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The flavan-3-ol fraction of cocoa powder suppressed changes associated with early-stage metabolic syndrome in high-fat diet-fed rats

Naomi Osakabe *, Junpei Hoshi, Naoto Kudo, Masahiro Shibata

Department of Bio-science and Engineering, Shibaura Institute of Technology Tokyo, Japan

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ABSTRACT

Aims: Previous epidemiological studies have suggested that ingestion of chocolate reduces the risk of cardiovascular disease. In the present study, we examined the effects of flavan-3-ols derived from cocoa on blood pressure, lipolysis, and thermogenesis in rats fed a high-fat diet and that showed early signs of metabolic syndrome.
Main methods: The rats were divided into three groups, and fed either normal diet (normal), 60% fat high-fat diet (HFD), or HFD containing 0.2% flavan-3-ols (HFD-flavan) for 4 weeks. At the end of the feeding period, blood pressure was measured and animals were sacrificed under anesthesia. Lipolysis and thermogenesis-related protein levels were measured in several tissues by Western blotting, and mitochondrial DNA copy number was measured by RT-PCR.
Key findings: Mean blood pressure and epididymal adipose tissue weight of HFD-flavan were significantly lower compared with those of HFD. Uncoupling protein (UCP)1 in brown adipose tissue and UCP3 in gastrocnemius of HFD-flavan were significantly increased compared with those of HFD group. Carnitine palmitoyltransferase (CPT) 2 levels in liver and medium-chain acyl-CoA dehydrogenase (MCAD) levels in gastrocnemius and liver were significantly increased by the supplementation of flavan-3-ols.
Significance: In addition to having hypotensive effects, flavan-3-ols enhance thermogenesis and lipolysis and consequently reduce white adipose tissue weight gain in response to high-fat diet feeding.

Introduction

Chocolate is known to be rich in flavan-3-ols, including the flavan 3-ol monomers (+)-catechin and (−)-epicatechin and the oligomer B-type procyanidins that are linked by C4–C8 bonds (Hammerstone et al., 1999; Hatano et al., 2002; Sanbongi et al., 1998). Previous meta-analyses suggested that ingestion of chocolate reduced the risk of cardiovascular disease, including coronary heart disease, myocardial infarction, and stroke (Buitrago-Lopez et al., 2011; Hooper et al., 2012). In addition, several randomized, controlled trials confirmed that dark chocolate rich in flavan-3-ols mitigated risk factors for metabolic syndrome such as hypertension (Taubert et al., 2007a, 2007b; Desch et al., 2010a, 2010b), vascular endothelial dysfunction (Engler et al., 2004; Schroeter et al., 2006), dyslipidemia (Baba et al., 2007a, 2007b), and glucose intolerance (Grassi et al., 2005a, 2005b). Several meta-analyses conducted after these clinical trials were consistent with the observation that chocolate reduced the risk of cardiovascular disease by mitigating these risk factors (Taubert et al., 2007a, 2007b; Hooper et al., 2008; Desch et al., 2010a, 2010b; Ried et al., 2010; Tokede et al., 2011; Shrime et al., 2011).

In our previous study, we confirmed that mean resting respiratory ratio (RER) was significantly reduced and mitochondrial DNA copy number was significantly increased in normal mice by repeated treatment with flavan-3-ols (Watanabe et al., 2014). In addition, repeated cocoa supplementation decreased blood pressure in rats fed a high-fat diet, and enhanced acetylcholine-dependent vasodilatation in the mesentery artery as determined by intravital microscopy (Osakabe and Shibata, 2012).

In the present study, we examined the effects of repeated treatment with flavan-3-ols derived from cocoa on blood pressure, lipolysis, and thermogenesis in rats fed a high-fat diet, which induced early-stage metabolic syndrome.

Materials and methods

Materials

The flavan 3-ol fraction was provided by Meiji Co., Ltd. (Tokyo, Japan). The flavan-3-ol fraction was prepared from cocoa powder using methods described in a previous report (Osakabe et al., 1998). Briefly, cocoa powder was defatted with n-hexane, and then the residue

* Corresponding author at: Department of Bio-science and Engineering, Shibaura Institute of Technology, 307 Fukasaku, Munumaku, Saitama 337-8570, Japan. Tel.: +81 720 6031; fax: +81 480 720 6011.
E-mail addresses: nao-osa@sic.shibaura-it.ac.jp (N. Osakabe), mf12054@sic.shibaura-it.ac.jp (J. Hoshi), mf13033@sic.shibaura-it.ac.jp (N. Kudo), shibatam@sic.shibaura-it.ac.jp (M. Shibata).

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was extracted with acetone. The n-butanol-dissolved fraction of the extract was applied to a Diaion HP2MG column (Mitsubishi Kasei Co., Ltd., Tokyo, Japan). The fraction eluted with 80% ethanol was collected, freeze-dried, and then used. This fraction contained 4.56% (+)-catechin, 6.43% (−)-epicatechin, 3.93% procyanidin B2, 2.36% procyanidin C1, and 1.45% cinnamtannin A2, as determined by HPLC. Catechins and procyanidins were also analyzed by HPLC (Natsume et al., 2000).

Animals and diets

The study was approved by the Animal Care and Use Committee of the Shibaura Institute of Technology. All animals received humane care under the guidelines of this institution. Eight-week-old male Wistar rats were obtained from Saitama Experimental Animal Co., Ltd. (Saitama, Japan). The rats were kept in a room with a regulated temperature of 23–25 ° C and controlled lighting (12 h light–dark cycles). The basal diet was MF obtained from the Oriental Yeast, Co., Ltd., Tokyo, Japan. The experimental diet was prepared using commercially available ingredients as shown in Table 1. The animals in the normal diet group (normal) were fed AIN-93 diet, the HFD group was fed a diet containing 60% fat, and the flavan-3-ol HFD diet group (HFD-flavan) was fed a diet containing 0.2% flavan-3-ols and 60% fat.

Experimental procedures

Four days after feeding of the basal diet, the animals were divided into 3 groups. The groups were fed either normal diet (n = 8), HFD (n = 7), or HFD-flavan (n = 8) for 4 weeks. At the end of this supplementation period, mean blood pressure and heart rate of each animal were measured by the tail-cuff method (MK-2000ST, Muromachi Kikai, Tokyo, Japan). After measurements had been completed, rats were sacrificed under pentobarbital anesthesia (6.5 mg/kg, i.p.). Tissue samples were then collected by dissection and snap frozen in liquid nitrogen and stored at −80 ° C until analysis.

Western blotting analysis

Each tissue was homogenized in microtubes with lysis buffer (CelLytic™ MT Cell Lysis Reagent, SIGMA-Aldrich Japan, Tokyo, Japan) containing protease inhibitor (SIGMA-Aldrich Co., St. Louis, USA) and 0.2% SDS. Protein concentration was measured by the Bradford method. A 50 μg aliquot of protein was separated by SDS-PAGE using a 4–12% Bis-Tris gel and then transferred onto a polyvinylidene difluoride membrane (Life Technology, Carlsbad, USA). The membranes were blocked with membrane-blocking reagent (GE Healthcare, Buckinghamshire, UK) for 1 h, and then incubated for 2 h with rabbit polyclonal primary antibodies against either UCP1 (1:2500; ab1983, Abcam, Cambridge, UK), UCP3 (1:2000; ab3477, Abcam), CPT-2 (1:500; sc-20671, Santa Cruz Biotechnology, Inc., Santa Cruz, USA), MCAD (1:1000; sc-98926, Santa Cruz Biotechnology, Inc.) or α-tubulin (1:2000; ab4074, Abcam). After the primary antibody reaction, the membranes were incubated for 1 h with the appropriate horseradish peroxidase-conjugated secondary antibody (1:100,000). Immunoreactivity was detected by chemiluminescence using the ECL Select™ Western Blotting Reagent (GE Healthcare, Buckinghamshire, UK). Fluorescence band images were analyzed using Just TLC (Liponics, Tokyo, Japan) analysis software. Each value was normalized to that of α-tubulin.

Measurement of mitochondrial copy number

To measure mitochondrial DNA (mtDNA) copy number, the total DNA was isolated from each organ using the QIAamp® DNA mini kit (QIAGEN Ltd., Tokyo, Japan), and 50 ng of total DNA was used for real-time PCR. PCR was performed using a Step One real-time PCR system (Applied Biosystems Japan Ltd., Tokyo, Japan). The primer and probe sets used were from the TaqMan® Gene Expression Assay (Applied Biosystems Foster City, USA; GAPDH, Rn01775763_g1; CYTB, Rn03296746_s1). The buffer used in the system was TaqMan® Gene Expression Master Mix (Applied Biosystems). The PCR cycling conditions were: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. Data analysis was based on measurement of the cycle threshold (C_T), which represents the PCR cycle count when fluorescence measurement reaches a target value. mtDNA copy number was expressed relative to nuclear DNA following amplification of the mitochondrial gene region (cytochrome b vs. the nuclear endogenous control region, GAPDH).

Data analysis and statistical methods

Data were expressed as means and standard deviations. Statistical analyses were performed using the Dunnett's test. P values less than 0.05 were considered significant.

Results

Body weight, amount of total food intake, tissue weight, heart rate and blood pressure

In Table 2, the results of the body weight, amount of total food intake, tissue weight (liver, epididymis adipose, posterior abdominal adipose, mesentery adipose, subcutaneous adipose, brown adipose, gastrocnemius and soleus), heart rate, and mean blood pressure of the animals at the end

Table 1
Formulation of experimental diet.

Ingredient	Normal (g/kg diet)	HFD (g/kg diet)	HFD-flavan (g/kg diet)
Cornstarch	465.692	–	–
Casein	140	256	256
Dextrinized cornstarch	155	160	160
Maltodextrin	–	60	60
Sucrose	100	55	55
Soybean oil	40	20	20
Lard	–	330	330
Cellulose	50	66.1	64.1
Mineral mix (AIN-93G-MX)	35	35	35
Vitamin mix (AIN-93-VX)	10	10	10
L-Cystine	1.8	3.6	3.6
Choline bitartrate	2.5	2.5	2.5
tert-Butylhydroquinone (TBHQ),	0.008	–	–
Calcium carbonate	–	1.8	1.8
Flavan-3-ols[a]	–	–	2

[a] Flavan-3-ols contained 4.56% of (+)-catechin, 6.43% of (−)-epicatechin, 3.93% of procyanidin B2, 2.36% of procyanidin C1, and 1.45% of cinnamtannin A2.

Table 2
Body weight, total food intake, tissue weight, blood pressure and heart rate of the rats in experimental groups.

	Normal (n = 8)	HFD (n = 7)	HFD-flavan (n = 8)
Body weight, g	327.6 ± 18.0	357.9 ± 14.0	353.1 ± 27.4
Total food intake, g	505.4 ± 28.0	407.1 ± 39.2	387.5 ± 42.0
Liver, g	8.26 ± 0.56	8.93 ± 0.72	8.85 ± 0.68
Epididymis adipose, g	4.21 ± 1.12*	7.06 ± 1.15	5.77 ± 0.93*
Posterior abdominal adipose, g	7.20 ± 1.49	10.75 ± 3.28	10.17 ± 0.93
Mesentery adipose, g	4.19 ± 1.45	5.59 ± 1.44	4.71 ± 1.06
Subcutaneous adipose, g	10.51 ± 2.23	12.65 ± 3.70	11.38 ± 3.49
Brown adipose, g	0.23 ± 0.04	0.26 ± 0.05	0.31 ± 0.06
Gastrocnemius, g	3.14 ± 0.23	3.21 ± 0.45	3.47 ± 0.46
Soleus, g	0.23 ± 0.04	0.27 ± 0.03	0.27 ± 0.07
Heart rate, bpm	443.8 ± 20.2	462.5 ± 6.6	444.9 ± 24.8
Mean blood pressure, mm Hg	122.0 ± 5.1	122.2 ± 1.1	112.1 ± 7.5*

Each value represents the mean and standard deviation. Significant difference from HFD, *$p < 0.05$.