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

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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

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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