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The combined administration of EGCG and caffeine induces not only suppression of fat accumulation but also anorexigenic action in mice

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ABSTRACT

To elucidate the anorexigenic action and inhibitory effect of fat accumulation by epigallocatechin gallate (EGCG) and caffeine, including the optimal combination ratio and mechanism, fifteen diets with several concentrations of EGCG and/or caffeine were administered to mice for eight weeks. The 0.1% EGCG + 0.1% caffeine group showed the strongest suppression of food intake and a remarkable reduction of body weight and fat accumulation; therefore, the ratio was determined to be the optimal combination ratio. Moreover, serum glucagon-like peptide-1 (GLP-1) level and hypothalamic gene expression of pro-opiomelanocortin (POMC) were promoted by 0.1% EGCG + 0.1% caffeine. In conclusion, the combined treatment of 0.1% EGCG + 0.1% caffeine induces not only suppression of fat accumulation but also strong anorexigenic action in mice. The anorexigenic effect may be brought about via inhibiting gastric motility by GLP-1 and upregulating POMC in the hypothalamus.

1. Introduction

Metabolic syndrome, which is driven primarily by obesity, has become a worldwide health problem (Eckel, Alberti, Grundy, & Zimmet, 2010). Obesity is caused by the imbalance between fat synthesis and energy consumption, and increases the risk for type 2 diabetes mellitus, hypertension, cardiovascular disease, dyslipidemia, osteoarthritis, and some cancers (Lavie, Milani, & Ventura, 2009). Overeating is one of the most common causes of obesity (Razzoli, Pearson, Crow, & Bartolomucci, 2017). The regulation of appetite and food satiety is complex, many factors such as the peripheral hormone signals, brain integration of satiety signals, energy and nutritional status are involved. The peripheral regulation includes the hormone signals, which are secreted by the gastrointestinal tract, adipose tissue, and pancreas (Druce & Bloom, 2006). Two well-known major anorexigenic gut hormones, GLP-1 and peptide YY (PYY), are secreted by gastrointestinal L cells and are released postprandial (De Silva & Bloom, 2012; Holst, 2007; Song, Aihara, Hashimoto, Kanazawa, & Mizuno, 2015). Leptin, another anorexigenic hormone, is secreted by adipocytes and is an important regulator of food intake and body weight (Ahima & Hileman, 2000). The other major regulating path of appetite and feed behavior is via the central nervous system (CNS) mainly in the hypothalamus and brain stem. It has been well reported that hypothalamic neurotransmitters, neuropeptide Y (NPY)/agouti-related peptide (AgRP) and POMC/cocaine and amphetamine-related transcript play critical roles in orexigenics and anorexigenics, respectively. POMC is a precursor of alpha-melanocyte stimulating hormone (α-MSH) and induces anorexigenic action through binding to melanocortin-4 receptors (MC4R) in the hypothalamus. In contrast, AgRP is known as an antagonist of MC4R and induces orexigenic action by suppressing the binding of α-MSH and MC4R. NPY is also an orexigenic neurotransmitter and NPY neurons are stimulated by starvation, which contributes to increased hunger by declines in circulating leptin and insulin and improvement of energy deficiency (Hainerová & Lebl, 2010).

Green tea is one of the most popular drinks consumed worldwide, especially in Asian countries. Regular consumption of green tea has many potential health benefits, such as preventing cancer (Kavanagh et al., 2001), decreasing risk of cardiovascular disease (Sueoka et al., 2001), antiviral (Weber, Ruzindana-Umunyana, Imbeault, & Sircar, 2003) and neuroprotective effects (Weinreb, Mandel, Amit, & Youdim, 2004). Sayama et al. reported that the administration of a diet containing 2% green tea powder significantly suppressed fat accumulation without suppression of food intake in mice (Sayama, Lin, Zheng, & Oguni, 2000). That report also showed that the administration of 4% GTP containing diet induced both a remarkable suppression of fat accumulation and a reduction of food intake. Further study by Zheng et al.

Abbreviations: GTP, green tea powder; TG, triglyceride; TC, total cholesterol; EGCG, epigallocatechin gallate; IPAT, intraperitoneal adipose tissues; GLP-1, glucagon-like peptide 1; PYY, peptide tyrosine tyrosine; CNS, central nervous system; NPY, neuropeptide Y; AgRP, agouti-related peptide; POMC, pro-opiomelanocortin; BBB, blood-brain barrier

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reported that the combination of 0.3% catechins and 0.05% caffeine inhibited fat accumulation similarly to the 2% green tea powder, but did not reduce food intake (Zheng, Sayama, Okubo, Juneja, & Oguni, 2004). It is considered that the combination of catechins and caffeine is the main anti-obesity components of green tea and the administration of higher concentration of catechins and caffeine might induce the suppression of both food intake and fat accumulation in mice.

Epigallocatechin gallate (EGCG) is a major green tea catechin. Prior studies have demonstrated significant anti-obesity effects for EGCG, showing that EGCG significantly decreased body weight gain and increased fecal lipids, while suppressing blood glucose, plasma cholesterol, and hepatic TG in high-fat diet-induced obese mice (Chen et al., 2011). Caffeine, another major component of green tea, is also known for its anti-obesity effects. Caffeine increases serum free fatty acid levels by inducing lipolysis and decreases serum and hepatic TG in high-fat fed rats (Kobayashi-Hattori, Mogi, Matsumoto, & Takita, 2005). Prior studies have also demonstrated that subcutaneous administration of caffeine upregulates the expression of UCP-1 in brown adipose tissue, which contributes to thermogenesis in obese mice (Kogure et al., 2002).

Dulloo et al. demonstrated that the combination of EGCG and caffeine treatment affect thermogenesis synergistically in rat interscapular brown tissue (Dulloo, Seydoux, Girardier, Chantre, & Vandermander, 2000). The administration of a diet containing 4% green tea powder suppressed not only fat accumulation but also food intake in mice (Sayama et al., 2000). Additionally, EGCG stimulated secretion of anorexigenic gut hormones from Caco-2 cells *in vitro* (Song et al., 2015). However, the anorexigenic or anti-obesity effects of the combination of EGCG and caffeine have not been evaluated in mice.

Therefore, this study investigated the anorexigenic and anti-obesity effects of EGCG and caffeine individually and in combination in mice. Furthermore, we identified the optimal ratio of these two compounds and examined the underlying mechanisms of their actions.

2. Materials and methods

2.1. Materials

EGCG (commercial grade, purity 98%) was provided by Hara Office (Tea Solutions, Hara Office Inc., Tokyo, Japan). Caffeine was purchased from Wako Pure Chemical Industries, Ltd. (purity 98.5%, Osaka, Japan).

2.2. Animals and diets

Female ddY mice (10-wk old, 30–32 g) were purchased from Japan SLC, Inc. (Hamamatsu, Japan). All mice were maintained at 24 ± 2 °C (room temperature), 50 ± 10% humidity, and a 12 h light-dark cycle (lights on from 08:00 to 20:00). All mice were fed a standard powdered chow for one week prior to administration of the experimental compounds. Animal experiments were conducted according to the guidelines of the Science Council of Japan and the Japanese Association for Laboratory Animal Science. The Animal Care and Use Committee at Shizuoka University approved all protocols prior to beginning (Permission Number: 29A-19).

A standard powdered laboratory chow (MF diet, Oriental Yeast Co. Ltd., Tokyo, Japan) was used for the control group. The composition of the standard chow per 100 g is as follows: moisture: 7.9 g, crude protein: 23.1 g, crude lipid: 5.1 g, crude ash: 5.8 g, crude fiber: 2.8 g, nitrogen-free extract: 55.3 g, vitamin mixture: 0.6 g, mineral mixture: 3.25 g, and calories: 359 kcal. EGCG and caffeine were mixed with the standard powdered chow at different concentrations singly or in combination. The starting concentrations of EGCG and caffeine mixed with diet were 0.1% EGCG and 0.05% caffeine, which corresponded to the concentrations in the 2% green tea powder diet used by Zheng et al. (2004) who reported that 2% green tea powder has anti-obesity effects. The following fifteen experimental diets with half or double concentrations of EGCG and/or caffeine (based on the basic concentrations) were used: 0.05% EGCG; 0.1% EGCG; 0.2% EGCG; 0.025% caffeine; 0.05% caffeine; 0.1% caffeine; 0.05% EGCG + 0.025% caffeine; 0.05% EGCG + 0.05% caffeine; 0.05% EGCG + 0.1% caffeine; 0.1% EGCG + 0.025% caffeine; 0.1% EGCG + 0.05% caffeine; 0.1% EGCG + 0.1% caffeine, 0.2% EGCG + 0.025% caffeine; 0.2% EGCG + 0.05% caffeine; and 0.2% EGCG + 0.1% caffeine.

2.3. Feeding experiments

2.3.1. Long-term administration experiment

Nine female ddY mice were divided into three cages used for each experimental group. The 15 experimental diets outlined above or the control diet and tap water were administered to mice *ad libitum* for 8 weeks. Food intake was measured using a feeder designed for the measurement of food consumption (Oriental Yeast Co., Ltd.). The amount of food intake per mouse in each cage was calculated by division the total food intake by three mice. Then, the food intake of each mouse was determined from the mean values of the calculated food intake from all cages. During the feeding period, body weight and food intake were measured once weekly. Among the 15 experimental groups, the 0.1% caffeine and 0.1% EGCG + 0.1% caffeine groups had markedly decreased food intake compared to the control group. Therefore, these experimental groups were used for further analysis. Eighteen mice per group were divided into six cages and fed the experimental diets as above.

2.3.2. Short-term administration experiment

During the long-term administration experiment, we found that food intake was decreased significantly ($P < 0.01$), even by the second week, among the control, 0.1% caffeine and 0.1% EGCG + 0.1% caffeine groups (each total food intake per mouse for two weeks was 65.81 ± 1.56 g, 58.57 ± 0.91 g, and 53.85 ± 1.24 g, respectively). Thus, to elucidate the anorexigenic mechanism of EGCG and caffeine, a short-term feeding experiment for two weeks was performed. Six female ddY mice per group were divided into two cages and further divided randomly into 0.1% caffeine, 0.1% EGCG + 0.1% caffeine, and control groups and fed the diets and tap water *ad libitum* for two weeks. The body weight and food intake were measured as described above.

2.4. Tissue collection and blood analysis

For the long-term administration test, mice fasted for 12 h, were euthanized by diethyl ether overdose and blood was collected via the heart. Serum was collected from the clotted blood by centrifugation at 825*g* for 15 min at 4 °C and stored at −80 °C until analysis. The liver, kidneys, spleen, adrenal glands, and intraperitoneal adipose tissue (IPAT) were excised and weighed. The liver was frozen in dry ice immediately after weighing and kept at −80 °C until analysis. Serum levels of TC and TG were measured with LabAssay™ Cholesterol kit and LabAssay™ Triglyceride kits (Wako Pure Chemical Industries, Ltd., Japan) according to the manufacturer's instructions.

For the short-term administration test, mice were euthanized by diethyl ether overdose without fasting, and blood and serum were collected as described above. The hypothalamus was excised and stored at −80 °C in RNA*later* solution (Applied Biosystems, New Jersey, USA) for subsequent RNA isolation. Serum levels of GLP-1, PYY, insulin, glucose, and leptin were determined by GLP-1 ELISA *Wako* (High Sensitive; capable of detecting both activated and deactivated isoforms) (Wako Pure Chemical Industries, Ltd., Japan), YK081 Mouse/Rat PYY EIA kit (Yanaihara Institute Inc., Japan), Mouse Insulin ELISA Kit (MIoBS; Morinaga Institute of Biological Science, Inc., Japan), Glucose CII-Test *Wako* (Wako Pure Chemical Industries, Ltd., Japan) and Mouse and Rat Leptin ELISA (BioVendor, R&D, Czech Republic), respectively, according to manufacturer's instructions.

2.5. Hepatic lipid analysis

Hepatic lipids in the liver of mice after the long-term administration experiment were extracted as described by Folch et al. (Folch, Lees, & Sloane Stanley, 1957). Hepatic TC and TG were quantified as described by Zak (Zak, 1957) and Fletcher (Fletcher, 1968), respectively.

2.6. RNA extraction and real-time PCR

Hypothalamic mRNA levels were measured in mice receiving short-term administration of experimental diets. Total hypothalamic RNA was isolated using TRIzol® Reagent (Invitrogen, CA, USA) extraction and purified using the MN RNA isolation kit (MACHEREY-NAGEL, Düren, Germany) according to the protocol provided by the manufacturer.

The extracted total RNA (1 μg for hypothalamus) was reverse transcribed to cDNA using SuperScript™ II Reverse Transcriptase (Invitrogen, CA, USA) following the manufacturer's instructions. cDNA was analyzed by real time polymerase chain reaction (real-time PCR) using FastStart Essential DNA Green Master Mix (Roche, Basel, Switzerland) according to the manufacturer's instructions. PCR was performed using a LightCycler® Nano system (Roche, Basel, Switzerland) according to the following specifications: the cDNA (20 μl) was amplified for 35 cycles of denaturation (95 °C for 60 s), annealing (55 °C for 60 s), and extension (72 °C for 45 s) for *POMC*, *AgRP*, and *NPY*; 35 cycles of denaturation (95 °C for 30 s), annealing (65 °C for 30 s), and extension (72 °C for 45 s) for *β-actin* (Ahima & Hileman, 2000). Primer sequences are shown in Table 1. mRNA levels for experimental genes were normalized to *β-actin*.

2.7. Statistical analysis

Values are presented as means ± standard errors. Dunnett's test was performed to determine the potential statistical significance of parameters measured in the long-term administration experiment. Tukey's test was used for the statistical analysis of food intake. A two-tailed Student's *t* test was used to analyze the data from the short-term administration experiment. Statistical significance was defined as $P < 0.05$. Statistical analysis software SPSS (SPSS Inc., Chicago, IL, USA) was used for all data analysis.

3. Results

3.1. Body, organs, and adipose tissue weights

To investigate the anti-obesity effects of EGCG and caffeine individually and in combination, fifteen treatments containing varying concentrations of EGCG and/or caffeine and standard chow were administered to mice for 8 weeks. Body weight gain, weights of organs and IPAT per body weight are shown in Table 2. All experimental groups had significantly decreased body weight gain relative to the control group. Since body weight was remarkably suppressed, we calculated the weights of organs and IPAT relative to body weight. Compared to the control group, there were no significant differences on liver, kidney, adrenal glands, or spleen weight relative to body weight.

Table 1
Real-time PCR primer sequences.

	Primer sequences (5′ → 3′)	
	Forward primer	Reverse primer
POMC	TGGTGCCTGGAGAGCAGCCAGTGC	TGGAGTAGGAGCGCTTGCCCTCG
AgRP	GAAGGCCTGACCAGGCTCTGTTCC	AAAGGCATTGAAGAAGCGGCAG
NPY	GCTTGAAGACCCTTCCATTGGTG	GGCGGAGTCCAGCCTAGTGG
β-actin	TCGTGCGTGACATCAAAGAG	TCTCCTTCTGCATCCTGTCA

The IPAT weight per body weight was not affected by the EGCG single treatments. However, significant decreases were observed in the following caffeine single treatments and EGCG/caffeine combination groups: 0.05% caffeine, 0.1% caffeine, 0.05% EGCG + 0.05% caffeine, 0.1% EGCG + 0.05% caffeine, 0.1% EGCG + 0.1% caffeine, 0.2% EGCG + 0.05% caffeine, and 0.2% EGCG + 0.1% caffeine.

3.2. Food intake

The total food intake per mouse after administration of experimental diets for 8 weeks is also shown in Table 2. Compared to the control group, the food intake was not affected by EGCG single treatments. In caffeine single treatment groups, an approximately 13% decrease was observed in the 0.1% caffeine group only. Contrastingly, food intake decreased by approximately 21%, 24%, and 18% in the groups receiving 0.05% EGCG + 0.1% caffeine, 0.1% EGCG + 0.1% caffeine, and 0.2% EGCG + 0.1% caffeine, respectively. The reduction in food intake in these combination treatment groups was stronger than that of 0.1% caffeine single treatment.

The food intake was remarkably suppressed in the experimental groups receiving highly concentrated combined diets and in the 0.1% caffeine single treatment group. To conduct a statistical analysis of food intake, an additional feeding experiment was carried out in the 0.1% caffeine and 0.1% EGCG + 0.1% caffeine groups, which showed significant reductions in food intake in preliminary studies. Both the 0.1% caffeine and 0.1% EGCG + 0.1% caffeine treatments significantly decreased food intake by 13.4% and 21.1%, respectively ($P < 0.05$) (Fig. 1). Moreover, the combination treatment with 0.1% EGCG + 0.1% caffeine decreased food intake by 8.9% relative to the caffeine single treatment group (Fig. 1).

3.3. Lipid levels in serum and liver

Table 3 summarizes concentrations of serum and liver lipids in mice administered experimental diets for eight weeks. Hepatic TG were significantly decreased in all experimental groups except for the EGCG single treatment groups. Significant reductions of liver TC were observed only in the 0.05% EGCG + 0.05% caffeine, 0.05% EGCG + 0.1% caffeine, and 0.1% EGCG + 0.025% caffeine combination treatment groups. None of the experimental treatments affected serum TC or TG.

3.4. Serum levels of anorexigenic hormones

Because the strongest inhibitory effect on food intake was observed in the 0.1% EGCG + 0.1% caffeine group, (Table 1 and Fig. 1), we used this treatment to investigate the mechanism for suppression of food intake. Mice were fed a control chow base or 0.1% EGCG + 0.1% caffeine-containing diet for two weeks, and serum hormone levels were measured.

As shown in Fig. 2, serum levels of both activated and deactivated isoforms of GLP-1 and PYY were measured. Serum GLP-1 (both active and deactivated isoforms) in the 0.1% EGCG + 0.1% caffeine group significantly increased approximately 129.8% relative to the control group. There was no significant difference between the control and the experimental group in serum PYY (both active and deactivated isoforms). The circulating level of leptin was significantly lower in the 0.1% EGCG + 0.1% caffeine group than in the control group ($P < 0.05$) (Fig. 2).

3.5. Serum insulin and glucose

Serum levels of insulin and glucose are summarized in Fig. 3. Serum insulin in the 0.1% EGCG + 0.1% caffeine group showed a strong increasing trend ($P = 0.07$) relative to the control group, although no statistical significance was found. However, non-fasting blood glucose was not affected by the combined treatment.

Table 2
Effect of EGCG and caffeine on the weights of body, organs, IPAT and food intake in mice.

	Control	0.05% EGCG	0.1% EGCG	0.2% EGCG	0.025% caffeine	0.05% caffeine
Body weight gain (g)	8.58 ± 0.69	5.93 ± 1.09*	6.09 ± 0.52*	4.45 ± 0.82*	4.18 ± 0.44*	4.81 ± 0.56*
Intraperitoneal adipose tissue (IPAT, mg/g BW)	35.11 ± 6.59	25.78 ± 5.56	34.35 ± 4.57	21.45 ± 6.20	20.04 ± 4.88	10.19 ± 1.96*
Liver (g/g BW)	0.0414 ± 0.0015	0.0374 ± 0.0007	0.0374 ± 0.0009	0.0400 ± 0.0009	0.0397 ± 0.0012	0.0397 ± 0.0009
Kidneys (mg/g BW)	12.09 ± 0.25	12.53 ± 0.40	11.82 ± 0.27	12.85 ± 0.60	13.63 ± 0.55	12.79 ± 0.27
Adrenal glands (mg/g BW)	0.296 ± 0.014	0.307 ± 0.021	0.317 ± 0.018	0.344 ± 0.023	0.349 ± 0.026	0.304 ± 0.021
Spleen (mg/g BW)	4.11 ± 0.21	4.15 ± 0.18	3.40 ± 0.17	4.01 ± 0.30	3.96 ± 0.36	3.45 ± 0.17
Food intake (g, 8 weeks)	278.75	281.74	262.80	300.92	290.75	283.80

	0.1% caffeine	0.05% EGCG + 0.025% caffeine	0.05% EGCG + 0.05% caffeine	0.05% EGCG + 0.1% caffeine	0.1% EGCG + 0.025% caffeine
Body weight gain (g)	3.28 ± 0.67*	2.99 ± 0.44*	3.48 ± 0.55*	4.28 ± 0.35*	5.11 ± 0.84*
Intraperitoneal adipose tissue (IPAT, mg/g BW)	12.99 ± 2.43*	20.77 ± 3.22	13.38 ± 3.42*	19.93 ± 2.18	25.55 ± 4.44
Liver (g/g BW)	0.0393 ± 0.0014	0.0417 ± 0.0014	0.0406 ± 0.0005	0.0394 ± 0.0011	0.0397 ± 0.0007
Kidneys (mg/g BW)	13.01 ± 0.33	13.36 ± 0.47	11.64 ± 0.45	11.30 ± 0.29	11.43 ± 0.54
Adrenal glands (mg/g BW)	0.310 ± 0.039	0.406 ± 0.053	0.315 ± 0.027	0.266 ± 0.018	0.368 ± 0.037
Spleen (mg/g BW)	3.44 ± 0.24	4.96 ± 0.57	3.76 ± 0.27	3.64 ± 0.23	3.82 ± 0.11
Food intake (g, 8 weeks)	242.46	286.42	277.17	221.21	282.36

	0.1% EGCG + 0.05% caffeine	0.1% EGCG + 0.1% caffeine	0.2% EGCG + 0.025% caffeine	0.2% EGCG + 0.05% caffeine	0.2% EGCG + 0.1% caffeine
Body weight gain (g)	3.87 ± 0.65*	2.90 ± 0.38*	4.76 ± 1.03*	4.23 ± 0.53*	3.51 ± 0.52*
Intraperitoneal adipose tissue (IPAT, mg/g BW)	12.46 ± 3.05*	14.75 ± 1.44*	17.96 ± 7.18*	13.88 ± 1.81*	16.74 ± 3.11*
Liver (g/g BW)	0.0377 ± 0.0016	0.0385 ± 0.0005	0.0382 ± 0.0017	0.0401 ± 0.0009	0.0400 ± 0.0011
Kidneys (mg/g BW)	10.38 ± 0.72	11.05 ± 0.12	12.11 ± 0.78	11.94 ± 0.38	11.11 ± 0.43
Adrenal glands (mg/g BW)	0.307 ± 0.020	0.298 ± 0.023	0.308 ± 0.014	0.284 ± 0.015	0.258 ± 0.014
Spleen (mg/g BW)	3.39 ± 0.12	3.44 ± 0.19	4.05 ± 0.33	3.44 ± 0.19	3.82 ± 0.32
Food intake (g, 8 weeks)	269.91	213.24	296.99	265.77	229.84

Values are means ± standard errors, n = 9 for BW (body weight), IPAT (intraperitoneal adipose tissue) and organs; n = 3 for food intake.
* Significant difference compare to the control ($P < 0.05$).

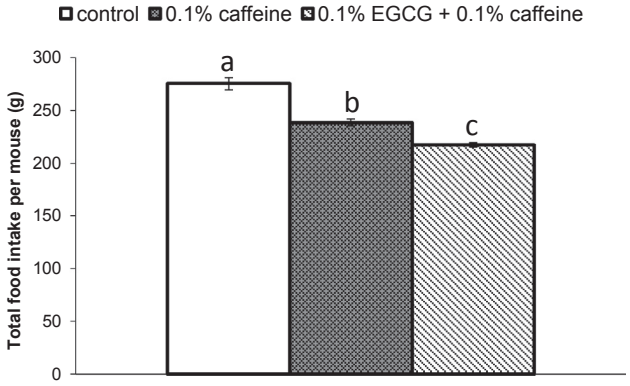

Fig. 1. Effect of EGCG and caffeine on food intake in mice. Food intake was calculated per mouse from the total expenditure for eight weeks by three mice in a cage. Values are means ± standard errors (n = 9). Significant difference between different letters ($P < 0.05$).

3.6. Expression of appetite regulatory genes in the central nervous system

We measured the mRNA expression of orexigenic NPY/AgRP and anorexigenic POMC to investigate the mechanism for suppression of food intake. The mRNA expression of *POMC* was significantly increased by the 0.1% EGCG + 0.1% caffeine treatment (Fig. 4). The mRNA expressions of *NPY* and *AgRP* were also increased significantly in the experimental group (Fig. 4).

4. Discussion

The present results clearly demonstrate that the combination of EGCG and caffeine treatments strongly suppresses body weight gain and fat accumulation in mice. Moreover, suppressive action on food intake was observed in these combined treatments. Specifically, the 0.1% EGCG + 0.1% caffeine group showed not only a remarkable suppression of body weight and fat accumulation, but also the strongest anorexigenic effect. Obesity and overeating are related closely, and the suppression of overeating was most effective in reducing obesity (Razzoli et al., 2017). Thus, we suggest that 0.1% EGCG + 0.1% caffeine is the optimal combination ratio, which affected both anti-obesity and anorexigenic actions, among the 15 experimental groups.

It has been reported that EGCG suppressed body weight and white adipose tissue mass in mice fed high fat diets (Chen et al., 2011; Lee, Kim, & Kim, 2009); however, the present study only showed the suppressive action on body weight, not on IPAT (Table 2). This suggests that the dose of EGCG in our study may not have been sufficient for the suppression of adipose tissue mass. Kobayashi-Hattori et al. demonstrated that caffeine treatment, which were the same doses used in the present study, markedly reduced body weight gain and body fat mass in high-fat diet-fed rats. Moreover, the caffeine treatments significantly decreased the hepatic TG level (Kobayashi-Hattori et al., 2005). This is consistent with our results (Table 2). Further, we also demonstrated that combinational administration of EGCG and caffeine also reduced body weight gain and IPAT, but no significant differences were found in reduction between the combined treatments and caffeine single treatments. Moreover, we observed the same pattern in hepatic TG level as well. Therefore, we suggest that caffeine may be responsible for the suppression of fat accumulation through the decreased hepatic TG level, and EGCG does not contribute to the action by caffeine.

Previous studies reported that intraperitoneal administration of EGCG in rats significantly reduced food intake and caffeine consumption reduced energy intake in male subjects (Kao, Hiipakka, & Liao, 2000; Westerterp-Plantenga, Diepvens, Joosen, Bérubé-Parent, & Tremblay, 2006). There was no anorexigenic activity by any EGCG single treatments (Table 2). On the contrary, 0.1% caffeine group showed a significant decrease on food intake (Fig. 1). Moreover, the

Table 3
Effect of EGCG and caffeine on the lipids levels in serum and liver in mice.

	Control	0.05% EGCG	0.1% EGCG	0.2% EGCG	0.025% caffeine	0.05% caffeine
TC (mg/dl)	126.21 ± 8.82	99.22 ± 6.96	95.22 ± 6.26	113.36 ± 9.20	112.16 ± 5.87	115.18 ± 5.34
TG (mg/dl)	146.44 ± 20.20	139.44 ± 18.23	111.29 ± 5.22	126.24 ± 13.94	142.02 ± 18.52	131.61 ± 21.07
TC index (mg/g liver)	20.18 ± 1.91	21.99 ± 2.27	23.36 ± 2.32	21.74 ± 3.00	18.41 ± 2.26	19.86 ± 2.65
TG index (mg/g liver)	109.27 ± 17.99	74.58 ± 21.82	74.02 ± 13.10	62.98 ± 11.74	28.45 ± 5.65*	48.77 ± 12.20*

	0.1% caffeine	0.05% EGCG + 0.025% caffeine	0.05% EGCG + 0.05% caffeine	0.05% EGCG + 0.1% caffeine	0.1% EGCG + 0.025% caffeine
TC (mg/dl)	103.40 ± 8.94	102.80 ± 4.55	115.99 ± 15.66	127.76 ± 5.68	118.24 ± 8.58
TG (mg/dl)	120.37 ± 25.75	111.72 ± 11.17	95.04 ± 8.23	120.45 ± 16.49	148.50 ± 14.17
TC index (mg/g liver)	18.45 ± 2.02	18.68 ± 2.31	13.43 ± 1.73*	12.78 ± 1.43*	13.73 ± 1.29*
TG index (mg/g liver)	19.30 ± 2.00*	51.93 ± 12.19*	33.37 ± 6.39*	34.78 ± 3.26*	41.32 ± 4.69*

	0.1% EGCG + 0.05% caffeine	0.1% EGCG + 0.1% caffeine	0.2% EGCG + 0.025% caffeine	0.2% EGCG + 0.05% caffeine	0.2% EGCG + 0.1% caffeine
TC (mg/dl)	127.63 ± 13.97	106.97 ± 10.90	115.47 ± 7.34	108.43 ± 8.02	102.48 ± 6.60
TG (mg/dl)	126.23 ± 18.53	109.37 ± 13.08	125.64 ± 17.73	116.86 ± 13.96	95.40 ± 13.30
TC index (mg/g liver)	15.33 ± 1.47	16.30 ± 0.81	19.08 ± 0.32	17.38 ± 1.09	15.65 ± 0.42
TG index (mg/g liver)	42.69 ± 5.43*	33.72 ± 9.47*	45.12 ± 12.46*	39.34 ± 8.60*	39.87 ± 8.99*

Values are means ± standard errors (n = 9).
* Significant difference compare to the control ($P < 0.05$).

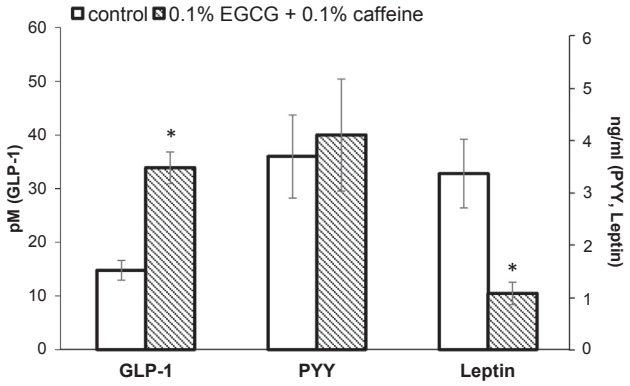

Fig. 2. Effect of EGCG and caffeine on serum levels of anorexigenic hormones in mice. Values are means ± standard errors, n = 5 for GLP-1 (glucagon-like peptide 1); n = 6 for PYY (peptide YY) and Leptin. *: Significant difference compared to the control ($P < 0.05$).

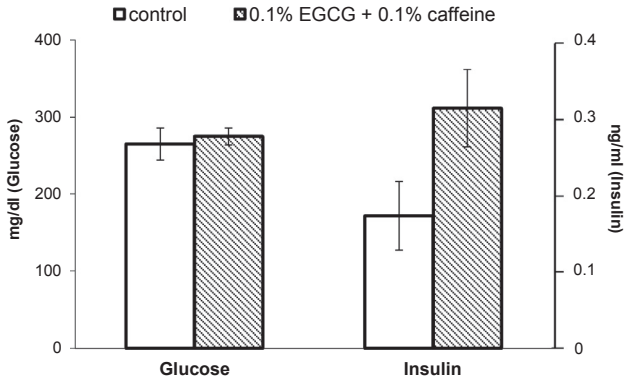

Fig. 3. Effect of EGCG and caffeine on serum levels of glucose and insulin in mice. Values are means ± standard errors (n = 6).

food intake of the 0.1% EGCG + 0.1% caffeine group was significantly lower than in the 0.1% caffeine group. These results clearly demonstrated that the anorexigenic action of caffeine was synergistically promoted by EGCG.

To investigate the mechanism of anorexigenic action by EGCG and

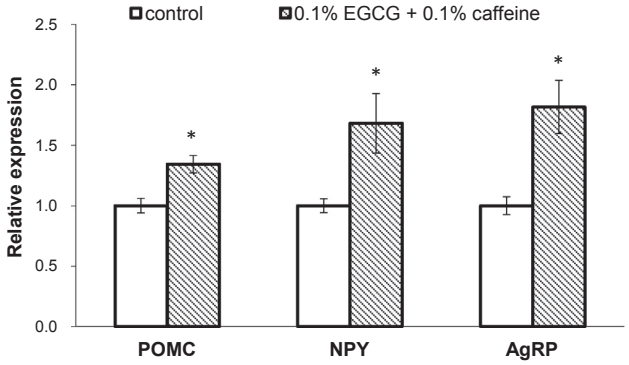

Fig. 4. Effect of EGCG and caffeine on mRNA expression of appetite regulation-related factors in the hypothalamus of mice. Values are means ± standard errors (n = 6). *: Significant difference compared to the control ($P < 0.05$).

caffeine, we examined several gut-brain-related appetite factors. As a result, we found a significant increase in circulating GLP-1 (both activated and deactivated isoforms) in mice receiving the 0.1% EGCG + 0.1% caffeine treatment, which showed the strongest suppressive effect on food intake. (Fig. 2). Song et al. reported that EGCG increased the expression of GLP-1 in Caco-2 cells (Song et al., 2015), which suggests that at least EGCG may be responsible for the increase of GLP-1 *in vivo* as well. Moreover, a previous report clarified that GLP-1 has anorexigenic action (De Silva & Bloom, 2012) Thus, it indicated that elevation of GLP-1 by EGCG and caffeine were involved in the anorexigenic action. As for their anorexigenic action, we consider two pathways. First, GLP-1 is known as the stimulator of insulin secretion (Holst, 2007). Insulin, a pancreatic peptide hormone, also known as an anorexigenic hormone besides regulating glucose level, can pass across the blood-brain barrier (BBB) and participate in regulation of food intake (Banks, Jaspan, & Kastin, 1997; Gray, Aylor, & Barrett, 2017). We observed a strong increasing trend in circulating insulin in mice treated with EGCG and caffeine (Fig. 3). This result suggests that increased GLP-1 may stimulate insulin secretion and that subsequently elevated insulin may be indirectly involved in the anorexigenic effects of EGCG and caffeine. Although the level of GLP-1 was increased, the glucose level was not changed. We considered that because the glucose level was measured under non-fasting conditions. In contrast, GLP-1 is reported that inhibits stomach functions, such as acid secretion and

gastric emptying, by suppressing central parasympathetic control and appetite suppression induced indirectly by these functions (Halim et al., 2017; Wettergren, Wøjdemann, & Holst, 1998). Accordingly, we considered that the anorexigenic action of the combination treatment of EGCG and caffeine is most likely to act via the above function of GLP-1.

The most important region of appetite regulation is located in the brain, specifically in the hypothalamus. These neurons integrate the signals from sensing circulating peripheral hormones or neuron activity inputs and transmit the information to downstream neurons in the CNS to regulate energy homeostatic factors, such as food intake and body weight. To investigate the mechanism for suppression of food intake, we analyzed hypothalamic levels of POMC, AgRP, and NPY, which are involved in the regulation of appetite. As the result, we found that mRNA expression of *POMC* was up-regulated by the combined treatment of EGCG and caffeine (Fig. 4). Moreover, *AgRP* and *NPY* were also significantly increased by treatment, despite food intake being significantly suppressed. In contrast, leptin, an adipocyte hormone that plays an important role in appetite regulation (Zhang et al., 1994) has been reported that the deficient or mutation of it leads to obesity in rodents and humans (Clément et al., 1998; de Lartigue, Ronveaux, & Raybould, 2014; Montague et al., 1997). Furthermore, it was reported that the mRNA expression of *AgRP* in ob/ob mice, which are leptin deficient and the obesity mouse model, was approximately 4.9 times higher than the normal mouse (Duan et al., 2007). The circulating level of leptin in the 0.1% EGCG + 0.1% caffeine treatment group was 70% lower than in the control group (Fig. 2), which was due to the remarkable reduction of adipose tissue by EGCG and caffeine (Table 2). Despite the reduction of the leptin level in the group, the hypothalamic *AgRP* was only 1.8 times higher compared to the control group (Fig. 4). Therefore, the treatment of 0.1% EGCG + 0.1% caffeine may diminish the overexpressing of *AgRP*. These results indicate that the upregulation of *POMC* expression and the attenuated expression of *AgRP* are strongly involved in anorexigenic action by 0.1% EGCG + 0.1% caffeine.

Experimental evidence suggests that EGCG and caffeine are able to cross the blood-brain barrier (McCall, Millington, & Wurtman, 1982; Nakagawa & Miyazawa, 1997). However, whether these two components regulate appetite through a peripheral endocrine mechanism or act directly on POMC and AgRP/NPY neurons remains unknown. The possibility should be considered, and further studies are required.

In conclusion, this study demonstrated that the combination of 0.1% EGCG and 0.1% caffeine has the strongest inhibitory effects on food intake and fat accumulation in mice. Moreover, we suggest that the anorexigenic mechanism of 0.1% EGCG + 0.1% may be due, in part, to the suppression of gastral actions by GLP-1 and the upregulation of POMC in the hypothalamic appetite center. Reduced food intake is responsible, at least in part, for the reduction of fat accumulation by these compounds. Additionally, these results suggest that a combined treatment of EGCG and caffeine might be available for the therapy of obesity accompanied by hyperphagia. Further studies including the protein levels of POMC, AgRP, and NPY are in progress to fully elucidate the repressive mechanisms of food intake and fat accumulation by EGCG and caffeine.

Ethics statement

I have read and adhere to the Publishing Ethics.

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K. Sayama and L. Liu designed this study; L. Liu performed all the experiments including data analysis and wrote the manuscript; and K. Sayama had the primary responsibility for the final content. All authors read and approved the final manuscript.

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Conflict of interest statement

The authors have declared no conflicts of interest.

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