SUPPLEMENTARY MATERIAL

Wheat alkylresorcinols reduce micellar solubility of cholesterol *in vitro* and increase cholesterol excretion in mice

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Abstract

Epidemiological studies have shown that the consumption of whole grains can reduce risk for metabolic disorders. We recently showed that chronic supplementation with wheat alkylresorcinols (ARs) prevents glucose intolerance and insulin resistance with hepatic lipid accumulation induced in mice by a high-fat high-sucrose diet (HFHSD). The present study examines the effects of ARs on the micellar solubility of cholesterol *in vitro*, as well as the effects of transient AR supplementation on fecal lipid excretion and plasma lipid levels in mice. We found that ARs formed bile micelles with taurocholate independently of phospholipids, and dose-dependently decreased the micellar solubility of cholesterol in a biliary micelle model. Transient AR supplementation with HFHSD increased fecal cholesterol and triglyceride contents and decreased plasma cholesterol concentrations. These suggest that one underlying mechanism through which ARs suppress diet-induced obesity is by interfering with the micellar cholesterol solubilisation in the digestive tract, which subsequently decreases cholesterol absorption.

Keywords: wheat alkylresorcinols; bile acids; micelle; cholesterol; obesity; hepatic lipid

Abbreviations: ANOVA, analyses of variance; ARs, alkylresorcinols; HFHSD, high-fat high-sucrose diet; HFHSD-AR, high-fat high-sucrose diet with 0.4% ARs; PBS, phosphate-buffered saline; PC, phosphatidylcholine.

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Experimental

Chemicals

Phosphatidylcholine, sodium taurocholate and porcine cholesterol esterase were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

Isolation of alkylresorcinols

Alkylresorcinols (ARs; 1,3-dihydroxy-5- alkylbenzene homologues with alkyl side chains of 15 – 25 carbon atoms) were isolated from wheat bran M (Fresh Food Service Co. Ltd., Tokyo, Japan) as described (Oishi et al. 2015). The homolog profile was reported in our recent report (Oishi et al. 2015).

Bile acid binding assay

Bile-acid binding was assayed as described (Ngamukote et al. 2011) with several modifications. Briefly, each AR was dissolved with or without 0.6 mM phosphatidylcholine (PC) in isopropanol and dried under nitrogen before adding 0.1 M phosphate-buffered saline (PBS) containing 2 mM taurocholic acid (pH 7.4). The mixtures were sonicated, incubated for 2 h at 37°C, passed through a 0.22- μ m filter, and then taurocholic acid concentrations were determined using Total Bile Acid-Test Wako kits (Wako Pure Chemical Industries Ltd., Osaka, Japan).

Micellar solubility of cholesterol

Micelles were prepared as described (Kirana et al. 2005) with several modifications. Briefly, each AR, 2 mM cholesterol, and 2.4 mM phosphatidylcholine were dissolved in isopropanol and dried under nitrogen before adding 0.1 M PBS containing 6.6 mM taurocholate salt (pH 7.4). The micellar suspension was sonicated, incubated for 24 h at 37°C, filtered through a 0.22- μ m filter and then cholesterol concentrations were determined using LabAssay Cholesterol kit (Wako Pure Chemical Industries, Ltd.).

Animal experiment

Ten-week-old male C57BL/6J mice (Japan SLC Inc., Hamamatsu, Japan) were fed with the CE-2 normal diet (Clea Japan Inc., Tokyo, Japan) *ad libitum* for one week under a 12-h light - 12-h dark cycle (LD 12:12; lights on at 0:00 and lights off at 12:00), deprived of food at 14:00 for 24 h and then fed with the same amount (2.5 g/mouse) of the F2HFHSD high-fat high-sucrose diet (Oriental Yeast Co. Ltd., Tokyo, Japan) (Oishi et al. 2015) without (HFHSD) or with (HFHSD-AR) 0.4%

ARs. Feces were collected 24 h later from each cage and stored at -80°C, and the mice were sacrificed by exsanguination under sevoflurane anesthesia. Dissected mouse tissues were rapidly frozen and stored in liquid nitrogen. The mice were maintained and the study proceeded under the approval of the Animal Care and Use Committee at the National Institute of Advanced Industrial Science and Technology (Permission No: 2014-020).

Measurement of blood, liver, and fecal lipids

Mouse blood collected in EDTA-coated tubes was immediately separated by centrifugation for 15 min at $5,800 \times g$. Platelet-poor plasma was collected and stored at -80°C. Hepatic and fecal lipids were extracted as described (Oishi et al. 2015). Triglyceride, total cholesterol and total bile acid concentrations were measured using LabAssay Triglyceride, LabAssay Cholesterol, and Total Bile Acid-Test Wako kits (Wako Pure Chemical Industries Ltd.), respectively.

Statistical analysis

All data are expressed as means $\pm$ standard error of the means (SEM) and were analyzed using Excel-Toukei 2010 software (Social Survey Research Information Co. Ltd., Tokyo, Japan). Data derived from experiments *in vitro* were statistically evaluated using one-way or two-way analyses of variance (ANOVA) followed by the Tukey-Kramer test. Differences between two groups were analyzed using unpaired Student's or Welch's t-tests. $P < 0.05$ was taken to indicate statistical significance.

References

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Table S1. Comparison of metabolic parameters of mice fed with high-fat high-sucrose diet with and without ARs.

	HFHSD	HFHSD-AR
Plasma		
Triglyceride (mg/dL)	50.0 ± 1.31	48.9 ± 2.15
Total cholesterol (mg/dL)	112.1 ± 8.46	76.6 ± 7.15*
Liver		
Total lipids (mg/g tissue)	22.4 ± 2.89	26.4 ± 3.38
Triglyceride (mg/g tissue)	8.96 ± 2.47	11.81 ± 2.23
Total cholesterol (mg/g tissue)	1.97 ± 0.163	2.55 ± 0.175*
Total bile acid (nmol/g tissue)	23.9 ± 1.79	26.9 ± 2.60
Feces		
Fecal excretion (g/kg BW)	5.08 ± 0.709	4.46 ± 0.414
Total lipids (mg/g feces)	102.9 ± 9.80	114.8 ± 8.92
Triglyceride (mg/g feces)	4.32 ± 0.39	7.89 ± 0.53†
Total cholesterol (mg/g feces)	6.19 ± 0.39	8.31 ± 0.73*
Total bile acids (μmol/g feces)	2.11 ± 0.37	2.40 ± 0.32

Data are shown as means ± SEM (n = 7 - 8 and 6 for HFHSD and HFHSD-AR, respectively). * $P < 0.05$, † $P < 0.01$ compared with HFHSD. HFHSD, high-fat high-sucrose diet; HFHSD-AR, high-fat high-sucrose diet with 0.4% ARs.