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Abstract

Molecular hydrogen ameliorates oxidative stress-associated diseases in animal models. We found that oral intake of hydrogen-rich water abolishes an immediate-type allergic reaction in mice. Using rat RBL-2H3 mast cells, we demonstrated that hydrogen attenuates phosphorylation of the FcεRI-associated Lyn and its downstream signal transduction, which subsequently inhibits the NADPH oxidase activity and reduces the generation of hydrogen peroxide. We also found that inhibition of NADPH oxidase attenuates phosphorylation of Lyn in mast cells, indicating the presence of a feed-forward loop that potentiates the allergic responses. Hydrogen accordingly inhibits all tested signaling molecule(s) in the loop. Hydrogen effects have been solely ascribed to exclusive removal of hydroxyl radical. In the immediate-type allergic reaction, hydrogen exerts its beneficial effect not by its radical scavenging activity but by modulating a specific signaling pathway. Effects of hydrogen in other diseases are possibly mediated by modulation of yet unidentified signaling pathways. Our studies also suggest that hydrogen is a gaseous signaling molecule like nitric oxide.

Keywords: molecular hydrogen; immediate-type allergic reaction; mast cell; FcεRI; signal transduction

Introduction

Type I allergy or immediate-type hypersensitivity is involved in a variety of allergic diseases such as bronchial asthma, rhinitis, conjunctivitis, pollinosis and urticaria. Mast cells are key effector cells in immunoglobulin E (IgE)-mediated immune responses including type I allergic reaction. High-affinity IgE receptor, FcεRI, expressed on the surface of mast cells is a heterotetrameric receptor composed of an IgE-binding α-subunit, β-subunit and two γ-subunits [1]. When antigens react with IgE molecules bound to FcεRI, aggregation of receptors and activation of signal transduction pathways take place, resulting in degranulation and release of preformed mediators, production of cytokines, and secretion of leukotriens [2].

In these two years, molecular hydrogen has been shown to exert beneficial effects in animal models of a number of oxidative stress-associated diseases. Inhalation of hydrogen gas is protective against cerebral infarction [3], hepatic ischemia/reperfusion injury [4], myocardial ischemia/reperfusion injury [5], neonatal hypoxic brain injury [6] and small intestinal transplantation-induced inflammation [7]. Similarly, oral intake of hydrogen-rich water is beneficial in stress-induced learning impairment [8], atherosclerosis [9], Parkinson's disease [10] and inflammatory bowel disease [11]. In humans, oral intake of hydrogen-rich water improves lipid and glucose metabolism in patients with diabetes and impaired glucose tolerance [12]. Based on the observations that hydrogen exclusively scavenges hydroxyl radical [3, 13], hydrogen effects in oxidative stress-associated diseases have been solely ascribed to the reduction of oxidative stress [3-12].

Immediate-type allergy is, however, not causally associated with oxidative stress,

and effects of hydrogen have not been reported to date. In the present study, we first demonstrated preventive effects of oral intake of hydrogen-rich water on type I allergic reaction in a mouse model. Using a mast cell culture model, we then elucidated that hydrogen attenuates degranulation through suppression of the FcεRI-mediated signal transduction. Effects of molecular hydrogen on signal transduction have not been documented to date. We propose that modulations of signaling pathways are essential underlying mechanisms of molecular hydrogen on a broad spectrum of diseases.

Materials and methods

Antibodies

Anti-dinitrophenol (DNP) IgE was from Yamasa (Tokyo, Japan). The antibodies to Akt, p-Akt, p44/42 MAP kinase (ERK1/2), p-p44/42 MAP kinase (Thr202/Tyr204), SAPK/JNK (JNK), p-SAPK/JNK (Thr183/Tyr185), p38 MAP kinase (p38), p-p38 MAP kinase (Thr180/Tyr182), Lyn, p-Lyn, $cPLA_2$, p-$cPLA_2$, p-PLCγ1, p-PLCγ2 and p-Syk were from Cell Signaling Technology (Beverly, CA). The antibodies against Syk, $p22^{phox}$, $p40^{phox}$, $p47^{phox}$, $p67^{phox}$, $gp91^{phox}$ and Rac were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-β-actin antibody was from Sigma (St. Louis, MO). Anti-FcεRIβ antibody was kindly provided by Dr. J. Rivera (NIH, Molecular Immunology and Inflammation Branch, Bethesda, MD).

Measurement of hydrogen concentrations

The hydrogen concentration was measured using an H2-N hydrogen needle sensor (Unisense, Aarhus, Denmark). We calibrated the sensor with hydrogen-saturated water (1.6 ppm). The water was made by bubbling 15 ml of water with 100% hydrogen gas for 30 min.

Animals and hydrogen treatment

Four-week-old male ICR mice (Japan SLC, Hamamatsu, Japan) were fed with CE-2 diets (Clea Japan, Tokyo, Japan) and with either hydrogen-rich or control water *ad libitum* for 2 or 4 weeks. Hydrogen-rich water was kindly provided by Blue Mercury Inc. (Tokyo, Japan). The control water was prepared by gently stirring hydrogen-rich water in open air for 24 h. The hydrogen concentration in hydrogen-rich water was approximately 1.0 ppm, while that in control water was the same as that in distilled water. In order to minimize the loss of hydrogen while feeding, the lid of glass vessel

was equipped with a metal tube containing a ball bearing. On every day basis, the vessels containing hydrogen-rich water given to mice were freshly prepared. 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.

Passive cutaneous anaphylaxis reaction

PCA reaction was performed according to the method previously described with a slight modification [14]. ICR mice were injected with 10 μg of anti-DNP IgE into two dorsal skin sites. Twenty-four hours later, mice received an intravenous injection of 200 μl of PBS containing 1 μg/μl of DNP conjugated with bovine serum albumin (DNP-BSA, Cosmo Bio, Tokyo, Japan) and 1% Evans blue (Sigma) through the tail vein. One hour later, mice were sacrificed and their dorsal skin was removed. After taking photographs of skin, a square area of skin (1 cm x 1 cm) was dissolved in 1N KOH at 37°C for 24 h and the extravasated Evans blue dye was extracted with a mixture of acetone and 0.2 M phosphoric acid (13:5). The amount of dye was determined optically at 620 nm.

Measurement of serum histamine levels

After PCA reaction, blood samples were taken from the abdominal aorta. The serum histamine levels were measured with the histamine ELISA kit (Shionogi, Osaka, Japan).

Cell culture and hydrogen treatment

Rat basophilic leukemia RBL-2H3 cells were obtained from Health Science Research Resource Bank (Tokyo, Japan). Cells were grown in Eagle's minimum essential medium containing 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA), 100 U/ml of penicillin and 100 μg/ml of streptomycin in a humidified atmosphere of 5% CO_2 at 37°C. For hydrogen treatment, RBL-2H3 cells were seeded

into 6- or 24-well plates and cultured for 4 h. Then, cells were incubated at 37°C for 24 h in the presence of IgE (0.45 μg/ml) either in culture medium containing molecular hydrogen (1.0 ppm) under a humidified condition of 75% H_2, 20% O_2 and 5% CO_2 or in culture medium under a humidified atmosphere of 95% air and 5% CO_2. We confirmed that the hydrogen concentration of the culture medium 24 h after hydrogen treatment was no less than 0.5 ppm.

β-Hexosaminidase release assay

After hydrogen treatment, cells were washed twice with Siraganian buffer (119 mM NaCl, 5 mM KCl, 0.4 mM $MgCl_2$, 25 mM PIPES, 40 mM NaOH, pH 7.2) and then 160 μl of Siraganian buffer containing 5.6 mM glucose, 1 mM $CaCl_2$ and 0.1% BSA was added. After incubation at 37ºC for 10 min, 20 μl of DNP-BSA was added at a final concentration of 10 μg/ml. Ten minutes later, reaction was terminated on ice and the supernatants were harvested by centrifugation. Fifty μl of the supernatants were transferred to a 96-well plate and mixed with 50 μl of 0.1 M citrate buffer (pH 4.5) including 1 mM p-nitrophenyl-N-acetyl-β-D-glucosaminide (Sigma). After incubation at 37ºC for 1 h, reaction was terminated by adding a stop buffer (0.1 M Na_2CO_3/$NaHCO_3$, pH 10.0). The absorbance was measured at 405 nm with an Immuno-Mini NJ-2300 colorimetric microplate reader (Nalge Nunc International, Tokyo, Japan).

Western blot analysis

Ten minutes after incubation with DNP-BSA in Siraganian buffer, cells were harvested by centrifugation. After washing twice in PBS, cells were resuspended in RIPA buffer containing the Complete protease inhibitor cocktail (Roche, Penzberg, Germany) and the Phosphatase inhibitor cocktails 1 and 2 (Sigma). The cytosolic and membrane

fractions were separated by using the ProteoExtract subcellular proteome extraction kit (Merk KGaA, Darmstdt, Germany). The lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto a PVDF membrane. Membranes were incubated with a primary antibody, followed by incubation with a horseradish peroxidase-conjugated secondary antibody. After washing, proteins were detected using the ECL chemiluminescence kit (GE Healthcare, Piscataway, NJ) and the LAS-4000 lumino-image analyzer (Fuji Film, Tokyo, Japan).

Measurement of intracellular calcium concentrations

After hydrogen treatment, cells were washed twice with PBS and incubated for 1 h with 1 ml of loading buffer containing Fluo-3AM (Calcium Kit-Fluo 3, Dojindo Laboratories, Kumamoto, Japan). Then, cells were washed twice with PBS and incubated in 900 μl of loading buffer for 10 min. Two minutes after adding 100 μl of DNP-BSA at a final concentration of 10 μg/ml, cells were washed twice with PBS and then 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, Hitachinaka, Japan).

Measurement of intracellular ROS, H_2O_2 and superoxide levels

Intracellular levels of ROS, H_2O_2 and superoxide were determined using cell-permeable fluorescent probes: CM-H_2DCF-DA detects superoxide, H_2O_2 and hydroxyl radical (Invitrogen); BES-H_2O_2 (Wako, Osaka, Japan), H_2O_2; and BES-So (Wako), superoxide. After hydrogen treatment, cells were washed twice with PBS and incubated with 10 μM CM-H_2DCF-DA, 25 μM BES-H_2O_2 or 25 μM BES-So for 1 h at 37°C. Then, cells were washed twice with PBS and stimulated by DNP-BSA at 10 μg/ml. Ten minutes later, 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).

Statistical analysis

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

Results

Oral intake of hydrogen-rich water attenuates PCA reaction in ICR mice.

PCA is an animal model of immediate-type allergic reaction, which has been widely used to evaluate effects of anti-allergic drugs [14]. We first examined effects of hydrogen on PCA reaction. ICR mice were fed with either hydrogen-rich or control water for 2 or 4 weeks followed by PCA assays. In mice treated with hydrogen-rich water, leakage of Evans blue dye from circulation to skin was efficiently abolished (Fig. 1A). As shown in Fig. 1B, pretreatment with hydrogen-rich water for 2 and 4 weeks decreased the amounts of dye to approximately 40% of those of control water-treated mice ($p < 0.05$ and $p < 0.01$, respectively). Consistent with these results, the serum histamine levels were markedly reduced in mice treated with hydrogen-rich water for 4 weeks compared with those treated with control water ($p < 0.01$) (Fig. 1C). Pretreatment for 2 weeks similarly exhibited a decrease of the serum histamine levels but without statistical significance. These results indicate that oral intake of hydrogen-rich water efficiently ameliorates type I allergic reaction.

Hydrogen inhibits the FcεRI-mediated signal transduction in mast cells.

In an attempt to elucidate molecular mechanisms underlying the inhibition by hydrogen of allergic reactions in mice, we investigated its effects on antigen-induced degranulation in IgE-sensitized rat basophilic leukemia RBL-2H3 cells representing mast cells. Hydrogen treatment significantly reduced the release of β-hexosaminidase, a marker of degranulation ($p < 0.05$), indicating that degranulation is suppressed by hydrogen in cultured cells as well as in mice (Fig. 2A). Antigen-induced aggregation of FcεRI receptors initiates activation of Lyn- and Syk-tyrosine kinases, which is followed

by activation of several signal transduction pathways including phospholipase Cγ (PLCγ), phosphatidylinositol 3-kinase (PI3-K)/Akt kinase, mitogen-activated protein kinase (MAPK) and calcium influx pathways, thereby leading to allergic responses including degranulation (see Fig. S1). Western blot analysis revealed no difference in the expression levels of FcεRIβ between cells treated with and without hydrogen (Fig. 2B). Hydrogen treatment, however, downregulated antigen-induced phosphorylation of Lyn as well as its downstream targets including Syk, PLCγ1, PLCγ2, Akt, extracellular signal–regulated kinases 1 and 2 (ERK1/2), c-Jun N-terminal kinase (JNK), p38 and cytosolic phospholipase A2 ($cPLA_2$). On the other hand, hydrogen had no effect on other signaling molecules such as apoptosis signal-regulating kinase 1 (ASK1) and nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IkBα). In addition, the intracellular calcium increase after antigen challenge was significantly lower in hydrogen-treated cells than in control cells ($p < 0.05$) (Fig. 2C). These results suggest that hydrogen attenuates phosphorylation of the most upstream FcεRI-associated Lyn and its downstream targets, which culminates in reduced degranulation of mast cells.

Hydrogen attenuates the NADPH oxidase activity in mast cells.

Activation of the FcεRI-mediated signaling pathway enhances the NADPH oxidase activity (see Fig. S1). This is mediated by activation of PLCγ-mediated intracellular calcium release and calcium influx, PI3-K/Akt signaling pathway, and Rac small G protein [15-18]. The activated Rac associates with the cytosolic subunits of NADPH oxidase ($p40^{phox}$, $p47^{phox}$, $p67^{phox}$). Subsequently, the complex translocates to the plasma

membrane and interacts with the membrane-bound subunits of NADPH oxidase ($p22^{phox}$, $gp91^{phox}$). Hydrogen is thus predicted to downregulate the NADPH oxidase activity. Indeed, hydrogen treatment of mast cells decreased the levels of the $p40^{phox}$, $p47^{phox}$, and $p67^{phox}$ subunits of NADPH oxidase as well as Rac in the membrane fraction (Fig. 3). Conversely, hydrogen elevated their levels in the cytosolic fraction, which supports the notion that hydrogen compromised translocation of these molecules to the membrane. These results indicate that hydrogen attenuates the NADPH oxidase activity.

Hydrogen decreases antigen-induced production of H_2O_2 in mast cells.

NADPH oxidase generates superoxide, which is immediately converted to hydrogen peroxide (H_2O_2). Measurement of DCF signals demonstrated that hydrogen efficiently decreases production of reactive oxygen species (ROS) in response to antigen challenge ($p < 0.05$) (Fig. 4). Further analysis using specific probes (BES-H_2O_2 and BES-So) disclosed that this is due to decreases of H_2O_2 and superoxide ($p < 0.05$) (Fig. 4). Hydrogen accordingly attenuates the NADPH oxidase activity (Fig. 3), and consequently reduces production of H_2O_2 (Fig. 4). This is unlikely to be mediated by direct scavenging of H_2O_2 by hydrogen, because hydrogen exclusively reduces hydroxyl radical but not superoxide or H_2O_2 *in vitro* and in cells [3, 13].

Inhibition of NADPH oxidase results in suppression of the FcεRI-mediated signal transduction in mast cells.

H_2O_2 generated by NADPH oxidase serves as a signaling molecule and facilitates the FcεRI-mediated signaling pathway [19]. The identity of the target molecule that H_2O_2 activates, however, remains elusive in mast cells. We thus sought for the target molecule

using an NADPH oxidase inhibitor, diphenyleneiodonium (DPI). Blockade of NADPH oxidase suppressed antigen-induced phosphorylation of Lyn and its downstream targets including Syk, PLCγ1, PLCγ2, Akt, ERK1/2, p38 and $cPLA_2$ (Fig. S2), indicating that the most upstream FcεRI-associated Lyn is the target of the feed-forward loop (Fig. S1).

Discussion

We demonstrated preventive effects of oral intake of hydrogen-rich water on type I allergic reaction in a mouse model and elucidated molecular mechanisms underlying the hydrogen effects. Hydrogen attenuated type I allergy in mice through suppression of the FcεRI-mediated signal transduction. Our studies suggest that hydrogen may be effective for a wide variety of allergic diseases such as bronchial asthma, rhinitis, conjunctivitis, pollinosis and urticaria in humans. However, before making a recommendation to patients, efficacy of hydrogen therapy on each of allergic diseases has to be validated by large-scale clinical trials.

We also proved that inhibition of NADPH oxidase attenuates phosphorylation of Lyn and its downstream targets in RBL-2H3 mast cells, indicating the presence of a feed-forward loop that that potentiates the allergic response. Hydrogen attenuated all the tested molecules within the feed-forward loop, as well as its downstream molecules. Although the presence of the loop prevents us from identifying the exact molecule(s) that hydrogen directly modulates (Fig. S1), one plausible explanation for the hydrogen effect on the signal transduction is that hydrogen compromises the initial step, the phosphorylation of Lyn, which subsequently attenuates molecules within the loop.

ROS cause damage to nucleic acids, proteins, and lipids, leading to cellular dysfunction [20]. Among them, hydroxyl radical is most toxic. H_2O_2 also exerts toxic effects at high concentrations, but acts as a signaling molecule at lower concentrations. Previous studies showing effects of hydrogen on oxidative stress-mediated diseases [3-12] exploits the unique property of hydrogen that can only reduce hydroxyl radical [3, 13]. In diseases where oxidative stress plays a less important role in the pathogenesis, however, beneficial effects of hydrogen can not be simply accounted for by the removal

of hydroxyl radical. The immediate-type allergic reaction is not causally associated with oxidative stress, and indeed the hydrogen effect resides not in its radical scavenging activity but in its modulating activity of a specific signaling pathway. Our results imply that hydrogen may ameliorate a wide variety of diseases, irrespective of their causal association with oxidative stress, through modulating yet unidentified signaling pathways.

Remarkable amounts of hydrogen are produced by intestinal bacteria such as *Escherichia coli* expressing hydrogenases [21]. Kajiya and colleagues report that hydrogen released from intestinal bacteria can suppress Concanavalin A-induced hepatitis in mice [22]. They suggest that hydrogen produced by commensal bacteria in intestinal flora may enhance the host's resistance to oxidative stress. Internally produced hydrogen as well as exogeneously administered hydrogen may serve as a modulator of signal transduction, thereby exerting biological effects in physiological and pathological conditions.

Nitric oxide (NO) [23], carbon monoxide (CO) [24], and hydrogen sulfide (H_2S) [25] are gaseous signaling molecules and play important roles in a variety of biological processes. For example, NO activates cytosolic guanylate cyclase and thereby increases the cyclic guanosine monophosphate (cGMP) concentration, which causes relaxation of smooth muscle and inhibition of platelet aggregation. In the current studies, we demonstrate that hydrogen attenuates the FcεRI-mediated signal transduction, which implies that hydrogen is the fourth gaseous signaling molecule after NO, CO, and H_2S.

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Competing Interests Statement

The authors declare no conflict of interest.

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Figure legends

Figure 1. Effects of oral intake of hydrogen-rich water on PCA reaction in ICR mice. ICR mice fed with either hydrogen-rich or control water for 2 or 4 weeks were given injection of anti-DNP IgE. Twenty-four hours later, PBS containing DNP-BSA and Evans blue dye were intravenously injected. (A) One hour later, mice were sacrificed and their dorsal skin was photographed. Photographs shown represent three independent experiments, each containing 6 mice per group. Sham represents mice injected with IgE and Evans blue dye but without DNP-BSA. (B) After taking photographs, the amounts of Evans blue dye extracted from skin were determined. The inhibition of PCA reaction was expressed as a percentage of Evans blue content in control water-fed mice. Values are mean ± SD of 6 mice per group. Asterisks indicate statistical significance as determined by Student's-t test (*$p < 0.05$, **$p < 0.01$). Note that values represent extravasated as well as intravascular Evans blue. (C) One hour after antigen stimulation, blood samples were taken and the serum histamine levels were determined. Values are mean ± SD of 6 mice per group. Asterisks indicate statistical significance as determined by Student's-t test (**$p < 0.01$, N.S.; not significant).

Figure 2. Effects of hydrogen on the FcεRI-mediated signal transduction in rat RBL-2H3 mast cells. RBL-2H3 cells were incubated in the presence of IgE in culture medium with or without hydrogen. (A) Twenty-four hours after treatment with or without hydrogen, cells were challenged with DNP-BSA in Siraganian buffer. Ten minutes later, the mounts of β-hexosaminidase in the supernatants were determined. Values are mean ± SD of three independent experiments. Asterisk indicates statistical significance as determined by Student's-t test (*$p < 0.05$). Hydrogen concentration in

the medium was initially 1.0 ppm, and was maintained at no less than 0.5 ppm in 24 h. (B) Twenty-four hours after treatment with or without hydrogen, cells were incubated with or without DNP-BSA in Siraganian buffer. Ten minutes later, cells were harvested and subjected to Western blot analysis for indicated proteins. A representative blot from three independent experiments is shown. Ag represents antigen. (C) Twenty-four hours after treatment with or without hydrogen, cells were incubated for 1 h with Fluo-3AM. Two minutes after incubation with or without DNP-BSA, cell lysates were prepared and subjected to measurement of intracellular calcium levels. Values are mean ± SD of three independent experiments. Statistical significance was determined by one way ANOVA and Fisher's-multiple range test ($p < 0.05$).

Figure 3. Effects of hydrogen on the NADPH oxidase activity in rat RBL-2H3 mast cells. RBL-2H3 cells were incubated in the presence of IgE in culture medium with or without hydrogen. Twenty-four hours after treatment with or without hydrogen, cells were incubated with or without DNP-BSA in Siraganian buffer. Ten minutes later, the cytosolic and membrane fractions were separated and subjected to Western blot analysis for indicated proteins.

Figure 4. Effects of hydrogen on intracellular ROS, H_2O_2 and superoxide levels in rat RBL-2H3 mast cells. RBL-2H3 cells were incubated in the presence of IgE in culture medium with or without hydrogen. Twenty-four hours after treatment with or without hydrogen, cells were incubated with 10 μM CM-H_2DCF-DA for detecting ROS (H_2O_2, superoxide, hydroxyl radicals), 25 μM BES-H_2O_2 for H_2O_2 or 25 μM BES-So for superoxide for 1 h in PBS. Then, cells were challenged with or without DNP-BSA.

Ten minutes later, cell lysates were harvested and subjected to measurement of intracellular ROS (A), H_2O_2 (B) and superoxide (C) levels. Values are mean ± SD of three independent experiments. Statistical significance was determined by one way ANOVA and Fisher's-multiple range test ($p < 0.05$).

Figure 1

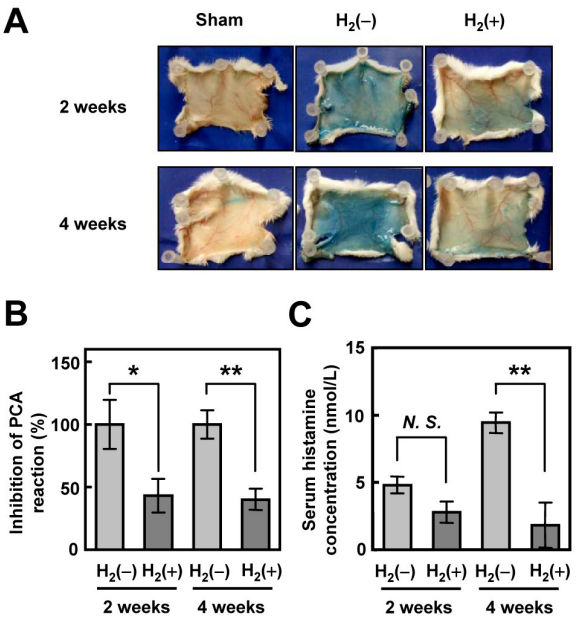

Figure 2

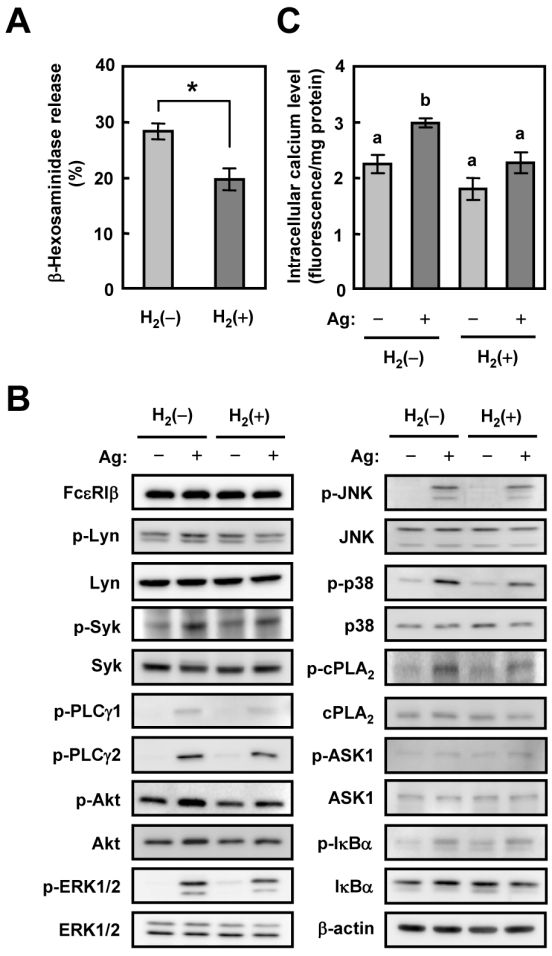

Figure 3

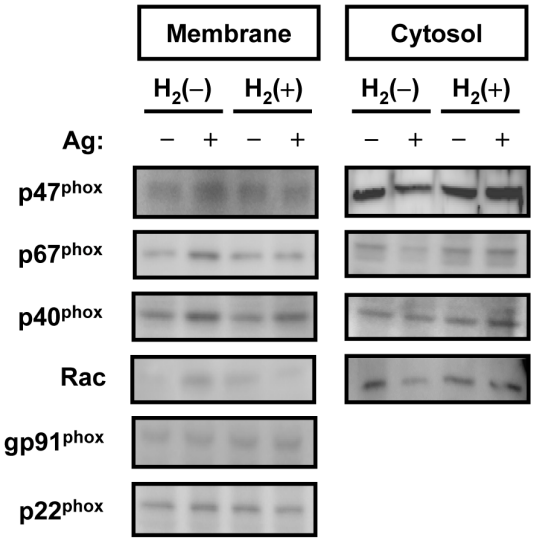

Figure 4