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Hydrogen produced in rat colon improves *in vivo* reduction–oxidation balance due to induced regeneration of α-tocopherol

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Abstract

We investigated whether non-digestible saccharide fermentation-derived hydrogen molecules (H_2) in rat colon could improve the *in vivo* reduction–oxidation (redox) balance via regeneration of α-tocopherol, by assessing their effect on hydroxyl radicals, the α-tocopherol concentration and the redox balance. In Expt 1, a Fenton reaction with phenylalanine (0 or 1·37 mmol/l of H_2) was conducted. In Expt 2, rats received intraperitoneally maize oil containing phorone (400 mg/kg) 7 d after drinking *ad libitum* water containing 0 or 4 % fructo-oligosaccharides (FOS) (groups CP and FP, respectively). In Expt 3, rats unable to synthesise ascorbic acid drank *ad libitum* for 14 d water with 240 mg ascorbic acid/l (group AC), 20 mg of ascorbic acid/l (group DC) or 20 mg of ascorbic acid/l and 4 % FOS (group DCF). In the Fenton reaction, H_2 reduced tyrosine produced from phenylalanine to 72 % when platinum was added and to 92 % when platinum was excluded. In Expt 2, liver glutathione was depleted by administration of phorone to rats. However, compared with CP, no change in the *m*-tyrosine concentration in the liver of FP was detected. In Expt 3, net H_2 excretion was higher in DCF than in the other rats after 3 d of the experiment. Furthermore, the concentrations of H_2 and α-tocopherol and the redox glutathione ratio in perirenal adipose tissue of rats were significantly higher in DCF than in DC. To summarise, in rat colon, fermentation-derived H_2 further shifted the redox balance towards a more reducing status in perirenal adipose tissue through increased regeneration of α-tocopherol.

Key words: **Hydrogen molecules: α-Tocopherol: Adipose tissue: Colon: Rats**

Regulation of oxidative stress caused by dietary components is crucial for promoting health because oxidative stress is a key factor involved in the development of certain diseases such as diabetes, ischaemia–reperfusion injury and arteriosclerosis[1–3]. However, a higher colonic production of hydrogen molecules (H_2) derived from fermentation *in vivo* of non-digestible saccharides in the colon mitigates oxidative stress because H_2 act as electron donors[4]. Although an *in vitro* study reported that H_2 purportedly aim to specifically scavenge hydroxyl radicals, the underlying mechanism remains unclear to date[5]. Hydroxyl radicals are the most detrimental reactive oxygen species and have a high reduction–oxidation (redox) potential (2340 mV)[6]. However, it still remains unclear whether or not H_2 exert an antioxidative effect *in vivo* by directly donating electrons to hydroxyl radicals. The difference in redox potential between H_2 and hydroxyl radicals is 2760 mV, as the potential of H_2 is –420 mV. As per the relationship between the redox potential and the Gibbs free-energy change achieved in the Nernst equation, electron donation by H_2 to hydroxyl radicals via one-step reaction should be accompanied by an immense release of Gibbs free energy ($\Delta G^{0'}$ – 267 kJ/mol), which corresponds to the free energy released by hydrolysis of about 9 mol of ATP ($\Delta G^{0'}$ – 30·5 kJ/mol)[7]. Should this reaction occur *in vivo*, cell damage could well be caused by electron donation from H_2. Therefore, it is generally assumed that electrons are not directly donated by H_2 to hydroxyl radicals.

H_2 is a hydrophobic, non-polar molecule and has a lower solubility in water (0·8 mmol/l at 20°C) than oxygen (1·34 mmol/l at 20°C), a similar hydrophobic, non-polar molecule. Being more soluble in oil and organic solvents than in water[8], H_2 is likely localised more frequently in biological membranes and adipose tissue. For example, in our previous study,

Abbreviations: AC, rats given water containing physiologically adequate ascorbic acid level (240 mg of ascorbic acid/l); CC, rats given water containing 240 mg of ascorbic acid/l; CP, phorone-treated rats given water containing 20 mg of ascorbic acid/l; DC, rats given water containing 20 mg of ascorbic acid/l; DCF, rats given water containing 20 mg of ascorbic acid/l and 4 % fructo-oligosaccharides; FOS, fructo-oligosaccharides; FP, phorone-treated rats given water containing 20 mg of ascorbic acid/l and 4 % FOS; H_2, hydrogen molecule; ODS, Osteogenic Disorder Shionogi; redox, reduction–oxidation; SD, Sprague–Dawley.

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we found that, in rats that were fed a diet of non-digestible saccharides, H_2 was produced in the colon and its concentration was greater in the adipose tissue than in other tissues. This phenomenon resulted in a reduced pro-inflammatory cytokine expression in fat tissues[9]. Therefore, we theorised that H_2 could exert an antioxidative effect by interacting with fat-soluble compounds.

α-Tocopherol is a compound with a redox potential of 370 mV[10]. It also acts as an electron donor to lipid peroxyl radicals produced *in vivo* by reactive oxygen species in membranes, resulting in *α*-tocopherol radicals[11]. These radicals are regenerated to *α*-tocopherol by electrons donated by ascorbic acid (redox potential of 80 mV[12]), which has a lower redox potential than do *α*-tocopherol radicals. The sparing effect of ascorbic acid on *α*-tocopherol can lead to effective mitigation of oxidative stress[13,14]. The difference in redox potential between H_2 and *α*-tocopherol radical is 790 mV, being the change in free energy during this electron donation of –76·1 kJ/mol. By contrast, the change in free energy during hydrolysis of phosphoenolpyruvic acid, a high-energy compound and an intermediate glucolysis product, is lower (–61·9 kJ/mol)[7]. Hence, donation of electrons by H_2 to *α*-tocopherol radicals can occur *in vivo*.

In the present study, to confirm if H_2 produced in the colon could directly scavenge hydroxyl radicals *in vivo*, we used glutathione-depleted rats fed fructo-oligosaccharides (FOS; an enhancer of colonic H_2 production) and measured the concentration of hepatic tyrosine isomers produced from phenylalanine by hydroxyl radicals. In addition, we analysed the adipose tissue of ascorbic acid-deficient rats fed FOS to assess if colonic H_2 regenerated *α*-tocopherol radicals to *α*-tocopherol.

Materials and methods

The present study was approved by the Shizuoka University Animal Use Committee (approval numbers: 29A-15 and 2018A-9). Animals were kept and cared for as per the Guidelines for the Care and Use of Laboratory Animals, Shizuoka University.

Samples

FOS (commercial name: Meioligo-P; 44 % 1-kestose, 46 % nystose and 10 % 1 F-*β*-fructofuranosylnystose) were purchased from Meiji Food Materia Co. Ltd.

Animals and diets

Five-week, male Sprague–Dawley (SD, mean body weight 130–150 g) and 6-week, male Osteogenic Disorder Shionogi (ODS, mean body weight 100–130 g) rats were purchased from Japan SLC (Haruno colony) and CLEA Japan, respectively. Rats were housed in individual cages with screen bottoms made of stainless steel and kept in a room maintained at 23 ± 2°C, with 50–70 % humidity, and in a 12 h light (07.00–19.00 hours)–12 h dark cycle. For all experiments, rats were first acclimatised for 7 d to the experimental settings. SD rats were given a basal, 25 % casein diet as previously reported[4] (online Supplementary Tables S1–S3) and water *ad libitum*. Unlike a previously reported work, in which rats were given a diet containing 300 mg of ascorbic acid/kg[15], ODS rats of the present work were fed the same diet as that of SD rats, but were given water containing 240 mg of ascorbic acid/l *ad libitum* for maximum growth.

In vitro effect of hydrogen molecule on hydroxyl radicals

In Expt 1, to determine if H_2 directly scavenges hydroxyl radicals, we examined the effect of H_2 from water on phenylalanine-derived tyrosine production, which is caused by hydroxyl radicals generated in the Fenton reaction. Supersaturated H_2 water was prepared using H_2 water 7.0 (ECOMO International)[16]. An aliquot of supersaturated H_2 water (0·05 ml) was placed into a 50-ml sealed vial for H_2 analysis. After incubation at 37°C for 3 min and to measure the H_2 concentration, 0·05 ml of the gaseous phase was withdrawn using a gas-tight syringe. Samples were analysed by GC (lower detection limit 0·10 μl/l; quantification range 0·30–50 μl/l; Biogas analyzer BAS-1000; Mitleben). The concentration of supersaturated H_2 water was found to be 2·73 mmol/l. Hydroxyl radicals were generated by the Fenton reaction described by Yoshimura *et al.*[17], with some modifications. The reaction mixture (1 ml) was as follows: 1·37 mmol H_2/l, 5 mmol HEPES buffer (pH 7·5)/l, 5 μmol ammonium iron (II) sulphate/l, 0·5 mmol EDTA/l, 0·5 mmol L-ascorbic acid/l and 1·25 mmol L-phenylalanine/l. To confirm the effect of catalysis on occurrence of reducing power of H_2, a platinum colloid solution (10 mmol/l, particle size: 1–4 nm; Renaissance Energy Research) was added to the above mixture in a final concentration of 50 μmol/l. As a control, water without H_2 was used. The Fenton reaction was initiated by adding H_2O_2 in a concentration of 2·5 mmol/l and kept at 25°C for 40 s. At 40 s post-initiation of reaction, the concentration of tyrosine was measured using an HPLC method reported by Kaur & Halliwell[18].

In vivo effect of hydrogen molecule on hydroxyl radicals

In Expt 2, to determine if colonic H_2 scavenges hydroxyl radicals *in vivo*, the effect of H_2 produced by fermentation of FOS in the colon of rats, during the conversion of phenylalanine to *m*-tyrosine, was assessed. Phenylalanine is converted to *m*-tyrosine by hydroxyl radicals generated when glutathione is depleted in the liver of rats. A two-way factorial design was not used because we previously found that the antioxidative effect of colonic H_2 was observed only in rats with oxidative stress[4]. After acclimatisation, eighteen rats were divided into three groups with comparable body weight and given the basal diet *ad libitum* for 7 d. Two rat groups were given tap water *ad libitum*, and the remaining group was given water containing 4 % FOS, also *ad libitum*. FOS was added to drinking water but not diet and given it to rats to avoid a decrease in FOS intake due to decreased consumption of diet. At the end of the experiment, the net H_2 excretion was measured for 5 min by the same method as we previously reported[4]. Rats in one of the groups given FOS-free tap water were then administered an intraperitoneal injection of maize oil (4 ml/kg, group CC). Separately, to deplete glutathione in the body, rats in the remaining groups were intraperitoneally injected 400 mg/kg of 2,6-dimethyl-2,5-heptadien-4-one (phorone) in maize oil (4 ml/kg injection, groups CP and

(4 %FOS water) FP)[19]. Three hours post-phorone injection, all rats were anaesthetised via inhalation with 2 % isoflurane. Under the effect of anaesthesia, 0·5 ml of blood was collected from the portal vein of rats into 5-ml sealed vials containing heparin, for H_2 analysis. In addition, 1 ml of blood from the portal vein was collected into separate, sealed heparin microtubes for plasma preparation. A 0·5 ml sample of the gaseous phase in sealed vials was withdrawn using a gas-tight syringe. Next, the concentration of portal H_2 was measured by the same method as we previously reported[4]. The liver of rats was perfused immediately after blood withdrawal with 20 ml of ice-cold saline solution through the portal vein. After perfusion, the median lobe of the liver was removed and rapidly dropped into liquid N_2. All samples were stored at –80°C until further analysis.

Effect of colonic hydrogen molecule on regeneration of α-tocopherol

In Expt 3, to determine if colonic H_2 exerts a suppressive effect on the oxidative stress via regeneration of α-tocopherol, we examined the α-tocopherol concentration and the redox balance in adipose tissue of ascorbic acid-deficient ODS rats as follows. After acclimatisation, rats were placed inside a sealed polypropylene chamber for 5 min and the excreted H_2 was captured. Next, GC (Biogas analyzer BAS-1000; Mitleben) was then used to determine the net H_2 excretion. Twenty-four ODS rats were divided into three groups with comparable body weight and net H_2 excretion. As in Expt 2, a two-way factorial design was not used. All rats were given the basal diet *ad libitum* for 14 d. In addition, rats were given water *ad libitum* for 14 d as follows: (1) water containing 240 mg of ascorbic acid/l (physiologically adequate ascorbic acid level; AC group), (2) water containing 20 mg of ascorbic acid/l (ascorbic acid-deficient; DC group), or (3) water containing 20 mg of ascorbic acid/l and 4 % FOS (ascorbic acid-deficient plus FOS; DCF group). FOS and ascorbic acid were added to drinking water but not diet and given them to rats to avoid a decrease in the intake of FOS and ascorbic acid due to decreased consumption of diet. After the start of the experiment, net H_2 excretion was measured again at days 3, 7, 10 and 14. At the end of the experiment and after measuring net H_2 excretion, all rats were anaesthetised via inhalation with 2 % isoflurane. Laparotomy was carried out on rats and the livers of the rats were perfused immediately after blood withdrawal with 20 ml of ice-cold saline solution through the portal vein. After perfusion, the median lobe of the liver was removed and rapidly dropped into liquid N_2. Liver samples were then stored at –80°C until further analysis. To analyse the concentrations of H_2, α-tocopherol and ascorbic acid, and the redox parameters, the perirenal adipose tissue was removed immediately. Approximately 0·5 g of perirenal adipose tissue was placed into 5-ml sealed vials for H_2 analysis. After incubation at 37°C for 3 min and to measure the H_2 concentration, a volume of 0·5 ml of the gaseous phase was withdrawn using a gas-tight syringe. Next, the concentration of H_2 in adipose tissue was measured using the above-mentioned GC method. The remaining of the adipose tissue was stored at –80°C until further analysis.

m-Tyrosine analysis

Liver samples (0·25 g) were homogenised with three volume of 5 % trichloric acid and centrifuged. The concentration of *m*-tyrosine in the resulting supernatant was determined by the method of Kaur & Halliwell[18].

Determination of ascorbic acid and α-tocopherol

The concentrations of ascorbic acid and α-tocopherol in the liver and adipose tissue were determined by the methods of Kishida *et al.*[20] and Abe *et al.*[21], respectively.

Glutathione analysis

The levels of GSH and GSSG in the liver and adipose tissue were determined by the method of Rahman *et al.*[22].

Antioxidant enzyme activity

The activity of glutathione peroxidase, glutathione reductase and superoxide dismutase in the adipose tissue was assessed by the method of Flohe & Gunzler[23], Carlberg & Mannervik[24] and Ukeda *et al.*[25], respectively.

Statistical analysis

To determine an adequate sample size to identify significant differences in the concentrations of liver *m*-tyrosine (Expt 2) and adipose α-tocopherol (Expt 3), a Student's *t* test power analysis was carried out using the G*Power statistical package version 3.1.9.3. The sample size was then calculated considering an α probability of 0·05 with a power of 0·80. The effect size was estimated using the results from a preliminary study at these premises (unpublished results). From the power analysis, it was determined that the required sample size per group was six (Expt 2) and eight (Expt 3) rats. Data were analysed for homogeneity of variances with Bartlett's test. To compare between DC and DCF groups, for data with equal variances, one-way ANOVA was used, followed by Student's *t* test. Data with unequal variances were first log-transformed. For data with unequal variances even after log-transformation, Welch's *t* test was used instead. Apart from the power analysis, all statistical analyses were carried out using SAS JMP software (version 13.2.1). Values obtained from the experiments are expressed as mean values with their standard errors, and statistical significance was defined as $P < 0·05$.

Results

In vitro effect of hydrogen molecule on hydroxyl radicals

In Expt 1, when H_2 was added to the reaction, total tyrosine produced in 40 s via the Fenton reaction from phenylalanine, with or without platinum, was 72 and 92 %, respectively, of that produced in a Fenton reaction with neither H_2 nor Pt (Table 1). It was then observed that, if maintaining the reactants at 25°C for 60 min during the Fenton reaction without Pt, unknown pink pigments, perhaps oxidised compounds, were produced, whereas when Pt was added to the reaction, these pigments were not detected (online Supplementary Fig. S1).

Table 1. Effect of hydrogen molecules (H_2) on hydroxyl radical-produced tyrosine originated from phenylalanine by the Fenton reaction (*n* 6) (nmol)*† (Mean values with their standard errors)

	H_2 (–)		H_2 (+)			
	Pt (–)		Pt (+)		Pt (–)	
	Mean	SEM	Mean	SEM	Mean	SEM
Total tyrosine	7·06	1·48	5·10	0·86	6·47	1·04
Para form	2·33	0·46	1·85	0·29	2·08	0·34
Meta form	1·61	0·35	1·11	0·18	1·54	0·25
Ortho form	3·10	0·65	2·14	0·39	2·85	0·46

* The Fenton reaction for H_2 (–) was carried out without H_2. Fenton reaction for H_2 (+) was carried out with a reaction system containing 1·37 mmol H_2 /l. Colloid Pt was added to the reaction system in a final concentration of 50 μmol/l.
† Data are indicated as the quantities of tyrosine produced in 40 s via the Fenton reaction.

Table 2. Effect of colonic hydrogen molecules (H_2) on tyrosine produced from phenylalanine by hydroxyl radicals in glutathione-depleted rats (*n* 6) (Mean values with their standard errors)

	CC		CP		FP	
	Mean	SEM	Mean	SEM	Mean	SEM
Food intake (g/7 d)	142	6	137	8	122	4
Body weight gain (g/7 d)	65	5	62	5	59	3
Final body weight (g)	261	9	257	8	256	5
Net H_2 excretion (μmol/5 min)	0·118	0·034	0·158	0·035	7·51†	2·20
Portal H_2 (μmol/l)	1·36	0·31	1·79	0·58	7·39†	1·1
Liver						
Weight (g/100 g of body weight)	4·40	0·08	3·94	0·06	4·06	0·07
Total glutathione (mmol/kg)	7·53	0·26	0·05*	0·01	0·14	0·03
m-Tyrosine (μmol/kg)	1·54	0·16	1·91‡	0·10	1·97	0·03

CC, untreated rat group given tap water only; CP, phorone-treated rat group given tap water only; FP, phorone-treated group given water containing 4 % fructo-oligosaccharides.
* Mean values were significantly different from those of CC ($P < 0·05$).
† Mean values were significantly different from those of CP ($P < 0·05$).
‡ Mean values tended to be different from those of CC ($P = 0·0793$).

In vivo *effect of hydrogen molecule on hydroxyl radicals*

In Expt 2, although neither food intake nor body weight differed between rat groups (Table 2), net H_2 excretion and portal H_2 concentration were significantly higher in FP than in CP. Nonetheless, no significant differences in liver weight were observed between rat groups, and administration of phorone to rats almost completely depleted glutathione in their liver. Moreover, although the concentration of *m*-tyrosine in the liver tended to be greater in CP than in CC ($P = 0·0793$), no significant differences were observed between treatment groups.

In vivo *effect of colonic hydrogen molecule on regeneration of α-tocopherol*

In Expt 3, neither food nor α-tocopherol intake differed between AC and DC, but food and α-tocopherol intake were significantly lower in DCF than in DC (Table 3). Ascorbic acid intake was significantly lower in DC than in AC, but did not differ between the DC and DCF. Compared with that of AC, the body weight gain of DCF was the lowest, followed by that of DC. Net H_2 excretion in DCF was higher than that in DC after 3 d of the experiment (Fig. 1). Furthermore, the AUC for 14 d for net H_2 excretion of DCF was 350 % that of DC (Table 3). Liver weight and the concentration of ascorbic acid were significantly lower in DC than in AC, but no significant differences were found between ascorbic acid-deficient groups. In addition, administration of FOS had no effect on the concentration of α-tocopherol in the liver of rats (Table 3).

Table 3. Food, ascorbic acid and α-tocopherol intake, body weight, net hydrogen molecules (H_2) excretion and condition parameters of perirenal adipose tissue in rats given different levels of ascorbic acid in tap water or tap water containing 4 % fructo-oligosaccharides (*n* 8) (Mean values with their standard errors)

	AC		DC		DCF	
	Mean	SEM	Mean	SEM	Mean	SEM
Food intake (g/14 d)	217	6	215	4	165†	8
Days 1–7 (g/7 d)	113	3	122	2	84†	4
Days 8–14 (g/7 d)	104	3	93	3	81	5
Ascorbic acid intake (mg/14 d)	71·6	2·0	5·82*	0·24	5·08	0·37
α-Tocopherol intake (mg/14 d)	15·1	0·4	15·0	0·3	11·5†	0·6
Body weight gain (g/14 d)	62	3	44*	3	29†	4
Final body weight	210	7	190	3	175	5
$AUC_{days\ 0–14}$ for net H_2 excretion (mmol)	10·5	1·6	7·53	1·36	26·0†	3·2
Liver						
Weight (g/100 g of body weight)	4·31	0·05	3·90*	0·05	4·09	0·15
Ascorbic acid (nmol/g)	68·9	4·7	9·19*	0·49	10·3	0·7
α-Tocopherol (nmol/g)	117	4	114	5	99·7	6·2
Perirenal adipose tissue						
Weight (g/100 g of body weight)	1·72	0·09	1·88	0·06	1·13†	0·09
H_2 (nmol/g)	1·41	0·31	0·73	0·13	3·26†	0·51
Ascorbic acid (nmol/g)	19·4	1·9	ND		ND	
α-Tocopherol (nmol/g)	18·3	5·3	16·6	4·9	68·9†	10·5

AC, rats given a physiologically adequate ascorbic acid level in water (240 mg of ascorbic acid/l); DC, rats given water containing 20 mg of ascorbic acid/l; DCF, rats given water containing 20 mg of ascorbic acid/l and 4 % fructo-oligosaccharides; $AUC_{days\ 0–14}$, AUC for 14 d; ND, not detected.
* Mean values were significantly different from those of AC ($P < 0·05$).
† Mean values were significantly different from those of DC ($P < 0·05$).

Finally, although administration of FOS did not significantly affect the activity of antioxidative glutathione peroxidase and superoxidase dismutase, glutathione reductase was significantly lower in DCF when compared with that in DC.

Discussion

Although it was previously reported that, *in vitro*, electrons are donated by H_2 to hydroxyl radicals[5], no evidence of such donation was found in the present study, neither *in vitro* nor *in vivo*.

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Table 4. Redox parameters in perirenal adipose tissue of rats given different levels of ascorbic acid in tap water or tap water containing 4 % fructo-oligosaccharides (*n* 8)
(Mean values with their standard errors)

	AC		DC		DCF	
	Mean	SEM	Mean	SEM	Mean	SEM
Total glutathione (nmol/g)	245	23	228	20	338†	31
Reduced form (nmol/g)	187	22	136	17	245†	21
Oxidised form (nmol/g)	43·9	3·9	59·6	6·8	70·9	13·5
Reduced:oxidised glutathione ratio	4·42	0·55	2·59*	0·50	4·64‡	1·00
Antioxidative enzyme activity						
Glutathione peroxidase (nmol/min/mg protein)	76·1	9·6	77·1	5·8	79·3	7·2
Glutathione reductase (nmol/min/mg protein)	66·3	7·8	73·6	4·3	58·1†	2·8
Superoxide dismutase (units/mg protein)	234	22	217	21	229	31

AC, rats given a physiologically adequate ascorbic acid level in water (240 mg of ascorbic acid/l); DC, rats given water containing 20 mg of ascorbic acid/l; DCF, rats given water containing 20 mg of ascorbic acid/l and 4 % fructo-oligosaccharides.
* Mean values were significantly different from those of AC ($P < 0·05$).
† Mean values were significantly different from those of DC ($P < 0·05$).
‡ Mean values tended to be different from those of DC ($P = 0·0960$).

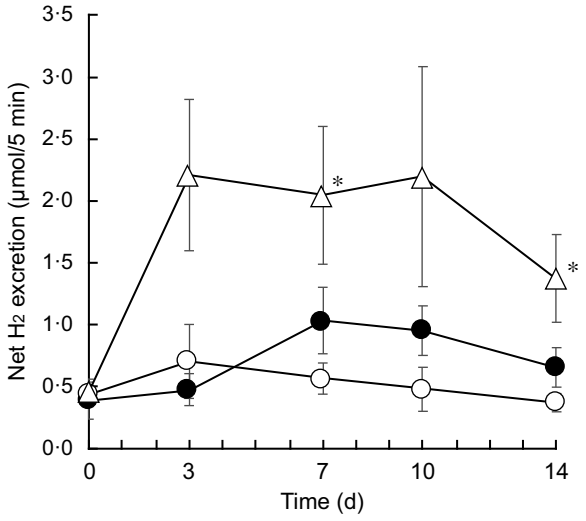

Fig. 1. Changes in net H_2 excretion of rats given water containing 0 and 4 % fructo-oligosaccharides and rats with normal intake of ascorbic acid. AC, rats given a physiologically adequate ascorbic acid level in water (240 mg of ascorbic acid/l); DC, rats given water containing 20 mg of ascorbic acid/l; DCF, rats given water containing 20 mg of ascorbic acid/l and 4 % fructo-oligosaccharides. * Mean values were significantly different from those of DC ($P < 0·05$). —●—, AC; —○—, DC; —△—, DCF.

Nonetheless, in the present work, we found that the concentration of α-tocopherol in perirenal adipose tissue was higher in ascorbic acid-deficient rats, which experienced enhanced H_2 production in the large intestine due to administration of FOS. In a previous study, we also reported an elevated concentration of H_2 in perirenal adipose tissue of rats given FOS[9]. A similar result was observed in the present study. Fat-soluble α-tocopherol donates electrons to lipid peroxyl radicals produced by reactive oxygen species in adipose tissue and biological membranes, which results in α-tocopherol radicals[11,26]. However, these α-tocopherol radicals are usually reconverted to α-tocopherol by ascorbic acid[13,14]. Therefore, the results in the present study likely demonstrate that not ascorbic acid, but H_2 derived from colonic fermentation contributed to regenerate α-tocopherol as an electron donor in ascorbic acid-deficient rats. Although glutathione acts as an electron donor in many types of cells, which is crucial for maintaining the redox balance, in rats of the present study, glutathione in perirenal adipose tissue was only about 5 % compared with that in the liver that we previously reported[4]. This result seems to imply that α-tocopherol, but not glutathione, is an important compound to maintain the balanced redox in the adipose tissue. Thus, since oxidative stress has been implicated in a number of adipose tissue accumulation-related conditions, an increased regeneration of α-tocopherol in the adipose tissue by H_2 produced in the colon via fermentation will likely help prevent from developing diabetes and atherosclerosis, for example[1,27].

Previous studies showed the possibility that chitosan oligosaccharides and inulin may suppress hepatic cytochrome P450 enzymes[28,29]. The decreased activities of these enzymes could shift the redox balance toward a more reducing status as cytochrome P450 enzymes are involved in increased oxidative stress. α-Tocopherol is metabolised by cytochrome P450 enzymes to be excreted[30]. Therefore, higher α-tocopherol concentration in the adipose tissue in ascorbic acid-deficient rats given FOS may be attributed to inhibited catabolism of α-tocopherol due to decreased activities of cytochrome P450 enzymes. However, whether this is caused by FOS intake and H_2 produced by colonic fermentation remains unclear. Further study needs to be performed to elucidate the involvement of colonic H_2 in α-tocopherol degradation.

Hydroxyl radicals react with many types of biological compounds such as lipids, proteins and DNA, leading to metabolic dysfunction. In addition, hydroxyl radicals also react with phenylalanine to produce *p*-, *m*- and *o*-tyrosine isomers. Apart from *p*-tyrosine, tyrosine isomers are not normally produced *in vivo*; thus, increased concentrations of *m*- and *o*-tyrosines result from overproduction of hydroxyl radicals. Glutathione depletion can increase oxidative stress and hence generate hydroxyl radicals[31,32]. Therefore, in the present study, administering phorone, a glutathione depletor, to rats, elevated the concentration of *m*-tyrosine, which expectedly increased hydroxyl radicals production. Interestingly, a high production of colonic H_2 did not reduce the *m*-tyrosine concentration. These results seem to suggest that H_2 derived from colonic fermentation are

unlikely to directly scavenge hydroxyl radicals accumulated after depletion of glutathione in the liver of rats. In a previous study, we showed that H_2 improved the redox balance of glutathione in hepatic ischaemia–reperfusion injured rat, which is an oxidative stress model[4]. Our previous data and those reported elsewhere showed that, compared with that in other tissues, the content of glutathione in the liver was substantially higher[9,33]. In addition, in the present work, the concentration of liver α-tocopherol did not change in rats experiencing a high production of colonic H_2. Therefore, unlike that in adipose tissue, glutathione in the liver likely plays a pivotal role in the regulation of the redox balance by H_2. Nonetheless, whilst the results of the present work are solid and promising, it cannot entirely be ruled out that there may be other pathways for direct reduction of hydroxyl radicals.

Ohsawa *et al.*[5] demonstrated that hydroxyphenyl fluorescein could be used to detect hydroxyl radicals and that H_2 specifically scavenged hydroxyl radicals *in vitro*. However, the results from both *in vitro* and *in vivo* experiments of the present work do not agree with those reported by Ohsawa *et al.* Whilst it remains unclear the reason for this discrepancy between the results, we can cautiously speculate that the use of different markers in the analysis may have biased the results. Indeed, hydroxyphenyl fluorescein is a highly specific probe for hydroxyl radical *in vitro*[34], but it is difficult to quantitatively use it as a marker in *in vivo* studies. In the present work, to assess the effect of colonic H_2 on hydroxyl radicals, we used instead tyrosine isomers as *in vivo* markers for hydroxyl radical detection. Although *p*-tyrosine is produced by phenylalanine hydroxylase *in vivo*, hydroxyl radicals randomly attack the aromatic ring of phenylalanine at different positions, resulting in production of *m*- and *o*-tyrosine in addition to *p*-tyrosine[35]. Using the same marker, the hydroxyl radical scavenging activity of certain antioxidative compounds has been evaluated in rats[36] and humans[37]. However, to the best of our knowledge, no study, thus far, has confirmed the ability of H_2 to scavenge hydroxyl radicals *in vivo*. Therefore, we believe that the present work is the first investigation to demonstrate the effect of colonic H_2 on hydroxyl radicals *in vivo*. Nonetheless, we suggest that further investigation *in vivo* on the direct scavenging of hydroxyl radicals by H_2 should be conducted.

According to the Nernst equation, the difference in redox potential between H_2 and hydroxyl radicals is 2760 mV and the electron donation between these molecules is likely accompanied by an exergonic reaction of 267 kJ/mol. In addition, the free energy occurring during the electron donation is estimated to produce approximately 9 mol ATP. Thus, the total energy released via direct donation of electrons by H_2 to hydroxyl radicals would likely cause tissue damage. Nonetheless, no detrimental effect of electron donation by H_2 has been reported in the literature to date. *In vivo* electron donation causing such an immense potential difference usually occurs multiple stepwise, not one stepwise, as the electron is transported from NADH to the oxygen molecule in the electron transport chain ($\Delta G^{0'}$ − 220 kJ/mol through five reaction steps[38]). By contrast, the difference in redox potential between H_2 and α-tocopherol radical is merely 790 mV, which corresponds to a relatively lower change in Gibbs free energy ($\Delta G^{0'}$ − 76·1 kJ/mol). This change in energy is similar to the amount of energy produced in the reaction from phosphoenolpyruvate to pyruvate. Therefore, H_2 likely donates electrons directly to α-tocopherol radicals *in vivo*. Moreover, in the present study, an increased α-tocopherol concentration in perirenal adipose tissue of FOS-fed rats seems to confirm this possibility. Considering the physiological concentrations of H_2, α-tocopherol radicals and α-tocopherol occurring during the electron donation to α-tocopherol radicals, the ΔG produced during this phenomenon is estimated to be about 10 kJ/mol, which seems to be physiologically acceptable.

In the present study, two strains (SD and ODS) of male rats were used. These were purchased from different breeders. Previous studies have shown differences in microbiota composition among breeders[39]. Therefore, the microbiota composition in the large intestine is estimated to be different between the SD and ODS rats used. Although H_2 was more produced by FOS intake in both rat strains in the present study, it remains unclear how much other fermentation products were produced. The involvement of these fermentation products in oxidative stress may not also be negligible. Different microbiota compositions could alter the fermentation pattern even when rats are fed the same diet. Several products from colon fermentation have been reported to have physiological effects. Among products by colonic fermentation, SCFA have been investigated extensively. In our previous study, increased caecal concentrations of these acids were observed by FOS intake in rats given the high-fat diet[9]. Butyrate is the primary energy source of colon cells and prevents inflammation[40]. Also, butyrate has been shown to protect against oxidative stress by modulating the activity of antioxidant enzymes such as catalase and glutathione S-transferase[41]. Therefore, the possibility that other products such as butyrate contribute to reducing α-tocopherol radical cannot be denied.

Finally, although redox balance was improved in rats given FOS in the present study, food intake and body weight gain for 14 d were slower in rats given the ascorbic acid-deficient diet and FOS (DCF group) than in those given the ascorbic acid-deficient diet (DC group). The administration of FOS with a low degree of polymerisation is assumed to increase the osmotic pressure in the stomach content. Higher osmotic pressure in the stomach content decreases food intake[42,43]. Therefore, increased osmotic pressure in the stomach content due to FOS intake may decrease food intake, resulting in slower growth.

In summary, our results show that H_2 derived from fermentation of non-digestible saccharides in the colon of rats, but not ascorbic acid, shifted the redox balance towards a more reducing status in adipose tissue via increased regeneration of α-tocopherol. In addition, the present work seems to suggest that H_2 do not directly scavenge hydroxyl radicals in the liver. Although the effect of H_2 on the regulation of the redox balance may not seem as strong as that of other antioxidative compounds such as glutathione, α-tocopherol and ascorbic acid, H_2 derived from colonic fermentation could well play an important role in maintaining a balanced redox via electron donation, that is, the regeneration of α-tocopherol. The present work should help advance our understanding of how non-digestible saccharides, including dietary fibre and resistant starch, prevent occurrence of lifestyle-related diseases, which are associated with redox imbalances.

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N. N. designed the research; Y. I., S. H., T. M. and N. N. conducted the research; S. I gave suggestions and information on α-tocopherol analysis. N. N. and Y. I. analysed the data; N. N. wrote the manuscript and was responsible for the final content. All authors were involved in designing the study, reviewing and interpreting the results and drafting the manuscript. In addition, all authors have read and approved the final document.

The authors declare that there are no conflicts of interest.

Supplementary material

For supplementary materials referred to in this article, please visit https://doi.org/10.1017/S0007114519003118

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