

# A combination of exercise and capsinoid supplementation additively suppresses diet-induced obesity by increasing energy expenditure in mice

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<sup>1</sup>Frontier Research Laboratories, Institute for Innovation, Ajinomoto Company, Incorporated, Suzuki-cho Kawasaki-ku, Kanagawa, Japan; and <sup>2</sup>University of California, San Francisco Diabetes Center and Department of Cell and Tissue Biology, University of California, San Francisco, California

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## Ohyama K, Nogusa Y, Suzuki K, Shinoda K, Kajimura S, Bannai M.

A combination of exercise and capsinoid supplementation additively suppresses diet-induced obesity by increasing energy expenditure in mice. *Am J Physiol Endocrinol Metab* 308: E315–E323, 2015. First published December 17, 2014; doi:10.1152/ajpendo.00354.2014.—Exercise effectively prevents the development of obesity and obesity-related diseases such as type 2 diabetes. Capsinoids (CSNs) are capsaicin analogs found in a nonpungent pepper that increase whole body energy expenditure. Although both exercise and CSNs have antiobesity functions, the effectiveness of exercise with CSN supplementation has not yet been investigated. Here, we examined whether the beneficial effects of exercise could be further enhanced by CSN supplementation in mice. Mice were randomly assigned to four groups: 1) high-fat diet (HFD, Control), 2) HFD containing 0.3% CSNs, 3) HFD with voluntary running wheel exercise (Exercise), and 4) HFD containing 0.3% CSNs with voluntary running wheel exercise (Exercise + CSN). After 8 wk of ingestion, blood and tissues were collected and analyzed. Although CSNs significantly suppressed body weight gain under the HFD, CSN supplementation with exercise additively decreased body weight gain and fat accumulation and increased whole body energy expenditure compared with exercise alone. Exercise together with CSN supplementation robustly improved metabolic profiles, including the plasma cholesterol level. Furthermore, this combination significantly prevented diet-induced liver steatosis and decreased the size of adipocyte cells in white adipose tissue. Exercise and CSNs significantly increased cAMP levels and PKA activity in brown adipose tissue (BAT), indicating an increase of lipolysis. Moreover, they significantly activated both the oxidative phosphorylation gene program and fatty acid oxidation in skeletal muscle. These results indicate that CSNs efficiently promote the antiobesity effect of exercise, in part by increasing energy expenditure via the activation of fat oxidation in skeletal muscle and lipolysis in BAT.

exercise; capsinoids; obesity; energy expenditure; fat oxidation

OBESITY IS RAPIDLY BECOMING a major global health problem (4) and is a major risk factor for several common diseases, including type 2 diabetes, cardiovascular diseases, and cancer (32). Obesity develops when energy intake chronically exceeds total energy expenditure (51). Conventional antiobesity strategies have focused on repressing energy intake by suppressing appetite or inhibiting intestinal fat absorption; however, increasing energy expenditure by activating the metabolic function of skeletal muscle or brown fat could serve as an alternative and effective antiobesity intervention, thereby avoiding the potential adverse effects associated with conventional antiobesity

therapies such as depression, oily bowel movements, and steatorrhea.

Exercise is one of the most efficient ways to prevent obesity and type 2 diabetes through an increase in energy expenditure (25, 27, 52). However, people in the developed world are becoming less physically active as a result of lifestyle changes and the nature of their work. Moreover, constraints such as lack of time, limited access, and injuries frequently become barriers to exercise (48). For example, a recent epidemiological study estimated that in a 1-yr period, 39% of women adopted some type of physical activity, but the attrition rate exceeded 30% within only months (46).

Capsaicin is the pungent component in chili pepper, and it is known to have an antiobesity effect (18, 20). Capsinoids (CSNs) are capsaicin analogs found in a nonpungent type of chili pepper, “CH-19 Sweet” (23, 24). CSNs differ from capsaicin in their chemical structure only at the center linkage of an ester bond, resulting in reduced (<0.1%) pungency, which facilitates daily intake, while maintaining the metabolic effect. Several studies have shown that CSNs increase energy expenditure and suppress body fat accumulation in mice (31). A single administration of capsiate, a CSN, was found to increase oxygen consumption, and chronic administration for 2 wk diminished fat accumulation in mice (31, 39). Moreover, chronic treatment with CSNs via their inclusion in a high-fat diet (HFD) for 12 wk was shown to dramatically suppress body weight gain and fat accumulation (Inoue N, Nogusa Y, Hara-Kimura Y, Okabe-Nogusa Y, Ohyama K, Tsukamoto-Yasui M, and Ono K, unpublished observations). In adult humans, a 4-wk treatment with CSNs was found to increase oxygen consumption, and a 12-wk treatment decreased the abdominal fat mass in subjects with body mass indexes (BMIs) of 25–35 (15, 50). Mechanistically, oral CSN supplements have been reported to activate transient receptor potential vanilloid subtype 1 (TRPV1) in the gut (14, 47), resulting in increased sympathetic efferent activity and thermogenesis (41). Activation of TRPV1 signaling has been reported to positively regulate exercise endurance and energy expenditure in mice (30).

We propose that the combination of dietary supplements with exercise may be effective and realistic for obesity control. Although there have been many investigations into the individual beneficial effects of exercise and dietary supplements, little is known about their combined effects. Thus we investigated the effects of daily intake of CSNs in combination with exercise on the development of obesity in C57BL/6J mice.

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Table 1. *Composition of the experimental diets*

	HFD, %	HFD + CSNs (0.3%)
Casein	20	20
Sucrose	10	10
Corn starch	22.48	22.48
$\alpha$ -Corn starch	7.47	7.47
L-Cysteine	0.3	0.3
Cellulose	5	5
Lard	30	30
Mineral mix	3.5	3.5
Vitamin mix	1	1
Choline bitartrate	0.25	0.25
CSNs	—	0.3

HFD, high-fat diet; CSNs, capsinoids.

## MATERIALS AND METHODS

**Animals and diets.** C57BL/6J male mice were purchased from Charles River (Kanagawa, Japan) at 7 wk of age and were housed in a controlled-lighting environment (lights on from 1600 to 0400) at  $25 \pm 1^\circ\text{C}$ . They were fed CRF-1 (Charles River, Kanagawa, Japan) for 2 wk to stabilize their metabolic condition. The mice were divided into two groups: one group was given free access to a running wheel (Ex group; wheel diameter, 14 cm; Melquest, Toyama, Japan) connected to a counter, and the other group could not access the wheel. After habituation to wheel running for 1 wk, the mice were divided into two groups by body weight, food intake, and wheel running counts. The mice were allowed ad libitum access to water and either the HFD or HFD supplemented with 0.3% (wt/wt) CSNs (Yoyu-Lab, Gunma, Japan), depending on the group. The compositions of the diets are listed in Table 1. Thus we prepared four groups: 1) sedentary-HFD [Control: Ex(-)/CSN(-)], 2) sedentary-CSNs [CSN: Ex(-)/CSN(+)], 3) voluntary running-HFD [Exercise: Ex(+)/CSN(-)], and 4) voluntary running-CSNs [Exercise + CSN: Ex(+)/CSN(+)]. The mice were maintained on these diets for 8 wk. Body weight, food intake and running distance were monitored twice a week. All animal protocols were approved by the Animal Committee of Ajinomoto.

**Sampling procedures.** At the end of the experiment, the mice were anesthetized with isoflurane, and blood samples were collected by aortic puncture after 3 h of fasting. The blood samples were centrifuged at 3,000 rpm for 20 min at  $4^\circ\text{C}$  and stored at  $-20^\circ\text{C}$  until analyzed. The organs [liver, mesenteric white adipose tissue (WAT), perirenal WAT, epididymal WAT, subcutaneous WAT, brown adipose tissue (BAT), gastrocnemius muscle, and soleus muscle] were excised and weighed. The organs were then immediately frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$ .

**Blood analysis.** The plasma levels of glucose and glutamic pyruvic transaminase (GPT) were determined using a Fuji-drychem device (Fujifilm, Tokyo, Japan). The plasma insulin levels and leptin levels were determined by an enzyme-linked immunosorbent assay (ELISA; Morinaga, Kanagawa, Japan). The plasma levels of triglycerides (TG)

and cholesterol were determined using the Wako TG-test kit (Wako, Osaka, Japan) and Cholesterol-E test kit (Wako), respectively.

**Liver lipid analysis.** Lipids in the liver and gastrocnemius muscle were extracted using the Folch partition method (10) with slight modifications as follows: a portion of the tissue was homogenized in 2.5 ml of methanol, and 5 ml of chloroform were added. The mixture was horizontally shaken for 10 min and extracted at  $4^\circ\text{C}$  overnight. The extracted samples were filtered using a Kiriya-rohto device (Kiriya, Tokyo, Japan), and the volume was increased to 8 ml with methanol:chloroform (2:1, vol/vol). Then, 1.6 ml of saline were added. The mixture was horizontally shaken for 10 min and centrifuged at 2,000 rpm for 5 min at  $4^\circ\text{C}$ . The supernatant was removed, and the lower chloroform phase was filled to 6 ml with chloroform. Next, 2.5 ml of the lipid fraction in this phase were dried and weighed. The dried lipids were dissolved in 1 ml of 10% Triton/isopropanol, and the TG and nonesterified fatty acid (NEFA) levels were determined using a Wako TG-test kit (Wako) and NEFA-C test kit (Wako), respectively.

**Quantitative real-time RT-PCR.** Total RNA was isolated from the gastrocnemius muscle and subcutaneous WAT using Ribozol (AMRESCO). cDNA was synthesized from 1  $\mu\text{g}$  of RNA using an iScript cDNA Synthesis Kit (Bio-Rad). After cDNA synthesis, quantitative real-time PCR was performed in 10  $\mu\text{l}$  of iTaq Fast SYBR Green Supermix (Bio-Rad) using a fluorometric thermal cycler (ViiA 7 System; Life Technologies). The reaction mixtures were incubated for an initial denaturation at  $95^\circ\text{C}$  for 10 s, followed by 45 cycles of  $95^\circ\text{C}$  for 5 s and  $60^\circ\text{C}$  for 20 s. The sequences of the sense and antisense primers used in the amplification are shown in Table 2. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or TATA box binding protein (TBP) was used as an internal control.

**Histology.** Subcutaneous WAT was fixed in 4% paraformaldehyde for 24 h at  $4^\circ\text{C}$ . The samples were then dehydrated, embedded in paraffin, sliced into 7- $\mu\text{m}$ -thick sections with a Leica RM2255 (Leica Microsystems; Vienna, Austria), deparaffinized, and rehydrated. The sections were then stained with hematoxylin and eosin (H&E).

**Respiratory gas analysis and measurement of activity.** With the use of a respiratory gas analysis system consisting of an acrylic metabolic chamber, gas analyzers, and a switching system (ARCO2000-RAT/ANI System; Arco, Chiba, Japan), the  $\text{O}_2$  and  $\text{CO}_2$  concentrations of gas sampled from each metabolic chamber were measured as described previously (39). Briefly, room air was constantly pumped through the chamber, and expired air was dried in a thin cotton column and then introduced into the gas analyzer. The respiratory quotient (RQ:  $\dot{V}\text{CO}_2/\dot{V}\text{O}_2$ ) was calculated according to Weir (56). Fat oxidation was calculated based on the  $\dot{V}\text{O}_2$  and carbon dioxide production. The spontaneous activity of the mice was simultaneously measured using an activity sensor (NS-AS01; Neuroscience, Tokyo, Japan). All measurements were performed in 4.5-min intervals.

**cAMP levels and PKA activity in BAT.** cAMP levels and PKA activity were determined using the cAMP Direct Immunoassay Kit (Abcam, Cambridge, UK) and PKA Kinase Activity Assay Kit (Abcam), respectively.

Table 2. *Primers used for real-time PCR analysis*

Gene	Sense	Antisense	Entrez Gene ID
Gapdh	CTGAGGACCAGGTTGCTCC	ACCACCCTGTGCTGTAGCC	14433
Tbp	ACCCTTCACCAATGACTCCTATG	TGACTGCAGCAAATCGCTTGG	21374
Aco	CATTGGCATGTGAGAACAG	AGCAAATCTGATGGCTTTGA	11430
Cox-1	CACATAAATCGGAGCCCCA	TTCATCCTGTTCCTGTCTCT	17708
Lpl	CCAGCAACATTATCCAGTGCTAG	CAGTTGATGAATCTGTCTCT	16956
Mead	GCTCGTGAGCACATTGAAAA	CATTGTCCAAAAGCCAAACC	11364
Pgc-1 $\alpha$	CACTACAGACACCGCACACA	AGGCTTCATAGCTGTCTGACC	19017
Ucp-1	CACCTTCCCCTGGACACT	CCCTAGGACACCTTTATACCT	22227
Cidea	ATCACAACCTGGCCTGGTTACG	TACTACCCGGTGTCCATTCT	12683
Cox8B	GAACCATGAAGCCAACGACT	GCGAAGTTCACAGTGGTTCC	12869

**Fatty acid oxidation in muscle.** The muscle fatty acid oxidation rate was determined in fresh muscle homogenate using a modification of the method of Kim et al. (21a). The oxidation rate of palmitate was measured by collecting and counting the  $^{14}\text{CO}_2$  produced during incubation. Twenty microliters of muscle homogenate were incubated with 380  $\mu\text{l}$  of reaction mixture (pH 8.0) for 1 h. The final concentrations of the reaction mixture were as follows in millimoles per liter: 100 sucrose, 10 Tris-HCl, 5 potassium phosphate, 80 potassium chloride, 1 magnesium chloride, 2 L-carnitine, 0.1 malate, 2 ATP, 0.05 coenzyme A (CoA), 1 dithiothreitol, 0.2 EDTA, 5 nicotinamide, 0.001 trichostatin A, and 0.3% bovine serum albumin. The substrate used was [ $^{14}\text{C}$ ]palmitate (0.4  $\mu\text{Ci}$ ) with 2 mM BSA. After 60 min of incubation at 37°C, 200  $\mu\text{l}$  of 1 M perchloric acid were injected to stop the reaction. The  $\text{CO}_2$  produced during the incubation was trapped by Whatman filter paper with 15  $\mu\text{l}$  of hyamine hydroxide. Then, the filter paper was transferred to a glass scintillation vial that contained 4 ml of Emulsifier-Safe. The average counts per minute were measured over 3 min.

**Statistical analysis.** The statistical analysis was performed using JMP version 9.0 (SAS Institute). The main and interaction effects of the CSNs and Ex on the metabolic parameters were determined by two-way ANOVA. Repeated-measures ANOVA with *t*-tests was used to compare the time courses. Other statistical comparisons utilized a two-tailed Student's *t*-test. The results are given as the means  $\pm$  SE unless otherwise stated.  $P < 0.05$  was considered significant throughout the study.

## RESULTS

**Effects of exercise and CSN supplementation on body weight gain and adiposity.** The metabolic effects of exercise and CSNs were evaluated in an 8-wk cohort of diet-induced obese mice. The mice given CSNs gained significantly less weight in both the normal and exercised groups under a HFD (Fig. 1A). The combination of exercise and CSNs dramatically (17%) suppressed weight gain at 56 days compared with that in the control [Ex(-)/CSN(-)] group. Body weight gain was significantly different between the Ex(-) and Ex(+) groups and between the CSN(-) and CSN(+) groups (Fig. 1B). The main effects of CSNs and exercise on final body weight gain were significant as determined by two-way ANOVA, whereas their interaction was not significant ( $P = 0.915$ ). The reduction of body mass was accounted for by a decrease in fat as reflected in the weights of various white fat deposits, including the epididymal, mesenteric, and perirenal WAT (Fig. 1C). However, heart weight was not significantly affected by either exercise or the CSN treatment. Consistent with these results, the combination of exercise and CSNs significantly improved metabolic profiles. The blood levels of glucose, insulin, leptin, and cholesterol were all significantly decreased by the combination of exercise and CSNs (Table 3).

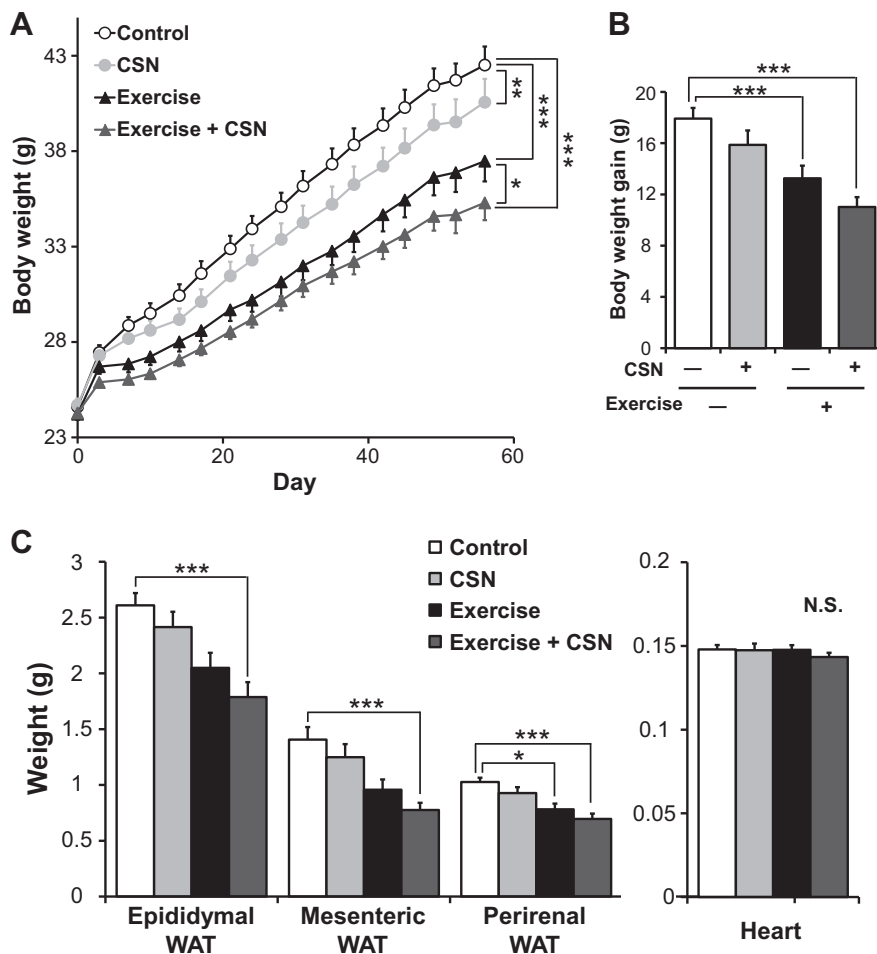


Fig. 1. Capsinoid (CSN) supplementation with exercise additively suppressed body weight gain and adiposity in diet-induced obesity. *A*: body weight development. *B*: body weight gain. *C*: tissue weight of C57BL/6J mice fed an high-fat diet (HFD, Control), HFD supplemented with CSNs, HFD together with voluntary exercise (Exercise), or HFD supplemented with CSNs in addition to voluntary exercise (Exercise + CSN) for 56 days. WAT, white adipose tissue. The values represent the means  $\pm$  SE ( $n = 7-8$ ). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

Table 3. Effects of CSN supplementation plus exercise on blood parameters

	Control	CSN	Exercise	Exercise + CSN	P Value
Blood glucose, mg/ml	31.45 ± 2.27	25.28 ± 0.90*	21.84 ± 0.80*	23.00 ± 1.14†	0.0003
Insulin, ng/ml	6.00 ± 1.08	3.89 ± 0.56	3.63 ± 0.53*	3.12 ± 0.63†	0.0481
Leptin, ng/ml	22.81 ± 1.45	21.47 ± 2.75	16.93 ± 3.61	12.03 ± 1.98†	0.0262
Cholesterol, mg/ml	18.79 ± 0.98	15.13 ± 1.15*	16.11 ± 0.40*	15.15 ± 0.44†	0.0120
TG, mg/ml	5.36 ± 0.63	5.51 ± 0.34	5.04 ± 0.47	4.70 ± 0.21	0.5755

Values represent the means ± SE ( $n = 7-8$ ). The levels of blood glucose, insulin, leptin, cholesterol, and triglyceride (TG) were measured in C57BL/6J mice fed a HFD (Control), HFD supplemented with CSNs, HFD together with voluntary exercise (Exercise), or HFD supplemented with CSNs in addition to voluntary exercise (Exercise + CSN) for 56 days.  $P$  values were calculated by one-way ANOVA. \* $P < 0.05$  and † $P < 0.01$ , significant difference compared with the control group.

**Effects of exercise and CSN supplementation on energy expenditure.** The critical parameter contributing to body weight control is the energy balance between caloric intake and energy expenditure (29). Food intake was not affected by CSNs in either the Ex(-) or Ex(+) groups (Fig. 2A). We then measured the energy expenditure, which comprises physical activity and thermogenesis. The physical activity (locomotive activity and running distance) of the mice during the treatment was not affected by the CSN supplementation (Fig. 2, B and C). However, the whole body oxygen consumption and fat oxidation were significantly increased by the combination of exercise and CSNs compared with the control group (Fig. 2, D and E).

**CSN supplementation with exercise improved HFD-induced liver steatosis.** Next, we examined the metabolic consequences of the antiobesity effects of exercise and CSNs. It is well known that a HFD induces liver steatosis; thus lipids should

accumulate in the liver. Therefore, we measured the lipid content in the liver, as assessed by the levels of total lipids, TG, and NEFA. Total lipid levels tended to be decreased by exercise or CSN treatment but not significantly. However, the combination of exercise and CSNs significantly reduced the total lipid content in the liver (Fig. 3A). Similar effects were observed for TG and NEFA levels (Fig. 3, B and C). Importantly, the combination of exercise and CSNs significantly reduced the total lipid, TG, and NEFA levels in the liver by 64, 46, and 78%, respectively (Fig. 3, A-C). The plasma levels of GPT, a sensitive parameter in diagnosing fatty liver in humans (21), were significantly reduced in the mice that exercised (Fig. 3D).

**CSN supplementation with exercise diminished cell size in subcutaneous WAT.** The morphological and biochemical changes in WAT in response to exercise and CSNs were examined. Metabolic improvements are strongly associated

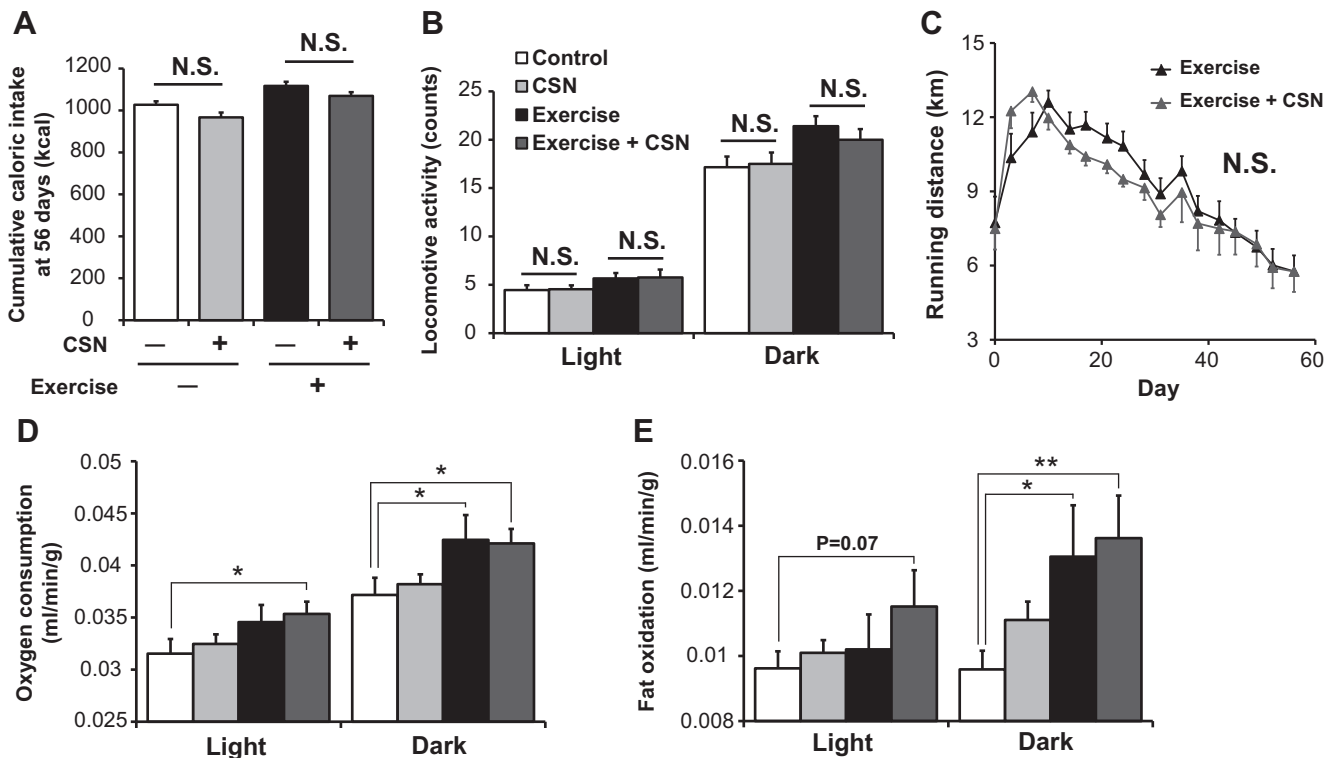


Fig. 2. CSN supplementation with exercise additively increased energy expenditure. A: food intake. B: locomotive activity. C: running distance. D: oxygen consumption. E: fat oxidation of C57BL/6J mice fed a HFD (Control), HFD supplemented with CSNs, HFD in addition to voluntary exercise (Exercise), or HFD supplemented with CSNs in addition to voluntary exercise (Exercise + CSN) for 56 days. The values represent the means ± SE ( $n = 7-8$ ). \* $P < 0.05$ , \*\* $P < 0.01$ .



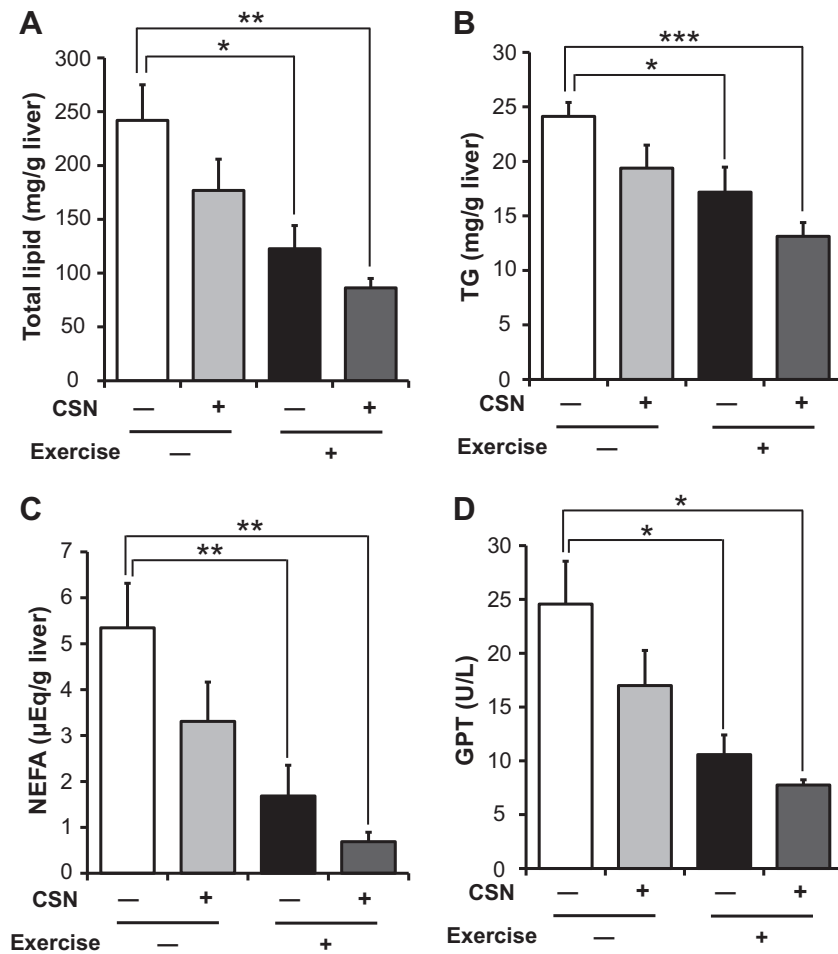


Fig. 3. CSN supplementation with exercise improved HFD-induced liver steatosis. *A*: hematoxylin and eosin (H&E) liver staining. *B–D*: liver levels of total lipids (*B*), triglycerides (TGs; *C*); nonesterified fatty acid (NEFA; *D*). *F*: glutamic pyruvic transaminase (GPT) levels in the plasma of C57BL/6J mice. Mice were fed a HFD (control), HFD supplemented with CSNs, HFD together with voluntary exercise (Exercise), or HFD supplemented with CSNs in addition to voluntary exercise (Exercise + CSN) for 56 days. The values represent the means  $\pm$  SE ( $n = 7–8$ ). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

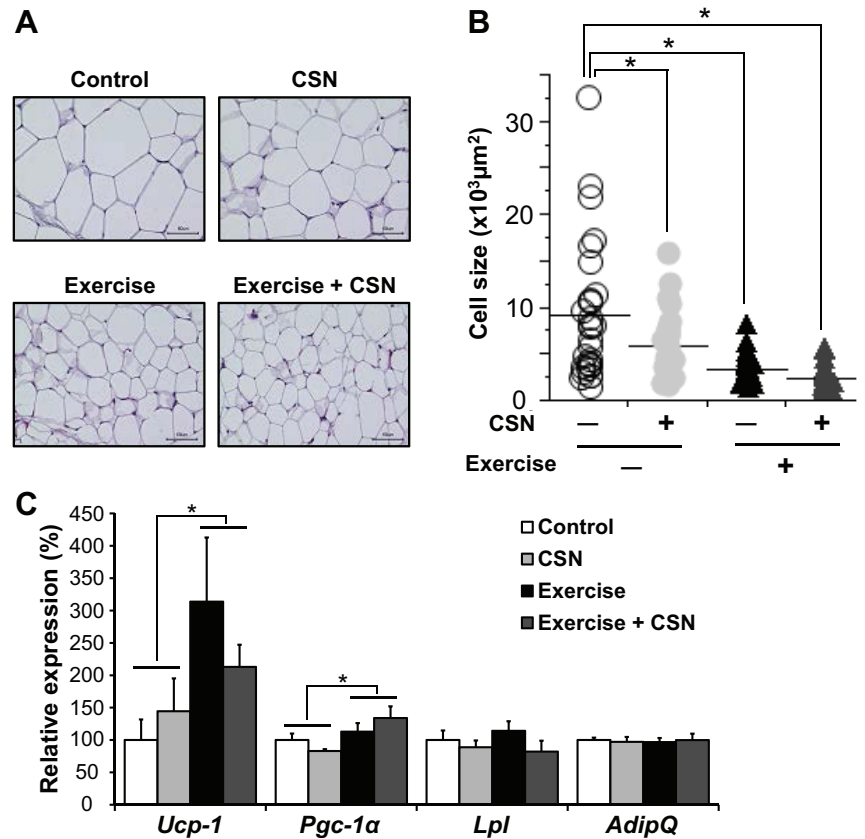
with a reduction in adipocyte size. Histological examination (H&E staining) of subcutaneous WAT revealed that the adipocyte size significantly decreased in the WAT from the mice that received both exercise and CSNs (Fig. 4A). To quantitatively assess the difference, we calculated the average cell size in a 1-mm field of view of H&E-stained tissue and observed that exercise and CSNs indeed significantly diminished cell size (Fig. 4B). Because we observed a significant increase in energy expenditure that was associated with exercise and CSN supplementation (Fig. 2C), we hypothesized that nonshivering thermogenesis through brown adipocytes may be altered. Thus we measured the mRNA expression levels of thermogenic genes [uncoupling protein-1 (*Ucp1*) and peroxisome proliferator-activated receptor gamma coactivator 1 $\alpha$  (*Pgc-1 $\alpha$* )] in WAT. As previously reported (5, 58), exercise significantly increased *Ucp-1* and *Pgc-1 $\alpha$*  expression (main effect  $P = 0.02$ ), although the effects of CSNs did not reach significance, with  $P$  values of 0.64 (*Ucp-1*) and 0.87 (*Pgc-1 $\alpha$* ) (Fig. 4C). However, the expression levels of lipoprotein lipase (*Lpl*), a key enzyme of lipolysis, and adiponectin (*AdipQ*), a differentiation marker, did not change among the groups (Fig. 4C).

*CSN supplementation combined with exercise increased lipolysis in BAT.* It is well known that the sympathetic nervous system (SNS) is activated during exercise (54). Additionally, it has been reported that CSNs increase BAT sympathetic nervous activity (41). We therefore hypothesized that CSN treat-

ment with exercise would additively activate the SNS. To address this hypothesis, we determined cAMP levels in BAT. Exercise significantly increased cAMP levels, and the combination of exercise and CSNs tended to further increase cAMP levels (Ex: 54%; Ex + CSN: 87%) in BAT (Fig. 5A). We further determined the PKA activity in BAT, because increased intercellular cAMP activates PKA. Similar to the effect on cAMP levels, the combination of exercise and CSNs resulted in the highest PKA activity (Fig. 5B).

*CSN supplementation combined with exercise increased fatty acid oxidation in the gastrocnemius muscle.* It is known that a HFD induces lipid accumulation not only in the liver but also in muscle. Exercise significantly decreased TG levels, and the combination of exercise and CSNs tended to further decrease TG levels (Ex: 37%; Ex + CSN: 42%) in the gastrocnemius muscle (Fig. 6A). Skeletal muscle is one of the most active organs in dissipating energy, and it plays a central role in systemic energy homeostasis. Exercise is well known to increase the expression of genes involved in fatty acid oxidation, such as acyl-CoA oxidase (*Aco*) and cytochrome *c* oxidase-1 (*Cox-1*), through the transcriptional pathway of *Pgc-1 $\alpha$*  (28, 45, 59). Hence, we hypothesized that exercise and CSNs would additively activate the expression of fat oxidative genes in muscle, which could contribute to increased energy expenditure. As shown in Fig. 5B, the combination of exercise and CSNs significantly increased the mRNA levels of *Lpl*, *Cox-1*,

Fig. 4. CSN supplementation with exercise decreased cell size in subcutaneous WAT. **A:** H&E staining of subcutaneous WAT. **B:** cell size of **A** (in  $\times 10^3 \mu\text{m}^2$ ). The sizes of 100 randomly chosen cells in representative H&E-stained slides ( $n = 5$ ) were quantified by ImageJ (1.48v). Each dot represents a cell, and the horizontal bar is the group mean. **C:** mRNA expression levels of uncoupling protein-1 (*Ucp-1*), peroxisome proliferator-activated receptor gamma coactivator-1 $\alpha$  (*Pgc-1 $\alpha$* ), lipoprotein lipase (*Lpl*), and adiponectin (*AdipQ*) in the subcutaneous WAT of C57BL/6J mice fed a HFD (Control), HFD supplemented with CSNs (CSN), HFD together with voluntary exercise (Exercise), or HFD supplemented with CSNs in addition to voluntary exercise (Exercise + CSN) for 56 days. The values represent the means  $\pm$  SE ( $n = 7-8$ ). \* $P < 0.05$ .

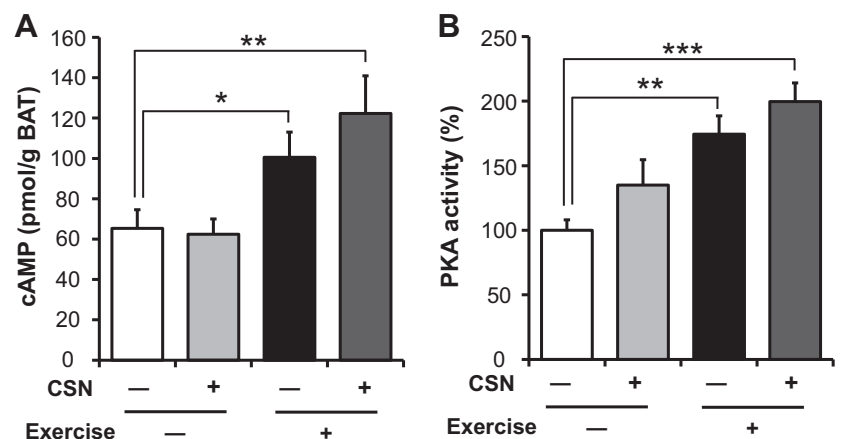


*Aco*, and medium-chain acyl dehydrogenase (*Mcad*) compared with the expression levels in the control group. Additionally, CSNs significantly increased the mRNA levels of *Cox-1* under the exercise conditions. By contrast, myosin heavy chain-1/β (*Mhc-1/β*) mRNA expression did not change (Fig. 6B), indicating that muscle fiber type changes did not occur in this setting. Furthermore, we directly determined the fatty acid oxidation rate in muscle using [<sup>14</sup>C]palmitate. The oxidation rate tended to be increased by the exercise or the CSN treatment but not significantly. However, the combination of exercise and CSNs significantly increased the oxidation rate in the gastrocnemius muscle (Fig. 6C).

## DISCUSSION

Exercise is important in treating obesity because of its capacity to increase energy expenditure (13, 17). Despite the well-documented health benefits of exercise, constraints such as lack of time, limited access, and injuries frequently become barriers to exercise (48). Hence, we propose that the combination of antiobesity food components with exercise may be effective in weight control by compensating for or adding to individual effects. Although there have been many investigations into the individual beneficial effects of exercise or food components, little is known about their combined effects on

Fig. 5. CSN supplementation with exercise increased cAMP levels and protein kinase A (PKA) activity in brown adipose tissue (BAT). **A:** level of cyclic AMP (cAMP). **B:** PKA activity in the BAT of C57BL/6J mice fed a HFD (Control), HFD supplemented with CSNs, HFD together with voluntary exercise (Exercise), or HFD supplemented with CSNs in addition to voluntary exercise (Exercise + CSN) for 56 days. The values represent the means  $\pm$  SE ( $n = 7-8$ ). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .



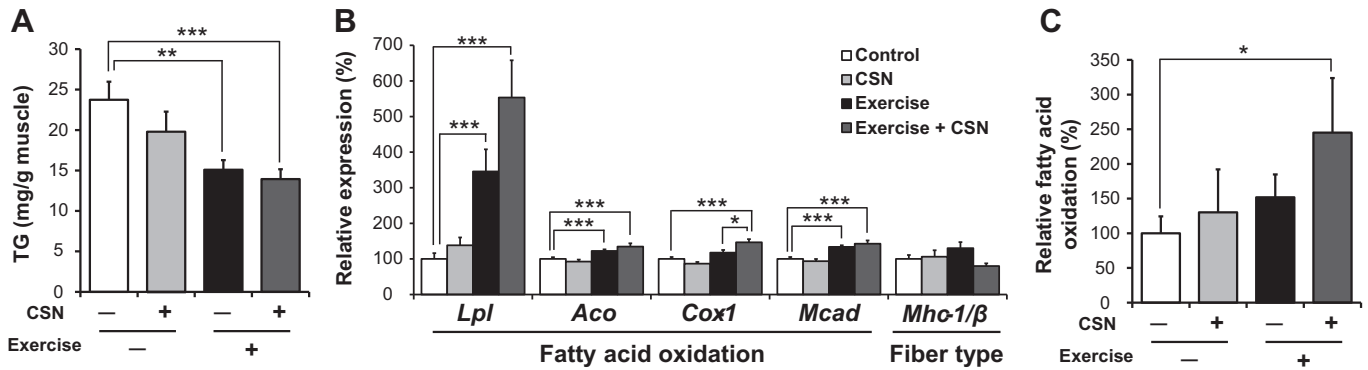


Fig. 6. CSN supplementation with exercise increased fatty acid oxidation by activating the oxidative phosphorylation (OXPHOS) gene program in the gastrocnemius muscle. *A*: TG levels in the gastrocnemius muscle. *B*: mRNA expression levels of lipoprotein lipase (*Lpl*), acyl-CoA oxidase (*Aco*), cytochrome *c* oxidase-1 (*Cox-1*), medium-chain acyl dehydrogenase (*Mcad*), and myosin heavy chain-1/β (*Mhc-1/β*) in the gastrocnemius muscle. *C*: fatty acid oxidation rate in the gastrocnemius muscle of C57BL/6J mice fed a HFD (Control), HFD supplemented with CSNs (CSN), HFD together with voluntary exercise (Exercise), or HFD supplemented with CSNs in addition to voluntary exercise (Exercise + CSN) for 56 days. The values represent the means  $\pm$  SE ( $n = 7-8$ ). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

obesity development. This study demonstrated that the combination of exercise and CSNs additively reduced adiposity (Fig. 1C). Furthermore, CSN supplementation with exercise improved the status of peripheral markers such as blood glucose, insulin, and leptin levels, providing additional supporting evidence for the additive effect (Table 3). Although several groups, including ours, have reported independent effects of CSNs on obesity (Inoue N, Nogusa Y, Hara-Kimura Y, Okabe-Nogusa Y, Ohshima K, Tsukamoto-Yasui M, and Ono K, unpublished observations; Refs. 31, 39), this report is the first to show that CSNs have an antiobesity effect under exercise conditions. The combination of exercise and tea catechin was also reported to be more effective than the effects of the individual components in suppressing obesity (33). Catechins, such as epigallocatechin gallate (EGCG), have antiobesity effects in mice and humans (22, 34, 35, 57). These observations support our finding that the combination of exercise and antiobesity food components has increased efficacy for suppressing obesity and that CSNs are a promising candidate for combining with exercise.

The antiobesity mechanisms of CSNs include increased energy expenditure (15, 39, 40). Given the established effect of exercise on energy expenditure, we expected that CSNs would provide an additive increase in energy expenditure. Indeed, the combination of exercise and CSNs increased whole body fat oxidation, whereas food intake and exercise amounts were unchanged (Fig. 2). Thus the combination of exercise and CSNs may conceivably suppress obesity via an increase in basal energy expenditure through a significant increase in fat oxidation that involves an increase in the expression of fatty acid oxidative genes in muscle (Fig. 5, *B* and *C*). Exercise is well known to upregulate *Pgc-1α* gene expression, and downstream genes are transcriptionally regulated by *Pgc-1α* (28, 45, 59). For example, swimming exercise resulted in a twofold increase in *Pgc-1α* mRNA and protein levels in the skeletal muscle of rats (2), and exercise training induced a marked increase in *Pgc-1α* mRNA content in humans (44). Additionally, chronic administration of CSNs has been shown to increase the expression of *Pgc-1α* and its target genes in the gastrocnemius muscle (Inoue N, Nogusa Y, Hara-Kimura Y, Okabe-Nogusa Y, Ohshima K, Tsukamoto-Yasui M, and Ono

K, unpublished observations). In the present study, the increase in *Pgc-1α* expression by only exercise or CSNs was smaller than that in previous reports ( $P = 0.15$  and  $P = 0.07$ ; data not shown), possibly because of differences in experimental conditions, such as follow-up duration or type of exercise (forced or voluntary). These results suggest that the increased fat oxidation in muscle induced by the combination of exercise and CSNs plays an important role in increasing the energy expenditure of the whole body.

Both single and chronic CSN supplementation protocols have been reported to increase energy expenditure (11, 15, 31, 39, 40, 50). This CSNs-dependent increase in energy expenditure may be mediated by activation of the SNS. CSNs increase BAT sympathetic nervous activity and the release of norepinephrine (41). Because the SNS is activated and the release of catecholamines increases during exercise (55), the release of catecholamines may be synergistically regulated by the combination of exercise with CSNs. Catecholamines released from sympathetic nerve terminals promote lipid mobilization and thermogenesis via  $\beta_3$ -adrenoreceptors (12, 36). In the present study the cAMP levels in BAT were additively increased by the combination of exercise and CSNs (Fig. 5A), which indicates that exercise and CSNs additively activated  $\beta_3$ -adrenoreceptors and increased cAMP levels, resulting in activation of PKA (Fig. 5B). Activated PKA phosphorylates hormone-sensitive lipase (HSL) and induces lipolysis (1, 8). Because fatty acids are a UCP-1 substrate (9), the combination of exercise and CSNs could induce energy expenditure and thermogenesis by driving UCP-1 activity.

UCP-1 is a thermogenic protein that produces heat by uncoupling electron transport from ATP production (6) during adaptive nonshivering thermogenesis (36). UCP-1 has recently attracted attention as a target for suppressing obesity. CSNs and exercise have been reported to increase UCP-1 levels in interscapular BAT (31, 37, 38). Furthermore, exercise has been reported to induce browning in subcutaneous WAT (5). However, in this research, we did not observe an increase in *Ucp-1* expression in interscapular BAT in response to the exercise and CSN treatment (data not shown). Moreover, we did not observe any differences in the mRNA levels in the subcutaneous WAT among the groups. These results suggest that the suppression of

obesity by the combination of exercise and CSNs does not involve an increase in the expression of *Ucp-1* in either interscapular BAT or subcutaneous WAT.

Recently, CSNs have been reported to activate the transient receptor potential cation channel, subfamily A, member 1 (TRPA1) and the TRPV1 channel (49). TRPA1 is sensitive to cold stimuli below 17°C (43) and to various reagents such as allyl isothiocyanate, cinnamaldehyde, and farnesyl thiosalicylic acid (3). However, the specific function of this protein has not yet been determined. Further studies are necessary to elucidate the role of the activation of TRPA1 by CSNs in the antiobesity effect.

Excessive accumulation of TG in the liver in response to a HFD has been reported to induce disorders such as fatty liver and to lead to liver cirrhosis and even hepatocellular cancer (54). In the present study, the combination of exercise and CSNs decreased the total lipid, TG, and NEFA levels in the liver (Fig. 3, A–C). Plasma cholesterol and GPT levels were also significantly reduced by the combination of exercise and CSNs (Table 3 and Fig. 3D). Previous studies have reported that exercise suppresses liver lipid accumulation (26), possibly via mechanisms involving increased fat oxidation and decreased lipogenesis in the liver (7, 42). Furthermore, capsiate was reported to reduce hepatic TG levels in rats fed a HFD (53), and CSNs were shown to reduce hepatic TG levels in mice fed a HFD (Inoue N, Nogusa Y, Hara-Kimura Y, Okabe-Nogusa Y, Ohyama K, Tsukamoto-Yasui M, and Ono K, unpublished observations). These data suggested that the combination of exercise and CSNs may suppress lipid accumulation by increasing fat oxidation in the liver. Hence, we measured the expression levels of genes involved in fatty acid oxidation in the liver but found that they were not changed in this cohort (data not shown). Therefore, the suppression of lipid accumulation is thought to be a secondary effect of increasing energy expenditure in muscle.

Some studies have reported that CSNs increase energy expenditure and suppress obesity in humans. Yoneshiro et al. (6) reported that CSNs acutely increase energy expenditure through the activation of BAT in humans and that chronic CSN treatment increases cold-induced thermogenesis in humans (61). Additionally, CSNs or CH-19 sweet pepper fruits containing CSNs were found to decrease visceral adiposity in subjects with BMIs of 25–35 kg/m<sup>2</sup> (19, 50). CSN has already been filed with the Food and Drug Administration as a new dietary ingredient. Combining exercise with CSNs in the human diet could thus be a powerful tool for fighting obesity.

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#### DISCLOSURES

K. Ohyama, Y. Nogusa, K. Suzuki, and M. Bannai are employees of Ajinomoto Co., Inc.

#### AUTHOR CONTRIBUTIONS

Author contributions: K.O., K. Suzuki, and M.B. conception and design of research; K.O. and Y.N. performed experiments; K.O. and K. Shinoda analyzed data; K.O. prepared figures; K.O. and M.B. drafted manuscript; K.O.,

S.K., and M.B. edited and revised manuscript; M.B. interpreted results of experiments; M.B. approved final version of manuscript.

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