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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)

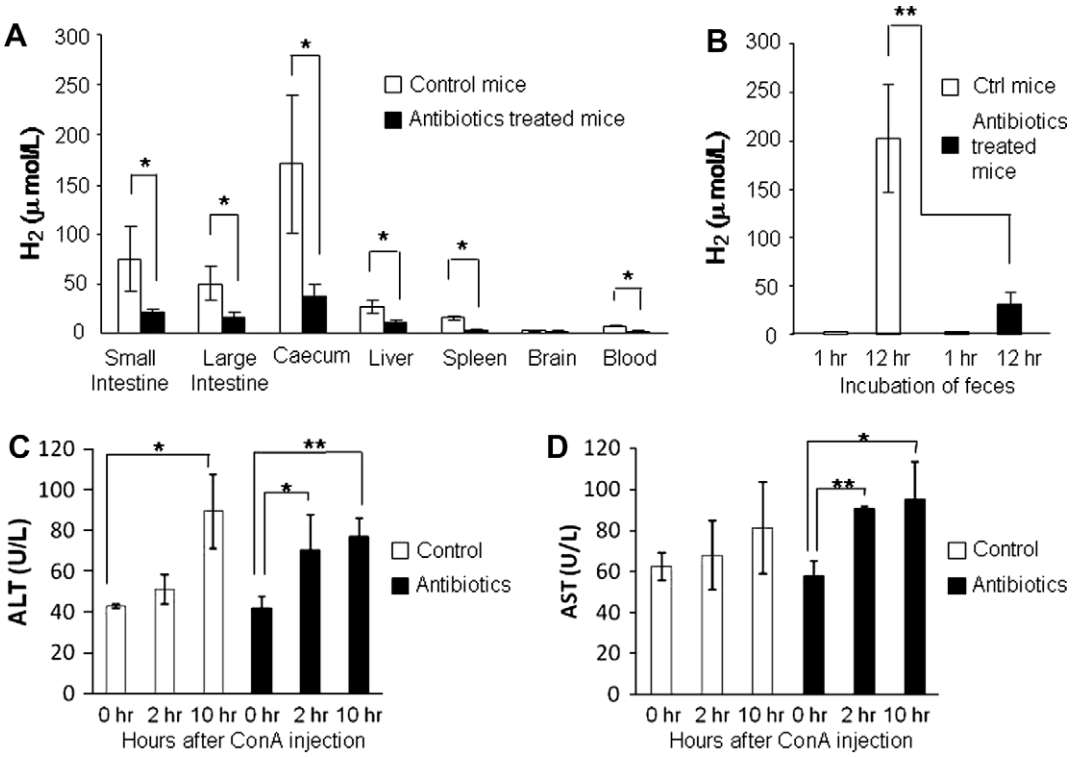


**Fig. 1.** Effects of systemic antibiotics treatment on the $H_2$ level in intestinal ducts and liver and the susceptibility of mice to ConA-induced hepatitis. (A) $H_2$ concentrations in different organs shown in the histogram were measured using a needle-type Hydrogen Sensor ($n$ = 3/group). (B) Fresh 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).