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Molecular Hydrogen Improves Obesity and Diabetes by Inducing Hepatic FGF21 and Stimulating Energy Metabolism in *db/db* Mice

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Recent extensive studies have revealed that molecular hydrogen (H_2) has great potential for improving oxidative stress-related diseases by inhaling H_2 gas, injecting saline with dissolved H_2, or drinking water with dissolved H_2 (H_2-water); however, little is known about the dynamic movement of H_2 in a body. First, we show that hepatic glycogen accumulates H_2 after oral administration of H_2-water, explaining why consumption of even a small amount of H_2 over a short span time efficiently improves various disease models. This finding was supported by an *in vitro* experiment in which glycogen solution maintained H_2. Next, we examined the benefit of *ad libitum* drinking H_2-water to type 2 diabetes using *db/db* obesity model mice lacking the functional leptin receptor. Drinking H_2-water reduced hepatic oxidative stress, and significantly alleviated fatty liver in *db/db* mice as well as high fat-diet-induced fatty liver in wild-type mice. Long-term drinking H_2-water significantly controlled fat and body weights, despite no increase in consumption of diet and water. Moreover, drinking H_2-water decreased levels of plasma glucose, insulin, and triglyceride, the effect of which on hyperglycemia was similar to diet restriction. To examine how drinking H_2-water improves obesity and metabolic parameters at the molecular level, we examined gene-expression profiles, and found enhanced expression of a hepatic hormone, fibroblast growth factor 21 (FGF21), which functions to enhance fatty acid and glucose expenditure. Indeed, H_2 stimulated energy metabolism as measured by oxygen consumption. The present results suggest the potential benefit of H_2 in improving obesity, diabetes, and metabolic syndrome.

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INTRODUCTION

Oxidative stress is involved in many lifestyle-related diseases, including diabetes, atherosclerosis, heart failure, Alzheimer's disease, and Parkinson diseases (1–6). Recent studies have revealed that molecular hydrogen (H_2) acts as a novel antioxidant and prevents or ameliorates diseases associated with oxidative stress in animal experiments (7–18) and clinical tests (19–22). The brain, heart, liver, and intestine were protected from oxidative stress by inhalation of 1–2% H_2 gas (7–11). Interestingly, instead of inhaling H_2 gas, drinking water with dissolved H_2 (H_2-water) protected the brain and kidney from oxidative stress (12–15). These studies strongly suggest the potential of H_2 as an effective therapeutic and preventive antioxidant; however, water dissolves H_2 at 0.8 mmol/l at saturated level. Thus, it has been an open question why consumption of even a small amount of H_2 is effective for various disease models.

Oxidative stress is one of the causes of type 2 diabetes (1–3). To examine whether H_2 has benefits on type 2 diabetes, we used *db/db* mice, in which oxidative stress is accumulated in the liver and leads to hyperglycemia and hyperlipidemia (23). *Db/db* mice lack a functional leptin receptor, and have been extensively studied as a model for type 2 diabetes (24,25).

In this study, we showed that H_2 can be accumulated in the liver with glycogen after oral administration. Next, chronic consumption of H_2-water reduced oxidative stress in the liver of *db/db* mice, and improved obesity and diabetes. As a mechanistic study, we showed that long-term consumption of H_2-water enhanced the expression of a hepatic hormone, fibroblast growth factor 21 (FGF21), which is a regulator of energy expenditure (26–29). These findings suggest the great potential for hydrogen therapy and prevention of metabolic syndrome.

METHODS AND PROCEDURES

Animals

Male Sprague–Dawley rats of 10 weeks of age and male C57BL/6 mice of 12 weeks of age were purchased from Nippon SLC (Hamamatsu, Shizuoka, Japan). Genetically diabetic male *db/db* mice (BKS.Cg +*Lepr*^{db}/+*Lepr*^{db}/Jcl) and their nondiabetic heterozygous *db*/+ littermates (BKS.Cg *m*+/+*Lepr*^{db}/Jcl) were purchased at 5 weeks of age from CLEA

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Japan, (Tokyo, Japan). Mice were given H_2-water from 6 weeks of age. For the diet-induced obesity study, C57BL/6 mice were given a high fat-diet (F2HFD1; Oriental Yeast, Tokyo, Japan) for 1 or 2 weeks. The care and use of laboratory animals were in accordance with the National Institutes of Health guidelines. This study was approved by the Animal Care and Use Committee of Nippon Medical School (Tokyo, Japan).

Hydrogen water

H_2-water was prepared as described previously (12). In brief, H_2 was dissolved in water under high pressure (0.4 MPa) to a supersaturated level and the saturated H_2-water was stored under atmospheric pressure in an aluminum bag with no dead volume. Saturated H_2-water was used as 100% H_2-water. H_2-water degassed by gentle stirring was used as control water. Saturated H_2-water was diluted with ninefold control water and used as 10% saturated H_2-water. Mice were given water freely using closed glass vessels equipped with an outlet line containing two ball bearings, which kept the water from being degassed. The vessel was freshly refilled with H_2-water at 2:00 PM every day.

Diet restriction

Diabetic mice were subjected to controlled dietary restriction, such that the daily diet consumption was equivalent to 80% or 50% of that of *ad libitum* diet consumption. Control groups were permitted free access to food and water throughout the experiment. In the diet-restricted group, mice were permitted *ad libitum* water, but food intake was restricted.

Measurement of H_2 concentration

H_2 concentration in rat liver tissue was measured using a needle-type hydrogen electrode (Unisense, Aarhus, Denmark). Rat received H_2-water orally by stomach gavage at 15 ml/kg. Throughout the experiment, the electrode current was measured with a picoammeter (Keithley, Cleveland, Ohio) and H_2 concentration was obtained from the calibration curve generated using known levels of H_2-saturated saline.

In an *in vitro* experiment, H_2 concentration in glycogen, glucose solutions, or drinking water in the glass vessel was measured using a needle-type hydrogen electrode as described above.

Sample collection and biochemical analysis

Mice were killed under anesthesia, blood was collected from the heart, and liver tissues were excised and frozen with liquid nitrogen or fixed with 4% paraformaldehyde for further analysis. The antioxidation effect of H_2 was determined by measuring lipid peroxides in the liver, using a malondialdehyde assay kit (Northwest Life Science Specialties, Vancouver, WA) and the level of lipid peroxides was expressed as nmol malondialdehyde (MDA)/mg protein. Plasma concentrations of total ketone bodies, triglyceride, and total cholesterol were determined with commercially available kits (Wako Pure Chemical Industries, Osaka, Japan). Plasma low-density lipoprotein-cholesterol and high-density lipoprotein-cholesterol were measured using kits (Sekisui Medical, Tokyo, Japan). Plasma glucose and nonesterified fatty acids were determined with kits available from Shino-Test (Tokyo, Japan) and Eiken Chemical (Tokyo, Japan), respectively. Plasma insulin was measured using an insulin ELISA kit (Morinaga Institute of Biological Science, Kanagawa, Japan).

Oil Red O staining

Mouse livers were fixed in 4% paraformaldehyde in phosphate-buffered saline, embedded, and cryosectioned 10 mm thick. The sections were rinsed with 60% isopropanol, stained with 0.25% Oil Red O solution, rinsed with 60% isopropanol, and mounted in aqueous mountant. The area of stained lipid (%) was calculated using the Image J program (ver 1.41; National Institutes of Health, Bethesda, MD) from four sections for each mouse.

Body fat composition analysis

For computed tomography analysis of body fat composition, mice were anesthetized with halothane in a mixture of nitrous oxide and oxygen (70%:30%, vol/vol) and then scanned using a LaTheta LCT-100, experimental animal computed tomography system (Aloka, Tokyo, Japan). Contiguous 1-mm slice images were used for quantitative assessment using LaTheta software (ver 1.00). Visceral fat, subcutaneous fat, and muscle were distinguished and evaluated quantitatively.

RNA isolation and reverse transcriptase-PCR

Total RNA was isolated from the liver tissue using an RNeasy Mini kit (QIAGEN, Valencia, CA). Complementary DNA generated by SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) was analyzed by quantitative PCR using Thermal Cycler Dice Real Time System TP800 (TAKARA BIO, Shiga, Japan). All samples were normalized to glyceraldehyde 3-phosphate dehydrogenase expression. Primer and probe sequences for each PCR are shown in **Table 1**.

Behavioral analysis

Movement activities were recorded in *db/+* and *db/db* mice automatically using a laboratory animal monitoring system (ACTIMO-100; Shinfactory, Fukuoka, Japan). Mice were housed individually and temperature was maintained at 22 °C. Food and water with or without hydrogen were available *ad libitum*. Mice were acclimatized to the chambers for 24 h before beginning recordings and then monitored for 48 h. Movement activity was measured as ambulatory counts from a record of consecutive adjacent infrared beam breaks. Cumulative ambulatory counts on the x- and y-axes were recorded every 10 min.

Indirect calorimetric analyses

Metabolic rate was measured by indirect calorimetric analysis in *db/+* and *db/db* mice using an open-circuit calorimeter (Oxymax; Columbus Instruments, Columbus, OH). Mice were housed individually in a chamber (20 × 10 × 12.7 cm) and temperature was maintained at 22 °C, with air flow of 0.5 l/min. Food and water with or without H_2 were available *ad libitum*. Mice were acclimatized to the chambers for 48 h before beginning recordings and then monitored for 48 h. VO_2 and VCO_2 were measured every 10 min using an electrochemical O_2 analyzer and a CO_2 sensor (Oxymax), and the respiratory exchange ratio was calculated as VCO_2/VO_2 (volume of CO_2 produced per volume of O_2 consumed (ml/kg/h)).

Statistical analysis

We performed statistical analysis using StatView software (SAS Institute) by applying an unpaired two-tailed Student's *t*-test and ANOVA followed by Fisher's exact test, as described previously (7). Differences were considered statistically significant at $P < 0.05$.

Table 1 Primers and probes for reverse transcriptase-PCR

Gene		Sequence
FGF21	F primer	5′-CCGCAGTCAGAAAGTCTCCT-3′
	R primer	5′-TCTGAAGCTGCAGGCCTCA-3′
	Probe	5′-AGCTCTCTATGGATCGCCTCACTTTGATCC-3′
PEPCK	F primer	5′-TGCTGCAGAACACAAGGGC-3′
	R primer	5′-TTTGCCGAAGTTGTAGCCG-3′
	Probe	5′-TCATCATGCACGACCCCTTTGCC-3′
G6PC	F primer	5′-CGCCATGCAAAGGACTAGGA-3′
	R primer	5′-AGGGCCGATGTCAACACCT-3′
	Probe	5′-TAAAGCCTCTGAAACCCATTGTGAGGCC-3′
GAPDH	F primer	5′-CATCACTGCCACCCAGAAGA-3′
	R primer	5′-ATGTTCTGGGCAGCC-3′
	Probe	5′-TGGATGGCCCCTCTGGAAAGCTG-3′

FGF21, fibroblast growth factor 21; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; G6PC, glucose-6-phosphatase, catalytic subunit; PEPCK, phosphoenolpyruvate carboxykinase.

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RESULTS

Molecular hydrogen is accumulated in the liver with glycogen

We monitored the dynamic movement of H_2 in the liver after oral administration of H_2-water. Rat received H_2-water orally by stomach gavage, and levels of H_2 in the liver were monitored by directly inserting a needle-type hydrogen sensor into the liver for an hour (**Figure 1a**). The H_2 concentration profile gave a peak 5 min after administration of H_2-water in both a fed and fasted liver; however, a great difference was found between the fed and fasted liver; the maximum H_2 level in the fed liver was twofold that of the fasted liver. Moreover, the fed liver maintained a considerable H_2 level for an hour, while that in the fasted liver returned to the basal level after 25 min.

Since a fed liver is rich in glycogen, we speculated that higher polymers of carbohydrates including glycogen have the capacity to maintain H_2. To verify this speculation, we examined saturated solubilities of H_2 in glycogen and glucose solutions by a hydrogen sensor. H_2 was dissolved in a glycogen or glucose solution by bubbling H_2 gas up to a saturated level (**Figure 1b**). Compared to the glucose solution and water, the glycogen solution dissolved a significantly higher amount of H_2. The hydrogen sensor was not influenced by glycogen and glucose (data not shown). In addition, H_2 was maintained for a longer time in saturated glycogen solution than in saturated glucose solution or water in a plastic tube with the lid off under atmospheric pressure (**Figure 1c**). The half-life was 19.1 ± 2.3, 30.9 ± 3.0, and 20.3 ± 0.4 min (mean ± s.d., $n = 3$) in water, 100 mg/ml glycogen and glucose solutions, respectively. The half-life of dissolving H_2 in the glycogen solution was prolonged 1.6-fold. Thus, it is concluded that H_2 can be accumulated and reserved in the liver with glycogen, suggesting that expenditure of glycogen should accompany the release of H_2.

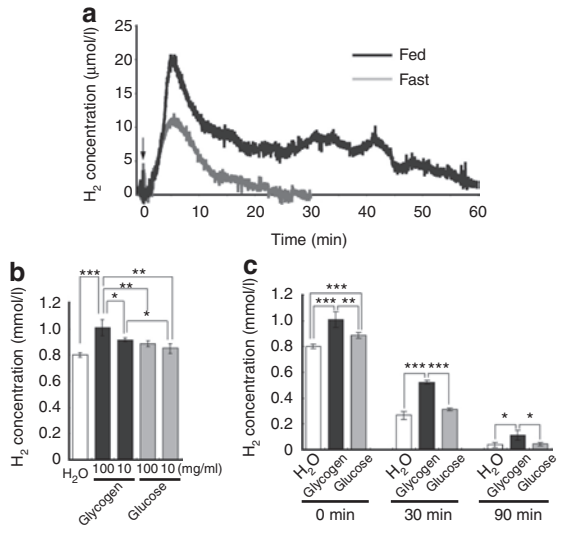

Figure 1 Hydrogen is accumulated and maintained in a fed liver and in glycogen solution *in vitro*. (**a**) The concentration of molecular hydrogen in the liver was monitored using a needle-type hydrogen sensor in fed or overnight-fasted rat liver. Rat received hydrogen water (0.8 mmol/l H_2 in water) orally by stomach gavage at 15 ml/kg. Arrow indicates the time point when rat was administered hydrogen water. (**b**) Saturated concentration of hydrogen in glycogen and glucose solutions, and water. Molecular hydrogen was dissolved in indicated solutions by bubbling H_2 gas to a saturated level. The concentration of hydrogen in solution was measured using a hydrogen sensor. Data are means ± s.d. ($n = 3$). *$P <$ 0.05; **$P < 0.01$; ***$P < 0.001$ compared with 100 or 10 mg/ml glycogen groups. (**c**) Concentration of hydrogen in water, 100 mg/ml glycogen solution and 100 mg/ml glucose solution after bubbling stopped. Hydrogen-saturated solutions were kept in a plastic tube with the lid off at 20 °C for 0, 30, or 90 min under atmospheric pressure. Concentration of hydrogen in solution was measured using a hydrogen sensor. Data are mean ±s.d. ($n = 3$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Consuming hydrogen water reduces oxidative stress in the liver and improves fatty liver

Since obesity is a proinflammatory disease, consumption of H_2-water may suppress obesity by acting as an anti-inflammatory. To examine the antioxidation effect of H_2 on the liver, we used obesity and type 2 diabetes model mice *db/db* lacking functional leptin receptors, because oxidative stress is accumulated in the liver. *Db/db* mice and their lean littermates drank H_2-water *ad libitum* for 3 months. H_2-water was exchanged for fresh saturated or 10% saturated water at 1400 h every day. The concentration of dissolved H_2 was measured as described in Methods and Procedures (**Figure 2a**). Since *db/db* mice drank much more water than wild-type mice, H_2 was degassed much faster into the air phase in the vessel used for *db/db* mice; however, a considerable amount of H_2 was maintained by this method.

The effect of H_2 on oxidative stress in the liver was examined as judged by the level of malondialdehyde (MDA), an oxidative stress marker derived from lipid peroxides. The MDA level in the liver of H_2-administered mice significantly fell to nearly the level in nondiabetes control mice, indicating that consumption of H_2-water *ad libitum* markedly suppressed oxidative stress (**Figure 2b**). A dotted pattern caused by the accumulation of fat disappeared in the liver of *db/db* mice with H_2, compared to control water administered *db/db* mice (**Figure 2c**, insets of upper panels). Oil red O staining indicated that H_2 administration significantly reduced neutral lipid accumulation in the livers of *db/db* mice (**Figure 2b**, lower panels and **Figure 2d**, left graph). Furthermore, even with short-term administration (1–2 weeks), H_2 significantly reduced fat accumulation in the liver of high fat-diet-induced obesity mice using the wild-type (**Figure 2d**, middle and right graph). These data clearly indicate that consumption of H_2 markedly reduces hepatic oxidative stress levels and improves fatty liver in *db/db* as well as diet-induced obesity mice.

Consuming hydrogen water suppressed body-weight gain and reduced plasma glucose and triglyceride levels

To investigate the effect of H_2 on the obesity of *db/db* mice, body-weight was monitored throughout the experimental period, and body fat mass at 18 weeks old was measured by computerized tomography. Mice were divided randomly into 3 groups. Group I (control) was allowed to freely drink water

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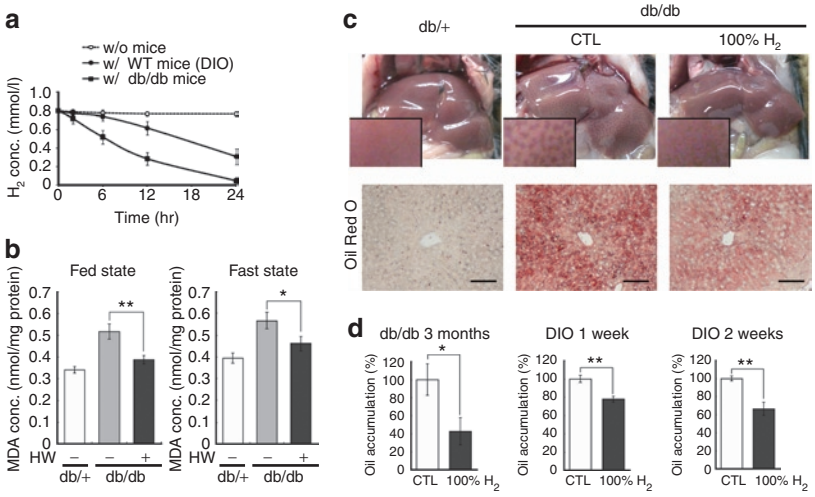

Figure 2 Consuming hydrogen suppresses oxidative stress in the liver and improves fatty liver. (**a**) Hydrogen concentration in a glass vessel described in Methods and Procedures section was measured without mice (open circle), showing that the equipment retains hydrogen. Profile of the hydrogen concentration in drinking water in a glass vessel given to *db/db* mice (closed square) or wild-type mice used for diet-induced obesity (DIO) (closed circle), indicating that hydrogen gas was released into the air phase by consuming water. Data are the mean ± s.d. ($n = 3$). (**b**) Malondialdehyde concentration in a fed or overnight-fasted liver was measured. The *db/+* and *db/db* mice were given water with or without hydrogen for 3 months. Data are mean ± s.e.m. ($n = 10$ for each *db/+* group and $n = 15$ for each *db/db* group). $^*P < 0.05$, $^{**}P < 0.01$. (**c**) The appearance and representative oil red O staining of the liver of *db/+* and *db/db* mice given water with and without hydrogen for 3 months, respectively. Scale bar: 100 μm. (**d**) Levels of fat accumulation in the liver. The *db/db* mice were given water with or without hydrogen for 3 months (left panel). DIO (high fat-diet induced obesity) mice were given water with or without hydrogen for 1 or 2 weeks (middle or right panel). Oil accumulation in the liver was calculated from oil red O staining using an image analysis program, Image J program. Data are mean ± s.e.m. ($n = 12$ for each *db/db* group and $n = 8$ for each DIO group). $^*P < 0.05$, $^{**}P < 0.01$.

without H_2. Group II and Group III were given H_2-water with 0.8 mmol/l (saturated H_2-water; 100%) and 0.08 mmol/l (10% saturated level of H_2), respectively. In the initial phase of the experiment, the weight gains of all group animals were similar, suggesting no toxic effect of H_2-water; however, while the control mice exhibited a progressive weight increase, the mice in both 100% and 10% H_2-administered groups achieved a modest weight increase (**Figure 3b**). Group II mice (100% H_2) were slimmer and their body-weight was significantly lower than that of Group I mice (control) at 18 weeks of age (**Figure 3a,b**). The suppression of body-weight gain was observed in mice drinking 10% H_2-water (Group III) (**Figure 3b**). Body fat was also substantially lower in 100% H_2-consuming mice (**Figure 3c,d**). Since the consumed amounts and volumes of diet and water did not differ among groups (**Figure 3e,f**), it is suggested that H_2 consumption stimulates energy metabolism to suppress the gain of fat and body weights.

Next, we performed biochemical examinations of blood. Plasma levels of glucose and insulin were significantly reduced in the 100% H_2-administered group and triglycerides were significantly lowered in both 100% and 10% H_2-administered groups (**Figure 4a–c**). Plasma total ketone bodies tended to increase in the 100% H_2-administered group (**Figure 4d**), while no change in plasma levels of free fatty acid, high-density lipoprotein-cholesterol, low-density lipoprotein-cholesterol-cholesterol, total cholesterol, and adiponectin was found (**Figure 4e–i**). These data demonstrate that the consumption of H_2 markedly improves obesity, hyperglycemia, and the plasma triglycerides of diabetic *db/db* mice.

Consuming hydrogen water shows a similar effect to diet restriction

Since *db/db* mice cannot regulate their appetite to eat excess diet by a deficient leptin receptor gene, diet restriction should be effective to improve obesity and diabetes (30). When *db/db* mice were subjected to controlled dietary restriction, the levels of plasma glucose, insulin, and triglycerides significantly fell (**Figure 5a–c**). When comparing the effects by drinking H_2-water and dietary restriction, the plasma glucose level of mice given 80% of *ad libitum* diet consumption was the same as that of H_2-administered mice (**Figure 5a**). Furthermore, an additive effect was observed when mice were given both H_2-water and a restricted diet (**Figure 5a**). A similar additive effect was seen in plasma triglyceride levels (**Figure 5c**). In particular, when both H_2-water and 80% diet restriction were given to *db/db* mice, the plasma triglyceride reached the level of control *db*/+ mice (**Figure 5c**).

Consuming hydrogen water increases hepatic mRNA level of FGF21

The liver plays an essential role in controlling blood glucose levels by modulating glucose catabolism and gluconeogenesis. To understand the mechanism of how H_2 regulates glucose and triglycerides, *db/db* mice given H_2-water for 3 months were subjected to expression analyses of genes related with the regulation of gluconeogenesis. Phosphoenolpyruvate carboxykinase catalyzes the initial step of gluconeogenesis, and glucose-6-phosphatase catalyzes the last committed step of this process (31). Data from real-time PCR revealed that H_2 administration had no influence on hepatic mRNA levels of

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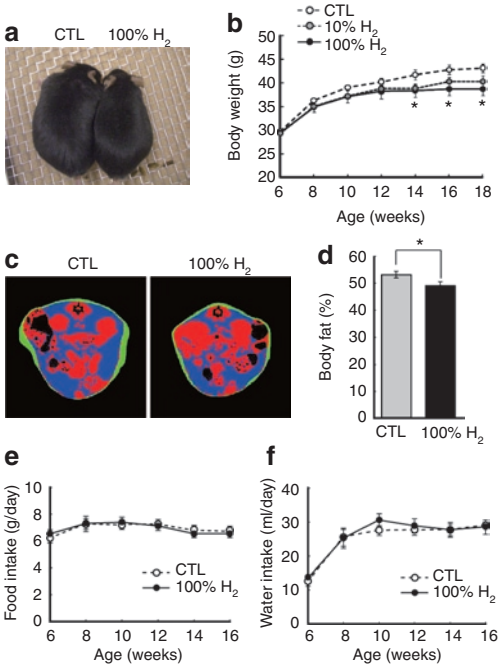

Figure 3 Consuming molecular hydrogen suppresses obesity. (**a**) The appearance of *db/db* mice given water with or without hydrogen for 3 months. Each average mouse was photographed. (**b**) Body weights of *db/db* mice given water with 100% (0.8 mmol/l) or 10% (0.08 mmol/l) hydrogen were examined every 2 weeks. Control mice (CTL) received water without hydrogen. $^*P < 0.05$; 100% H_2 vs. control group ($n = 9$ for H_2 and $n = 6$ for control group). (**c**) Representative CT images of abdominal part of average *db/db* mice drinking water with or without hydrogen for 3 months. Blue, green, and red represent visceral fat, subcutaneous fat, and muscle, respectively. (**d**) Total body fat composition of *db/db* mice calculated by the integration of fat area in each section from CT scan images. Data are mean ± s.e.m. ($n = 15$). $^*P < 0.05$. (**e**) Food and (**f**) water intake of *db/db* mice was measured every 2 weeks throughout the experiment.

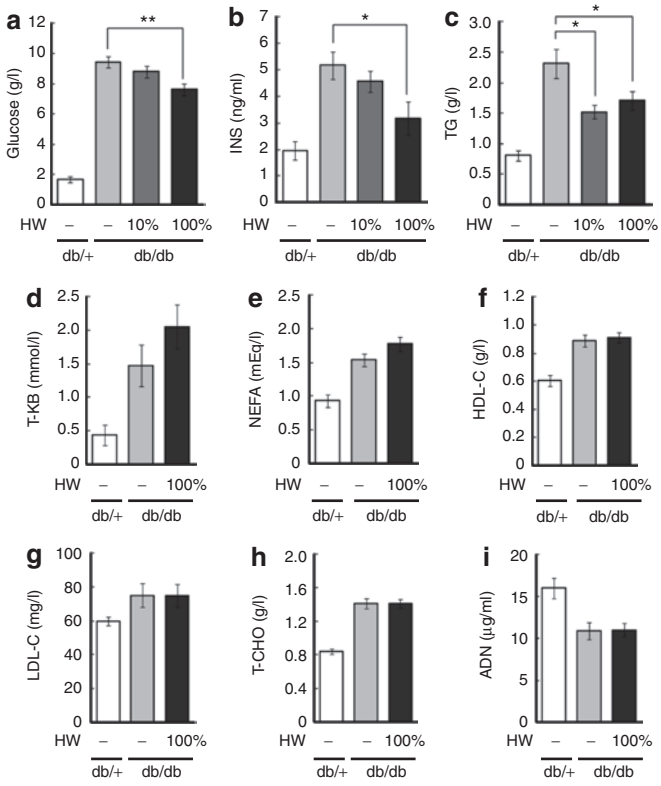

Figure 4 Consuming molecular hydrogen suppresses hyperglycemia, hyperinsulinemia, and plasma triglyceride level. *Db/db* mice were given water with 100% (0.8 mmol/l) or 10% (0.08 mmol/l) hydrogen for 3 months. *Db/+* and *db/db* mice were given water without hydrogen (HW) for the same period as controls. Biochemical analyses were performed to obtain plasma parameters of *db/+* and *db/db* mice. (**a**) Plasma concentration of glucose, (**b**) insulin, (**c**) triglyceride (TG), (**d**) total ketone bodies (T-KB), (**e**) free fatty acids (NEFA), (**f**) high-density lipoprotein-cholesterol (HDL-C), (**g**) low-density lipoprotein-cholesterol-cholesterol (LDL-C), (**h**) total cholesterol (T-CHO), and (**i**) adiponectin (ADN) are shown as mean ± s.e.m. ($n = 15$). $^*P < 0.05$, $^{**}P < 0.01$.

phosphoenolpyruvate carboxykinase and glucose-6-phosphatase, catalytic subunit (G6PC) (**Figure 6c,d**).

Since FGF21, an atypical member of the fibroblast growth factor family, contributes to energy metabolism, we focused on the gene expression of FGF21 after integrated study of the gene expression. H_2 administration induced hepatic mRNA levels of FGF21, regardless of a fed or fasted liver (**Figure 6a,b**). These results indicate that, at least in part, the induction of hepatic FGF21 contributes to the lowering effect on plasma glucose and triglyceride levels.

Drinking hydrogen water stimulates energy metabolism

To verify whether drinking H_2-water stimulates energy metabolism, we examined oxygen (O_2) consumption and carbon dioxide (CO_2) production. First, we compared physical ability by detecting the movement of mice with infrared beams. Although *db/db* mice was apparently less active in light and dark than control *db/+* mice, no difference was found between *db/db* mice with and without H_2-water (**Figure 7a,b**).

Db/db mice with or without H_2-water consumed less O_2 and produced less CO_2 than *db/+* control mice (**Figure 7c–f**). Moreover, it is significant that H_2-drinking *db/db* mice consumed more O_2, 10%, and produced more CO_2, 10%, than *db/db* mice without H_2-water during both night and day (**Figure 7c–f**). Since respiratory exchange rates (VCO_2/VO_2) did not differ between *db/db* mice with and without H_2-water, the carbon source for energy production was not changed to stimulate energy metabolism (**Figure 7g,h**).

Thus, drinking H_2-water suppresses the gain of fat and body weights and improves metabolic parameters by stimulating energy metabolism.

DISCUSSION

In this study, we show a novel benefit of H_2, it may be useful on therapy for and prevention of obesity and diabetes. So far, many reports have confirmed that consumption of H_2 reduces oxidative stress in various disease models and clinical tests (7,8,10–16,19). Clinical tests revealed that drinking H_2-water reduced oxidative stress makers in patients with type 2 diabetes (19) or subjects with potential metabolic syndrome (20), and influenced glucose (19) and cholesterol metabolism (20).

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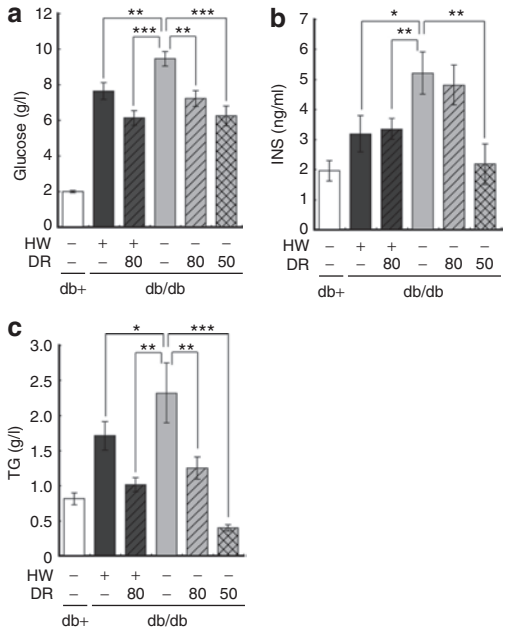

Figure 5 The effect of hydrogen is similar to that of dietary restriction. *Db/db* mice were given water with or without hydrogen (HW) and subjected to dietary restriction (DR). Control groups were permitted free access to food and water throughout the experiment. In diet-restricted groups, mice were permitted *ad libitum* water, but food intake was restricted to 80% or 50% of that of *ad libitum* diet consumption. Plasma concentrations of (**a**) glucose, (**b**) insulin (INS), and (**c**) triglyceride (TG) were measured after 3-month treatment. Data are mean ± s.e.m. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; compared with HW (–)/DR (–) group (n = 8–15).

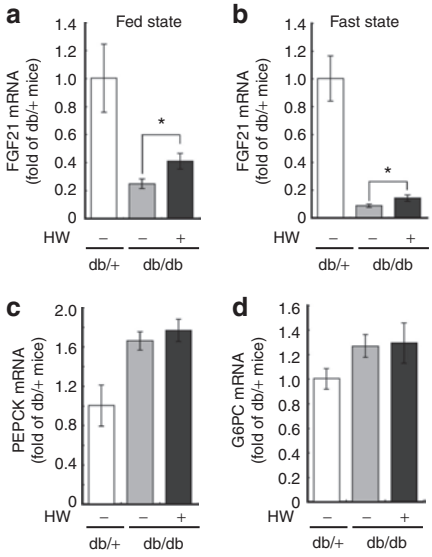

Figure 6 Chronic effects of hydrogen on fibroblast growth factor 21 (FGF21) gene expression in the liver. *Db/+* and *db/db* mice were given water with or without hydrogen for 3 months. (**a**) The gene expression of FGF21 with fed liver, (**b**) FGF21 with fast liver, (**c**) Phosphoenolpyruvate carboxykinase (PEPCK), and (**d**) glucose-6-phosphatase, catalytic subunit (G6PC) were measured. Data are mean ± s.e.m. $^{*}P < 0.05$; control vs. hydrogen water in *db/db* mice (n = 15).

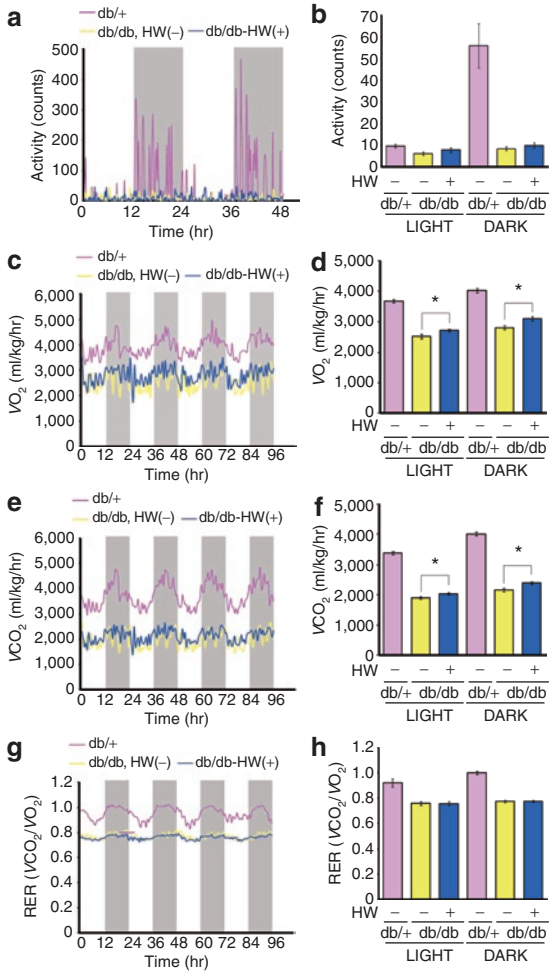

Figure 7 Molecular hydrogen increases oxygen consumption and carbon dioxide production without influencing movement activities. (**a** and **b**) movement activity, (**c** and **d**) oxygen consumption, (**e** and **f**) carbon dioxide production, and (**g** and **h**) RER (respiratory exchanging rate) in *db/+* and *db/db* mice given water with or without 100% (0.8 mmol/l) hydrogen for 3 months. (**a**, **c**, **e**, and **g**) Representative profiles for each parameter. Gray area represent dark phase. (**b**, **d**, **f**, and **h**) Data are the mean ± s.e.m. (n = 9 and n = 6, for *db/db* and for *db/+* groups, respectively). $^{*}P < 0.05$.

The mitochondria are a major source of reactive oxygen species during energy production metabolism and H_2 directly protects mitochondria that are exposed to reactive oxygen species (7). Thus, it may be reasonable that mitochondrial energy metabolism, especially fatty acid metabolism, functions against oxidative stress to efficiently expend glucose and fatty acid.

Initially, H_2 was dissolved in culture media and shown to protect cells and organelles by directly reacting highly active reactive oxygen species (7). Next, the brain, heart, liver, and intestine were protected from oxidative stress by inhalation of H_2 gas (7–11). Interestingly, instead of inhaling H_2 gas, H_2-water was effective in protecting the brain and kidney from oxidative stress (12–15) and decreased oxidative stress markers of patients with diabetes and potential metabolic syndrome (19,20). Drinking H_2-water is the most convenient method to

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consume H_2; however, when H_2 is consumed from H_2-water, a very limited amount of H_2 can be consumed, because H_2 is dissolved in water at a saturated level of only 0.8 mmol/l under atmospheric pressure. After H_2-water is consumed, most H_2 in blood becomes undetectable within 30 min (14), probably *via* expiration from the lungs; thus, it has been an open question why H_2-water is effective despite its small amount and short exposure. In this study, it was found that H_2 can be accumulated and reserved in the liver with glycogen, which at least partly explains this question.

Moreover, it has been reported that H_2 acts as an anti-inflammatory and antiallergic regulator by inducing inflammatory cytokines, such as tumor necrosis factor-α, interleukin-6 and some phosphorylating signal factors (9,10,14,32). On the other hand, the concept that obesity is a proinflammatory disease has been accepted (33); thus, consumption of H_2-water may suppress obesity by acting as an anti-inflammatory.

Alternatively, we found that consumption of H_2-water enhanced the expression of FGF21. Since it has not been shown that H_2 directly regulates transcription, H_2 is indirectly involved in FGF21 expression. Since FGF21 is a metabolic hormone that improves insulin sensitivity and glucose clearance, reduces plasma triglyceride concentrations and suppresses weight gain when fed a high-fat diet (26–29), all the findings shown in this study were elucidated by the enhanced expression of FGF21.

Indeed, we revealed that drinking H_2-water stimulates energy metabolism as measured by O_2 consumption and CO_2 production. The enhancement of energy metabolism may fully elucidate why consumption of H_2-water suppresses the gain of fat and body weights and improves metabolic parameters; however, it remains unknown whether the induction of FGF21 fully elucidates the enhancement of energy metabolism. Moreover, the relationships among the reduction of oxidative stress, induction of FGF21 expression and stimulation of energy metabolism are still unclear. It should be analyzed whether these relationships are direct or indirect. It might be valuable to examine the relationship of obesity with oxidative stress. Although the primary target of H_2 essentially remains unknown, these findings provide a clue to understand the mechanism of chronic treatment with H_2-water.

Finally, we would like to emphasize that the novel benefit of H_2 in therapeutic and preventive applications for metabolic syndrome could be achieved by the most convenient way, H_2-water.

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DISCLOSURE

The authors declared no conflict of interest.

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