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Hydrogen from intestinal bacteria is protective for Concanavalin A-induced hepatitis

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ABSTRACT

It is well known that some intestinal bacteria, such as *Escherichia coli*, can produce a remarkable amount of molecular hydrogen (H_2). Although the antioxidant effects of H_2 are well documented, the present study examined whether H_2 released from intestinally colonized bacteria could affect Concanavalin A (ConA)-induced mouse hepatitis. Systemic antibiotics significantly decreased the level of H_2 in both liver and intestines along with suppression of intestinal bacteria. As determined by the levels of AST, ALT, TNF-α and IFN-γ in serum, suppression of intestinal bacterial flora by antibiotics increased the severity of ConA-induced hepatitis, while reconstitution of intestinal flora with H_2-producing *E. coli*, but not H_2-deficient mutant *E. coli*, down-regulated the ConA-induced liver inflammation. Furthermore, *in vitro* production of both TNF-α and IFN-γ by ConA-stimulated spleen lymphocytes was significantly inhibited by the introduction of H_2. These results indicate that H_2 released from intestinal bacteria can suppress inflammation induced in liver by ConA.

Introduction

The antioxidant effects of water dissolved with molecular hydrogen (H_2) was demonstrated in the mouse model of brain injury induced by ischemia reperfusion [1]. Following this study, several other reports also demonstrated that H_2 could suppress tissue injury in organs, such as liver, intestine and heart [2–4], caused by oxidative stress following ischemia reperfusion. Since a close link between inflammation and oxidative stress is well recognized, as each one activates the other, an efficient antioxidant agent should also suppress the inflammation induced in tissue-destructive diseases. However, few reports documenting the anti-inflammatory aspects of H_2 can be found.

Importantly, in past studies using animal models, H_2 has been exogenously applied in the form of gas or dissolved in water supplied to the animals [1–4]. However, it is also true that some intestinal bacteria, such as *Escherichia coli* (*E. coli*), can produce H_2 as a result of their possession of hydrogenases [5]. If, indeed, H_2 is released by intestinal bacteria [6], such internally produced H_2 should affect the host's resistance to oxidative as well as inflammatory stresses. Again, however, no studies have thus far addressed the effects of H_2, as produced by intestinal bacteria, on the host's resistance to inflammatory stimuli.

Concanavalin A (ConA) is a hemagglutinin that agglutinates blood erythrocytes and a mitogen which predominantly stimulates T cells. Therefore, it causes acute inflammation by the infiltration of activated lymphocytes, which results in massive necrotic tissue injury of hepatocytes accompanied by intrasinusoidal hemostasis [7,8]. Accordingly, ConA-induced hepatitis has been used as an experimental murine model that mirrors most of the pathogenic properties of human autoimmune hepatitis [9]. The resistance to ConA-induced hepatitis by athymic nude mice and SCID mice clearly demonstrates the permissive role T cells play in the induction of hepatic injury induced by ConA [10,11]. Although the tissue injury caused by ConA is limited to the liver [11], the underlying mechanism that explains such organ specificity is still unclear. Nevertheless, ConA-mediated T cell activation also increases the blood level of proinflammatory cytokines, including tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ), which are released from activated T cells and considered to play critical roles

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in the development of ConA-induced hepatic inflammation [12–14].

Using a mouse model of acute hepatitis induced by Concanavalin A, the present study examined (1) the amount of H_2 released from bacteria colonized in the intestines and (2) the effects of H_2 released from intestinal bacteria on the inflammation induced in liver.

Materials and methods

Animals

C57BL/6j mice (8- to 10-week-old males) were kept in a conventional room with a 12-h light-dark cycle at constant temperature. The experimental procedures employed in this study were approved by the Forsyth IACUC.

Establishment of GFP-expressing E. coli

Escherichia coli strain W3110 (ATCC 27325) and its *hypF* deletion mutant strain PMD23, which does not produce H_2, were used in this study (Supplementary Material 1; accessible online). HypF is indispensable for the synthesis of active hydrogenase because its absence results in >95% decrease in hydrogenase activity [15,16]. Using electroporation, both strains of *E. coli* were transfected with pGFPuv-vector (Clontech, Mountain View, CA) possessing an Ampicillin-resistant gene (Amp^r) in the promoter. The resulting two strains, *E. coli* $W3110^{gfp+}$ (Amp^r+/GFP+/HypF+) and *E. coli* $PMD23^{gfp+}$ (Amp^r+/GFP+/HypF−) were cultured in Luria–Bertani (LB) broth containing Ampicillin (100 μg/ml).

Measurement of molecular hydrogen

The molecular hydrogen (H_2) produced in organs of mice was measured using a needle-type Hydrogen Sensor (Unisense A/S, Aarhus, Denmark) following the method published by Hayashida et al. [3]. Immediately after mice were sacrificed under CO_2 inhalation, the needle-type Hydrogen Sensor was placed to the pilot paths prepared in organs by a 25-G needle. Otherwise, the Hydrogen Sensor was directly placed into blood sampled by cardiac puncture. The standard positive concentration of H_2 was prepared by saturation of H_2 gas in water (781 μM at 25 °C or 721 μM at 37 °C) at an atmospheric pressure, while non-treated control water was used for H_2 amount 0 μM. The diffusion factor of H_2 was always taken into account and adjusted (e.g., 0.7 μM/min from sampled blood in a plastic tube).

Generation of H_2 dissolved water

High purity H_2 gas (Airgas, Salem, NH) was ejected into water or culture medium until H_2 concentration reached to saturation (780 μM, at 25 °C). Then, H_2 at appropriate concentration was prepared by dilution. The saturated H_2 in water showed pH 7.6 and very high redox potential (ORP level −511 mV).

Concanavalin A-induced acute hepatitis model

Experimental Protocol-A. (1) Animals were supplied with water containing an antibiotics cocktail (Sulfamethoxazole, 8 mg/ml, and Trimethoprim, 1.6 mg/ml) or control antibiotics-free water ad libitum for 3 days. (2) For two additional days, both groups of animals were rested with antibiotics-free water ad libitum. (3)

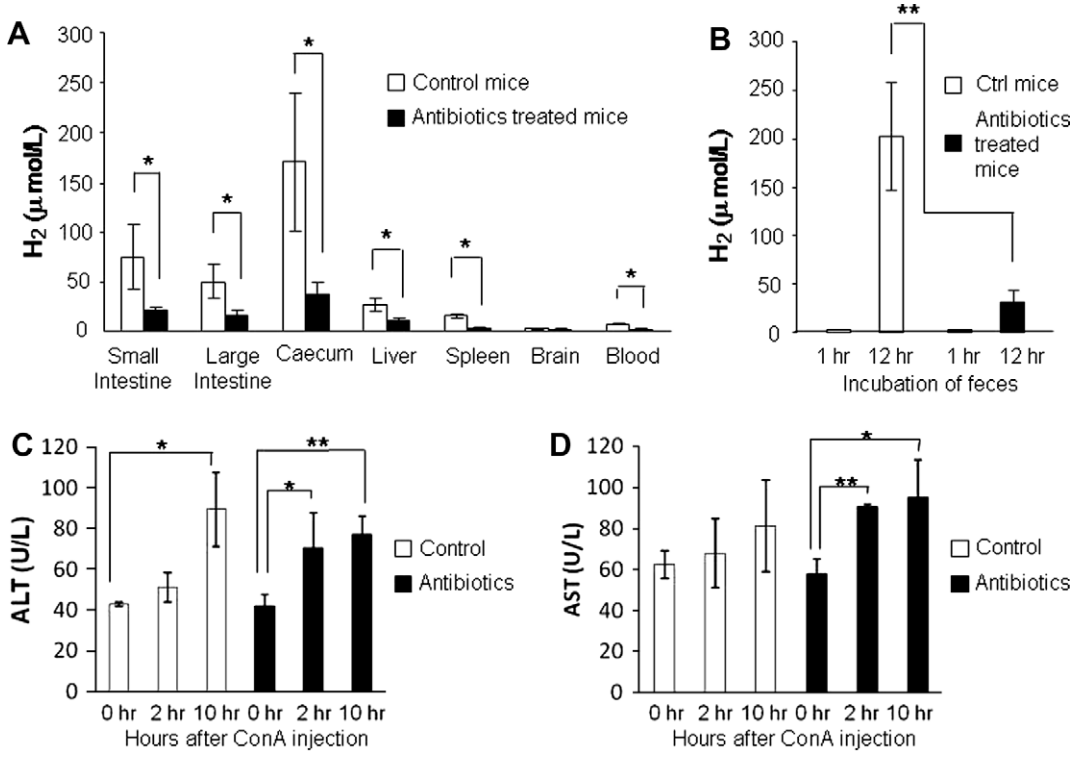

Fig. 1. Effects of systemic antibiotics treatment on the H_2 level in intestinal ducts and liver and the susceptibility of mice to ConA-induced hepatitis. (A) H_2 concentrations in different organs shown in the histogram were measured using a needle-type Hydrogen Sensor (n = 3/group). (B) Fresh fecal samples collected from the mice treated with or without antibiotics for 3 days followed by a 2-day resting period (feces, 20 mg/10 ml of LB broth, n = 3/group) were incubated for 1 h or 12 h at 37 °C, followed by measurement of H_2 in the bacterial culture. (C and D) ConA (15 mg/kg) was injected i.v. to the mice which were pretreated with or without antibiotics (Sulfamethoxazole, 8 mg/ml, and Trimethoprim, 1.6 mg/ml) for 3 days followed by a 2-day resting period with antibiotics-free water. The levels of ALT (C) and AST (D) in blood serum were measured. Data are shown as the mean ± SD of five mice per group. $^{*}p < 0.05$, $^{**}p < 0.01$: values differ significantly (t-test).

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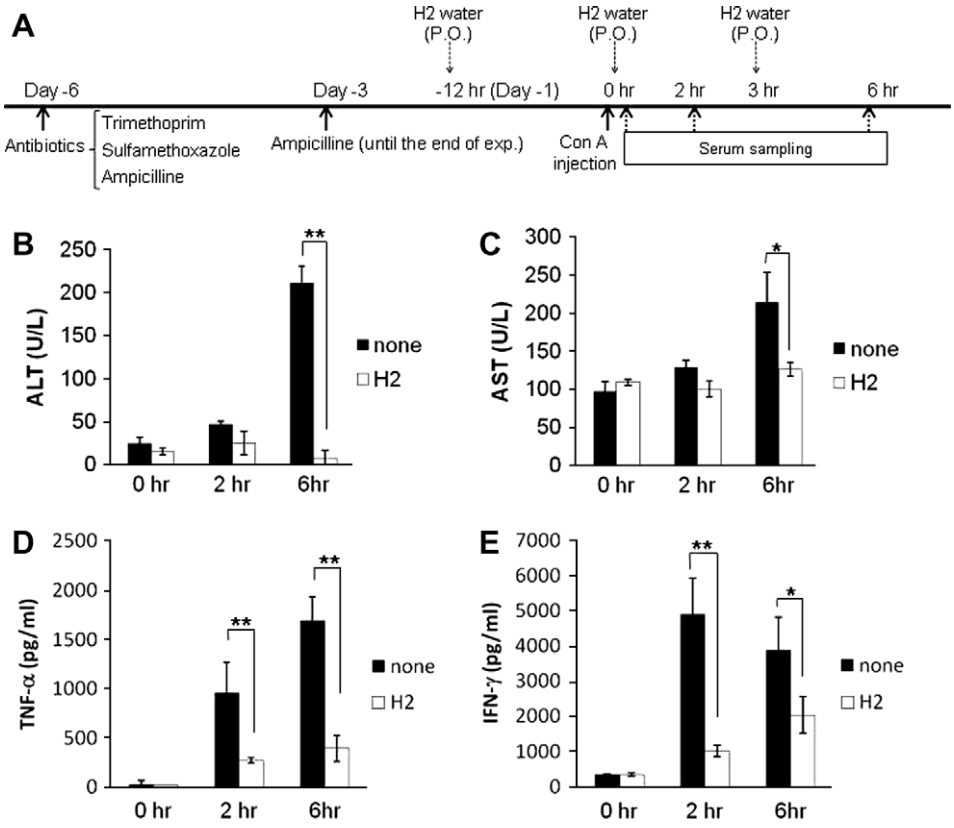

Fig. 2. Effects of exogenously applied H_2 on ConA-induced liver injury of C57BL/6j mice which were pretreated with antibiotics. (A) Diagram of Experimental Protocol-B: details are described in Materials and methods. The levels of ALT (C), AST (D), TNF-α (E) and IFN-γ (F) collected from mice at 0, 2 and 6 h after ConA injection were measured and presented in histograms. Columns and bars in each histogram (C, D, E and F) indicate mean ± SD of respective values (n = 5/group). $^{*}p < 0.05$, $^{**}p < 0.01$: values differ significantly (t-test).

ConA (Sigma, St. Louis, MO, 15 mg/kg; saline solution) was injected i.v. to both groups of mice, and ALT and AST in serum was monitored at 0, 2 and 10 h afterwards.

Experimental Protocol-B. (1) Animals were supplied with water containing an antibiotics cocktail (Sulfamethoxazole, 4 mg/ml; Trimethoprim, 0.8 mg/ml; and Ampicillin, 0.1 mg/ml) ad libitum for 3 days. (2) For three additional days, the animals were kept with drinking water containing Ampicillin (0.1 mg/ml) ad libitum. (3) ConA (15 mg/kg, saline solution) was injected to two groups of mice: (a) those receiving H_2-enriched water (780 μM, pH 7.6, 1 ml/mouse [p.o.], n = 5/group) or (b) those receiving control water (1 ml/mouse [p.o.], n = 5/group) at 12 h prior to ConA injection and 0 and 3 h after ConA injection. After ConA injection, both groups were still supplied with drinking water containing Ampicillin. The diagram of Experimental Protocol-B is shown in Fig. 2A.

Experimental Protocol-C. (1) Animals were supplied with water containing the same cocktail of three antibiotics as indicated in Protocol-B for 3 days. (2) For three additional days, the animals were kept with water containing Ampicillin (1 mg/ml) ad libitum. (3) ConA (15 mg/kg, saline solution) was injected to two groups of mice: (a) those reconstituted with *E. coli* W3110^{gfp+} (n = 5/group) or (b) those colonized with PMD23^{gfp+} (n = 5/group). The two strains of *E. coli* growing in the mid-log phase were harvested and applied (10^9 bacteria/100 μl saline with 5% carboxymethyl cellulose/mouse [p.o.]) using a Popper feeding needle at 2 days prior to ConA injection. Even after ConA injection, both groups were supplied with drinking water containing Ampicillin. The diagram of Experimental Protocol-C is shown in Fig. 3A.

Measurement of liver inflammation biomarkers and proinflammatory cytokines

The extent of liver injury was analyzed by determining the serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) using detection kits and following the manufacturer's instructions (Biotron Diagnostics, Hemet, CA). Quantification of proinflammatory cytokines, TNF-α and IFN-γ, was performed by enzyme-linked immunosorbent assay (ELISA) kits (PeproTech, Rocky Hill, NJ).

Analysis of liver histopathology

The left lobes of the livers sampled from sacrificed mice 10 h following ConA injection were processed for histological analysis by hematoxylin and eosin (H&E)-staining.

In vitro analyses of proliferation of spleen lymphocytes and their production of proinflammatory cytokines

The mononuclear lymphocytes were isolated from the spleen of C57BL/6j mice by a density gradient centrifugation using Histopaque (Sigma). In a 96-well plate, the lymphocytes (2×10^5/well) were pretreated with H_2 dissolved in RPMI medium supplemented with 10% FBS, L-glutamine and antibiotics (H_2 at concentrations of 175, 350 and 700 μM). The cells in the 96-well plate were then reacted with or without ConA (1 μg/ml) for 24 h, and the culture supernatants were subjected to ELISA for detection of TNF-α and IFN-γ. The spleen lymphocytes in the 96-well plate were further

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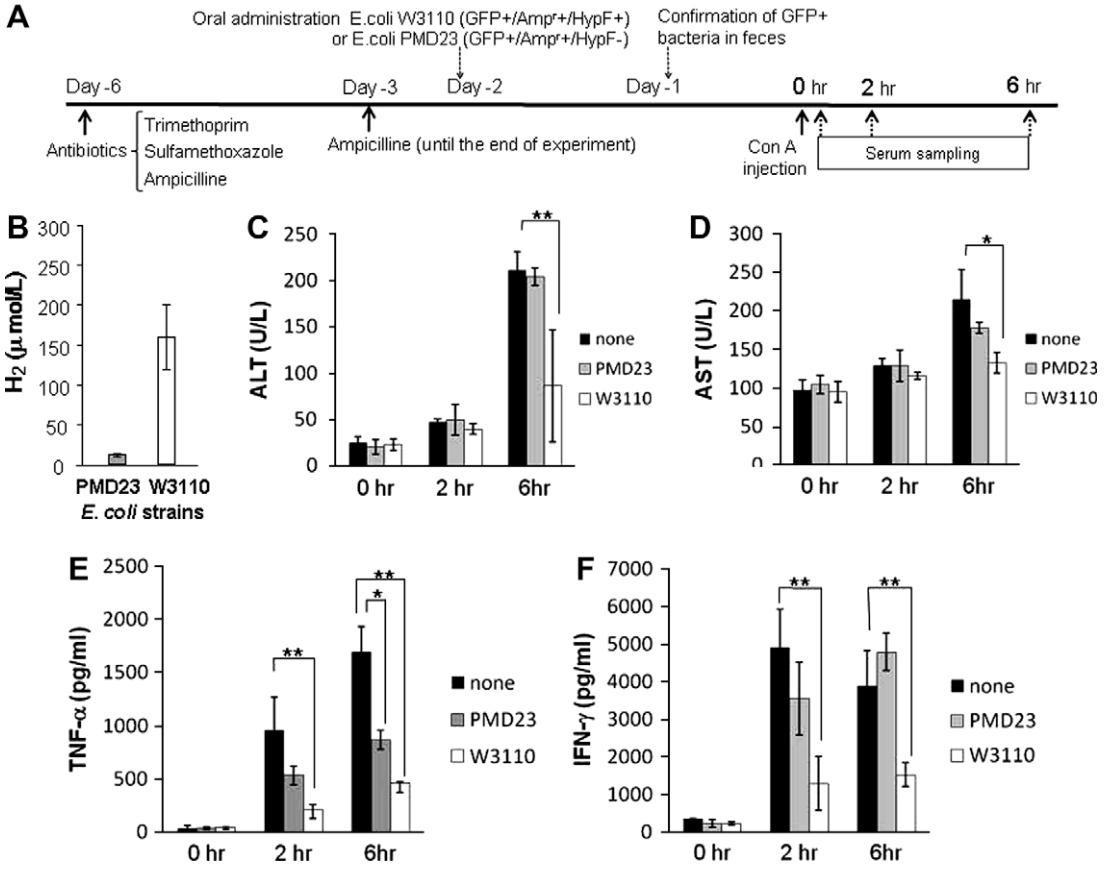

Fig. 3. Reconstitution of intestinal duct with H_2-producing *E. coli*, but not H_2-deficient *E. coli*, can down-regulate the ConA-induced liver injury in C57BL/6 mice pretreated with antibiotics. (A) Experimental Protocol-C: details are described in Materials and methods. (B) Level of H_2 production by *E. coli* strain PMD23 (Amp^r+/GFP+/HypF−) or W3110 (Amp^r+/GFP+/HypF+), as cultured in LB broth supplemented with Ampicillin (100 μg/ml) for 12 h. The levels of ALT (B), AST (C), TNF-α (D) and IFN-γ (E) collected from mice at 0, 2 and 6 h after ConA injection were measured and are shown in histograms as mean ± SD of respective values (n = 5/group). $^{*}p < 0.05$, $^{**}p < 0.01$: Significantly different by t-test.

incubated with [^{3}H] thymidine (0.5 μCi) for the last 16 h of a total 48 h culture, and the radioactivity incorporated in the cells under proliferation (cpm) was monitored by a radio scintillation counter.

Results

H_2 is produced in the intestine of animals as a byproduct of carbohydrate fermentation [17]. It was also demonstrated that H_2 concentrations in live mouse stomach or livers (about 20–80 μM) are over 20 times greater than the apparent whole-cell K_m for hydrogen [6,18]. Based on this evidence, we hypothesized that such elevated level of H_2 in abdominal organs is derived from intestinal bacteria. To test this premise, mice were treated with or without antibiotics (Sulfamethoxazole and Trimethoprim) for 3 days, followed by a 2-day resting period with antibiotics-free water. Thereafter, the effect of antibiotics in suppressing intestinal flora was confirmed by the culture of fresh feces in red blood agar plate (control non-treated mice, $1.6 \pm 0.5 \times \mathrm{Log}_{10}9$ CFU/g; antibiotics-treated mice, $7.0 \pm 6.1 \times \mathrm{Log}_{10}7$ CFU/g). Fig. 1A shows the amount of H_2 in different organs. The amount of H_2 detected in the caecum was highest, followed, in descending order, by small intestine, large intestine, liver, spleen and blood. A trace level of H_2 was detected in the brain. The systemic treatment of mice with antibiotics (Sulfamethoxazole and Trimethoprim) significantly decreased the amount of H_2 detected in all organs tested. The *ex vivo* culture of fresh fecal matter sampled from the mice treated with antibiotics also showed significantly lower H_2 production than the sample collected from control non-treated mice (Fig. 1B). These data demonstrate the antibiotics-dependent change of H_2, as measured *in situ* of mouse organs and by *ex vivo* feces culture, and indicate that H_2 in intestinal ducts, as well as liver and spleen, is directly derived from resident bacteria.

To explore whether the presence of commensal bacteria, which produce H_2 in intestinal ducts, affects the susceptibility of mice to ConA-induced liver injury, ConA (15 mg/kg) was injected i.v. to the mice which were pretreated with or without antibiotics (Experimental Protocol-A). Baseline levels of ALT and AST showed no difference between the mouse groups pretreated with or without antibiotics (Fig. 1C and D), indicating that antibiotics did not cause liver damage. The levels of ALT and AST in blood serum measured at 2 h were significantly elevated in mice receiving antibiotics, but did not differ from the control baseline level measured at 0 h (Fig. 1C and D). Histo-morphological analysis of liver also demonstrated that the level of tissue damage was worse in antibiotics-treated mice compared to control non-treated mice (see Supplementary Data 1), suggesting that antibiotics treatment increased the susceptibility of mice to ConA-induced hepatitis. In other words, without antibiotics, the presence of intestinal bacterial flora seems to give sufficient protection against the development of ConA-induced hepatitis.

If H_2 produced by intestinal bacteria is responsible for the protection of liver from ConA-induced inflammation, then the exogenous supplement of antibiotics-treated mice with H_2 should down-regulate the level of inflammatory responses to ConA challenge in the antibiotics-treated mice. To test this premise, the antibiotics-treated mice received water dissolved with H_2 (p.o.) (Experimental Protocol-B). As expected, exogenously applied H_2 by the oral route

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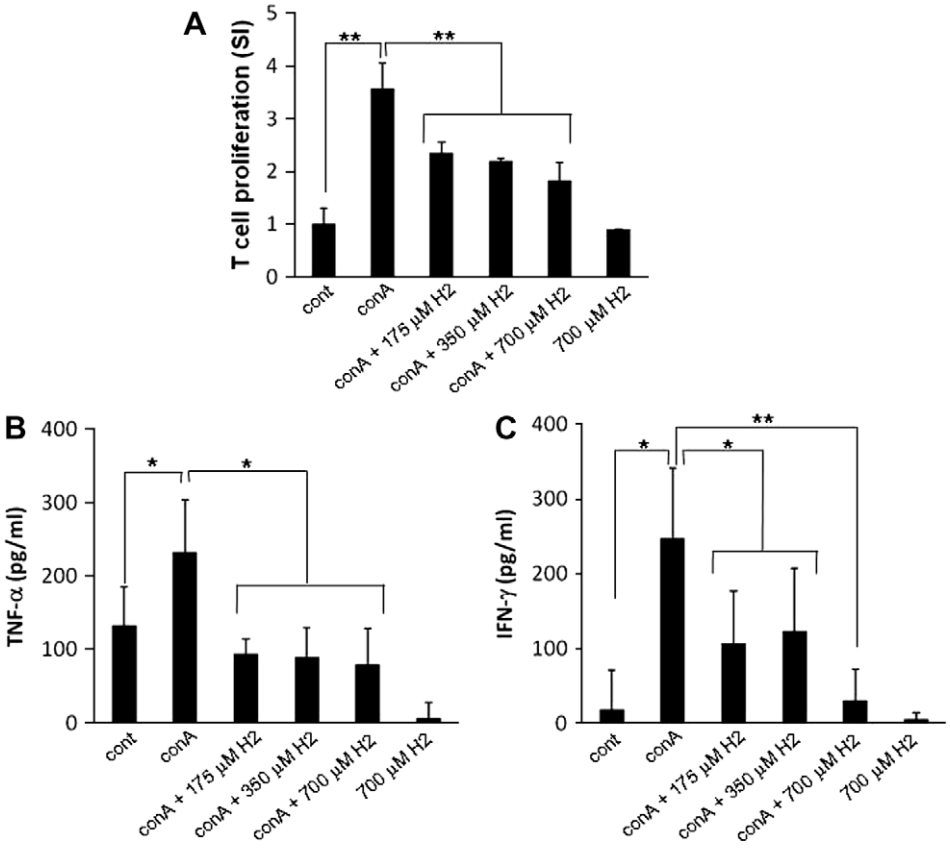

Fig. 4. *In vitro* effects of H_2 on the inflammatory responses by lymphocytes stimulated with ConA. Mononuclear lymphocytes isolated from C57BL/6 mouse spleen were pretreated with medium dissolved with H_2 at concentrations of 175, 350 and 700 μM for 1 h in a 96-well plate. The cells were then reacted with or without ConA (1 μg/ml) for 24 h to measure the expressions of proinflammatory cytokines using ELISA or for 48 h to assess the proliferation by [^{3}H] thymidine incorporation assay. Proliferation of lymphocytes (A) and production of TNF-α and IFN-γ in culture supernatant (B and C) are shown in histograms. SI (Stimulation Index): ratio of cpm for stimulated cells to the cpm for unstimulated cells. Columns and bars indicate mean ± SD of respective values of three different cultures. $^{*}p < 0.05$, $^{**}p < 0.01$: values differ significantly (*t*-test).

significantly suppressed the inflammatory ALT and AST biomarkers in antibiotics-treated mice measured at 6 h from ConA injection (Fig. 2B amd C). Importantly, the proinflammatory cytokines in serum, TNF-α and IFN-γ, which are produced by activated T cells, were also significantly down-regulated by application of H_2 in antibiotics-treated mice (Fig. 2D and E).

In order to examine the effects of H_2 derived from intestinal bacteria on ConA-induced liver injury, mice pretreated with antibiotics were reconstituted by two different strains of *E. coli*, i.e., (1) H_2-producing *E. coli* strain W3110^{gfp+} or (2) H_2-deficient *E. coli* strain PMD23^{gfp+}; then, ConA was injected i.v. (Fig. 3A, Experimental Protocol-C; Fig. 3B, H_2 production by W3110^{gfp+} and PMD23^{gfp+}). The colonization of both strains of *E. coli* in the mice which received the drinking water with Ampicillin was confirmed by the recovery of GFP+ bacteria from the feces of mice as cultured on agar plate containing Ampicillin. An elevated amount of H_2 was detected in the small and large intestines, caecum and liver of mice that were colonized with W3110^{gfp+}, whereas mice colonized with the PMD23^{gfp+} retained a low level of H_2 in those organs (see Supplementary Data 2; accessible online). Compared to the PMD23^{gfp+}-harboring- or control-mice, the levels of ALT and AST in the sera collected at 6 h after ConA injection were significantly lower in the W3110 strain mice (Fig. 3C and D). The serum levels of TNF-α and IFN-γ were also significantly suppressed in the mice harboring the W3110^{gfp+} compared to the PMD23^{gfp+}-harboring- or control-mice (Fig. 3E and F). Therefore, based on the results from Experimental Protocols-A, -B, and -C, H_2 released from intestinal bacteria seems to play a role in the suppression of the inflammation induced in liver by ConA injection.

It is thought that TNF-α and IFN-γ released from activated T cells cause hepatic tissue damage in the ConA-induced hepatitis model [12–14]. Therefore, to address whether H_2 can affect TNF-α and IFN-γ production in ConA-stimulated T cells, spleen mononuclear lymphocytes (MNL) were stimulated *in vitro* with ConA in the presence or absence of H_2. As shown in Fig. 4, the presence of H_2 in the culture medium significantly suppressed the proliferation of MNL (Fig. 4A), as well as the production of TNF-α and IFN-γ (Fig. 4B and C, respectively), compared to the stimulation of MNL with ConA in the absence of H_2. It is noteworthy that H_2 alone neither affected the proliferation nor the production of IFN-γ by non-stimulated MNL. Consequently, this *in vitro* study strongly supported the premise that H_2 can suppress ConA-mediated T cell activation which results in the tissue-destructive production of TNF-α and IFN-γ.

Discussion

Accumulated lines of evidence have suggested that intestinal resident bacteria possess a host protective function in the context of their commensal host relationship [19,20]. However, the underlying mechanism supporting such bacteria-mediated host protective function has been unclear. Some studies revealed that the intestinal blood system of germ-free mice is poorly vasculated compared to that of conventional mice, suggesting that intestinal commensal bacteria can affect the host development of homeostatic angiogenesis [21]. Since, however, H_2 produced from intestinal resident bacteria was shown to elicit an anti-inflammatory effect on Concanavalin A-induced hepatitis in mice, the present study demonstrated a novel anti-inflammatory mechanism mediated by intestinally colonized bacteria. If H_2 released from intestinal bacteria does play a role in the suppression of inflammation

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induced in liver by ConA injection, as demonstrated in our Protocols-A, -B, and -C, then it is plausible that that the micro-capillary network promoted by commensal bacteria facilitates the transportation of H_2 through the blood stream.

It is noteworthy that the anti-inflammatory effect of H_2 administered orally was higher than that of H_2 released from intestinal bacteria. The reverse is normally true since H_2 is constantly released from bacteria present in intestinal digestive content (about 1 g/mouse), whereas total water consumption is about 2 ml/day/mouse, and all H_2 from drinking water is immediately diffused from the stomach. Therefore, the relatively low anti-inflammatory potency of H_2 released from intestinal bacteria in our study might be most plausibly attributed to the scavenging of H_2 by other bacteria present deep inside the intestinal mucosa or in the stomach, such as *Helicobacter hepaticus* which is reported to consume significant amounts of H_2 [6]. To prove this hypothesis, detailed profiling of bacteria that either produce or consume H_2 in the oral gastrointestinal mucosa is required.

Although most previous studies examining the biological effects of H_2 addressed the oxidative tissue injury caused by ischemia reperfusion of organs, such as liver and brain [1–4], it has been unclear if H_2 can also affect the inflammation elicited by the activation of lymphocytes. Therefore, the novelty of this study derives from the finding that molecular hydrogen (H_2) produced from commensal bacteria seemed to suppress the tissue-destructive production of proinflammatory cytokines, TNF-α and IFN-γ, from the ConA-stimulated lymphocytes. Moreover, ROS can activate TNF-α expression by up-regulation of the NF-kB signaling pathway [22], while, at the same time, it can activate NADPH–Oxygenase (NOX) expression that generates ROS from NADPH [23]. Thus, both inflammation and oxidation processes are reciprocally related. Such multiplicity of cross reactions between ROS and inflammation indicates that the H_2-mediated suppression of TNF-α and IFN-γ from ConA-stimulated lymphocytes may also involve antioxidant effects by H_2.

In summary, the present study indicates that H_2 released from intestinally colonized bacteria can suppress inflammation induced in liver by Concanavalin A and that systemic antibiotics treatment may alter the number of host protective commensal bacterial flora in the intestines, ultimately resulting in a reduced concentration of H_2 present in the liver. Since most mammalians lack the catabolic enzyme to generate H_2, intestinal bacteria are the only possible source of protective H_2 in the liver. In fact, one of the roles of commensal bacteria in host defense may be defined by the ability of resident flora to produce anti-inflammatory H_2. Thus, exogenous factors, such as the introduction of antibiotics, may affect the functional amount of H_2 and, consequently, the organism's susceptibility to disease.

Acknowledgment

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.06.024.

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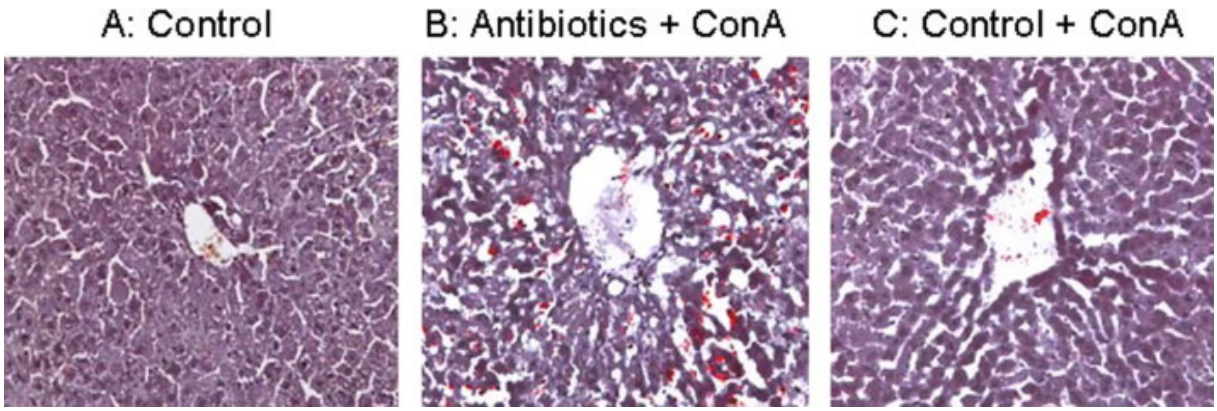
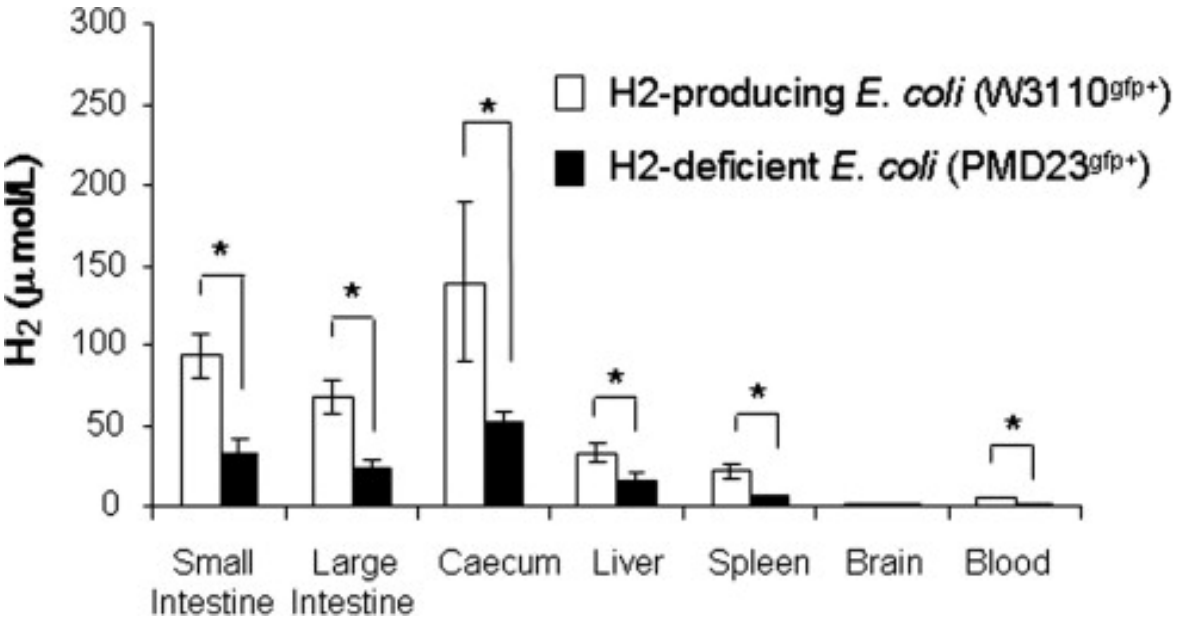


Supplementary data. Histological evaluation of mouse livers. Livers sampled from; (A) a normal mouse that did not receive antibiotics, (B) antibiotics-treated mouse 10 h after Con A injection, and (E) control non-treated mouse 10 h after Con A injection, were sectioned and stained with hematoxylin–eosin (HE) (original magnification 200×).



Supplementary data. Effects of reconstituted *E. coli* strains on the H2 concentration in different organs. Mice pretreated with antibiotics were reconstituted by two different strains of *E. coli*, i.e., (1) H2-producing *E. coli* strain W3110^{gfp+} or (2) H2-deficient *E. coli* strain PMD23^{gfp+} (Experimental Protocol-C). H2 concentrations in different organs were measured at Day-1 using a needle-type Hydrogen Sensor (n = 3/group). $^{*}p < 0.05$: values differ significantly between the columns indicated by a bracket (t-test) .

Bacterial strains. A wild type *Escherichia coli* strain W3110 (ATCC 27325) and a *hypF* deletion derivative, strain PMD23, that is defective in all three hydrogenase activities of the bacterium were used in this study. Strain PMD23 was constructed using the methods described by Datsenko and Wanner (1). For construction of strain PMD23, two PCR primers that also contain 50 bases long *hypF* DNA flanking the ends of the region to be deleted were used to amplify a kanamycin resistance gene cassette in plasmid pKD4 (ATGGCAAAAAACACATC TTGCGGT GTCCAAC TGCGTATTCGTGGCAAAGTGTGTAGGCTGGAGCTGCTTC and CACCCGCCGGTAAACTCTGTGGAAAGAGCAATGTGAAATCAGCGAGATAACATATG AATATCCTCCTTAGT; underlined, *hypF* sequence). The resulting PCR product was introduced into *E. coli* strain BW25113 (1) by electroporation and kanamycin-resistant transformants were selected on rich medium with kanamycin (50 mg/L) and tested for fermentative H_2 production (2). All kanamycin-resistant transformants tested were defective for H_2 production. Using phage P1 (3) the Δ*hypF*-FRT-kan^R-FRT mutation was transduced to wild type *E. coli* strain W3110 (strain PMD22). The kanamycin gene cassette in strain PMD22 was removed as described by Datsenko and Wanner (1) that left one FRT sequence at the site of deletion. The resulting kanamycin-sensitive strain, PMD23 carries a deletion of 2088 bp of the 2253 bp long *hypF* gene.

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