Biochemical and Biophysical Research Communications 411 (2011) 143–149

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Molecular hydrogen inhibits lipopolysaccharide/interferon γ-induced nitric oxide production through modulation of signal transduction in macrophages

Tomohiro Itoh [a,b], Nanako Hamada [a], Riyako Terazawa [a], Mikako Ito [c], Kinji Ohno [c], Masatoshi Ichihara [d], Yoshinori Nozawa [a,e], Masafumi Ito [a,*]

[a] Department of Longevity and Aging Research, Gifu International Institute of Biotechnology, 1-1 Naka-fudogaoka, Kakamigahara, Gifu 504-0838, Japan
[b] Faculty of Agriculture, Kinki University, 3327-204 Nakamachi, Nara 631-8505, Japan
[c] Division of Neurogenetics, Center for Neurological Diseases and Cancer, Nagoya University Graduate School of Medicine, 65 Tsurumai, Showa-ku, Nagoya, Aichi 466-8550, Japan
[d] Department of Biomedical Sciences, College of Life and Health Sciences, Chubu University, 1200 Matsumoto-cho, Kasugai, Aichi 487-8501, Japan
[e] Department of Food and Health, Tokai Gakuin University, 5-68 Naka-kirinocho, Kakamigahara, Gifu 504-8511, Japan

ARTICLE INFO

Article history:
Received 7 June 2011
Available online 23 June 2011

Keywords:
Molecular hydrogen
Lipopolysaccharide/interferon γ
Macrophage
Signal transduction
Inflammatory arthritis

ABSTRACT

Molecular hydrogen has been reported to be effective for a variety of disorders and its effects have been ascribed to the reduction of oxidative stress. However, we have recently demonstrated that hydrogen inhibits type I allergy through modulating intracellular signal transduction. In the present study, we examined the hydrogen effects on lipopolysaccharide/interferon γ LPS/IFNγ-induced nitric oxide (NO) production in murine macrophage RAW264 cells. Treatment with hydrogen reduced LPS/IFNγ-induced NO release, which was associated with a diminished induction of inducible isoform of nitric oxide synthase (iNOS). Hydrogen treatment inhibited LPS/IFNγ-induced phosphorylation of apoptosis signal-regulating kinase 1 (ASK1) and its downstream signaling molecules, p38 MAP kinase and JNK, as well as IκBα, but did not affect activation of NADPH oxidase and production of reactive oxygen species (ROS). As ROS is an upstream activator of ASK1, inhibition of ASK1 by hydrogen without suppressing ROS implies that a potential target molecule of hydrogen should be located at the receptor or immediately downstream of it. These results suggested a role for molecular hydrogen as a signal modulator. Finally, oral intake of hydrogen-rich water alleviated anti-type II collagen antibody-induced arthritis in mice, a model for human rheumatoid arthritis. Taken together, our studies indicate that hydrogen inhibits LPS/IFNγ-induced NO production through modulation of signal transduction in macrophages and ameliorates inflammatory arthritis in mice, providing the molecular basis for hydrogen effects on inflammation and a functional interaction between two gaseous signaling molecules, NO and molecular hydrogen.

1. Introduction

Accumulating evidence suggest that molecular hydrogen is effective for a number of disorders including oxidative stress-related diseases and inflammatory diseases [1]. In animal disease models, inhalation of hydrogen gas protects against cerebral infarction [2], myocardial infarction, hepatic ischemia, neonatal hypoxic brain injury, small intestine and lung transplantation, zymosan-induced inflammation, inflammatory bowel disease and sepsis. Oral intake of hydrogen-rich water exerts beneficial effects on stress-induced learning impairment, atherosclerosis, Parkinson's disease, kidney transplantation and hearing disturbance. Infusion of hydrogen-rich saline also alleviates acute pancreatitis, spinal cord injury and obstructive jaundice. In humans, oral intake of hydrogen-rich water improves lipid and glucose metabolism in patients with diabetes and impaired glucose tolerance. In most of studies, hydrogen effects have been ascribed to the reduction of oxidative stress.

We have recently demonstrated a preventive effect of oral intake of hydrogen-rich water on type I allergy in a mouse model, which is not causally associated with oxidative stress [3]. In cultured mast cells, we investigated the underlying mechanisms and found that hydrogen attenuates degranulation by inhibiting the high affinity IgE receptor (FcεRI)-mediated signal transduction but not by reducing oxidative stress. Based on these observations, we proposed that modulation of signaling pathways may be an essential mechanism underlying hydrogen effects on a broad spectrum of diseases and that hydrogen may be a gaseous signaling molecule like nitric oxide (NO).

NO is involved in a variety of important physiological processes such as vasodilatation, neurotransmission and host defense against

* Corresponding author. Fax: +81 58 371 4412.
E-mail address: mito@giib.or.jp (M. Ito).

0006-291X/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2011.06.116

invading pathogens [4]. However, an excessive amount of NO is detrimental, resulting in rheumatoid arthritis, gastritis, bowel inflammation and bronchitis [5,6]. In macrophages, NO is synthesized by inducible isoform of nitric oxide synthase (iNOS), which catalyzes the reaction of L-arginine to L-citrulline and NO, in response to various stimuli such as lipopolysaccharide (LPS), interferon (IFN), tumor necrosis factor α (TNFα) and interleukin 1β (IL1β) [7]. LPS binds to the cell surface receptor CD14, which triggers activation of toll like receptor 4 (TLR4) and the downstream signaling molecules such as IκB and mitogen-activated protein kinases (MAPKs) including c-Jun NH_2-terminal protein kinase (JNK), p38 MAP kinase and extracellular signal-regulated kinase (ERK) [8]. TLR4 signaling activates transcription factors such as nuclear factor kappa B (NFκB), activator protein 1 (AP1) and ELK1, culminating in the expression of pro-inflammatory genes including iNOS, cyclooxygenase 2 (COX2), TNFα and IFNβ. On the other hand, IFNβ and INFγ, respectively, bind to type I and type II IFN receptors expressed on the surface of macrophages, and activate Janus kinase (JAK)–signal transducers and activators of transcription (STAT) signaling, resulting in up-regulation of IFN regulatory factor 1 (IRF1) [9]. Both IRF1 and STAT1 bind to the iNOS promoter and enhance production of NO.

Previous reports have demonstrated that hydrogen treatment attenuates inflammation in animal models of inflammatory diseases such as zymosan-induced inflammation [10] and inflammatory bowel disease [11], but the underlying molecular mechanisms are not yet understood. According to our recent findings [3], we hypothesized that hydrogen might modulate the inflammatory signal transduction and that there might be a functional interaction between two gaseous signaling molecules, NO and molecular hydrogen. In the present study, we examined the effects of hydrogen on LPS/IFNγ-induced signal transduction and NO production in murine RAW264 macrophage cells. We also studied the hydrogen effects on anti-type II collagen antibody-induced arthritis in mice, a model for human rheumatoid arthritis.

2. Materials and methods

2.1. Antibodies

The antibodies to p-ASK1 (Ser967/Thr845), AKT, p-AKT, p44/42 MAP kinase (ERK1/2), p-p44/42 MAP kinase (Thr202/204), SAPK/JNK, p-SAPK/JNK (Thr180/Tyr204), p38 MAP kinase, p-p38 MAP kinase (Thr180/Tyr182), iNOS, COX2 TAK1, p-TAK1 (Ser412/Thr184/187), IκBα, p-IκBα (Ser32/36), NFκB p65, STAT1α and p-STAT1α (Tyr701) were purchased from Cell Signaling Technology (Beverly, CA, USA). The antibodies against $p22^{phox}$, $p47^{phox}$, $p67^{phox}$ and $gp91^{phox}$ were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-ASK1, -histone H3 and -β-actin antibodies were obtained from Abcam (Cambridge, MA, USA), Upstate (Lake Placid, NY, USA) and Sigma–Aldrich (St. Louis, MO, USA), respectively.

2.2. Cell culture and hydrogen treatment

Murine macrophage RAW264 cells were purchased from RIKEN BioResource Center (Tsukuba, Japan) and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml of penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C. Hydrogen treatment was performed as described previously with a slight modification [3]. Briefly, cells seeded onto multi-well plates were incubated at 37 °C under a humidified condition of 75% H_2, 20% O_2 and 5% CO_2, or 95% air and 5% CO_2 in a small aluminum bag. After 24 h incubation in the presence of hydrogen, the hydrogen concentration in the culture media was about 0.3 ppm as measured by using the H_2–N hydrogen needle sensor (Unisense, Aarhus, Denmark). After treatment with or without hydrogen for 24 h, cells were treated with or without LPS (final concentration, 200 ng/ml) (Sigma–Aldrich) and IFNγ (final concentration, 25 ng/ml) (Millipore, Bedford, MA, USA), which was followed by incubation in the presence or absence of hydrogen.

2.3. Measurement of nitric oxide production

Cell culture media were centrifuged at 4 °C for 5 min and the supernatant was subjected to measurement of the amount of nitrite, a stable metabolite of NO, using the Griess reagent kit (Promega, Madison, WI, USA).

2.4. Western blot analysis

Whole cell extracts were prepared by lysing in RIPA buffer containing the complete protease inhibitor cocktail and the phosphatase inhibitor cocktail (Roche, Penzberg, Germany). The cytosolic and nuclear fractions were separated by the NE-PER nuclear and cytoplasmic extraction kit (Thermo Fisher Scientific, Waltham, MA, USA). The cytosolic and membrane fractions were isolated using the ProteoExtract subcellular proteome extraction kit (Merk KGaA, Darmstdt, Germany). Samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and electroblotted onto PVDF membranes. Membranes were incubated with a primary antibody, followed by incubation with a horseradish peroxidase-conjugated secondary antibody. Immunolabeled proteins were detected using the ECL chemiluminescence kit (GE Healthcare, Piscataway, NJ, USA) and the LAS-4000 lumino-image analyzer (Fujifilm, Tokyo, Japan).

2.5. Quantitative RT-PCR

Total RNA was extracted from cells by the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) followed by DNase I treatment. cDNA was synthesized using the PrimeScript reagent kit (Takara Bio, Ohtsu, Japan) and subjected to quantitative RT-PCR using the Thermal Cycler Dice real-time PCR system (TP800, Takara Bio). Primers for *iNOS* and *GAPDH* were purchased from Takara Bio. The expression level of *iNOS* gene was determined using the comparative C_t method and normalized to that of *GAPDH*. The PCR consisted of 45 cycles (95 °C for 10 s, 60 °C for 40 s and 72 °C for 1 s) after an initial denaturation step (95 °C for 10 min).

2.6. Measurement of intracellular ROS levels

Intracellular levels of reactive oxygen species (ROS) were determined using a cell-permeable fluorescent probe, CM-H_2DCF-DA (Invitrogen). Cells were incubated with 10 μM CM-H_2DCF-DA for 1 h at 37 °C. After treatment, cells were washed twice with PBS and lysed in RIPA buffer. The absorbance of the lysates was measured with excitation at 490 nm and emission at 530 nm using the MTP-600 fluorometric imaging plate reader (Corona Electric, Ibaraki, Japan).

2.7. Hydrogen treatment of mice

Five-weeks-old female BALB/c Cr Slc mice (Japan SLC, Hamamatsu, Japan) were fed with either hydrogen-rich or control water *ad libitum*, as described previously [3]. Hydrogen-rich water packed in aluminum pouches was purchased from Blue Mercury (Tokyo, Japan). The hydrogen concentration of the hydrogen-rich water was approximately 1.0 ppm. The control water was prepared by gently stirring the hydrogen-rich water in open air for 24 h. This study was approved by the Animal Use Committee of the Gifu

International Institute of Biotechnology and the animals were maintained according to the guidelines for the care of laboratory animals of the Gifu International Institute of Biotechnology.

2.8. Anti-type II collagen antibody-induced arthritis in mice

Inflammatory arthritis was induced using the arthritogenic mouse monoclonal anti-type II collagen 5 clone antibody cocktail (Iwai Chemical, Tokyo, Japan). Seven-weeks-old mice, which were fed with either hydrogen-rich or control water for 2 weeks, were injected intravenously with 1 mg of the arthritogenic cocktail. Hydrogen treatment continued after the injection. Three days later, 25 μg of LPS (*Escherichia coli* O111:B4) was injected intraperitoneally. Two weeks after the antibody injection, we took photographs of the hind and front paws, evaluated the arthritis scores and measured the foot volume of hind paws using the plethysmometer (MK550M, Muromachi-kikai, Tokyo, Japan). The arthritis score was determined by grading each of four paws on a 0–4 scale [12]. Thus, the total arthritis score of a given mouse varies in the range 0–16.

2.9. Statistical analysis

All data were analyzed using Student's *t*-test or two-way ANOVA followed by Fisher's multiple range test.

3. Results

3.1. Hydrogen inhibits LPS/IFNγ-induced NO release from RAW264 macrophage cells

Hydrogen treatment for 24 h did not affect cell viability and proliferation (data not shown). In order to explore possible interaction between NO and hydrogen, we examined the effects of hydrogen on LPS/IFNγ-induced NO release from murine RAW264 macrophage cells. Treatment with hydrogen significantly reduced the NO levels in the culture media, the inhibitory effect being more pronounced at 12 h than at 6 h after LPS/IFNγ stimulation (Fig. 1A). These results suggest that there exists a functional relationship between NO and hydrogen.

3.2. Hydrogen inhibits LPS/IFNγ-induced iNOS expression

Stimulation with LPS/IFNγ up-regulates expression of pro-inflammatory genes such as iNOS and COX2. As shown in Fig. 1B, LPS/IFNγ stimulation resulted in a robust increase in protein expression of iNOS and COX2 at 6 h after treatment, which was markedly suppressed by treatment with hydrogen. Consistent with these findings, quantitative RT-PCR demonstrated that hydrogen inhibits LPS/IFNγ-induced mRNA expression of *iNOS* at 3 h after stimulation (Fig. 1C). These results indicate that hydrogen is capable of inhibiting LPS/IFNγ-induced expression of iNOS, which may account for suppression by hydrogen of LPS/IFNγ-induced NO release from macrophage cells (Fig. 1A).

3.3. Hydrogen inhibits LPS/IFNγ-mediated signal transduction

LPS signaling enhances phosphorylation of MAPKs and IκBα, and thereby activates transcription factors such as AP1, ELK1 and NFκB, whereas IFNγ signaling increases expression of IRF1 via activation of JAK–STAT signaling. These transcription factors activated or up-regulated by LPS/IFNγ stimulation bind to the iNOS promoter and enhance NO production. LPS/IFNγ stimulation enhanced phosphorylation of MAPKs including p38, JNK and ERK as well as AKT and STAT1α (Fig. 2A). Hydrogen treatment inhibited LPS/IFNγ-induced phosphorylation of p38 and JNK, but did not affect that of ERK, AKT and STAT1α.

Phosphorylation of IκB proteins leads to its degradation and NFκB translocation into the nucleus. As shown in Fig. 2B, LPS/IFNγ stimulation enhanced phosphorylation of IκBα and reduced its cytosolic level, which was associated with a decrease in NFκB p65 subunit in the cytosol and its increase in the nuclei. Treatment with hydrogen suppressed the LPS/IFNγ-induced activation of the NFκB pathway.

Taken together, these results suggest that hydrogen suppresses LPS/IFNγ-mediated signal transduction by inhibiting phosphorylation of p38, JNK and IκBα, resulting in reduced iNOS expression and NO production.

3.4. Hydrogen inhibits LPS/IFNγ-induced phosphorylation of ASK1

Among protein kinases activated by LPS/IFNγ, p38, JNK and IκBα were specifically inhibited by hydrogen (Fig. 2A and B). Apoptosis signal-regulating kinase 1 (ASK1), which is activated by endotoxins such as LPS, has been shown to activate both p38 and JNK MAPKs [13]. We thus investigated whether ASK1 phosphorylation is affected by hydrogen. As shown in Fig. 2C, phosphorylation of ASK1 at Ser967 and Thr845 caused by LPS/IFNγ stimulation was attenuated by hydrogen treatment. In contrast, LPS/IFNγ-induced phosphorylation of TGFβ-activated kinase 1 (TAK1) at Ser412 and Thr184/187, which, as well as ASK1, has been implicated in TNF receptor associated factor (TRAF)-dependent signaling pathways [14], was not affected by treatment with hydrogen. These results indicate that hydrogen inhibits LPS/IFNγ-induced phosphorylation of ASK1.

3.5. Hydrogen does not affect LPS/IFNγ-induced NOX activation and ROS production

It has been shown that ASK1 is activated by endotoxins such as LPS through ROS production, which in turn activates p38 and JNK MAPKs [15]. Furthermore, a direct link between ASK1 and NADPH oxidase (NOX) has been reported [16]. Here we investigated whether inhibition by hydrogen of LPS/IFNγ-induced ASK activation is mediated by suppression of NOX activation and ROS production. As shown in Fig. 3A, hydrogen treatment did not affect LPS/IFNγ-induced ROS production. For NOX activation, in response to LPS/IFNγ stimulation, the levels of the cytosolic subunits of NOX, $p47^{phox}$ and $p67^{phox}$, were decreased in the cytosolic fraction and increased in the membrane fraction (Fig. 3B). However, treatment with hydrogen did not influence the LPS/IFNγ-induced translocation of $p47^{phox}$ and $p67^{phox}$ to the membranes. These results suggest that hydrogen does not affect LPS/IFNγ-induced NOX activation and ROS production.

3.6. Oral intake of hydrogen-rich water ameliorates anti-type II collagen antibody-induced arthritis in mice

The findings that hydrogen suppressed LPS/IFNγ-induced NO production in cultured macrophage cells prompted us to examine whether oral intake of hydrogen-rich water could ameliorate anti-type II collagen antibody-induced arthritis in mice, a model for human rheumatoid arthritis [17]. In this mouse disease model, following the injection of anti-type II collagen-specific monoclonal antibody, LPS is injected to increase the incidence and severity of the disease. As shown in Fig. 4A, erythema and swelling of the hind and front paws were alleviated in mice treated with hydrogen-rich water compared with those treated with control water. The arthritis score was significantly lower in hydrogen-rich water-treated mice than in control water-treated mice (Fig. 4B). Both left and right hind paw volumes were decreased in hydrogen-treated mice

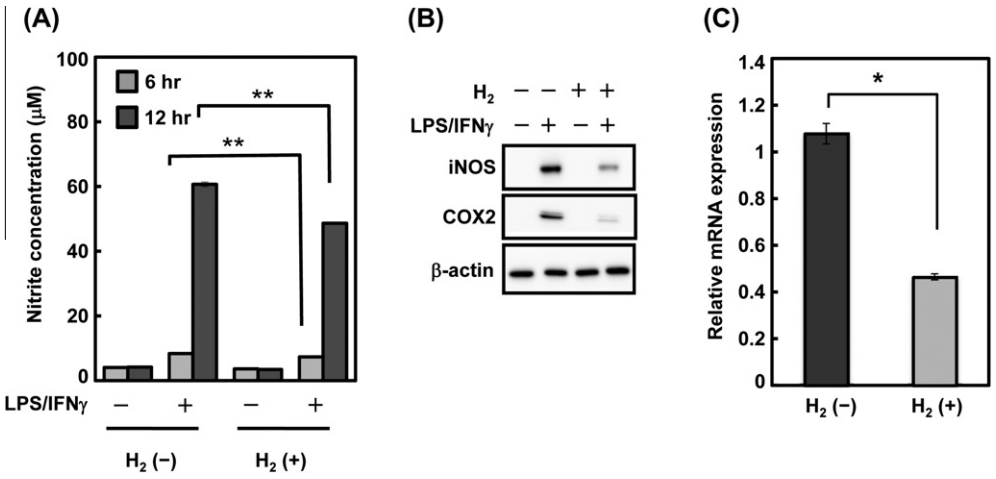

Fig. 1. Effects of hydrogen treatment on LPS/IFNγ-induced NO release and iNOS expression in RAW264 cells. RAW264 cells were incubated for 24 h in the presence or absence of hydrogen and then treated with or without LPS and IFNγ. (A) After incubation in the presence or absence of hydrogen for additional 6 and 12 h, cell culture media were harvested for measurement of nitrite, a stable metabolite of NO (mean ± SD, $n = 9$). Asterisks indicate statistical significance as determined by Student's t-test ($^{**}p < 0.01$). (B) After incubation in the presence or absence of hydrogen for additional 6 h, cell lysates were harvested and subjected to Western blot analysis for iNOS, COX2 and β-actin. A representative blot from three independent experiments is shown. (C) After incubation in the presence or absence of hydrogen for additional 3 h, total RNA was harvested and subjected to quantitative RT-PCR for *iNOS* (mean ± SD, $n = 3$). Asterisks indicate statistical significance as determined by Student's t-test ($^{*}p < 0.05$).

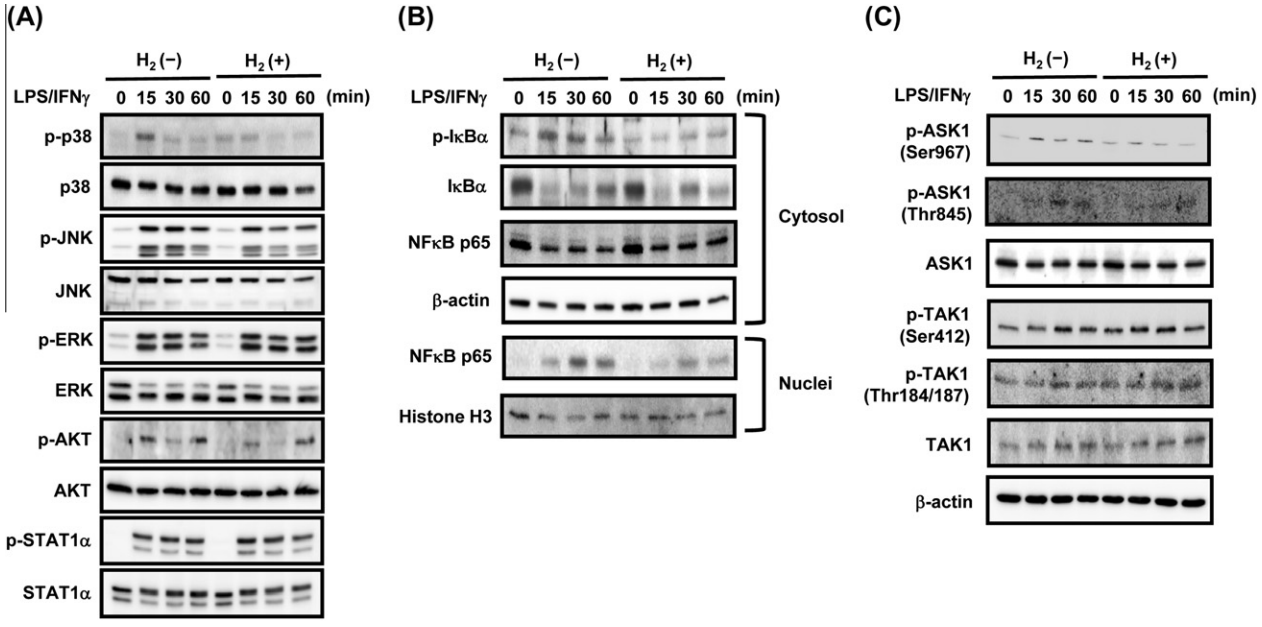

Fig. 2. Effects of hydrogen on LPS/IFNγ-mediated signal transduction in RAW264 macrophage cells. RAW264 macrophage cells were incubated for 24 h in the presence or absence of hydrogen and then treated with or without LPS and IFNγ. After incubation in the presence or absence of hydrogen for indicated time periods, cell lysates (A) and (C) and the cytosolic and nuclear fractions (B) were harvested and subjected to Western blot analysis for indicated proteins. A representative blot from three independent experiments is shown.

compared with control mice, but the statistical significance was observed only for the left hind paw ($p < 0.05$) (Fig. 4C). These results suggest that oral intake of hydrogen-rich water suppresses inflammation and alleviates arthritis in mice.

4. Discussion

Numerous papers have been published showing the efficacy of hydrogen treatment [1] since the first report in 2007 [2], in which specific scavenging of hydroxyl radical has been proposed as a mechanism accounting for the hydrogen effect. Most studies demonstrate reduced oxidative stress by hydrogen and assume that this is a major mechanism underlying the hydrogen effects. However, in type I allergy, hydrogen suppresses phosphorylation of FcεRI-associated Lyn and its downstream signaling molecules, which subsequently inhibits the NOX activity and reduces the generation of hydrogen peroxide [3]. Thus, we concluded that reduction of ROS by hydrogen in type I allergy is the consequence of inhibition of signal transduction, but not of direct radical scavenging activity.

Based on these findings, we hypothesized that hydrogen may ameliorate a wide variety of diseases, irrespective of their causal association with oxidative stress, through modulating yet

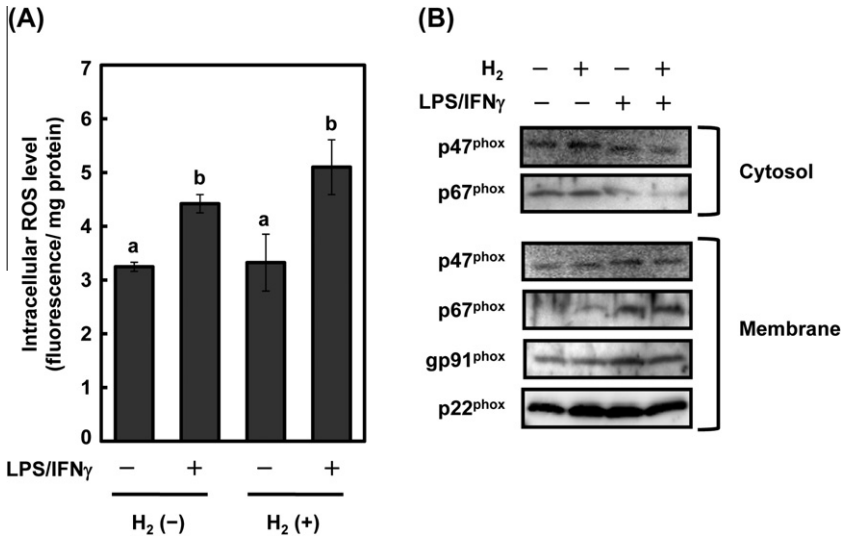

Fig. 3. Effects of hydrogen on LPS/IFNγ-induced NOX activation and ROS production in RAW264 macrophage cells. RAW264 macrophage cells were incubated for 24 h in the presence or absence of hydrogen. (A) Cells were incubated with 10 μM CM-H_2DCF-DA for 1 h in PBS and then treated with or without LPS/IFNγ. Three hours after incubation in the presence or absence of hydrogen, cell lysates were harvested and subjected to measurement of intracellular ROS (mean ± SD, $n = 6$). Statistical significance was determined by two-way ANOVA and Fisher's multiple range test ($p < 0.05$). (B) Cells were treated with or without LPS/IFNγ and then cultured in the presence or absence of hydrogen. Three hours later, the cytosolic and membrane fractions were separated and subjected to Western blot analysis for indicated proteins. A representative blot from three independent experiments is shown.

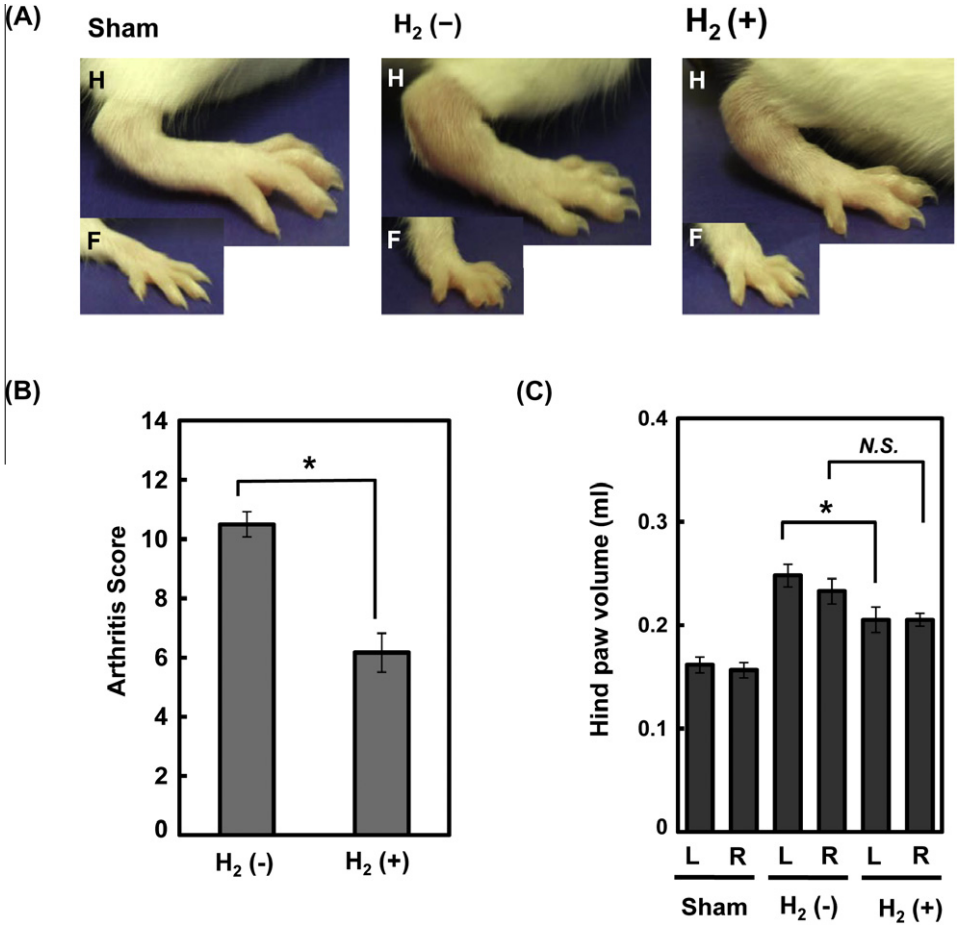

Fig. 4. Effects of oral intake of hydrogen-rich water on anti-type II collagen antibody-induced arthritis in mice. After treatment with or without hydrogen for 2 weeks, BALB/c Cr Slc female mice were injected intravenously with 1 mg of the arthritogenic mouse monoclonal anti-type II collagen antibody cocktail. Three days later, 25 μg of LPS was injected intraperitoneally. Two weeks after the antibody injection, photographs of the hind and front paws were taken (A), the arthritis score was evaluated (B), and the foot volume of hind paws was measured using the plethysmometer (C). Values are expressed as mean ± SD ($n = 5$). Asterisks indicate statistical significance as determined by Student's t-test ($^*p < 0.05$). N.S., not statistically significant; L, left; R, right.

unidentified signaling pathways and also that hydrogen may functionally interact with other gaseous signaling molecule, NO, carbon monoxide (CO) and hydrogen sulfide (H_2S). In an attempt to corroborate our hypotheses, we focused on inflammation because of the following reasons. First, it has been reported that hydrogen alleviates several inflammatory diseases [10,11], the mechanism of which, however, remains unknown. Second, in the inflammatory processes, LPS/IFNγ-induced activation of signal transduction leads to expression of iNOS, resulting in up-regulation of NO.

In the present study, we demonstrated that hydrogen suppresses LPS/IFNγ-induced NO release from macrophage cells (Fig. 1A), which was due to inhibition of LPS/IFNγ-induced expression of iNOS (Fig. 1B and C). We also revealed that LPS/IFNγ-induced phosphorylation of p38, JNK and IκBα was specifically suppressed by hydrogen (Fig. 2A and B). These results suggest that hydrogen may reduce binding to the iNOS promoter of several transcription factors such as AP1 and NFκB via inhibition of signal transduction.

Since our results showed that LPS/IFNγ-induced activation of p38 and JNK is inhibited by hydrogen, we investigated the effects of hydrogen on phosphorylation of ASK1, which is an upstream signaling molecule of both kinases [13]. Indeed, ASK1 phosphorylation was inhibited by hydrogen (Fig. 2C). We also asked if hydrogen directly inhibits phosphorylation of ASK1 *in vitro*, but found that ASK1 phosphorylation is not changed by the presence of hydrogen (Supplementary Fig. 1). On the other hand, ROS-dependent activation of the TRAF6–ASK1–p38 pathway is selectively required for TLR4-mediated innate immunity [15]. In addition, ASK1 is an important effector of NOX in the redox signaling [16]. We thus examined the effects of hydrogen on LPS/IFNγ-induced activation of NOX and subsequent production of ROS, but neither of them was affected by hydrogen treatment (Fig. 3).

In addition to ASK1, hydrogen strongly inhibited the NFκB signaling (Fig. 2B) as represented by a marked decrease in LPS/IFNγ-induced expression of COX2 as well as iNOS (Fig. 1B). In TLR4 signaling, LPS induces interaction of TLR4 with TRAF6, an E3 ubiquitin–protein ligase, and promotes TRAF6 auto-ubiquitination, leading to ubiquitination and activation of TAK1 [18]. TAK1 phosphorylates IκB kinases, which in turn phosphorylate IκB, resulting in IκB degradation and NFκB translocation into the nucleus. TAK1 also activates JNK and p38 MAP kinases by phosphorylating MKK4/7 and MKK3/6, respectively. We therefore investigated if hydrogen could inhibit LPS/IFNγ-induced phosphorylation of TAK1, but TAK1 activation was not affected by treatment with hydrogen (Fig. 2C).

Taken together, we demonstrated that hydrogen inhibits LPS/IFNγ-induced phosphorylation of ASK1 and its downstream signaling molecules (p38 and JNK) as well as IκBα in macrophage cells. Although we were unable to identify the exact molecule(s) that hydrogen directly modulates, we could narrow down the potential target sites. In TLR4 signaling, after formation of the LPS/CD14/TLR4 ligand receptor complex, MyD88 and IRAK as well as TRAF6 and TAK1 are recruited, and their interaction mediates downstream signaling. Our studies suggest that hydrogen modulates molecular events at the receptor or immediately downstream of it. In type I allergy, although the presence of the feed-forward loop in the FcεRI-mediated signal transduction prevented us from identifying direct target(s) of hydrogen, we proposed as a plausible mechanism that hydrogen may compromise the initial step of signal transduction, phosphorylation of Lyn kinase [3]. Together with the findings from the present studies, it is tempting to speculate that molecular hydrogen acts at or around the receptors. Nevertheless, the hypothesis remains to be proved by further studies.

In cultured macrophage cells, we showed that hydrogen inhibits TLR4 signaling that plays a critical role in induction of pro-inflammatory genes, providing the molecular bases for the hydrogen effects on inflammatory diseases. Finally, we studied *in vivo* effects of hydrogen on inflammation using a mouse model for human rheumatoid arthritis. It was found that oral intake of hydrogen-rich water suppresses inflammation and ameliorates anti-type II collagen antibody-induced arthritis (Fig. 4). Although we and others have demonstrated beneficial effects of hydrogen on inflammatory diseases in animal models, its efficacy in humans needs to be established in clinical trials.

We confirmed that hydrogen is capable of modulating signal transduction and suggested a role for molecular hydrogen as a signal modulator. Since a number of functional interactions among NO, CO and H_2S have been reported [19], it is conceivable that hydrogen may interact with other gas molecules. Indeed, we demonstrated a functional interaction between NO and hydrogen and elucidated the underlying mechanisms. Future studies will provide information about interactions among four gaseous signaling molecules and their physiological significance.

Competing interest statement

The authors declare no conflict of interest.

Acknowledgments

This work was supported by Grant for Biological Research from Gifu prefecture, Japan (Masafumi Ito) and Grants-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology, Japan (Masafumi Ito).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.06.116.

References

[1] S. Ohta, A. Nakao, K. Ohno, The 2011 Medical Molecular Hydrogen Symposium: An inaugural symposium of the journal Medical Gas Research, Med. Gas Res. 1 (2011) 10.
[2] I. Ohsawa, M. Ishikawa, K. Takahashi, M. Watanabe, K. Nishimaki, K. Yamagata, K. Katsura, Y. Katayama, S. Asoh, S. Ohta, Hydrogen acts as a therapeutic antioxidant by selectively reducing cytotoxic oxygen radicals, Nat. Med. 13 (2007) 688–694.
[3] T. Itoh, Y. Fujita, M. Ito, A. Masuda, K. Ohno, M. Ichihara, T. Kojima, Y. Nozawa, M. Ito, Molecular hydrogen suppresses FcepsilonRI-mediated signal transduction and prevents degranulation of mast cells, Biochem. Biophys. Res. Commun. 389 (2009) 651–656.
[4] C. Bogdan, Nitric oxide and the immune response, Nat. Immunol. 2 (2001) 907–916.
[5] S.B. Abramson, A.R. Amin, R.M. Clancy, M. Attur, The role of nitric oxide in tissue destruction, Best Pract. Res. Clin. Rheumatol. 15 (2001) 831–845.
[6] M.A. Gassull, Review article: the role of nutrition in the treatment of inflammatory bowel disease, Aliment. Pharmacol. Ther. 20 (Suppl. 4) (2004) 79–83.
[7] C. Nathan, Q.W. Xie, Regulation of biosynthesis of nitric oxide, J. Biol. Chem. 269 (1994) 13725–13728.
[8] E.D. Chan, D.W. Riches, IFN-gamma + LPS induction of iNOS is modulated by ERK, JNK/SAPK, and p38(mapk) in a mouse macrophage cell line, Am. J. Physiol. Cell Physiol. 280 (2001) C441–C450.
[9] A.T. Jacobs, L.J. Ignarro, Lipopolysaccharide-induced expression of interferon-beta mediates the timing of inducible nitric-oxide synthase induction in RAW 264.7 macrophages, J. Biol. Chem. 276 (2001) 47950–47957.
[10] K. Xie, Y. Yu, Z. Zhang, W. Liu, Y. Pei, L. Xiong, L. Hou, G. Wang, Hydrogen gas improves survival rate and organ damage in zymosan-induced generalized inflammation model, Shock 34 (2010) 495–501.
[11] M. Kajiya, M.J. Silva, K. Sato, K. Ouhara, T. Kawai, Hydrogen mediates suppression of colon inflammation induced by dextran sodium sulfate, Biochem. Biophys. Res. Commun. 386 (2009) 11–15.
[12] E. Douni, P.P. Sfikakis, S. Haralambous, P. Fernandes, G. Kollias, Attenuation of inflammatory polyarthritis in TNF transgenic mice by diacerein: comparative analysis with dexamethasone, methotrexate and anti-TNF protocols, Arthritis Res. Ther. 6 (2004) R65–R72.
[13] H. Nagai, T. Noguchi, K. Takeda, H. Ichijo, Pathophysiological roles of ASK1–MAP kinase signaling pathways, J. Biochem. Mol. Biol. 40 (2007) 1–6.
[14] M. Landstrom, The TAK1–TRAF6 signalling pathway, Int. J. Biochem. Cell Biol. 42 (2010) 585–589.

[15] A. Matsuzawa, K. Saegusa, T. Noguchi, C. Sadamitsu, H. Nishitoh, S. Nagai, S. Koyasu, K. Matsumoto, K. Takeda, H. Ichijo, ROS-dependent activation of the TRAF6–ASK1–p38 pathway is selectively required for TLR4-mediated innate immunity, Nat. Immunol. 6 (2005) 587–592.
[16] F. Jiang, Y. Zhang, G.J. Dusting, NADPH oxidase-mediated redox signaling: roles in cellular stress response, stress tolerance, and tissue repair, Pharmacol. Rev. 63 (2011) 218–242.
[17] K. Terato, D.S. Harper, M.M. Griffiths, D.L. Hasty, X.J. Ye, M.A. Cremer, J.M. Seyer, Collagen-induced arthritis in mice: synergistic effect of *E. coli* lipopolysaccharide bypasses epitope specificity in the induction of arthritis with monoclonal antibodies to type II collagen, Autoimmunity 22 (1995) 137–147.
[18] C. Wang, L. Deng, M. Hong, G.R. Akkaraju, J. Inoue, Z.J. Chen, TAK1 is a ubiquitin-dependent kinase of MKK and IKK, Nature 412 (2001) 346–351.
[19] M. Kajimura, R. Fukuda, R.M. Bateman, T. Yamamoto, M. Suematsu, Interactions of multiple gas-transducing systems: hallmarks and uncertainties of CO, NO, and H_2S gas biology, Antioxid. Redox Signal. 13 (2010) 157–192.