/ml; $p<0.01$). In addition, the mortality of the pups also increased to 62.7% (number of death/total pups, 79/126). Furthermore, the level of TNF-α in the fetal serum of the LPS group was also significantly elevated to 105.6 ± 37.7 pg/ml, compared with that of the Control group, 11.55 ± 1.62 pg/ml ($p<0.05$). Maternal administration of H_2 decreased the mortality of pups to 31.5% (number of death/total pups, 35/111, $p<0.01$). As shown in Fig. 1A, the H_2 concentration was significantly higher in the fetal head and maternal blood of the HW+LPS group compared to that of the Control and LPS groups (fetal head, $p<0.01$ and $p<0.01$; maternal blood, $p<0.001$ and $p<0.001$, respectively). Treatment with LPS alone did not have a significant effect on the H_2 concentration.

The levels of TNF-α, IL-1β and IL-6 mRNA expression in the LPS group significantly increased 7.0- ($p<0.05$), 4.4- ($p<0.01$) and 13.3- ($p<0.001$) fold compared to those in the Control group, respectively (Fig. 1B). Maternal HW administration completely attenuated the LPS-induced increase in the mRNA expression of TNF-α ($p<0.05$), IL-1β ($p<0.01$) and IL-6 ($p<0.05$) (Fig. 1B).

The analyses of oxidative damage in the brains of the pups 8 h after LPS injection were performed with immunohistochemical staining for 8OHdG and 4HNE, which are markers of oxidative DNA damage and lipid peroxidation, respectively (Fig. 2A). Compared to the Control group, the number of 8OHdG-positive cells

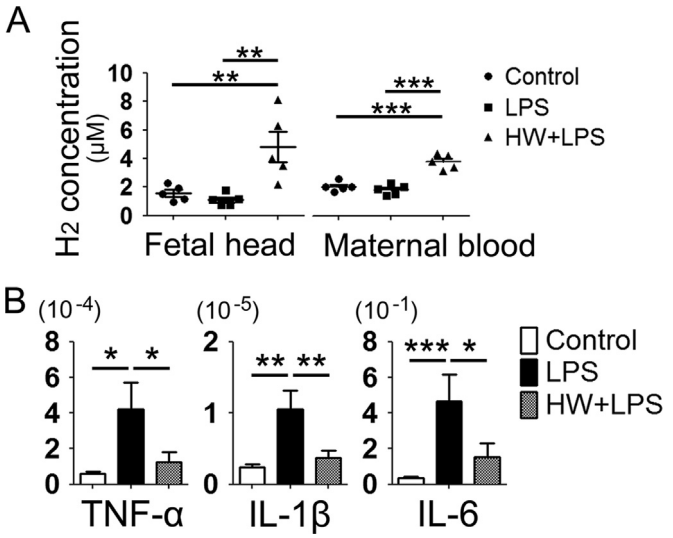

Fig. 1. The H_2 concentrations *in vivo* and the effects of H_2 on LPS-induced TNF-α, IL-1β and IL-6 mRNA expression in the mouse pup cortex: (A) maternal administration of hydrogen water significantly increased the H_2 concentration in the fetal head and maternal blood. The results are expressed as the means ± SEM ($n=5$ in each group). Data were analyzed by one-way ANOVA with Tukey's HSD test. $^{**}p<0.01$, $^{***}p<0.001$. (B) The mRNA expression levels of TNF-α, IL-1β and IL-6 were determined by qRT-PCR 6 h after LPS injection. The data were normalized to the expression of β-actin. The results are expressed as the means ± SEM ($n=25$ in each group). The data were analyzed by the non-parametric Kruskal–Wallis ANOVA with Dunn's test. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

(Fig. 2B, $p<0.001$) and the 4HNE-histological score (Fig. 2C, $p<0.001$) strikingly increased in the LPS group. The severity of the LPS-induced oxidative damage was completely reduced in the HW+LPS group, as evidenced by the immunohistochemical staining of 8OHdG (Fig. 2A, panels a–c and Fig. 2B, $p<0.01$) and 4HNE (Fig. 2A, panels d–f and Fig. 2C, $p<0.05$). Concurrently, LPS treatment caused activation of microglia in the cortices of the pups as indicated by the increased levels of Iba1, which is a 147-amino-acid calcium-binding protein widely used as a marker for microglia [37] (Fig. 3A). In the Control group, only a few Iba1-positive cells were detected, and most of these cells were in the resting state with small somas (Fig. 3A, panel a). Significantly increased number of activated microglia showing bright staining of an elongated cell body was found in the cortex of pups after LPS injection (Fig. 3A, panel b). Compared to number of Iba1-positive cells in the Control group, that was observed approximately 3.3-fold increase in the LPS group (Fig. 3B, $p<0.001$). H_2 treatment completely reduced both the number of Iba1-positive cells (Fig. 3B, $p<0.01$) and the activation of microglia following LPS injection (Fig. 3A, panel c).

3.2. H_2 treatment prevented the ROS generation induced by LPS and pro-inflammatory cytokines in primary microglia cells

As shown in Fig. 4A, approximately 2-fold increase in iROS was detectable 0.5 h after any addition of LPS ($p<0.05$), TNF-α ($p<0.01$), IL-6 ($p<0.05$) or IL-1β ($p<0.05$). Treatment with H_2 completely prevented iROS generation, regardless of any stimulus added (Fig. 4A, LPS; $p<0.001$, TNF-α; $p<0.05$, IL-6; $p<0.05$, IL-1β; $p<0.05$). The level of extracellular superoxide was remarkably enhanced 2 h after LPS treatment (Fig. 4C, $p<0.001$). Treatment with H_2 significantly prevented LPS-induced superoxide generation (Fig. 4C, $p<0.001$). The effect of H_2 on gene expressions of antioxidant enzymes, such as superoxide dismutase (SOD)1, SOD2, glutathione peroxidase (Gpx)1, Gpx4, and catalase (Cat) in microglia was also investigated; however, none of the gene expressions was significantly altered (data not shown).

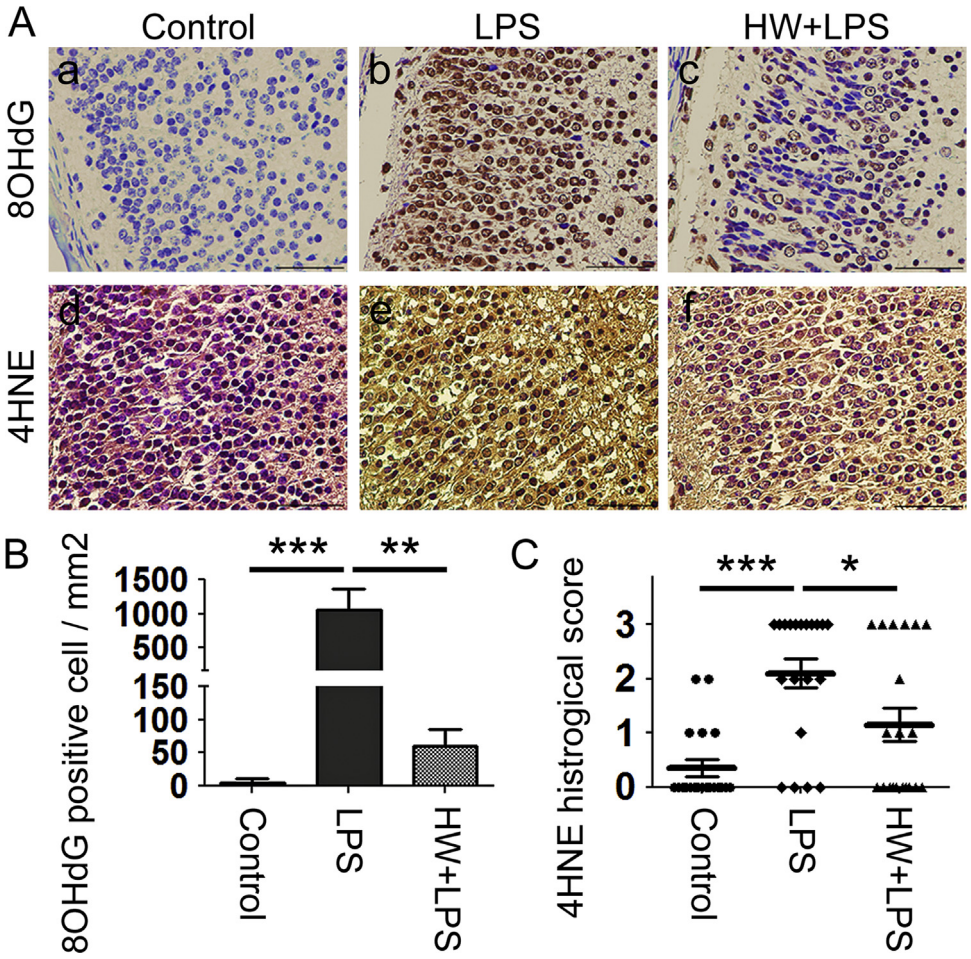

Fig. 2. The effects of H_2 on LPS-induced oxidative damage in the brain of pups: (A) representative images of 8OHdG- (upper panels) and 4HNE- (lower panels) immunostaining of sagittal sections of brains from pups taken 8 h after LPS injection. Scale bars = 50 μm. (B, C) The quantitation of the number of 8OHdG-positive cells (B), and the grading of the 4HNE-histological score (C) were performed as described in Section 2. The results are expressed as the means ± SEM (n = 20 in each group). Data were analyzed by a one-way ANOVA with Tukey's HSD test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

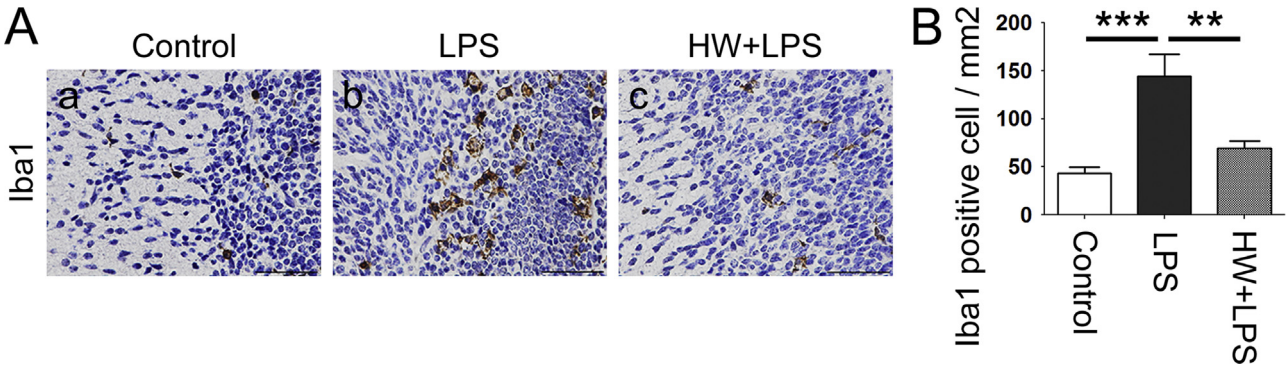

Fig. 3. The effects of H_2 on the LPS-induced microglial activation in the brain of pups: (A) representative images of Iba1-immunostaining of sagittal sections of brains from pups taken 8 h after LPS injection. Scale bars = 50 μm. (B) The quantitation of the number of Iba1-positive cells was performed as described in Section 2. The results are expressed as the means ± SEM (n = 20 in each group). Data were analyzed by a one-way ANOVA with Tukey's HSD test. $^{**}p < 0.01$, $^{***}p < 0.001$.

We also incubated microglia using serially diluted HM in two-fold steps from 1/2 to 1/64 with control medium, and a significant suppressive effect on LPS-induced iROS generation at the higher concentrations than the 1/32 dilution was observed and that effect was detected in a concentration dependent manner (Fig. 4B). The H_2 concentrations of the 1/32 and 1/64 diluted HM was 6.08 ± 0.97 μM (n = 5) and 2.92 ± 0.56 μM (n = 5), respectively.

3.3. *H_2 treatment suppressed the LPS-induced inflammatory response in primary microglia cells*

As shown in Fig. 5, stimulation of microglia with LPS for 3 and 24 h led to marked increases in TNF-α ($p < 0.001$ and $p < 0.05$, respectively), IL-1β ($p < 0.001$ and $p < 0.05$, respectively), IL-6 ($p < 0.01$, and $p < 0.001$, respectively) and inducible nitric oxide

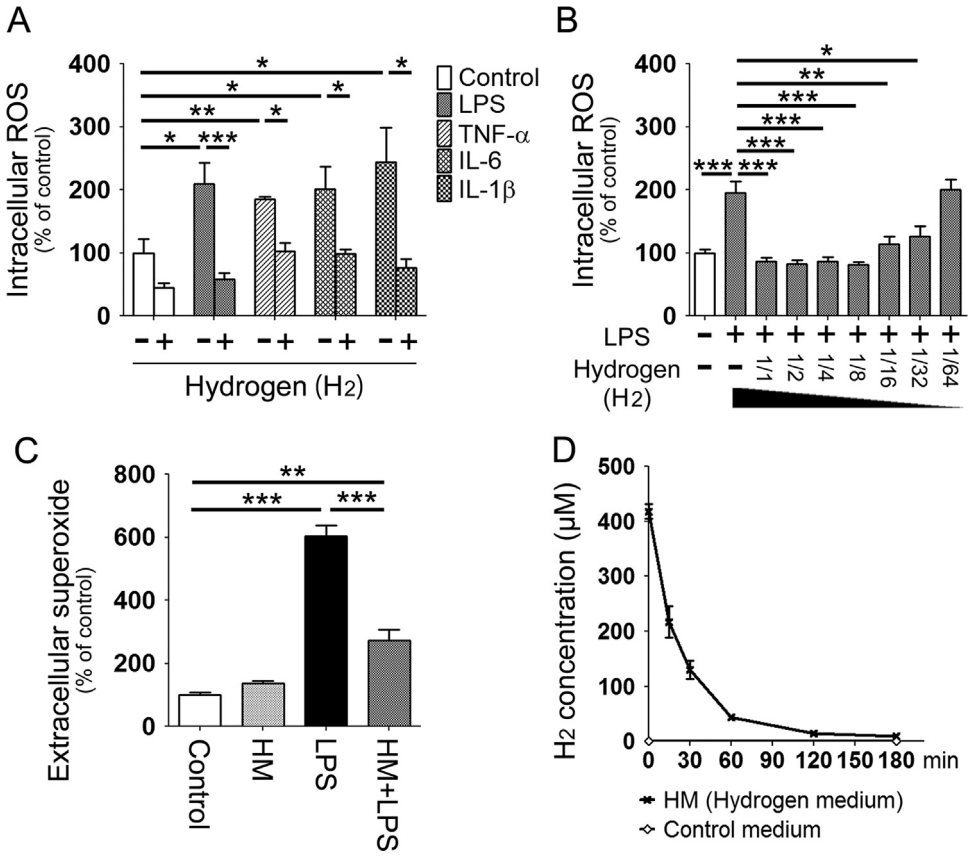

Fig. 4. The H_2 concentrations *in vitro* and the effects of H_2 on the microglial ROS induced by LPS and pro-inflammatory cytokines in primary microglial culture: (A, B) The levels of iROS were determined 0.5 h after the addition of LPS,TNF-α, IL-6 and IL-1β with or without HM (A), and of LPS with HM serially diluted in two-fold steps (B). (C) The levels of extracellular superoxide were determined 2 h after the addition of LPS with or without HM. The results are expressed as the % of the control group without H_2 treatment, and the data are the means ± SEM ($n=5$ in each group). The data were analyzed by a one-way ANOVA with Tukey's HSD test. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. (D) The time course of the H_2 concentration in HM.

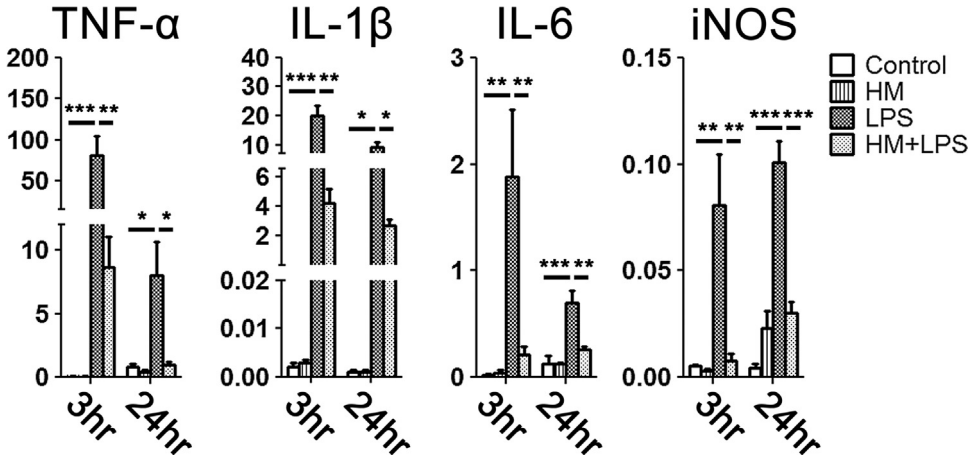

Fig. 5. The effects of H_2 on the LPS-induced expression of pro-inflammatory cytokines and iNOS in primary microglial culture. The mRNA expression levels of TNF-α, IL-1β, IL-6 and iNOS were determined by qRT-PCR at 3 and 24 h after the addition of LPS. The data were normalized to the expression of β-actin. The results are expressed as the means ± SEM ($n=5$ in each group). The data were analyzed with a one-way ANOVA with Tukey's HSD test. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

synthase (iNOS) ($p<0.01$ and $p<0.001$, respectively) mRNA expression. Those increases in the expression of TNF-α (3 h; $p<0.01$, 24 hours; $p<0.05$), IL-1β (3 h; $p<0.01$, 24 h; $p<0.05$), IL-6 (3 h; $p<0.01$, 24 h; $p<0.01$) and iNOS (for 3 h; $p<0.01$, 24 h; $p<0.001$) were significantly attenuated by H_2 treatment.

3.4. H_2 treatment suppressed LPS-induced microglial neurotoxicity

As shown in Fig. 6A (panel a), unstimulated neurons stained with an anti-MAP2 antibody possessed intact cell bodies and dendrites. Supernatants collected from microglia treated with LPS induced neuronal cell damage, with the survival rates decreased to 30.4% of the Control (Fig. 6A, panel c and Fig. 6B, $p<0.01$). Treatment with H_2 rescued 78.0% of the neurons from LPS-induced microglial neurotoxicity (Fig. 6A, panel d and Fig. 6B, $p<0.05$). Furthermore, in the MTS assay, the neuronal viability incubated with supernatant collected from microglia treated with LPS was significantly reduced to 50.9% of the Control (Fig. 6C, $p<0.01$).

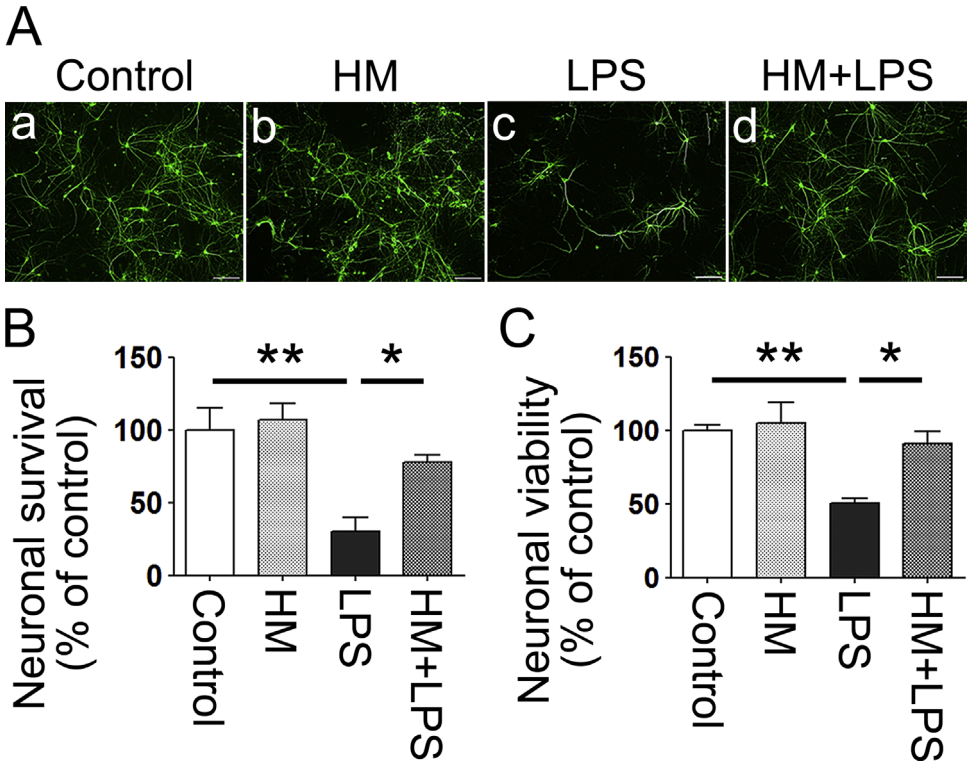

Fig. 6. The neuroprotective effects of H_2 on LPS-induced microglial neurotoxicity: (A) representative images of neurons stained for MAP2. Scale bars = 100 μm. (B) The neuronal survival rate was assessed by described in Section 2. The results are expressed as the % of the control group, and data are the means ± SEM (n = 4 in each group). (C) The neuronal viability was assessed by the MTS assay. The results are expressed as the % of the Control, and data are the means ± SEM (n = 5 in each group). The data were analyzed with a one-way ANOVA with Tukey's HSD test. $^{*}p < 0.05$, $^{**}p < 0.01$.

Table 2
List of genes.

Name	Ratio	Genebank No.
Fgf1	−4.11	NM_010197
Lepr	−3.45	NM_001122899
Fgfr2	−1.92	NM_010207
Gja5	−1.48	NM_001271628
Gja8	−1.22	NM_008123
Cacna1c	−1.03	NM_001256002

These genes were selected from the DNA microarray data, which could be a concern given the suppression of microglial activation induced by H_2 treatment.

Treatment with H_2 alone did not have any effect on the cell viability, but significantly attenuated the LPS-induced decrease in neuronal viability (Fig. 6C; LPS, 50.9 ± 3.0% vs. HM+ LPS, 91.5 ± 8.1%; $p < 0.05$).

3.5. H_2 treatment reduced the LPS-induced gene expressions, which are related to microglial activation

Comparison of mRNA expression between LPS-treated microglia either exposed or not exposed to H_2 revealed that the expressions of 956 among total 24,644 genes were changed by 2.0-fold or more. Among the genes above, calcium channel, voltage-dependent, L-type, alpha 1C subunit (Cacna1c), gap junction protein, alpha 5 (Gja5), gap junction protein, alpha 8 (Gja8), fibroblast growth factor 1 (Fgf1), fibroblast growth factor receptor 2 (Fgfr2), and leptin receptor (Lepr) have been reported to have important roles in microglial activation (Table 2) [38–43]. The expressions of Cacna1c ($p < 0.05$), Gja5 ($p < 0.05$), Gja8 ($p < 0.05$), Fgf1 ($p < 0.05$), Fgfr2 ($p < 0.01$), and Lepr ($p < 0.01$) were significantly down-regulated by H_2 treatment, and that results were consistent with the microarray results (Fig. 7).

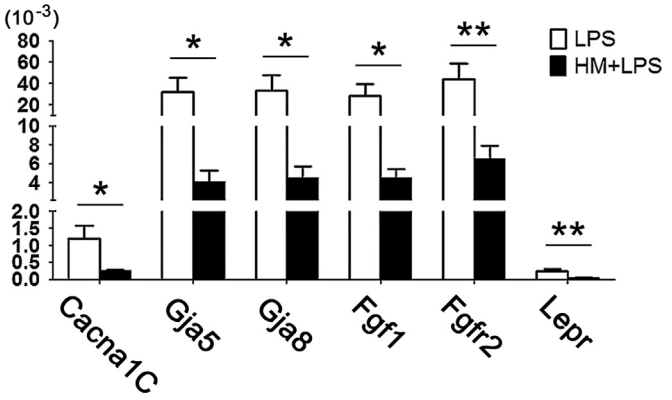

Fig. 7. The effects of H_2 on LPS-induced gene expression. The mRNA expression levels of Cacna1c, Gja5, Gja8, Fgf1, Fgfr2 and Lepr, selected from the DNA microarray data, were determined by qRT-PCR 3 h after the addition of LPS. The data were normalized to the expression of β-actin. The results are expressed as the means ± SEM (n = 7 in each group). The data were analyzed with a Mann–Whitney U-test. $^{*}p < 0.05$, $^{**}p < 0.01$.

4. Discussion

We have shown that fetal microglia are activated by maternal inflammation and play a role in fetal brain injury. This is the first report to demonstrate that the fetal brain injury could be ameliorated by maternal administration of H_2 via its effects on fetal microglia in a FIRS model.

In this study, we used a mouse model of the fetal brain injury by performing a maternal intraperitoneal injection of LPS. In a recent review, TNF-α, IL-1β and IL-6 were found to be elevated in postmortem brains with FIRS [3]. In the present mouse model, all of them significantly increased in the fetal cortex. These cytokines

increase the permeability of the blood–brain barrier, allowing the passage of microbial products such as LPS and cytokines, which activate microglia.

The precursor of microglia is known to invade the brain on E9.5 [44]. Thus, activated microglia also play a critical role in the brain injury of immature fetuses by producing pro-inflammatory cytokines, ROS and nitrogen species, which cause neuronal loss [3,16]. Activated microglia and oxidative damage to both DNA and lipids significantly increased in our mouse model compared with the control mice. These findings were consistent with the results of a previous report using rats [45]. In addition, elevation of the fetal serum IL-6 level, which is part of the definition for FIRS [7], was also confirmed. In this model, the mortality of pups also increased, consistent with clinical features of FIRS [17]. Those findings suggested that the characteristics of pups in our mouse model are somewhat analogous to those of FIRS. Then, maternal administration of H_2 decreased the mortality of pups, and suppressed the mRNA expression of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 in fetal brain, which were similar findings to be reported in another organ [46]. The levels of markers of oxidative damage were also markedly reduced by H_2. Those results were consistent with the previous reports with a rat model of Alzheimer's disease [47]. Moreover, we found that the activation of microglia was also repressed by H_2.

Then, we investigated particularly the effect of H_2 on microglia using primary cultured microglia to understand the molecular mechanisms. *In vivo*, the mean concentration of H_2 in the fetal brain after maternal administration of HW was 4.8 μM (range: 2.2–8.1) (Fig. 1A), which approximately corresponded to the minimum effective concentration of H_2 for microglia *in vitro*, 6.1 μM, although further investigations on the appropriate concentration of H_2 should be required. Pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1β, all of which were observed to increase in the fetal brains of the LPS group, resulted in a significant increase in rapid iROS generation in primary microglial cells as well as LPS exposure. H_2 completely reduced these induction of iROS. It is known that iROS act as second messengers that lead to pro-inflammatory cytokine induction by activating nuclear factor-κB (NF-κB) [48]. Previous studies proved that antioxidants inhibit NF-κB activation and the expression of inflammatory cytokines by inhibiting iROS generation [49]. H_2 was also reported to modulate a role of NF-κB in LPS-induced inflammatory response [47,50,51]. In primary microglia, we also found that H_2 significantly attenuated the expression of pro-inflammatory cytokines including TNF-α, IL-1β, IL-6 and iNOS 3 and 24 h after LPS treatment. It was similar to a previous report on the H_2 effects in macrophages [52]. To our knowledge, the present study is the first to provide the evidence that the activation of microglia was suppressed by the maternal administration of H_2 in an animal model of fetal brain injury. We have also first reported the response of primary microglia cells to H_2 exposure. The previous study reported that H_2 had almost no effect on the LPS induced pro-inflammatory cytokines in a microglia cell line, BV-2 cells [53], which is inconsistent with our results. We think that it is related with that these cell lines do not always mimic the reaction of primary microglia in culture or in the brain [54,55]. Others reported that only 17% of the LPS regulated genes in primary microglia were consistent with those expressed in the BV-2 cells [56]. In addition, conditioned medium from activated microglia decreased the number of neuronal cells, but the H_2 treatment apparently attenuated this reduction. Based on those results, the suppression of microglia by H_2 was demonstrated to reduce microglial neurotoxicity, which would result in reduction of oxidative damage observed in fetal brains of the HW+LPS group. LPS-induced iNOS expression and superoxide release are known mechanisms by which activated microglia kill neurons [57]. In this study, H_2 reduced LPS-induced iNOS expression and superoxide release. However, H_2 cannot react with superoxide directly [22]. Thus, our data suggested that H_2 suppressed the release of superoxide from microglia and inhibited microglial ROS generator activity. However, H_2 could not alter the antioxidant defense-related gene expression in the microglia. This suggested that H_2 treatment not only protected against oxidative stress by its hydroxyl radical-scavenging activity, but also suppressed LPS-induced microglial neurotoxicity by blunting superoxide release. Currently, we are investigating the role of H_2 in the augmentation of antioxidant defenses in cells other than microglia, comprising the brain tissue.

H_2 has a number of advantages as a "novel" antioxidant: it rapidly diffuses into tissues and cells, and does not disturb metabolic redox reactions or affect ROS, such as superoxide and hydrogen peroxide, which function in cell signaling [20]. There have been reports of hydrogen peroxide acting as a signaling agent in the nigrostriatal pathway and in the basal ganglia neurons [58]. In the light of this finding, the characteristic of H_2 not deleting hydrogen peroxide is an advantage for its administration during the period of neural development.

Although the molecular target of H_2 remains unknown, Cacna1c, Gja5, Gja8, Fgf1, Fgfr2 and Lepr, which could be associated with microglial activation, were first identified in this study as genes affected by H_2: their expressions were reduced by H_2. Cacna1c encodes an alpha-1 subunit of a voltage-dependent L-type calcium channel. Its blockade has been reported to be neuroprotective, associated with the inhibition of LPS-induced TNF-α and IL-1β [38] and the inhibition of neurotoxic secretions of microglia [39], which concords with the effect of H_2 observed in this study. A previous study showed that FGF1 potentiated microglial activation through FGFR2 IIIb, a spliced variant of FGFR2 [41]. The reduced expression of Fgf1 and Fgfr2 by H_2 might result in the suppression of this signaling. In addition, microglia reportedly lead to the production of IL-1β via leptin action [42]. Gja5 and Gja8 are members of the connexin gene family, which are gap-junction genes. A recent report has suggested that abnormally activated microglia secrete glutamate, a potently neurotoxic factor, via gap-junction hemi-channels on the cell surface. Administration of gap-junction inhibitors was reported to improve stroke and Alzheimer's disease symptoms by suppressing excessive microglial glutamate release [40]. Previously, H_2 was reported to reduce the levels of connexin43 (Gja1) and connexin30 (Gjb6), predominant gap-junction proteins in astrocytes and oligodendrocytes, in the rat hippocampus after cerebral ischemia [59]. The present study was the first to show that H_2 influenced the expressions of all these molecules in the microglia. These findings provide novel insights into the molecular action of H_2.

We previously reported that maternal H_2 intake ameliorated fetal hippocampal damage in a rat model of *in utero* ischemia–reperfusion with a high level of H_2 in the fetal brain [24]. However, maternal administration also increased H_2 concentration in the maternal blood in the present study. Thus, in addition to the direct effect of H_2 on the fetal brain, H_2 could have a suppressive effect on maternal inflammation. Recently, we have reported that IL-6 level in the amniotic fluid, as a result of maternal inflammatory response, had a tendency to be reduced by H_2 [60]. Increased level of IL-6 in the amniotic fluid, a marker of intrauterine inflammation, is known to be associated with perinatal adverse outcomes [61]. Therefore, H_2 could have a protective effect on the fetal brain partially via suppression of intrauterine inflammation in dams, although further investigation should be required.

There are several potential limitations associated with our study. First, in the fetal mouse brain, the volume of white matter is very small. This is very different from the human fetal brain. Thus, in this study, we mainly evaluated fetal cortex. Further investigations using large animals, such as sheep, will be needed to

determine the effects on white matter injury. Second, the long-term neurological outcome was not been assessed to determine whether H_2 confers beneficial outcomes in terms of the fetal brain injury induced by perinatal inflammation. In this model, the mortality of pups was relatively high, and evaluating only survivors would lead to biased results. Thus, we are planning studies to evaluate the long-term prognosis in modified models. For the clinical application, whether H_2 must be applied before the infection or delayed administration could have a similar effect should be examined. Further studies are going to establish the most appropriate and effective administration time and dose of H_2.

In summary, we demonstrated that maternal administration of H_2 markedly protected the fetal brain in a mouse model of fetal brain injury via its anti-inflammatory and anti-oxidative effects. Moreover, we provided the first demonstration of the suppressive effect of H_2 on LPS- and pro-inflammatory cytokine-induced activation of microglia via the reduction of ROS and inflammatory molecules, which are the key regulators of fetal brain injury. Those suppression of these processes leads to neuroprotection. Furthermore, several genes affected by H_2 were identified in microglia. These would partially reveal the mechanisms underlying the protective effect of H_2 against perinatal brain injury via microglia activity. The results of the present study suggest that maternal oral intake of HW is a promising strategy for the prevention of fetal brain injury induced by FIRS.

Conflict of interest

The authors have no conflicts of interest to declare in association with this study.

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