Inhalation of molecular hydrogen increases breath acetone excretion during submaximal exercise: a randomized, single-blinded, placebo-controlled study

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Abstract

Aerobic exercise is widely accepted as a beneficial option for reducing fat in humans. Recently, it has been suggested that molecular hydrogen (H_2) augments mitochondrial oxidative phosphorylation. Therefore, the hypothesis that inhaling H_2 could facilitate lipid metabolism during aerobic exercise was investigated in the current study by measuring the breath acetone levels, which could be used as non-invasive indicators of lipid metabolism. This study aimed to investigate the effect of inhaling H_2 on breath acetone output during submaximal exercise using a randomized, single-blinded, placebo-controlled, and cross-over experimental design. After taking a 20-minute baseline measurement, breath acetone levels were measured in ten male subjects who performed a 60% peak oxygen uptake-intensity cycling exercise for 20 minutes while inhaling either 1% H_2 or a control gas. In another experiment, six male subjects remained in a sitting position for 45 minutes while inhaling either 1% H_2 or a control gas. H_2 significantly augmented breath acetone and enhanced oxygen uptake during exercise ($P < 0.01$). However, it did not significantly change oxidative stress or antioxidant activity responses to exercise, nor did it significantly alter the breath acetone or oxygen uptake during prolonged resting states. These results suggest that inhaling H_2 gas promotes an exercise-induced increase in hepatic lipid metabolism. The study was approved by the Ethical Committee of Chubu University, Japan (approved No. 260086-2) on March 29, 2018.

Key words: aerobic exercise; antioxidant activity; hepatic lipid metabolism; hydrogen gas; ketone bodies; mitochondrial oxidative phosphorylation; obesity; oxidative stress; reactive oxygen species; seated rest

doi: 10.4103/2045-9912.296038
How to cite this article: Hori A, Ichihara M, Kimura H, Ogata H, Kondo T, Hotta N. Inhalation of molecular hydrogen increases breath acetone excretion during submaximal exercise: a randomized, single-blinded, placebo-controlled study. Med Gas Res. 2020;10(3):96-102.

Introduction

Obesity causes various disease complications and is generally recognized as an international health hazard.[1,2] Physical exercise, e.g., aerobic exercise, is known to be effective in reducing obesity.[3] However, mitochondrial oxidative phosphorylation, which is activated during exercise, increases reactive oxygen species (ROS), and results in an enhancement of oxidative stress.[4,5]

Mitochondrial ROS has been reported to impair mitochondrial functions.[6-8] In fact, ROS-induced mitochondrial dysfunction has been suggested to cause an excessive accumulation of fat.[9] Thus, it has been suggested that exercise-induced oxidative stress inhibits lipid metabolism during exercise.[10]

Recently, many studies have shown that molecular hydrogen (H_2) has beneficial biological effects that attenuate oxidative stress and/or intensify mitochondrial function.[11-13] Originally, Ohsawa et al.[14] reported that H_2 could protect cells and tissues against oxidative stress by selectively reducing ROS. Kawamura et al.[15] suggested that H_2 indirectly scavenges ROS by inducing nuclear factor-E2-related factor 2. Murakami et al.[16] demonstrated that H_2 enhanced mitochondrial activity, indicating that it increases oxidative phosphorylation. Conversely, results from that same study suggested that H_2 induces mild oxidative stress, and plays a hormesis effect, protecting mitochondria against exacerbated oxidative stress. As for the effects on lipid metabolism, Kamimura et al.[17] showed that intake of H_2 water induced the expression of fibroblast growth factor 21 and proposed that intake of H_2 water could lead to enhanced ketogenesis and lipolysis of adipose tissue[18]; the authors actually showed that H_2-induced fibroblast growth factor 21 augmented free fatty acid and glucose consumption and improved obesity in mice. Based on these studies, we hypothesised that H_2 would enhance an exercise-induced increase in lipid metabolism.

To non-invasively assess lipid metabolism in humans, recent studies have measured breath acetone[19,20], which is one of the ketone bodies produced from acetyl-coenzyme A (CoA) in hepatic mitochondria during lipid metabolism. Therefore, this study aimed to elucidate the effects of H_2 gas inhalation on breath acetone excretion during submaximal-intensity cycling exercise.

Participants and Methods

Participants

Twelve healthy men (height 174.5 ± 6.0 cm, age 21.8 ± 5.8 years, weight 67.7 ± 7.6 kg) volunteered to participate in this study. All participants were informed of the experimental protocol and the possible risks involved in this study before providing written consent. This study was approved by the Ethical Committee of Chubu University, Japan (approved No. 260086-2) on March 29, 2018.

Experimental protocol

This study consisted of two experimental groups: submaximal-

intensity exercise experiment (SEE) and seated rest experiment (SRE) (**Figure 1**). We adopted randomized, single-blinded, placebo-controlled, and cross-over design for each experiment. During each experiment, exhaled breath and blood samples were collected to detect changes in breath acetone excretion ($V_{Acetone}$) and oxidative stress, respectively. Experiments were performed between 9:00 a.m. and 11:00 a.m.

In the SEE, 10 of 12 subjects participated and came to the laboratory on three separate occasions. Participants first performed an incremental cycling exercise test to evaluate peak oxygen uptake (VO_{2peak}). On the 2nd and 3rd days, participants performed a submaximal cycling exercise with the workload calculated based on VO_{2peak} while inhaling one of two kinds of gas, H_2 containing air (H_2 trial) or artificial air (control trial), during each trial. The H_2 gas contained 1% H_2, 21% O_2, and 0% CO_2 (N_2 balance) and the artificial air did not contain H_2.

In the SRE, 6 of 12 subjects participated and visited the laboratory on two different days to perform two trials. During each trial participants rested in a sitting position for 35 minutes while inhaling either H_2 containing air or artificial air.

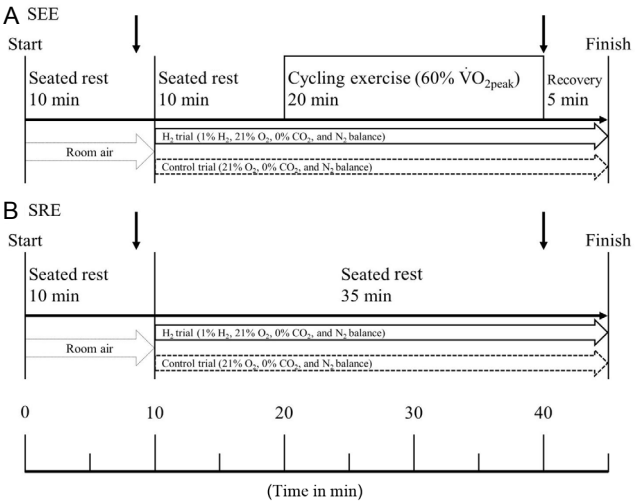

Figure 1: Design of the submaximal-intensity exercise experiment (SEE, A) and seated rest experiment (SRE, B).
Note: Black down arrows indicate the time points for blood sampling to evaluate oxidative stress and antioxidant activity. Recovery indicates subjects rested in a sitting position on the bicycle ergometer after the cycling exercise. VO_{2peak}: Peak oxygen uptake.

Measurement of VO_{2peak}

VO_{2peak} was determined during ramp incremental exercise using a bicycle ergometer (Aerobike 75XLIII; Combi Wellness Corporation, Tokyo, Japan) to determine the relative load of the submaximal cycling exercise in the SEE. The workload was gradually increased by 20 W every 1 minute after a 3-minute warm-up at 0 W. The subjects maintained a pedalling cadence of 60 r/min during the test. We terminated the exercise when the subject was unable to maintain a pedaling rate above 50 r/min and was unable to return to 60 r/min despite verbal exhortation. VO_2 was measured on a breath-by-breath basis using a metabolic gas analyzer (AE-310S; Minato Medical Science, Osaka, Japan). VO_{2peak} was defined as a 20-second averaged peak value of VO_2 during the exercise.

SEE and SRE

Figure 1 shows the SEE and SRE protocols. Subjects were instructed to fast for approximately 13 hours before performing the SEE. Participants were provided similar diets on the day before performing trials to minimize dietary influences (number of calories, and fat, protein, and carbohydrate energy ratios were 9586 ± 1360 kJ, 31 ± 7%, 14 ± 4%, and 55 ± 6%, respectively, for the H_2 trial and for 9573 ± 1402 kJ, 32 ± 8%, 14 ± 4%, and 54 ± 6%, respectively, for the control trial; $P > 0.34$, paired *t*-test). Subjects performed a 20-minute submaximal cycling exercise after 20-minute seated rest using the same bicycle ergometer used in the incremental exercise test. The workload corresponded to the intensity at 60% of VO_{2peak} and the pedalling cadence was kept constant at 60 r/min. This intensity was used to maximize lipid metabolism[21] and to increase oxidative stress.[22] Subjects started to inhale the experimental air 10 minutes after beginning seated rest until the end of the SEE.

Subjects fasted for approximately 13 hours before starting the experiment and had the same meal the day before both trials (8025 ± 2084 kJ; fat, protein, and carbohydrate energy ratios: 29 ± 6%, 16 ± 5%, and 55 ± 9%). Subjects started to inhale the experimental air (H_2 or artificial air) 10 minutes after beginning seated rest until the end of the SRE for 35 minutes in the same way as SEE (**Figure 1B**).

Measurement of breath acetone excretion

Figure 2 details the experimental setup for measuring $V_{Acetone}$. Gas (H_2 gas or artificial air) from a cylinder was buffered in a 200-L Douglas bag. Subjects inhaled the gas through a one-way valve (Hans Rudolph, Kansas City, KS, USA) and respiratory mask (Minato Medical Science). Exhaled breath was passed through a hot-wire flow meter (Minato Medical Science) to measure minute ventilation (V_E) on a breath by breath basis, then collected in a 50-L Douglas bag for 1 minute at rest and 30 seconds during and after exercise for measuring acetone concentration. We also continuously sampled exhaled breath at 150 mL/min immediately after the expiratory gas passed thorough the flow meter for continuous measurement of O_2 and CO_2 concentrations using a metabolic gas analyzer (AE-310S) in which VO_2, carbon dioxide output (VCO_2) and heart rate from electrocardiogram were calculated.

Acetone concentration was determined using the gas chromatographic method (VOC1; Figaro Engineering, Osaka, Japan). $V_{Acetone}$ was calculated from the product of V_E and acetone concentration because the drastic increase in ventilation during exercise enhances the dilution of exhaled breath and decreases the breath acetone concentration.[23,24]

Evaluation of oxidative stress and antioxidant activity

We collected blood samples from the subjects' fingertips at rest before exposure to the H_2 and artificial air gases in both the SEE and SRE. Blood samples were taken again immediately after the end of the exercise and 40 minutes after the beginning of the experiment in the SEE and SRE, respectively.

Blood samples were centrifuged to obtain plasma, and oxidative stress and antioxidant activity were measured by diacron-reactive oxygen metabolites (d-ROMs) and biologi-

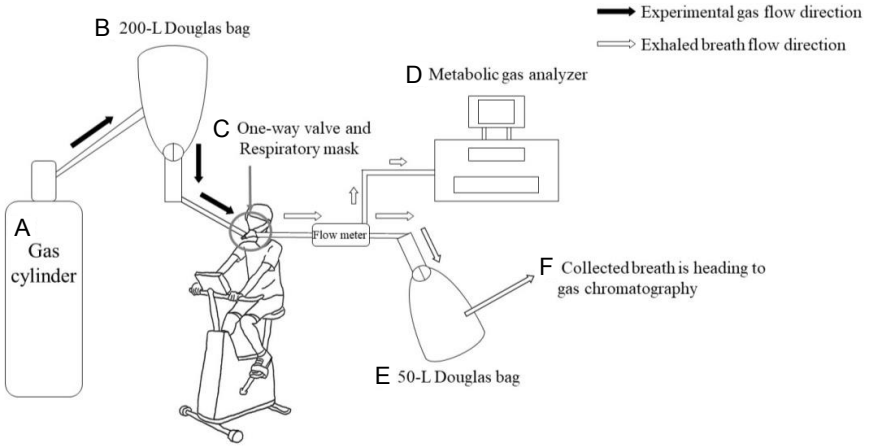

Figure 2: Schema of the submaximal-intensity exercise experiment (SEE) setup.
Note: (A, B) The experimental gas (H_2 gas or artificial air as the control gas) was supplied using a gas cylinder (A) and buffered in a 200-L Douglas bag (B). (C) Subjects inhaled the gas through a one-way valve and a respiratory mask. (D) Exhaled breath was sampled at a rate of 150 mL/min and respiratory parameters were detected by a metabolic gas analyzer. (E, F) Exhaled gas was collected using a 50-L Douglas bag (E) to measure breath acetone concentration, which was measured by gas chromatography (F).

cal antioxidant potential (BAP) tests using a Free Radical Elective Evaluator (FREE Carrio Duo; Wismerll, Tokyo, Japan).[25-27] The d-ROMs test measures the blood concentration of hydroperoxides according to the optical measurement method.[28] The values are expressed in UCARR, which are arbitrary units (1 UCARR corresponds 0.08 mg/dL H_2O_2).[27] The BAP test evaluates biological antioxidant activity in plasma by measuring the degree of decolourisation of the BAP solution caused by reduction of Fe^{3+} to Fe^{2+} ions by antioxidants.[25,29]

Statistical analysis

An a priori statistical power analysis was performed to determine the sample size needed for the study, using the G* Power 3.1.9.7 software (Heinrich-Heine-Universität, Düsseldorf, Germany). The primary outcome variable in this study was the change in VAcetone during exercise. For this analysis, it was determined that a minimal sample size of 6 subjects was needed to achieve a statistical power of more than 80% (1–β), required to reject the null hypothesis, with an effect size of 0.25 and an α error rate of 0.05, using a two-way repeated measures analysis of variance. In SEE, we recruited 10 participants, assuming potential subject attrition (e.g. due to dropouts). A two-way repeated measures analysis of variance was used for evaluation of significance. If a significant interaction and/or a main effect was observed, then Bonferroni's multiple comparisons test was also performed to identify the specific differences. For pairwise comparisons, a paired *t*-test or Wilcoxon signed-rank test was adopted. Statistical analyses were performed using SPSS 24.0 for Windows (IBM, Armonk, NY, USA) and StatView 5.0 (SAS Institute, Cary, NC, USA); the significance level for all tests was set at 5%. Data are presented as mean ± standard error (SE).

Results

Effects of H_2 gas inhalation on $V_{Acetone}$, respiratory and circulatory parameters

The time-course changes in respiratory and circulatory parameters and $V_{Acetone}$ are shown in **Figure 3**. Significant effects with time were observed in all indicators. In both trials, VO_2, VCO_2, heart rate, and V_E increased significantly during and after the exercise compared with the ambient air baseline. In the H_2 trial, $V_{Acetone}$ was significantly increased at 2, 3, 4, 5, 7, and 20 minutes during exercise. $V_{Acetone}$ in the control trial was also significantly increased during and 2 minutes after exercise. A significant trial-by-time interaction was detected in both VCO_2 and V_E. The H_2 trial significantly increased VCO_2 compared with the control trial at all-time points during exercise except 1 minute. Furthermore, inhalation of H_2 gas significantly increased V_E compared with that of the control trial at 5, 10, and 15 minutes during exercise. A significant interaction was also observed in VO_2 and the main effect of trial in VO_2 tended to be significant. Moreover, VO_2 in the H_2 trial was significantly higher than in the control trial at 3 minutes during exercise.

Importantly, the H_2 trial significantly augmented $V_{Acetone}$ response to exercise compared with the control trial. This result was confirmed by a significant trial-by-time interaction, though no significantly different time points were detected between the H_2 and control trials. We further confirmed that the rate of increase from the rest to exercise steady-state value, defined as the average of 15 and 20 minutes in the exercise, was significantly higher in the H_2 trial than the control trial (P = 0.02, 1563 ± 325% and 1148 ± 140%, respectively) as determined by a Wilcoxon signed-rank test.

Since inhalation of H_2 gas significantly augmented $V_{Acetone}$ during submaximal exercise in the SEE (**Figure 3**), we investigated whether inhalation of H_2 gas facilitated $V_{Acetone}$ without any physical exercise. However, neither significant interactions nor main effects were detected in any parameters during the SRE (**Table 1**).

Effects of H_2 gas inhalation on oxidative stress and antioxidant activity

In the SEE, no significant effect on changes in d-ROMs, as an index of oxidative stress levels, was detected (**Figure 4A**). The exercise significantly increased BAP as an index of antioxidant potential (**Figure 4B**); however, inhaling the H_2 gas did not significantly affect changes from rest to exercise. In SRE, inhalation of H_2 gas could not significantly change d-ROMs or BAP during 35-minute seated rest (**Figure 4C** and **D**).

Discussion

This investigation aimed to clarify the effects of inhaling 1% H_2 gas on breath acetone excretion during submaximal cycling exercise. First, H_2 gas significantly augmented $V_{Acetone}$ respons-

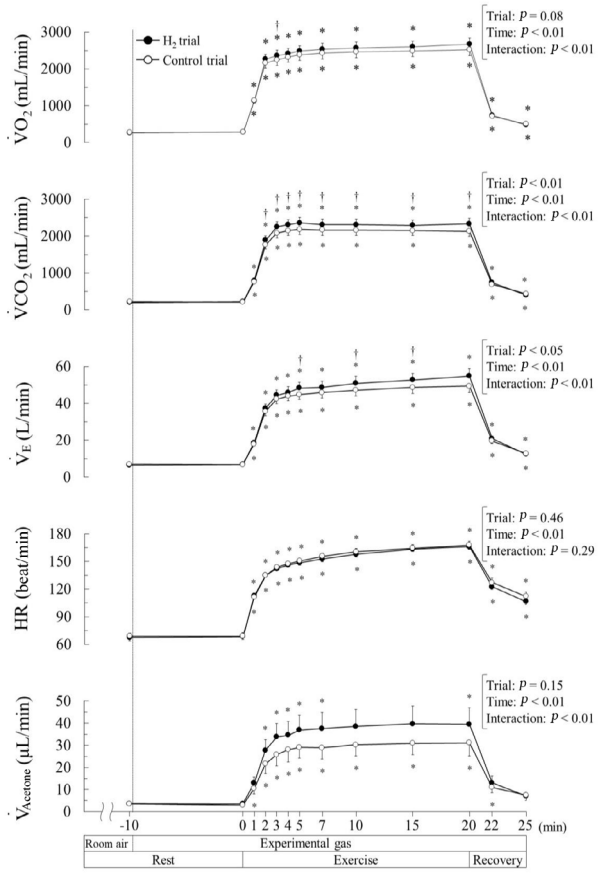

Figure 3: Changes in respiratory and circulatory parameters and acetone excretion ($V_{Acetone}$) during 20 minutes submaximal-intensity exercise experiment (SEE).

Note: Before exposure to the experimental gas, subjects inhaled ambient room air for 10 minutes during seated rest to establish a baseline. VO_2: oxygen uptake; VCO_2: carbon dioxide output; V_E: minute ventilation; HR: heart rate. Data are expressed as mean ± SE. *P < 0.05, *vs.* baseline; †P < 0.05, *vs.* control trial (two-way repeated measures analysis of variance followed by Bonferroni's multiple comparisons). Some error bars are smaller than the symbols.

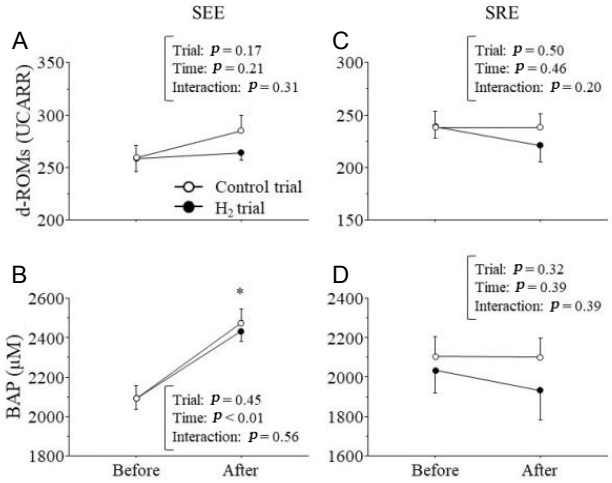

Figure 4: Changes in oxidative stress and antioxidant activity during submaximal-intensity exercise experiment (SEE, A and B) and seated rest experiment (SRE, C and D).

Note: Blood samples were collected at rest before inhaling experimental gas and immediately after the end of each experimental trial for both the SEE and SRE. d-ROMs: diacron-reactive oxygen metabolites, an index of oxidative stress level; BAP: biological antioxidant potential, an index of antioxidant activity. Data are expressed as mean ± SE. *P < 0.05, *vs.* baseline.

es during exercise. Second, H_2 gas slightly, but significantly, enhanced VO_2 responses to exercise. Third, H_2 gas did not significantly change oxidative stress and antioxidant activity responses to exercise. Fourth, H_2 gas did not significantly alter $V_{Acetone}$ or VO_2 in the resting states. To our knowledge, this is the first study implying that H_2 might enhance lipid metabolism during exercise in healthy humans.

Possible mechanism(s) underlying H_2 gas-induced augmentation of $V_{Acetone}$

In the present study, we found that inhalation of H_2 gas enhanced $V_{Acetone}$ during submaximal exercise. This result suggests H_2 gas strengthens hepatic lipid metabolism because acetone is produced by spontaneous decarboxylation of acetoacetate, originating from acetyl-CoA produced from β-oxidation of free fatty acid.[30] There are at least two possible mechanisms by which hepatic lipid metabolism could be increased by H_2 gas: (1) increasing adipocyte degradation and (2) augmenting mitochondrial-lipid metabolism.

In the case of adipocyte degradation, enzymes that are important for lipolysis, hormone-sensitive lipase, and adipose triglyceride lipase are activated by exercise[31] and inhibited by insulin.[32,33] However, some studies have shown that intakes of H_2 water can decrease blood insulin levels.[17,34] Hence, in the present study, H_2 might have inhibited inactivation of the hormone-sensitive and triglyceride lipases by suppressing increases in the insulin level, thereby enhancing the lipolysis. Alternatively, other exercise-induced lipolysis-related proteins, such as perilipin and CGI-58,[31,35] might also have been influenced by H_2 and further study is required to investigate these possibilities. Collectively, inhaling H_2 gas may have contributed to $V_{Acetone}$ augmentation during exercise as a result of accelerated lipolysis.

It is also possible that H_2 increased mitochondrial-lipid metabolism; inhalation of H_2 gas slightly, but significantly, increased VO_2 response during exercise in the present study. This result suggests that H_2 enhanced mitochondrial oxidative phosphorylation (it was assumed that VCO_2 was elevated in proportion to the increased VO_2 and the elevation of VCO_2 altered V_E response via chemoreflex). Since exercise enhances hepatic oxidative stress,[36-38] H_2 might have contributed to inhibition in ROS- and/or oxidative stress-induced impairment of mitochondrial function by directly or indirectly reducing oxidative stress. Previous studies have demonstrated that H_2 migrates into and accumulates in the liver after gas administration.[17] However, in the present study, H_2 gas did not significantly change the oxidative stress and antioxidant activity responses to exercise. Therefore, it is likely that this mechanism did not operate, at least in the situation described in the present study.

Although the detailed mechanism is still under discussion, H_2 could intensify mitochondrial function itself and, consequently, overall energy metabolism.[16,39-41] For instance, Cui et al.[39] demonstrated that H_2 treatment reduces the loss of mitochondrial membrane potential, indicating that H_2 protects mitochondrial function. Other studies have implied that

Table 1: Changes in respiratory and circulatory parameters and acetone excretion ($V_{Acetone}$) during 45 minutes seated rest experiment

		Experimental gas inhalation time (min)						Main effect		
	Room air	10	15	20	25	30	35	Trial	Time	Interaction
VO_2 (mL/min)								*P*=0.29	*P*=0.33	*P*=0.81
H_2	285±7	310±26	301±29	321±42	289±14	348±46	348±45			
Control	278±17	289±10	285±24	293±23	273±24	307±13	304±30			
VCO_2 (mL/min)								*P*=0.12	*P*=0.40	*P*=0.49
H_2	204±13	205±6	207±14	213±19	213±19	193±17	212±10			
Control	278±17	279±17	280±17	281±17	282±17	283±17	284±17			
V_E (L/min)								*P*=0.29	*P*=0.12	*P*=0.58
H_2	6.4±0.4	6.8±0.7	6.6±0.6	6.5±0.6	6.2±0.4	7.0±0.8	7.1±0.8			
Control	5.8±0.3	6.1±0.2	6.3±0.3	6.5±0.4	6.0±0.4	6.5±0.4	6.5±0.4			
Heart rate (beat/min)								*P*=0.73	*P*=0.42	*P*=0.56
H_2	66±5	66±4	65±4	65±4	62±4	66±5	67±3			
Control	63±4	64±3	66±3	65±4	64±4	65±4	65±3			
$V_{Acetone}$ (μL/min)								*P*=0.65	*P*=0.11	*P*=0.66
H_2	2.5±0.7	2.5±0.9	3.0±1.1	3.1±1.1	3.0±1.1	3.2±1.2	3.2±1.2			
Control	3.5±1.0	3.6±1.0	3.5±1.0	3.7±1.3	3.3±0.9	4.0±1.3	3.9±1.1			

Note: Subjects inhaled the experimental gas (H_2 or control) for 35 minutes after inhaling room air for 10 minutes. $V_{Acetone}$: breath acetone excretion; VO_2: oxygen uptake; VCO_2: carbon dioxide output; V_E: minutes ventilation. Data are expressed as mean ± SE, and analyzed by two-way repeated measures analysis of variance followed by Bonferroni's multiple comparisons.

H_2 promotes mitochondrial ATP production by producing a hydrogen gradient.[41]

Sirtuin 3 (Sirt3), which is localized in mitochondria, facilitates fatty-acid oxidation by deacetylation of long-chain acyl-CoA dehydrogenase, which catalyzes β-oxidation of fatty acids.[42] Moreover, Sirt3 was shown to increase the production of ketone bodies during fasting by deacetylation of 3-hydroxy-3-methylglutaryl CoA synthase 2.[43] As evidence suggests that intake of H_2 water inhibits down-regulation of Sirt3,[44] inhaled H_2 gas might enhance the production of ketone bodies by increasing levels of Sirt3 during exercise. In addition, Lee et al.[45] observed that H_2 activates adenosine monophosphate-activated protein kinase, which promotes fatty acid uptake and oxidation.[46]

Taken together, this suggests that inhaled H_2 gas might have reinforced the mitochondrial lipid metabolism, at least in the liver where H_2 was accumulated at high concentrations,[17] and consequently augmented $V_{Acetone}$ during exercise. However, it should be noted that some previous studies found that H_2 intensified mitochondrial functions adopted chronic intake of H_2.[17,40,44] Further animal research is needed to identify how H_2 acutely affects mitochondrial metabolism.

In the present study, we adopted 1% as the concentration of H_2 gas for inhalation, based on evidence showing the beneficial effects of 1% H_2 gas.[14,47,48] In contrast, a previous investigation demonstrated that inhalation of 2% and 4% H_2 gas suppressed hepatic cell death to a greater extent compared to 1% H_2 gas.[47] Therefore, it is possible that the inhalation of a concentration of H_2 gas that is higher than 1% could increase hepatic metabolism further. Additional studies are thus required to elucidate whether $V_{Acetone}$ is augmented in a H_2 concentration-dependent manner.

To our knowledge, no studies have investigated the acute effects of H_2 inhalation on energy metabolism at rest in healthy humans. In the present study, inhalation of H_2 gas did not change $V_{Acetone}$ and VO_2 during rest in the SRE experiment. This suggests at least, that the 'acute' effects of H_2 on hepatic metabolism might require "exercise"-induced increases in lipolysis and/or in mitochondrial metabolism. However, Nakai et al.[49] showed that 4-week administration of H_2-supplemented water up-regulated hepatic metabolism-related genes in healthy rats. Indeed, one explanation for inhalation of H_2 gas not changing $V_{Acetone}$ and VO_2 during rest might be an insufficient duration of H_2 inhalation. A limitation of the present study is the lack of a direct evidence to demonstrate the mechanisms that inhalation of H_2 gas augmented $V_{Acetone}$ during exercise. Further studies to clarify the effect of "chronic" inhalation of H_2 on hepatic metabolism in humans and explore the mechanism of H_2 gas inhalation are required.

Clinical implications

The incidence of obesity is increasing globally and it is considered an international health problem.[1,2] Furthermore, high body mass index was estimated to cause about 4.0 million deaths globally in 2015.[50] It is well known that exercise therapy, especially aerobic exercise, is effective in improving obesity.[3] The present study suggests that H_2 inhalation may enhance lipid metabolism in the liver during exercise and potentially intensify the effect of aerobic exercise on improving obesity.

Inhalation of H_2 during exercise augments $V_{Acetone}$ as well as β-hydroxybutyrate, presumably due to corresponding increases in β-hydroxybutyrate and breath acetone.[20] Recently, β-hydroxybutyrate was shown to have signalling functions related to antioxidant and anti-inflammatory effects.[51,52] Further, Newman et al.[53] showed that a ketogenic diet improves cognition and lifespan in mice; nutritional ketosis may also improve exercise performance, adaptive response to exercise, and recovery from exercise.[30] Considering these findings, an

increase in ketone bodies due to inhalation of H_2 gas during exercise might provide beneficial effects for exercise performance and general health in addition to the enhancement of lipid metabolism.

Conclusion

We demonstrated that inhalation of H_2 gas increased breath acetone excretion during submaximal-intensity cycling exercise. This result suggests that inhalation of H_2 gas facilitates hepatic lipid metabolism during exercise.

Acknowledgements
We thank Ryota Masuda (Chubu University) for providing technical assistance and Haruka Yamaguchi, RD (Chubu University) for her expert calorie calculation.

Author contributions
AH, MI, and NH: decided conception and design of research; AH, HK, and NH: performed experiments and analyzed data; AH, MI, HK, HO, TK, and NH: interpreted results of experiments; AH and NH: prepared figures; AH, MI, and NH: drafted manuscript; AH, MI, HK, HO, TK, and NH: approved final version of manuscript.

Conflicts of interest
We have no competing interest to declare.

Financial support
None.

Institutional review board statement
The study was approved by the Ethical Committee of Chubu University (approved No. 260086-2) on March 29, 2018.

Declaration of patient consent
The authors certify that they have obtained participant consent forms. In the form, the participants have given their consent for their images and other clinical information to be reported in the journal. The participants understand that their names and initials will not be published.

Biostatistics statement
The statistical methods of this study were reviewed by the biostatistician of the Chubu University, Japan.

Data sharing statement
Datasets analyzed during the current study are available from the corresponding author on reasonable request.

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Peer review
Externally peer reviewed.

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Received: February 3, 2020
Accepted: March 3, 2020
ublished Online:September 30, 2020