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A Combination of Caffeine, Arginine, Soy Isoflavones, and L-Carnitine Enhances Both Lipolysis and Fatty Acid Oxidation in 3T3-L1 and HepG2 Cells in Vitro and in KK Mice in Vivo[1]

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Abstract

To develop an anti-obesity agent containing dietary components, we focused on the mechanisms that enhance both lipolysis and fatty acid oxidation. Caffeine and arginine (CA), a nonselective adenosine-receptor antagonist and an inducer of lipolytic hormone, respectively, were used to stimulate lipolysis. Soy isoflavones and L-carnitine (SL), stimulators of carnitine palmitoyl transferase 1A and a cofactor for β-oxidation of fatty acids, respectively, were used to enhance fatty acid oxidation. Effects of a combination of CA and SL (CASL) on lipid metabolism were studied in vitro and in vivo. During 3T3-L1 differentiation, lipid accumulation was significantly lower in cells treated with CASL (50 μmol/L, 1 mmol/L, 1 μmol/L, and 1 mmol/L, respectively) compared with each alone. Lipolysis was also significantly greater in 3T3-L1 adipocytes treated with CASL (50 μmol/L, 1 mmol/L, 10 μmol/L and 0.5 mmol/L, respectively) compared with each alone. In addition, treatment with higher concentrations of CASL (2 mmol/L, 1 mmol/L, 10 μmol/L, and 1 mmol/L, respectively) significantly enhanced β-oxidation in HepG2 cells. The effects of CASL-containing diets (250 mg, 6 g, 200 mg, and 1.5 g/kg diet, respectively) were studied in vivo. When KK mice were food deprived for 48 h and subsequently refed a fat-free diet for 72 h, hepatic triglyceride (TG) accumulation was significantly lower in mice fed CASL compared with the control mice. In addition, after obese KK mice were fed a low-fat diet for 2 wk, adipose tissue weights were significantly lower in those fed CASL, but not CA or SL alone, compared with the control mice. Plasma and liver TG levels were also lower in mice fed CASL than in the control mice. These results suggest that CASL is effective for controlling obesity. J. Nutr. 137: 2252–2257, 2007.

Introduction

Obesity has become prevalent worldwide as a result of changes in lifestyle, especially in eating habits. It is associated with a number of serious medical complications, such as coronary heart disease, type 2 diabetes, nonalcoholic fatty liver, and certain cancers (1–3). Therefore, prevention and treatment of obesity are relevant to health promotion.

Lipolysis and fatty acid oxidation are important mechanisms involved in reducing body fat. Caffeine and arginine (CA)[4] are lipolysis stimulants, acting as a nonselective adenosine-receptor antagonist (4) and an inducer of lipolytic hormones (5), respectively. We have previously demonstrated that a mixture containing CA is effective in reducing adipose tissue mass as well as improving disorders in lipid metabolism in non-insulin-dependent diabetic KK mice (6). Ingestion of the mixture reduced percent body fat, triceps skinfold thickness, and serum triglyceride (TG) levels in healthy subjects with high percent body fat and was effective in reducing visceral fat in obese subjects (7).

Carnitine palmitoyl transferase (CPT) 1, a rate-limiting enzyme of fatty acid oxidation, catalyzes the esterification of long-chain acyl-CoA to L-carnitine to transport it into the mitochondria. Stimulation of CPT1 activity has been shown to increase energy utilization and fatty acid oxidation in diet-induced obese mice (8). We previously showed that soy isoflavone increases CPT1A enzyme activity and that cotreatment of L-carnitine and genistein additively increases CPT1A enzyme activity in a hepatocyte cell line (9). We have also shown that genistein enhances the expression of genes involved in fatty acid catabolism through activation of peroxisome proliferator-activated receptor α and downregulates sterol regulatory element-binding protein 1-regulated gene expressions through

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[4] Abbreviations used: CA, caffeine and arginine; CASL, a combination of caffeine, arginine, soy isoflavones, and L-carnitine; CPT, carnitine palmitoyl transferase; FBS, fetal bovine serum; MSI, mixture of soy isoflavones; P10, Fujiflavone P10; SL, soy isoflavones and L-carnitine; TG, triglyceride.

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the inhibition of site-1 protease expression in the HepG2 cell line (10,11). Consequently, soy isoflavones and L-carnitine (SL) effectively suppress obesity in mice fed a high-fat diet (12).

Langin (13) suggested utilizing molecules that stimulate lipolysis and oxidation of the released fatty acids as a first approach to decreasing fat stores. On this basis, the combined effects of CA, stimulants for lipolysis, and SL, stimulants for fatty acid oxidation, were investigated in in vitro and in vivo studies. We examined the effects of a combination of CA and SL (CASL) on lipolysis and fatty acid oxidation. In addition, fatty acid synthesis, another potential pathway in obesity therapy (14), and adiponectin, which is inversely associated with and has protective effects against obesity and non-insulin-dependent diabetes (15–20), were examined. Moreover, the anti-obesity effects of CASL were studied.

In in vitro studies, the effects of CASL on lipid accumulation, lipolysis, and adipocyte-specific adiponectin expression were examined in the well-characterized mouse 3T3-L1 preadipocyte cell line. Fatty acid oxidation was studied in the HepG2 cell line from the liver, where substantial fatty acid oxidation occurs. CPT1A promoter activity was examined in the Huh7 hepatoma cell line, which can be stably transfected with a CPT1A promoter reporter gene construct.

In in vivo studies, the effects of CASL were investigated in non-insulin-dependent diabetic KK mice. Hepatic lipogenesis was examined in KK mice subjected to food deprivation and refeeding of a no-fat diet to augment lipogenic enzyme activity, in which dietary components with anti-lipogenic activity have been effective when given during a refeeding period (6,21). Lipolysis was evaluated by measuring the epinephrine-induced changes in the plasma glycerol level (22) following acute administration of CASL to obese KK mice, by which sensitivity to epinephrine is potentially blunted (23,24). The anti-obesity effects of CASL were examined in obese KK mice during the dietary weight loss period when an anti-obesity agent is often used.

Materials and Methods

Dietary components. Caffeine, extracted from coffee beans (>98.5% purity), was purchased from Shiratori Pharmaceutical. Arginine was purchased from Kyowa Hakko. Partially purified isoflavone extracted from soy germ and spray-dried with dextrin [Fujiflavone P10 (P10), Fujicco] was used as a source of soy isoflavones in animal experiments. P10 contains 10% glycoside isoflavones that convert to active form, aglycone type isoflavones in the digestive tract. The composition of isoflavones was ~50% daidzin, 40% glycitin, and 10% genistin. Aglycone-type isoflavones were used in cell culture experiments. To obtain a mixture of soy isoflavones (MSI) comparable to P10, daidzein, glycitein, and genistein (Sigma-Aldrich) were mixed at a molar ratio of 5:4:1. L-Carnitine from Sigma was used in cell culture experiments and that from Lonza Japan was used in animal experiments. Cell culture experiments were carried out at suboptimal to optimal concentrations of each dietary component, which were determined by pilot experiments. Animal experiments were carried out at the doses of each dietary component previously studied (6,12).

3T3-L1 differentiation. DMEM, fetal bovine serum (FBS), and calf serum were purchased from Invitrogen and other reagents were purchased from Sigma-Aldrich. Mouse 3T3-L1 (ATCC CL173) preadipocyte cells were maintained in DMEM containing 10% calf serum. For differentiation, the medium was replaced with DMEM containing 10% FBS, 10 mg/L insulin, 0.5 mmol/L 3-isobutyl-1-methylxanthine, and 1 μmol/L dexamethasone at 2 d post-confluence. After 2 d, the medium was changed to DMEM containing 10 mg/L insulin and 10% FBS every 2 d for 8 d.

Lipid accumulation. 3T3-L1 adipocytes were differentiated with or without the dietary components [50 μmol/L caffeine; 1 mmol/L arginine; MSI (0.5 μmol/L daidzein, 0.1 μmol/L genistein, and 0.4 μmol/L glycitein); 1 mmol/L L-carnitine; alone or combined] during the last 8 d of culture. Intracellular lipid accumulation was determined by Sudan II staining according to the method previously described (25).

Adiponectin. 3T3-L1 adipocytes differentiated under the same conditions as the lipid accumulation study were used. The cells were washed with PBS and lysed on ice in radioimmunoprecipitation buffer, pH 8.5. Forty micrograms of total protein was analyzed under reducing conditions on 12% SDS/polyacrylamide gels and blotted onto nitrocellulose membranes. The blot was incubated with an anti-adiponectin (Upstate Biotechnology) and anti-β-actin (Sigma-Aldrich) antibody. The reaction products were detected by chemiluminescence with the ECL Western Blotting starter kit (Amersham Biosciences) according to the manufacturer's instructions. Anti-β-actin antibody was used to assess equal loading of the protein. Band intensities were measured using ImageMaster 2D (Amersham Pharmacia Biotech).

Lipolysis. Differentiated 3T3-L1 adipocytes were subjected to serum starvation for 48 h to synchronize the status of whole cells in low-glucose DMEM containing 2% (w:v) fatty acid-free bovine serum albumin (Sigma-Aldrich). During the last 24 h of serum starvation, the cells were treated with the dietary components [50 μmol/L caffeine; 1 mmol/L arginine; MSI (5 μmol/L daidzein, 1 μmol/L genistein, and 4 μmol/L glycitein); 0.5 mmol/L L-carnitine; alone or combined]. Glycerol content of the incubation medium was determined using a colorimetric assay (GPO-Trinder, Sigma-Aldrich). Protein content was measured using a BCA protein assay (Pierce).

Fatty acid oxidation. HepG2 cells were seeded in high-glucose DMEM containing 10% FBS at a density of 1.0×10^6 cells per T75 flask and grown for 24 h. Then the cells were incubated in serum-free DMEM with or without the dietary components [2 mmol/L caffeine; 1 mmol/L arginine; MSI (5 μmol/L daidzein, 1 μmol/L genistein, and 4 μmol/L glycitein); 1 mmol/L L-carnitine; alone or combined] in 6-well plates for another 24 h. The cells were washed and incubated in low-glucose DMEM containing 2% (w:v) fatty acid-free bovine serum albumin, 0.3 mmol/L L-carnitine, and ^{3}H-palmitic acid (111 kBq per well) for 3 h. Excess ^{3}H-palmitic acid in the medium was removed by trichloroacetic acid precipitation. The supernatant was extracted twice with chloroform:methanol (2:1) and then counted for ^3H_2O production. Total counts were normalized by total protein content.

CPT1A promoter assay. Huh7 cells were transfected with a CPT1A promoter reporter gene construct as described previously (9). The cells were cultured in 96-well microplates (2.0×10^5 cells/well) in the presence or absence of dietary components [2 mmol/L caffeine; 1 mmol/L arginine; MSI (5 μmol/L daidzein, 1 μmol/L genistein, and 4 μmol/L glycitein); 1 mmol/L L-carnitine; alone or combined] for 24 h. Luciferase assay was carried out according to the previous method (9).

Mice. All animal experiments in this study were approved by the Ethical Committee for Animal Experiments of House Wellness Foods Corporation and carried out in its facility. Five-week-old male KK/Ta mice were purchased from Clea Japan. They consumed a nonpurified diet (CE-2; Clea Japan) ad libitum for 1–2 wk to acclimatize or for 15 wk to induce obesity. The mice were allowed free access to food and tap water and housed in individual cages. The animal room was maintained at 23 ± 1°C, 55 ± 5% humidity, and 12-h-light/-dark cycle.

Basal diets. The compositions of a fat-free diet, a high-fat diet (19.5 kJ/g), and a low-fat diet (12.9 kJ/g) used in this study are shown in **Table 1**. Casein, corn oil, cellulose powder, and Harper mineral and vitamin mixtures (26) were purchased from Oriental Yeast. We purchased cornstarch from Japan Cornstarch and Choline bitartrate from Wako Pure Chemical.

TABLE 1 Composition of basal diets

Ingredient	Fat-free diet	Low-fat diet	High-fat diet
		g/kg	
Casein	180	180	180
Cornstarch	734	534	534
Corn oil	—	25	200
Cellulose powder	25	200	25
Harper mineral mixture (26)	50	50	50
Harper vitamin mixture (26)	10	10	10
Choline bitartrate	1	1	1

Lipogenesis in the liver. Seven-week-old male KK mice were food deprived for 48 h and then divided into 4 groups. The control group was refed a fat-free diet and the experimental groups were refed that diet supplemented with CA (250 mg and 6 g/kg diet) with SL (2 g P10 and 1.5 g/kg diet) or with CASL (250 mg, 6 g, 2 g P10, and 1.5 g/kg diet) for 72 h. After refeeding, the livers were removed and stored at −20°C for measurement of lipid contents. Hepatic lipid was extracted according to the method of Folch et al. (27). We determined TG contents using commercially available kits (Triglyceride-Test Wako).

Epinephrine-induced lipolysis. Twenty-week-old obese male KK mice were divided into 4 groups. Lipolysis was induced by subcutaneous injection of 500 μg/kg epinephrine (Wako). CA (2.5 mg and 60 mg per mouse) was i.g. administered 60 min prior to the epinephrine injection. SL (20 mg P10 and 15 mg per mouse), CASL (2.5, 60, 20 mg P10, and 15 mg per mouse), or saline were i.g. administered 2 d, 1 d, and 60 min prior to the epinephrine injection. Before and 30 min after the epinephrine injection, blood was sampled from the orbital sinus. After delipidation of the plasma, glycerol concentration was measured using commercially available kits (Triglyceride-Test Wako).

High-fat diet-induced obesity. Obesity was induced in KK mice (6 wk old) by feeding a high-fat diet for 3 wk, and then the mice with comparable body weight were divided into 4 treatment groups and the remainder was kept to consume a high-fat diet just for reference. The control group was fed a low-fat diet and the experimental groups were fed a low-fat diet supplemented with CA (250 mg and 6 g/kg diet), SL (2 g P10 and 1.5 g/kg diet), or CASL (250 mg, 6 g, 2 g P10, and 1.5 g/kg diet) for 2 wk. Body weight and energy intake were measured every 2–3 d. After feeding the low-fat diet, subcutaneous, epididymal, perirenal/retroperitoneal, and mesenteric adipose tissues and livers were removed and weighed. The livers were used for analysis of TG concentration. Preliminary examination for adiponectin expression of epididymal fat tissues was carried out in mice excluding the heaviest 2 and the lightest 1 in each group. The fat tissues were rinsed in PBS and homogenized on ice in radioimmunoprecipitation buffer with a glass homogenizer. After removal of cell debris by centrifugation, the homogenate containing 50 μg of protein was analyzed for adiponectin expression by the method described in the cell culture study. Before and after feeding the low-fat diet, blood was sampled from the orbital sinus and the plasma TG concentration was measured using Triglyceride-Test Wako.

Statistical analysis. Data are expressed as means ± SD. All statistical analyses were performed using Dr. SPSS software version 8.0J (SPSS Japan). Data from the control and dietary component-treated groups were analyzed by one-way ANOVA. The Games-Howell test was used as a post hoc test for in vitro studies, because the assumption of homoscedasticity was not met. The Tukey's honestly significant difference test was used as a post hoc test for in vivo studies. Significance level was set at $P < 0.05$.

Results

In vitro effect on lipid accumulation. Lipid accumulation in 3T3-L1 cells after differentiation to adipocytes was significantly lower in cells treated with caffeine or MSI than in control cells, but arginine or L-carnitine had no effect on the lipid accumulation (**Table 2**). The degree of inhibition of lipid accumulation by CASL was significantly higher than that by caffeine or MSI (Table 2), suggesting that there was combined effect of the dietary components on lipid accumulation. Despite a similar inhibition of lipid accumulation, the expression of adiponectin protein was lower in cells treated with caffeine but higher in cells treated with MSI than that of control cells (Table 2). Enhancement of adiponectin protein expression by CASL was comparable to that by MSI alone (Table 2). Thus, caffeine did not inhibit adiponectin expression when used as a constituent of CASL.

In vitro effect on lipolysis. When the differentiated 3T3-L1 adipocytes were treated with the dietary components, the degree of lipolysis in cells treated with a suboptimal concentration of caffeine, but not with the other 3 components, was significantly higher than that of control cells under the experimental conditions used (Table 2). Unexpectedly strong lipolysis was observed when we used CASL; lipolysis induced by CASL was much higher than that induced by each component alone (Table 2).

In vitro effect on fatty acid oxidation. The degree of oxidation of palmitic acid was significantly higher in cells pretreated with L-carnitine or MSI, but not in those pretreated with caffeine or arginine, than in control cells (**Table 3**). The degree of fatty acid oxidation was significantly higher in cells pretreated with CASL, but not with L-carnitine, than in those pretreated with caffeine, arginine, or MSI (Table 3). Promoter activity of CPT1A, a rate-limiting enzyme of fatty acid oxidation, was notably higher in cells treated with MSI than in control cells, as shown in our previous study (9). However, the activity was comparable between cells treated with MSI and those

TABLE 2 Effects of caffeine, arginine, MSI, and L-carnitine, alone or combined, on lipid accumulation, adiponectin protein expression, and lipolysis in 3T3-L1 adipocytes[1]

	Control	Caffeine	Arginine	MSI	L-Carnitine	CASL
Stained oil droplets, *absorbance at A_{490}*	2.50 ± 0.09^{a}	1.98 ± 0.01^{c}	2.40 ± 0.04^{a}	2.08 ± 0.03^{b}	2.38 ± 0.02^{a}	1.67 ± 0.02^{d}
Adiponectin band intensity, *% of control*	100, 100	8, 9	23, 30	286, 259	26, 35	168, 158
Glycerol, *mmol/mg protein*	1.44 ± 0.03^{c}	1.56 ± 0.01^{b}	0.51 ± 0.03^{d}	0.60 ± 0.37^{bcd}	0.87 ± 0.22^{bcd}	2.72 ± 0.08^{a}

[1] Values are means ± SD, $n = 3$ (stained oil droplets), 4 (glycerol) or 2 independent measures (adiponectin). Means in a row with superscripts without a common letter differ, $P < 0.05$.

TABLE 3 Effects of caffeine, arginine, MSI, and L-carnitine, alone or combined, on fatty acid oxidation in HepG2 cells and CPT1A promoter activity in CPT1A promoter-transfected Huh7 cells[1]

	Control	Caffeine	Arginine	MSI	L-Carnitine	CASL
Production of ^3H_2O from ^3H-palmitic acid, *kBq/mg protein*	3.40 ± 0.67[c]	4.20 ± 0.38[bc]	4.73 ± 1.42[bc]	4.83 ± 0.25[b]	6.67 ± 1.58[ab]	9.22 ± 1.53[a]
CPT1A promoter activity, *relative light unit*	539 ± 59[b]	—	—	3104 ± 299[a]	—	3174 ± 441[a]

[1] Values are means ± SD, $n = 5$. Means in a row without a common letter differ, $P < 0.05$.

treated with CASL; thus, no combined effect was observed (Table 3).

In vivo effect on lipogenesis. KK mice were food deprived for 48 h and then refed a fat-free diet for 72 h, which enhanced lipogenesis during the refeeding period. Lipogenesis in the liver was significantly lower in mice given CA than in control mice, similarly to that observed in our previous study (6). In contrast, SL did not affect lipogenesis in the liver (**Table 4**). The degree of inhibition of hepatic lipogenesis by CASL was comparable to that by CA (Table 4).

In vivo effect on lipolysis. In vivo lipolysis was evaluated by the increase in plasma glycerol after injection of the lipolytic hormone, epinephrine. Plasma glycerol levels 30 min after epinephrine injection tended to be higher ($P = 0.07$) in mice administered CASL (0.52 ± 0.13 mmol/L), but not in mice administered CA (0.43 ± 0.08 mmol/L) or SL (0.41 ± 0.11 mmol/L), than in control mice (0.38 ± 0.04 mmol/L). However, the increased plasma glycerol over 30 min following epinephrine injection did not differ between the 4 groups (data not shown). The combined effect of the dietary components on lipolysis seen in vitro (Table 2) was not observed in vivo.

Effect of CASL on obesity. After feeding a high-fat diet for 3 wk, we used obese KK mice to evaluate the anti-obesity action of the dietary components admixed with a low-fat diet. Body weight change was comparable in the mice given CA, SL, and control diets; however, it was significantly lower in mice given CASL than in control mice, despite no difference in food intake (**Table 5**). Similar to the results of body weight change, subcutaneous or mesenteric adipose tissue weight was significantly lower in mice given CASL, but not in mice given CA or SL, than in control mice (Table 5). Increased plasma TG levels after feeding a low-fat diet were significantly lower in mice given SL and CASL than in control mice (**Table 6**). Similarly, TG levels in the liver were significantly lower in mice given CASL, but not in mice given CA or the SL, than in control mice (Table 6). Adiponectin protein expression in epididymal fat tissue did not differ between groups (Table 6).

Discussion

We evaluated the combined effects of CA and SL, stimulants for lipolysis and fatty acid oxidation, respectively, on obesity. When CASL was applied to 3T3-L1 and HepG2 cells, inhibition of lipid accumulation and enhancement of lipolysis and fatty acid oxidation were observed (Tables 2 and 3). We also administered CASL to obese KK mice. As seen in in vitro studies, CASL significantly inhibited lipogenesis in the liver (Tables 4). Consequently, CASL, but not CA or SL alone, significantly decreased body weight and adipose tissue weight in obese KK mice (Table 5). These results suggest that CASL could be an effective prophylaxis for controlling obesity.

As another useful approach in the treatment of obesity, normal exercise is adopted to stimulate lipolysis and fatty acid oxidation. However, the capacity to utilize fat during exercise is impaired in obese subjects (28,29). Blunted lipolysis and fat oxidation occur after stimulation of β2-adrenoceptors and correlate with genetic variation in the β2-adrenoceptor gene in obese subjects (23,30). Similarly, whole body lipolytic sensitivity to epinephrine is blunted in upper-body obese women (24). Plasma glycerol levels tended to be higher ($P = 0.07$) after epinephrine injection in CASL-treated obese KK mice despite their putative unresponsiveness to epinephrine. Given the potent induction of lipolysis in 3T3-L1 cells, CASL might help with weight control in obese subjects treated with exercise through amelioration of fat utilization.

If lipolysis stimulants act excessively, a higher level of plasma free fatty acid may occur, as is seen in upper-body obesity. The abnormality in lipolysis is considered to be a cause of certain metabolic defects such as dyslipidemia and insulin resistance in upper-body obesity (31). However, a mixture containing CA

TABLE 4 Effects of CA, SL, and CASL on TG accumulation in the liver of food-deprived and refed KK mice[1]

	Control	CA	SL	CASL
Body weight, *g*				
Baseline	23.6 ± 1.2	23.6 ± 1.3	23.0 ± 0.8	22.1 ± 1.3
d 3	28.0 ± 0.8	26.6 ± 1.6	27.8 ± 1.5	26.2 ± 1.4
Weight gain, *g/3 d*	4.4 ± 1.0	2.9 ± 2.6	4.7 ± 1.9	4.1 ± 1.9
Food intake, *g/3 d*	13.4 ± 1.1	12.3 ± 1.5	13.5 ± 1.0	12.5 ± 1.1
Liver weight, *g*	1.59 ± 0.14[a]	1.35 ± 0.17[b]	1.47 ± 0.17[ab]	1.30 ± 0.13[b]
Liver TG, *mmol/liver*	16.4 ± 2.0[a]	8.0 ± 2.0[b]	14.7 ± 2.1[a]	7.6 ± 1.6[b]

[1] Values are means ± SD, $n = 7$. Means in a row with superscripts without a common letter differ, $P < 0.05$.

TABLE 5 Effects of CA, SL, and CASL on body weight and adipose tissue weight of KK mice fed a low-fat diet for 2 wk following feeding a high-fat diet for 3 wk[1]

	Control	CA	SL	CASL	High fat[2]
Body weight, *g*					
Before	33.8 ± 1.1	33.9 ± 0.6	33.7 ± 1.1	33.5 ± 0.9	(35.6 ± 2.1)
After	34.9 ± 1.4	34.7 ± 1.5	34.9 ± 1.3	32.9 ± 2.1	(38.4 ± 2.1)
Weight change, *g/2 wk*	1.1 ± 0.7[a]	0.9 ± 1.2[ab]	1.2 ± 0.8[a]	−0.7 ± 1.6[b]	(2.8 ± 0.4)
Food intake, *g/d*	4.7 ± 0.3	4.7 ± 0.4	4.5 ± 0.3	4.5 ± 0.2	(3.7 ± 0.2)
Energy intake, *kJ/d*	60.2 ± 3.7	60.7 ± 5.6	58.3 ± 3.3	57.7 ± 2.2	(72.0 ± 3.3)
Adipose tissue weight, *g*					
Subcutaneous	0.33 ± 0.05[a]	0.29 ± 0.02[ab]	0.33 ± 0.03[a]	0.25 ± 0.05[b]	(0.44 ± 0.04)
Epididymal	1.20 ± 0.16[ab]	1.13 ± 0.12[ab]	1.26 ± 0.12[a]	0.98 ± 0.24[b]	(1.59 ± 0.16)
Perirenal/retroperitoneal	0.47 ± 0.09[ab]	0.42 ± 0.08[ab]	0.52 ± 0.11[a]	0.34 ± 0.10[b]	(0.72 ± 0.18)
Mesenteric	0.63 ± 0.06[a]	0.57 ± 0.08[ab]	0.65 ± 0.07[a]	0.48 ± 0.13[b]	(0.85 ± 0.14)

[1] Values are means ± SD, $n = 7$. Means in a row with superscripts without a common letter differ, $P < 0.05$.
[2] Data from mice fed a high-fat diet during the period of low-fat diet feeding are shown for reference, $n = 5$.

was effective in ameliorating obesity and insulin resistance in non-insulin-dependent diabetic KK mice (6), suggesting no association with an adverse reaction. Nevertheless, treatment of 3T3-L1 cells with caffeine clearly reduced expression of adiponectin (Table 2), which might cause an adverse reaction in vivo under some circumstances. However, because the in vitro effect of caffeine on adiponectin expression was diminished in the case of its combined use (Table 2) and significant reduction of adiponectin expression was not observed in adipose tissue of CASL-treated KK mice (Table 6), CASL could possibly control obesity without any adverse reaction.

Plasma TG levels were significantly higher in KK mice after feeding a low-fat diet than in those before that feeding ($P < 0.001$ in each group; Table 6). Subjects in other studies who consumed a low-fat, high-carbohydrate diet increased their plasma TG (31,32). This increase is thought to be primarily due to decreased efficiency of TG clearance in normal subjects and increased production of VLDL from the liver in insulin-resistant subjects (33). The increased plasma TG due to a low-fat diet were significantly suppressed by SL and CASL (Table 6), suggesting there was increased TG clearance in mice given SL through stimulation of fatty acid oxidation by SL. Although CA could lower plasma TG levels via suppression of lipogenesis in the liver, the contribution of this mechanism to the TG-lowering effect of CASL was not clear, because the increased plasma TG did not significantly differ between the control and CA groups.

We selected CA as lipolysis stimulants of dietary components and lipolytic activity was confirmed by in vitro study in caffeine but not in arginine, possibly due to an indirect action of arginine on lipolysis (Table 2). However, in vivo lipolytic activity of CA was not confirmed. In contrast, significant inhibition of lipogenesis in the liver was observed in mice given CA (Table 4). It is possible that the inhibition of lipogenesis by CA and stimulation of fatty acid oxidation by SL act together cooperatively in the control of obesity. In fact, an anti-obesity agent, C75, has been reported to target 2 potential pathways, fatty acid synthesis and fatty acid oxidation, in obesity therapy (14). To clarify the contribution of lipogenesis inhibition on the control of obesity by the combination, further examinations are needed.

In conclusion, the combined effects of CASL on lipid accumulation, lipolysis, and fatty acid oxidation were confirmed by our in vitro study and CASL significantly inhibited lipogenesis in the liver and reduced body weight, adipose tissue weight, and TG levels in the plasma and liver in obese KK mice given a low-fat diet. Our results indicate that the administration of a combination of dietary components that stimulate either lipolysis or fatty acid oxidation could be a beneficial approach in controlling obesity.

TABLE 6 Effects of CA, SL, and CASL on TG in plasma and liver and adiponectin protein expression in epididymal fat tissue of KK mice fed a low-fat diet for 2 wk following feeding a high-fat diet for 3 wk[1]

	Control	CA	SL	CASL	High fat[4]
Plasma TG, *mmol/L*					
Before	1.27 ± 0.18	1.27 ± 0.29	1.28 ± 0.25	1.28 ± 0.21	(1.50 ± 0.54)
After	2.80 ± 0.41[a]	2.36 ± 0.37[ab]	2.22 ± 0.42[b]	1.98 ± 0.28[b]	(1.45 ± 0.51)
Changes in plasma TG,[2] *mmol/L*	1.53 ± 0.37[a]	1.09 ± 0.30[ab]	0.94 ± 0.52[b]	0.70 ± 0.24[b]	(−0.05 ± 0.60)
Liver weight, *g*	1.17 ± 0.15	1.16 ± 0.12	1.14 ± 0.09	1.12 ± 0.13	(1.33 ± 0.22)
Liver TG, *mmol/liver*	4.23 ± 0.79[a]	3.73 ± 0.55[ab]	3.86 ± 0.66[a]	2.78 ± 0.96[b]	(3.53 ± 0.69)
Adiponectin protein expression,[3] % *of control*	100 ± 20	55 ± 50	70 ± 5	71 ± 21	—

[1] Values are means ± SD, $n = 7$ unless otherwise noted. Means in a row with superscripts without a common letter differ, $P < 0.05$.
[2] To convert to mg/dL, multiply by 88.57.
[3] $n = 4$.
[4] Data from mice fed a high-fat diet during the period of low-fat diet feeding are shown for reference, $n = 5$.

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