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Original Article

Effects of oral intake of hydrogen water on liver fibrogenesis in mice

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Aim: Liver fibrosis is the universal consequence of chronic liver diseases. Sustained hepatocyte injury initiates an inflammatory response, thereby activating hepatic stellate cells, the principal fibrogenic cells in the liver. Reactive oxygen species are involved in liver injury and are a promising target for treating liver fibrosis. Hydrogen water is reported to have potential as a therapeutic tool for reactive oxygen species-associated disorders. This study aimed to investigate the effects of hydrogen water on liver fibrogenesis and the mechanisms underlying these effects.

Methods: C57BL/6 mice were fed with hydrogen water or control water, and subjected to carbon tetrachloride, thioacetamide and bile duct ligation treatments to induce liver fibrosis. Hepatocytes and hepatic stellate cells were isolated from mice and cultured with or without hydrogen to test the effects of hydrogen on reactive oxygen species-induced hepatocyte injuries or hepatic stellate cell activation.

Results: Oral intake of hydrogen water significantly suppressed liver fibrogenesis in the carbon tetrachloride and thioacetamide models, but these effects were not seen in the bile duct ligation model. Treatment of isolated hepatocyte with 1 μg/mL antimycin A generated hydroxyl radicals. Culturing in the hydrogen-rich medium selectively suppressed the generation of hydroxyl radicals in hepatocytes and significantly suppressed hepatocyte death induced by antimycin A; however, it did not suppress hepatic stellate cell activation.

Conclusion: We conclude that hydrogen water protects hepatocytes from injury by scavenging hydroxyl radicals and thereby suppresses liver fibrogenesis in mice.

Key words: hydrogen, hydrogen water, hydroxyl radical, liver cirrhosis, liver fibrosis, liver injury

INTRODUCTION

LIVER FIBROSIS IS a common cause of death worldwide.[1] Advanced liver fibrosis disrupts normal liver architecture, causing hepatocellular dysfunction and portal hypertension. To date, no effective hepatic antifibrotic therapies are available.

The hepatic stellate cell (HSC) is the major fibrogenic cell type in the liver.[2] Sustained hepatocyte injuries such as those caused by hepatitis viruses and alcohol, lead to the activation of HSC and hepatocyte death, which is induced by the activation of inflammatory mediators such as transforming growth factor (TGF)-β1.[3] HSC are activated to differentiate into myofibroblast-like cells, which promote collagen deposition.

Reactive oxygen species (ROS) play an important role in hepatic fibrosis.[4] Galli *et al.* reported that the superoxide anion ($O\cdot_{2^-}$) produced by xanthine and xanthine oxidase induced the proliferation of HSC.[5] On the other hand, Sandra *et al.* reported that rather than directly inducing HSC proliferation, hydrogen peroxide (H_2O_2) and $O\cdot_{2^-}$ inhibited the proliferation of these cells.[6] To date, it is unclear how ROS contribute to the development of liver fibrogenesis.

Oxidative stress arises from the strong cellular oxidizing potential of ROS or free radicals. Most of the $O\cdot_{2^-}$ is generated in the mitochondria by electron leakage from the electron-transport chain and the Krebs cycle.[7,8] $O\cdot_{2^-}$ is also generated by metabolic oxidases, including nicotinamide adenine dinucleotide phosphate oxidase and

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xanthine oxidase.[5,9] Superoxide dismutase converts $O\cdot_2^-$ into H_2O_2. Excess $O\cdot_2^-$ reduces transition metal ions, such as Fe^{3+} and Cu^{2+} and the reduced forms can react with H_2O_2 to produce hydroxyl radicals ($OH\cdot$) by the Fenton reaction. $OH\cdot$ is the strongest ROS and reacts indiscriminately with nucleic acids, lipids and proteins.[10]

Recent studies have shown that hydrogen acts as an antioxidant. A study by Ohsawa *et al.* on a model of brain ischemia–reperfusion injury revealed that hydrogen was a selective antioxidant for $OH\cdot$.[11] The beneficial effect of hydrogen was also reported recently in various models of liver injury, such as ischemia–reperfusion injury and injury induced by galactosamine lipopolysaccharide.[12] Sun *et al.* reported that i.p. injection with hydrogen-rich saline was also effective in a model of carbon tetrachloride (CCl_4)-induced liver fibrosis.[12] However, they examined the effects only in a hepatic injury model, and the pathophysiological mechanism by which hydrogen-rich saline attenuates liver fibrosis remains to be elucidated. Further, the applicability of hydrogen would be more widespread if orally ingested hydrogen water (H_2-water) could effectively suppress liver fibrogenesis.

This study aimed to investigate the effects of H_2-water on various liver injury models and the mechanisms underlying these effects.

METHODS

Animals and treatment

MALE C57BL/6 MICE were purchased from Clea Japan (Tokyo, Japan). The mice were treated with CCl_4, thioacetamide (TAA) or bile duct ligation (BDL) to induce liver fibrosis. The mice were fed with H_2-water or control water while liver fibrosis was induced. In the CCl_4 model, the 7-week-old mice were injected i.p. with 0.5 μL of CCl_4 per gram mouse weight twice a week for 6 weeks ($n = 20$ for hydrogen water and control water group).[13] Corn oil was injected as a control ($n = 3$ for each group). In the TAA model, the mice were injected i.p. with escalating dose of TAA twice a week for 6 weeks (first dose, 100 μg/g; weeks 1–2, 200 μg/g; week 3, 4300 μg/g; weeks 4–6, 400 μg/g) ($n = 6$ for each group).[14] Normal saline was injected as a control ($n = 3$ for each group). BDL was performed by surgical ligation of the common hepatic bile duct with 6-0 nylon under pentobarbital anesthesia (50 mg/kg) ($n = 6$ for each group). Sham-operated mice underwent a laparotomy without ligation of the common bile duct ($n = 3$ for each group). The animals were killed on postoperative day 21. The animal protocols were approved by the Animal Research Committee of Kyoto University, and all experiments were conducted in accordance with Guidelines for the Care and Use of Laboratory Animals promulgated by the National Institute of Health.

H_2-water and hydrogen-rich medium

Hydrogen water at the concentration of 1.24 mg/L and control water were provided in 300-mL aluminum pouches supplied by I'rom Pharmaceutical (Tokyo, Japan). The mice were provided water ad libitum through closed glass vessels (I'rom Pharmaceutical) equipped with an outlet line containing two ball bearings, which prevented the degassing of the water. The vessel was freshly refilled with H_2-water every day.

The hydrogen-rich medium was prepared according to a method described previously.[15] In brief, hydrogen gas was dissolved directly into the media by bubbling the gas into the medium.

Isolation and culture of hepatocytes

Hepatocytes were isolated from mouse livers by a previously described method.[16] The isolated hepatocytes were cultured on six-well plates coated with type 1 collagen, at a cell density of 5×10^5 cells/well, in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Isolation and culturing of HSC

Hepatic stellate cells were isolated from mouse livers by a method described previously.[17,18] In brief, liver cells were dispersed with 0.025% PronaseE (Merck, Darmstadt, Germany) and 0.025% collagenase (Wako, Osaka, Japan). The cell suspension was centrifuged through 9.7% Nycodenz (Nycomed Pharm, Oslo, Norway) cushion. The HSC-enriched band was transferred into DMEM, supplemented with 10% fetal bovine serum and antibiotics. The isolated HSC was incubated in a CO_2 incubator at 37°C. The purity of HSC was consistently more than 98% as judged by presence of lipid droplets and autofluorescence.

To analyze whether the hydrogen-rich medium suppresses HSC activation, the isolated HSC were cultured in hydrogen-rich medium for 5 days, during which the medium was changed every day.

ROS induction with antimycin A and detection of ROS

To examine the effects of ROS induction for hepatocytes, the medium was replaced with serum-free

DMEM 6 h after plating. Twelve hours later, 1 μg/mL antimycin A (AMA; Sigma-Aldrich, St Louis, MO, USA) was added, and the cells were incubated for 1 h. Then, the cells were stained with bisbenzimide H33342 fluorochrome dihydrochloride dimethylsulfoxide solution (Hoechst 33342; Nacalai Tesque, Kyoto, Japan) and propidium iodide (PI; Calbiochem, San Diego, CA, USA) and examined under a fluorescent microscope. Five non-overlapping fields at ×200 magnifications were photographed at random. PI positive cells were considered as dead cells, and the PI positive ratio was calculated as the percentage of PI positive cells among the Hoechst 33342 positive cells. For the detection of $OH\cdot$, $O\cdot_2^-$ and H_2O_2, the cells were incubated with MitoSOX, 2′,7′-dichlorodihydrofluorescein (CM-H_2DCFDA) and hydroxyphenyl fluorescein (HPF; Nikken Seil, Shizuoka Japan), respectively.[19,20]

To examine whether HSC activation is accelerated by ROS, the medium was replaced with serum-free DMEM 24 h after plating. Twenty-four hours later, 1 μg/mL AMA (Sigma-Aldrich) was added, and the cells were incubated for 1 h. The cells were observed with a BIOREVO BZ-9000 digital microscope (Keyence, Osaka, Japan) at ×200 magnifications.

Immunohistochemistry

The specimens were fixed in 4% paraformaldehyde and embedded in optimal cutting temperature compound. Slices of the specimens at 4-μm thickness were obtained, and the antigen was retrieved by incubation in citric acid buffer at 90°C for 20 min. After being blocked, the sections were incubated with primary antibody-recognizing α-smooth muscle actin (α-SMA; no. ab5694; Abcam, Cambridge, UK) at 1:200 dilution overnight at 4°C and then incubated with antibody-recognizing rabbit immunoglobulin (Ig)G labeled by Alexa594 (Invitrogen, Tokyo, Japan) at room temperature for 1 h.

For the detection of 3-nitrotyrosine, the sections were incubated for 1 h in a working solution of mouse IgG blocking reagent from the MOM Immunodetection kit (Vector, Burlingame, CA, USA), and then incubated sequentially with 3-nitrotyrosine-recognizing primary antibody (Mab5404; Merck) at 1:100 dilution overnight at 4°C, MOM biotinylated antimouse IgG reagent for 10 min and VECTASTAIN ABC reagent for 5 min. The sections were examined after incubation with Liquid DAB Substrate Chromogen System (Dako, Glostrup, Denmark) for 3 min.

Liver histology

Formalin-fixed, paraffin-embedded specimens were sliced at 4-μm thickness and mounted on silanized glass slides. The slides were stained with Sirius red.[21] Images of five non-overlapping fields were randomly selected and captured at ×200 magnifications. Sirius red staining was quantified by image analysis with National Institutes of Health image (Image J).

Hydroxyproline contents

Liver tissues were homogenized in ice-cold distilled water (1 mL). One hundred and twenty-five microliters of 50% tricarboxylic acid (TCA) was added and the homogenates were further incubated on ice for 30 min. The precipitated pellets were hydrolyzed for 24 h at 110°C in 6 N HCL. Samples were filtered and neutralized with 10 N NaOH. Hydrolysates were then oxidized with chloramine-T (Sigma, St Louis, MO, USA) for 25 min at room temperature. The reaction mixture was incubated in Ehrich's perchloric acid solution at 65°C for 20 min, and then cooled at room temperature. Sample absorbance was measured at 560 nm. Purified hydroxyproline (Sigma) was used to set a standard. Hydroxyproline content was expressed as nanograms of hydroxyproline per milligram of liver.

ROS analysis for the liver specimens

Accumulation of ROS in the liver was assessed by staining freshly frozen liver sections with HPF. In brief, the freshly frozen liver specimens were sliced at 4-μm thickness and were incubated with HPF diluted at 1:200 for 15 min at 37°C.

The malondialdehyde (MDA) level was determined according to the thiobarbituric acid method using an NWLSS Malondialdehyde Assay Kit (Northwest Life Science Specialties, Vancouver, WA, USA).

Glutathione (GSH) level was determined with a GSH quantification assay kit (Dojindo, Tokyo, Japan).

Biochemical measurements were carried out at room temperature using a spectrophotometer (Molecular Devices, Tokyo, Japan).

Western blotting

The protocol for western blotting was described previously.[16] The primary antibodies and dilutions were as follows: anti-α-SMA antibody (no. ab5694; Abcam) at 1:1000; and anti-α-tubulin antibody (no. CP06; Calbiochem) at 1:1000.

Quantitative reverse transcription polymerase chain reaction analysis

Total RNA was extracted from the liver sample or isolated HSC by RNeasy Mini Kit with on-column DNA digestion (Qiagen, Tokyo, Japan). Total RNA was reverse transcribed to complementary DNA by using the Omniscript RT Kit (Qiagen, Valencia, CA, USA). Quantitative real-time reverse transcription polymerase chain reaction (RT–PCR) was performed using SYBR Green Master reaction mix on a LightCycler 480 II (Roche Diagnostics, Basel, Switzerland). The relative abundance of the target genes was obtained by calculating against a standard curve and normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for liver samples, and to 18S ribosomal RNA for HSC samples, as the internal control. The primer sequences used were: Col-1α1, forward 5′-GCT CCT CTT AGG GGC CAC T-3′, reverse 5′-CCA CGT CTC ACC ATT GGG G-3′; α-SMA, forward 5′-GTC CCA GAC ATC AGG GAG TAA-3′, reverse 5′-TCG GAT ACT TCA GCG TCA GGA-3′; TGF-β1, forward 5′-CCG CAA CAA CGC CAT CTA TG-3′, reverse 5′-CCC GAA TGT CTG ACG TAT TGG AAG-3′; GAPDH, forward 5′-AGG TCG GTG TGA ACG GAT TTG-3′, reverse 5′-TGT AGA CCA TGT AGT TGA GGT CA-3′; and 18 s ribosomal RNA, forward 5′-AGT CCC TGC CCT TTG TAC ACA-3′, reverse 5′-CGA TCC GAG GGC CTC ACT A-3′.

Statistical analysis

Each quantitative dataset was presented as mean ± standard error of the mean, and statistically analyzed with two-tailed Student's *t*-test followed by a post-hoc Fisher's protected least significant difference test.

RESULTS

Oral intake of H_2-water suppressed liver fibrogenesis in models of CCl_4- and TAA-induced liver injury

IN THE CCL_4 model, hepatic hydroxyproline content was significantly lower in the H_2-water group than the control water group (295.0 vs 382.6 ng/mg liver; $P < 0.05$) (Fig. 1a). Liver fibrosis, assessed by the intensity of Sirius red staining, was inhibited in the H_2-water group (Fig. 1b). Sirius red staining showed that the fibrotic area with collagen deposit was 8.163% in the control water group and 4.666% in the H_2-water group ($P < 0.0001$) (Fig. 1b). The inhibitory effect of H_2-water on liver fibrogenesis were further evaluated by RT–PCR for collagen1α1 and α-SMA. In the H_2-water group and control water group, the mRNA levels of collagen1α1 were 4.77 and 11.55 times that in the control ($P < 0.05$), while those of α-SMA were 2.15 and 3.92 times that in the control ($P < 0.05$) (Fig. 1c), respectively. Western blotting showed that the α-SMA levels in the H_2-water group and control water group was 1.64 and 2.87 times that in the control, respectively ($P < 0.01$) (Fig. 1d). H_2-water also suppressed the mRNA level of TGF-β1 (3.03 vs 1.74 times as control; $P < 0.05$) in the liver.

In the TAA model, H_2-water drinking suppressed liver fibrogenesis, as assessed by the hydroxyproline content (232.7 vs 287.1 ng/mg liver; $P < 0.05$) (Fig. 2a), Sirius red staining (2.563% vs 4.271%; $P < 0.01$) (Fig. 2b), RT–PCR for collagen1α1 (7.88 vs 59.4 times that in the control; $P < 0.01$) and α-SMA (2.24 vs 26.62 times that in the control; $P < 0.01$) (Fig. 2c), and western blotting for α-SMA (4.86 vs 9.96 times that in the control; $P < 0.01$) (Fig. 2d). H_2-water suppressed the mRNA level of TGF-β1 (3.37 vs 2.07 times in the control; $P < 0.05$) in the liver.

H_2-water oral intake suppressed generation of hydroxyl radicals in the liver

Hydroxyphenyl fluorescein staining for the detection of OH· on freshly frozen liver sections in the CCl_4 model showed a significantly smaller area of HPF staining in the H_2-water group (Fig. 3a) than in the control water group; this indicated that orally ingested H_2-water had a scavenging effect on OH· in the liver. These results demonstrated that CCl_4 injection induced the release of OH· and that H_2-water scavenged OH·. In the TAA model, HPF staining was present in a significantly smaller area in the H_2-water group (Fig. 3b).

Malondialdehyde levels in the H_2-water group were significantly lower in the CCl_4 model (3.89 vs 1.57 times that in the control) and TAA model (2.17 vs 1.41 times that in the control) (Fig. 3c). These results supports the antioxidative effect of H_2-water on OH· which generates lipid peroxidation. H_2-water did not change 3-nitrotyrosine expression (Fig. 4a,b) and GSH level (Fig. 4c).

Inhibition of CCl_4- or TAA-induced liver fibrosis by H_2-water was associated with less hepatocyte damage

The H_2-water group showed significantly lower transaminase levels in CCl_4 model at day 1 after CCl_4 injection (aspartate aminotransferase [AST], 2532 ± 697 [H_2-water] vs 5133 ± 908 IU/L [control water]; alanine aminotransferase [ALT], 3441 ± 790 [H_2-water] vs 6756 ± 1166 IU/L [control water]) and in TAA model at day 1 after TAA injection (AST, 1066 ± 460 [H_2-water] vs 2906 ± 834 IU/L [control water]; ALT, 1197 ± 442 [H_2-water] vs 4018 ± 997 IU/L [control water]).

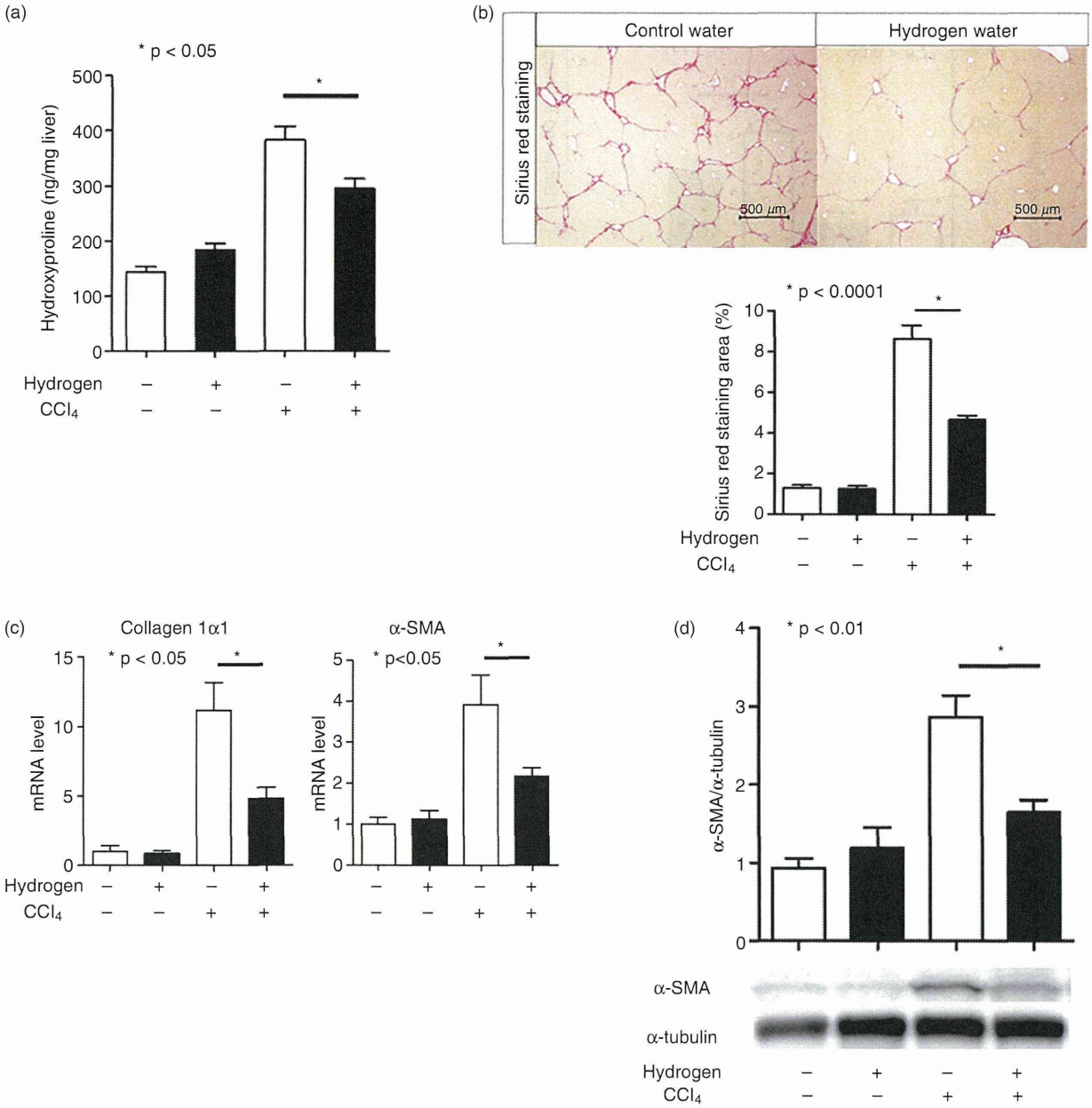

Figure 1 Oral intake of H_2-water suppresses carbon tetrachloride (CCl_4)-induced liver fibrogenesis in mice. C57BL/6 mice were subjected to repeated injections with CCl_4 and fed with hydrogen water (H_2-water) or control water. The hepatic hydroxyproline content was significantly lower in the H_2-water group than in the control water group (295.0 vs 382.6 ng/mg liver; $P < 0.05$) (a). Liver fibrosis, which was assessed by Sirius red staining, was inhibited in the H_2-water group. Fibrotic area with collagen deposit was 8.163% in the control water group and 4.666% in the H_2-water group ($P < 0.0001$), as determined by Sirius red staining (b). The mRNA levels of collagen1α1 in the H_2-water group and the control water group was 4.77 and 11.15 times that in the control, respectively ($P < 0.05$). The mRNA level of α-smooth muscle actin (α-SMA) was 2.15 times that in the controls in the H_2-water group and 3.92 times that in the controls in the control water group ($P < 0.05$) (c). Western blotting for α-SMA in the H_2-water group and the control water group were 1.64 and 2.87 times that in the control, respectively ($P < 0.01$) (d).

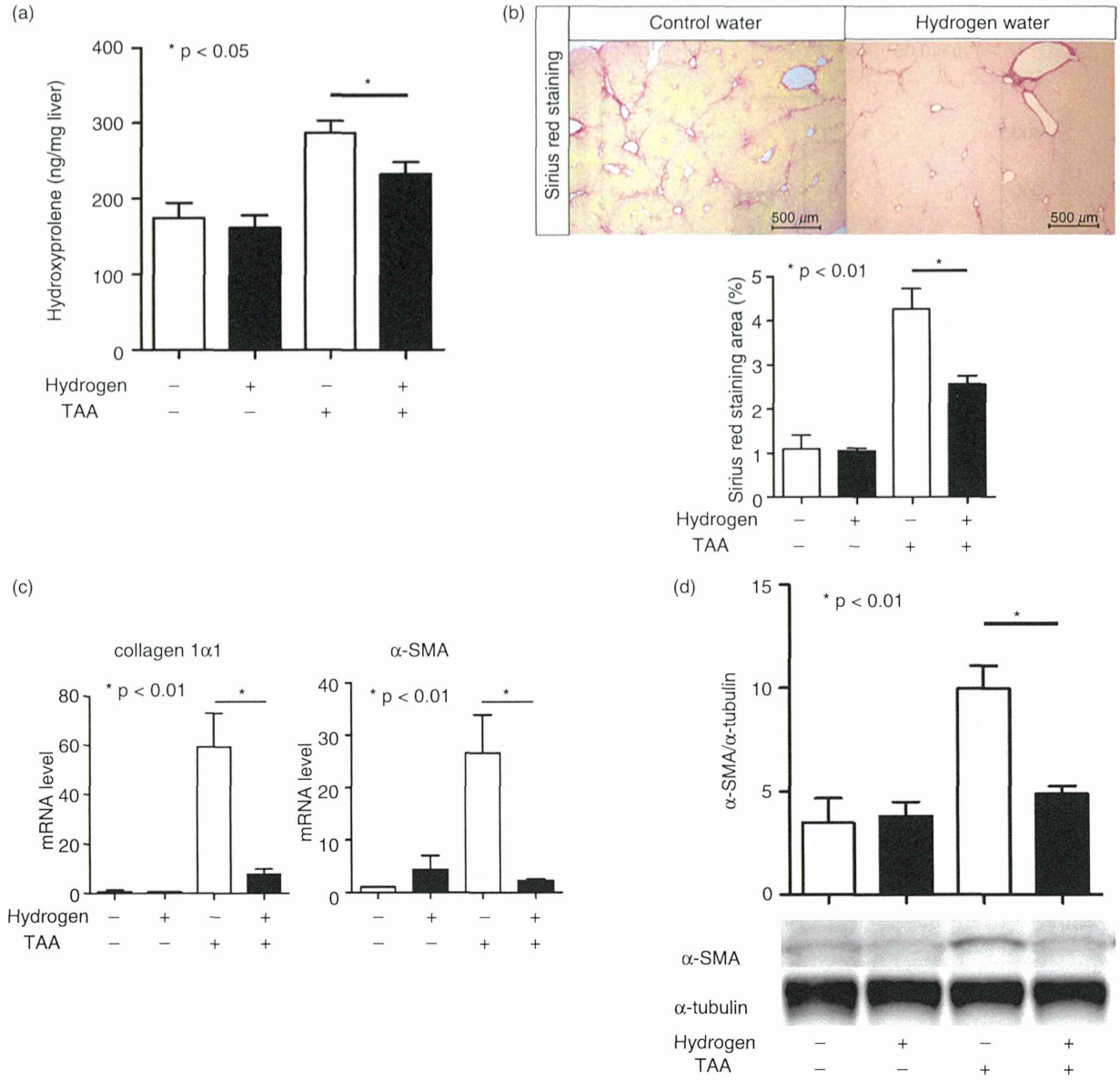

Figure 2 Oral intake of hydrogen water (H_2-water) suppresses thioacetamide (TAA)-induced liver fibrogenesis in mice. C57BL/6 mice were subjected to repeated injection with TAA and were fed with H_2-water or control water. Oral intake of H_2-water suppressed liver fibrogenesis, as assessed by hydroxyproline content (232.7 vs 287.1 ng/mg liver; $P < 0.05$) (a), Sirius red staining (b) (2.563 vs 4.271%; $P < 0.01$), reverse transcription polymerase chain reaction for collagen1α1 (7.88 vs 59.4 times that in the control; $P < 0.01$) and α-smooth muscle actin (α-SMA) (2.24 vs 26.62 times that in the control; $P < 0.01$) (c), and western blotting for α-SMA (4.86 vs 9.96 times that in the control; $P < 0.01$) (d).

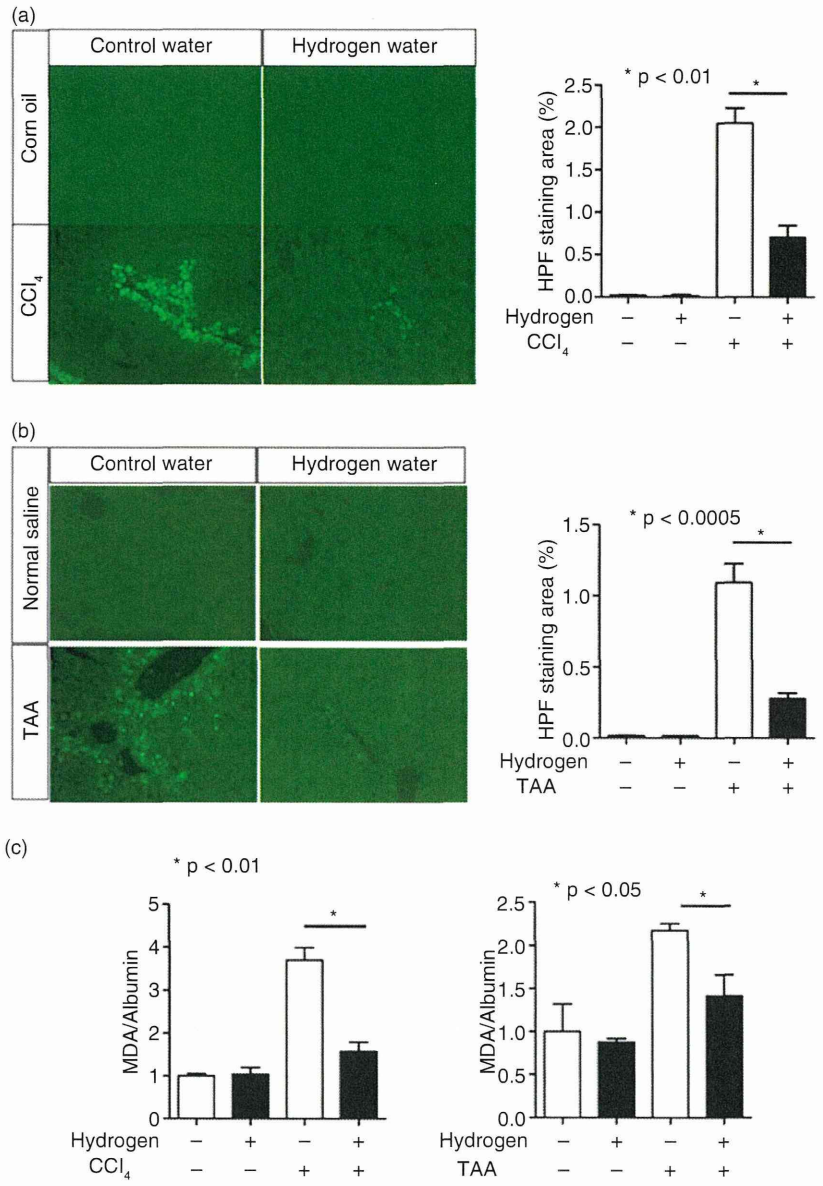

Figure 3 Oral intake of hydrogen water (H_2-water) suppresses generation of OH· in the liver. C57BL/6 mice were subjected to repeated injections of carbon tetrachloride (CCl_4) and were fed with H_2-water or control water. Generation of OH· in the liver in the CCl_4 model was assessed by hydroxyphenyl fluorescein (HPF) staining of freshly frozen liver sections. Samples from the H_2-water group had a significant smaller area of HPF staining compared to those from the control group (0.702% vs 2.051%, $P < 0.01$) (a). In the thioacetamide (TAA) model, the H_2-water group also showed a significantly smaller area of HPF staining compared to the control group (0.278% vs 1.092%, $P < 0.001$) (b). Malondialdehyde (MDA) levels in the H_2-water group were significantly lower in the CCl_4 model (3.89 vs 1.57 times that in the control) and TAA model (2.17 vs 1.41 times that in the control) (c).

Oral intake of H_2-water did not suppress liver fibrogenesis in the BDL-induced liver injury model, in which OH· was not generated

In the BDL model, no difference in hydroxyproline contents was observed between the H_2-water group and the control water group (Fig. 5a). This result was further confirmed by assessment of the mRNA expression of collagen1α1 and α-SMA (Fig. 5b) and Sirius red staining (Fig. 5c). HPF staining for the detection of OH· in freshly frozen liver sections in the BDL model showed no HPF staining in either the H_2-water or the control water groups (Fig. 5d).

Hydrogen-rich medium suppressed the generation of hydroxyl radicals and hepatocyte death induced by AMA

Antimycin A, a complex III inhibitor in the respiratory transduction system, induces the release of $O\cdot_2^-$. Hepatocytes were isolated from C57BL/6 mice and treated

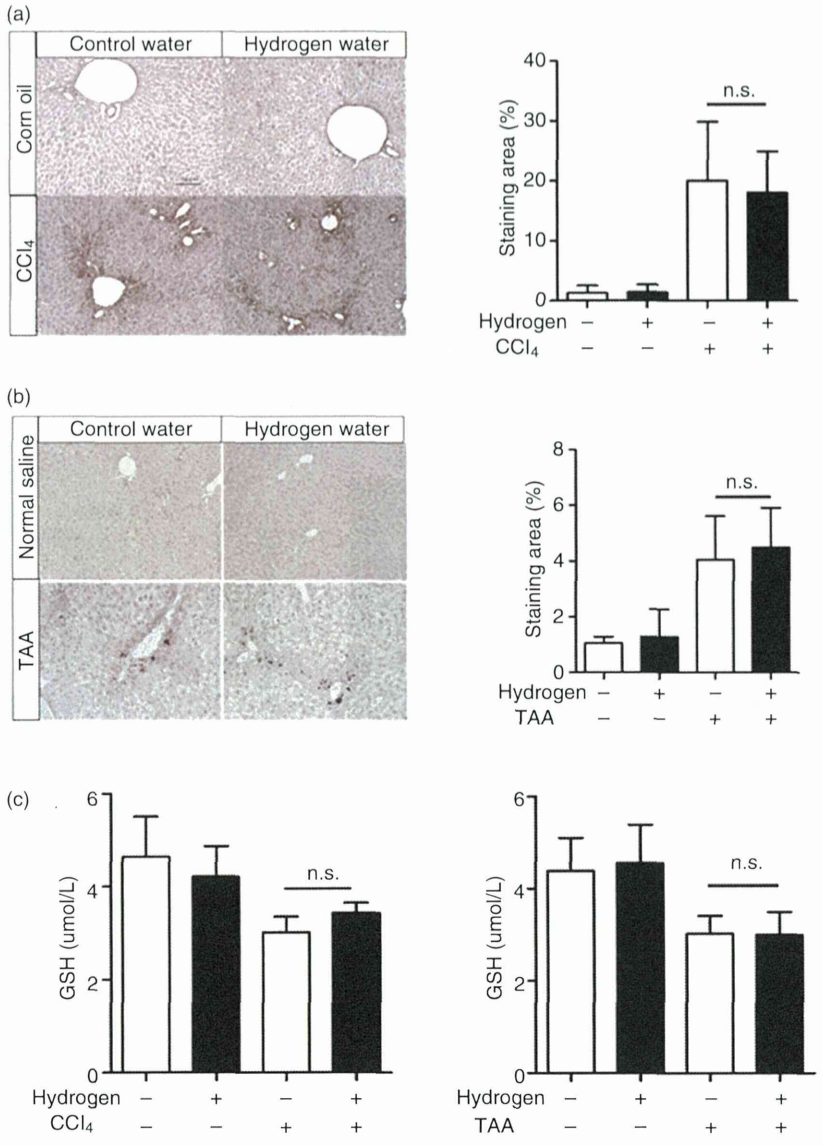

Figure 4 Oral intake of hydrogen water (H_2-water) did not affect 3-nitrotyrosine expression and glutathione (GSH). The 3-nitrotyrosine level in the liver did not differ in the carbon tetrachloride (CCl_4) model (20.1 vs 18.6 times that in the control) (a) and thioacetamide (TAA) model (4.0 vs 4.5 times that in the control) (b). GSH level in the liver did not show any difference between the control water group and the hydrogen water group both in the CCl_4 model (3.02 vs 3.44 μmol/L) and in the TAA model (3.02 vs 2.99 μmol/L) (c).

with 1 μg/mL of AMA. The addition of AMA increased levels of OH·, H_2O_2 and $O\cdot_{2^-}$, as evaluated by the intensity of fluorescence signals emitted by the oxidized forms of HPF (Fig. 6a), CM-H_2DCFDA (Fig. 6b) and MitoSOX (Fig. 6c), respectively. Culturing in hydrogen-rich medium suppressed the generation of OH· (Fig. 6a) induced by AMA, but did not affect the increased level of H_2O_2 (Fig. 6b) or $O\cdot_{2^-}$ (Fig. 6c), indicating that the hydrogen-rich medium selectively scavenged OH·.

Hepatocyte death induced by AMA was suppressed in the presence of hydrogen in the medium (13.2% and 26.8% for hydrogen-rich medium and control medium, respectively; $P < 0.001$) (Fig. 6d).

Hydrogen-rich medium did not suppress culture activation of HSC

We assessed the effect of culturing in hydrogen-rich medium on the activation of HSC, which are the principal fibrogenic cells in the liver. HSC are known to be activated when cultured on a plastic dish. RT–PCR for collagen1α1 and α-SMA of HSC that were cultured on a plastic dish for 5 days revealed that the hydrogen-rich medium did not suppress the activation of cultured

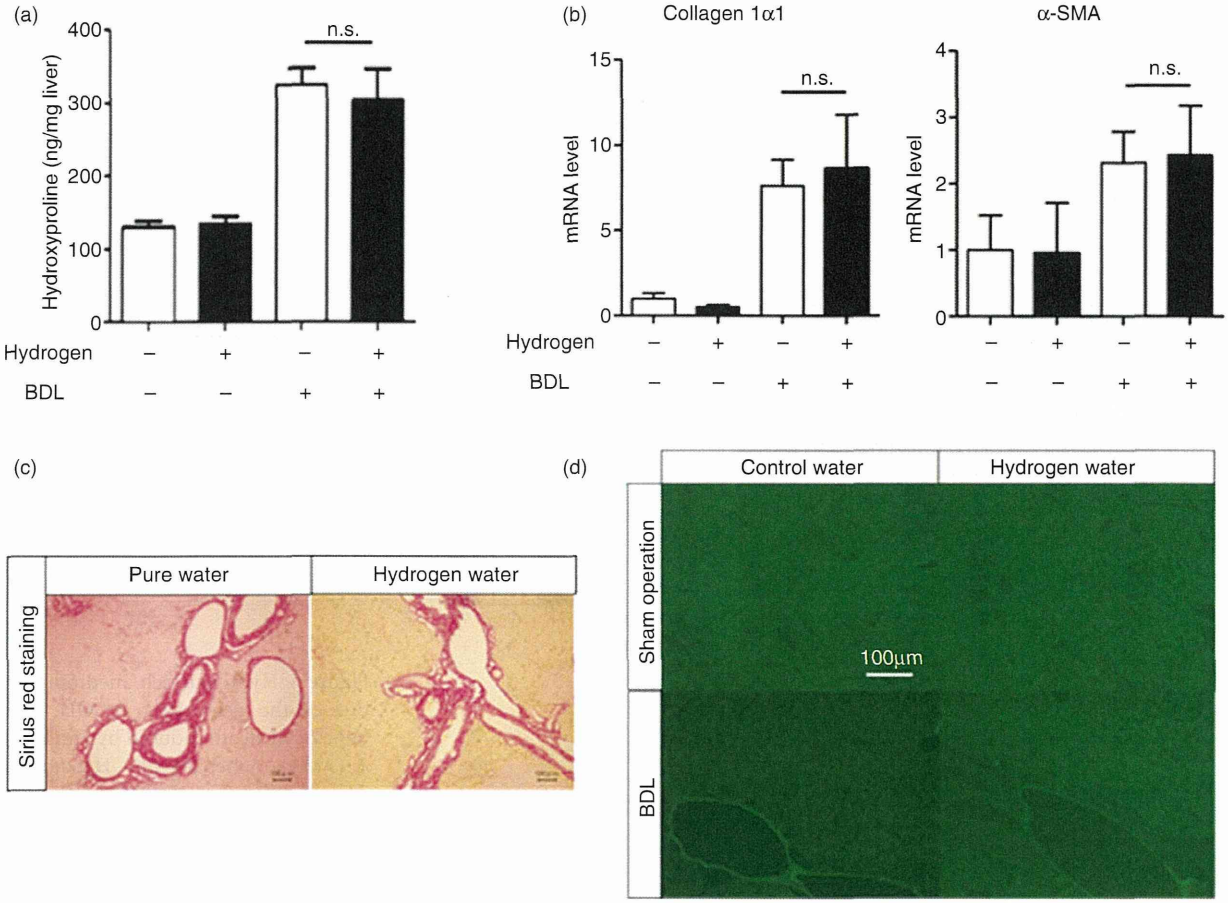

Figure 5 Oral intake of hydrogen water (H_2-water) did not suppress liver fibrogenesis in the bile duct ligation (BDL)-induced liver injury model, in which OH· was not generated. In the BDL model, hydroxyproline contents did not show any difference between the H_2-water group and the control group (a). The mRNA levels of collagen1α1 and α-smooth muscle actin (α-SMA) did not differ in the H_2-water group and the control water group (b). Sirius red staining also did not show any suppressive effect of H_2-water on liver fibrosis (c). Sirius red staining also did not show any suppressive effect of H_2-water on liver fibrosis (c). Hydroxyphenyl fluorescein staining of freshly frozen liver sections in the BDL model did not show any signs of OH· in both the H_2-water and control water groups (d).

HSC (Fig. 7a). The activated HSC showed traces of $O\cdot_{2^-}$, as detected by MitoSOX staining (Fig. 7b). The activated HSC contained H_2O_2, as evidenced by vivid staining with CM-H_2DCFDA (Fig. 7c), suggesting that culturing in the hydrogen-rich medium did not suppress the accumulation of H_2O_2. The HSC did not contain OH·, as evaluated by HPF staining (Fig. 7d). These results imply that OH· is not associated with the activation of cultured HSC and H_2-water does not seem to target HSC.

Hydrogen-rich medium did not suppress or accelerate HSC activation induced by AMA

Isolated HSC were cultured with or without hydrogen-rich medium in the presence of AMA to assess the effect of hydrogen on HSC activation under excess oxidative stress. Culturing in the hydrogen-rich medium did not affect the mRNA expression levels of the activation markers for HSC (collagen1α1 and α-SMA) (Fig. 8a). The increased intracellular $O\cdot_{2^-}$ levels induced by AMA

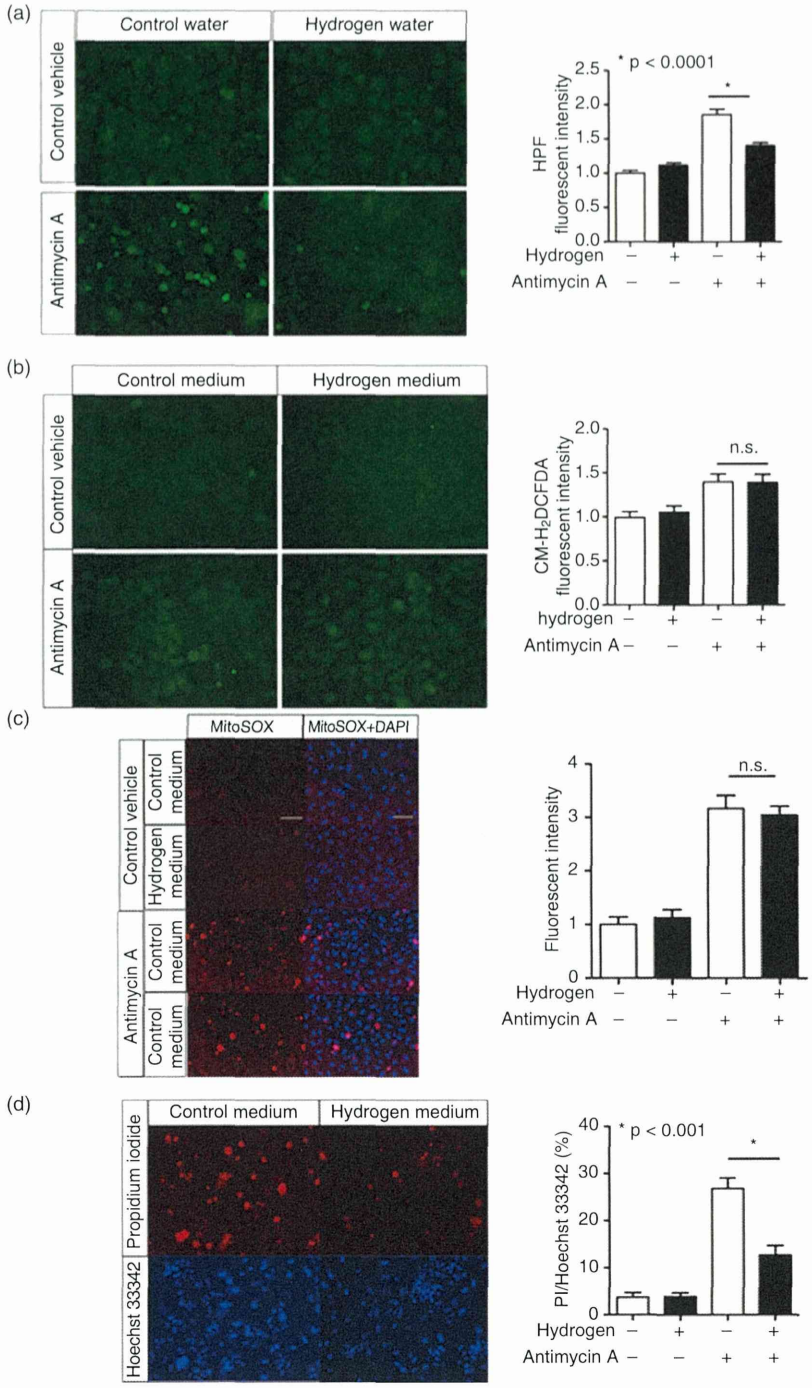

Figure 6 Hydrogen-rich medium suppresses the generation of OH· and hepatocyte death induced by antimycin A (AMA) in hepatocytes. Hepatocytes were isolated from C57BL/6 mice and treated with 1 μg/mL of AMA. Addition of AMA increased the intracellular levels of OH·, H_2O_2 and O_{2}^{-}, as evaluated by measuring fluorescent signals emitted by the oxidized forms of hydroxyphenyl fluorescein (HPF) (a), 2′,7′-dichlorodihydrofluorescein (CM-H_2DCFDA) (b) and MitoSOX (c), respectively. Hydrogen-rich medium suppressed the generation of OH· (a), but not of H_2O_2 (d) and O_{2}^{-} (c). Hepatocytes were stained with Hoechst 33342 and propidium iodide (PI) and observed by fluorescent microscopy (d). AMA induced hepatocyte death. The induction of hepatocyte death was suppressed by culturing in hydrogen-rich medium (13.2% in hydrogen-rich medium vs 26.8% in control medium; $P < 0.001$) (d). DAPI, 4′,6′-diamidino-2-phenylindole dihydrochloride.

stimulation and detected by MitoSOX were not suppressed by culturing in hydrogen-rich medium (Fig. 8b). The H_2O_2 level, detected by CM-H_2DCFDA was not altered by AMA stimulation (Fig. 8c). Moreover, we could not detect intracellular OH·, even after AMA treatment (Fig. 8d). These results suggest that hydrogen did not exert its antifibrotic effect through direct action on HSC.