Nootkatone, a characteristic constituent of grapefruit, stimulates energy metabolism and prevents diet-induced obesity by activating AMPK

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AMPK-activated protein kinase (AMPK) is a serine/threonine kinase that is implicated in the control of energy metabolism, and is considered to be a molecular target for the suppression of obesity and the treatment of metabolic syndrome. Here, we identified and characterized nootkatone, a constituent of grapefruit, as a naturally occurring AMPK activator. Nootkatone induced an increase in AMPKα1 and α2 activity along with an increase in the AMP/ATP ratio and an increase the phosphorylation of AMPKα and the downstream target, acetyl-CoA carboxylase (ACC), in C2C12 cells. Nootkatone-induced activation of AMPK was possibly mediated both by LKB1 and CaMKK. Nootkatone also upregulated PGC-1α in C2C12 cells and C57BL/6J mouse muscle. In addition, administration of nootkatone (200 mg/kg body weight) significantly enhanced AMPK activity, accompanied by LKB1, AMPK, and ACC phosphorylation in the liver and muscle of mice. Whole-body energy expenditure evaluated by indirect calorimetry was also increased by nootkatone administration. Long-term intake of diets containing 0.1% to 0.3% (w/w) nootkatone significantly reduced high-fat and high-sucrose diet-induced body weight gain, abdominal fat accumulation, and the development of hyperglycemia, hyperinsulinemia, and hyperleptinemia in C57BL/6J mice. Furthermore, endurance capacity, evaluated as swimming time to exhaustion in BALB/c mice, was 21% longer in mice fed 0.2% nootkatone than in control mice. These findings indicate that long-term intake of nootkatone is beneficial towards preventing obesity and improving physical performance, and that these effects are due, at least in part, to enhanced energy metabolism through AMPK activation in skeletal muscle and liver.
Keywords: AMP-activated protein kinase; obesity; exercise; energy metabolism; nootkatone
Introduction

Obesity, as a part of metabolic syndrome, is a well-known risk factor for the development of diabetes, hyperlipidemia, hypertension, and coronary heart disease, and is one of the most important health problems in the world (5). AMP-activated protein kinase (AMPK) plays a key role in regulating energy metabolism and is an attractive molecular target for suppressing obesity and treating metabolic syndrome.

AMPK is a serine/threonine protein kinase that regulates energy homeostasis in most tissues (17,27). AMPK is phosphorylated and activated by upstream kinases, including LKB1, which was originally identified as a tumor-suppressor protein, and CaMKK (2,8,12,33). AMPK activation results in the phosphorylation and inhibition of acetyl-CoA carboxylase (ACC) activity, and the loss of inhibition of carnitine palmitoyl transferase-1, leading to an increase in fatty acid oxidation. AMPK activity is also involved in glucose homeostasis by regulating glucose uptake, glycogen synthesis, and the expression of gluconeogenesis-related molecules, such as phosphoenolpyruvate carboxykinase and glucose-6-phosphatase (29). The AMPK pathway in muscle is also activated by exercise to generate energy (31). In addition, chronic activation of AMPK by 5-aminimidazole-4-carboxamide riboside increases the expression of glucose transporter-4, mitochondrial enzyme activity, and glycogen content (10,32), all of which are also increased in response to exercise, suggesting that the effects of exercise can be reproduced, at least in part, by treatment with AMPK activators. Narkar et al. recently reported that 5-aminimidazole-4-carboxamide riboside treatment induced the expression of metabolic genes and enhanced running endurance in mice, and proposed that AMPK activators may be useful as exercise
mimetic drugs (24). Based on the important and favorable role of AMPK in energy homeostasis, the development of specific small-molecule drugs affecting AMPK is considered a promising approach for the treatment of obesity and metabolic syndrome.

Some AMPK activators have been reported (4,35,36). The efficacy of a widely-used drug for anti-type 2 diabetes mellitus, metformin, is mediated by AMPK activation, and leads to increased fatty acid oxidation and decreased glucose production (36). In addition, much attention has recently focused on the potential use of food components for the treatment of obesity and related diseases. Resveratrol, a natural polyphenol that is present in grapes, activates AMPK and has beneficial effects towards suppressing obesity and atherosclerosis (35). In the course of our studies on bioactive natural compounds (20,21,23), we found that nootkatone \([4,4a,5,6,7,8\text{-hexahydro}-6\text{-isopropenyl}-4,4a\text{-dimethyl}-2(3H)\text{-naphthalenone}]\), a constituent of grapefruit, activates AMPK in \textit{vitro} and in \textit{vivo}. A whole grapefruit contains approximately 100 mg nootkatone, mainly in the rind. Little is known, however, about its biologic and nutritional effects. In the present study, we first characterized the effects of nootkatone on the AMPK signaling pathway in C2C12 and Hepa 1-6 cells. In addition, to elucidate the physiologic consequences of AMPK activation by nootkatone, we investigated the effect of nootkatone on whole-body energy metabolism and diet-induced obesity in C57BL/6J mice, and on swimming endurance capacity in BALB/c mice.
Materials and Methods

Cell culture. C2C12 (mouse myoblasts), Hepa 1-6 (mouse hepatoma), and Hela cells were purchased from Dainippon Sumitomo Pharma (Osaka, Japan). Cells were maintained in DMEM supplemented with 10% FBS and 10 ml/l antibiotic-antimicotic mixture (GIBCO, Grand Island, NY) in an atmosphere of 95% air/5% CO₂ at 37°C. For differentiation into myotubes, C2C12 myoblasts were grown to subconfluence in flasks and the culture medium was replaced with DMEM containing 2% heat-inactivated horse serum (GIBCO) for 5 days. All cells were cultured in serum-free medium overnight and exposed to nootkatone (>98%; Alfa Aesar, Ward Hill, MA) for the indicated period.

Western blot analysis. Cells were treated with nootkatone and the lysates were prepared as described previously (22). The livers and muscles were homogenized in ice-cold lysis buffer using a motor-driven pestle in a microcentrifuge tube. The homogenates were kept on ice for 30 min and then centrifuged at 16000 x g for 15 min at 4°C. The supernatants were removed and their protein concentrations determined. Equal amounts of protein were separated by SDS-PAGE and transferred to Immobilon polyvinylidene transfer membranes. Western blotting was performed with anti-phospho-AMPKα, anti-phospho-ACC, anti-phospho-LKB1, anti-AMPK, anti-ACC, anti-LKB1, anti-PGC-1α, and anti-α-tubulin (Cell Signaling, Beverley, MA) primary antibodies, and horseradish peroxidase-labeled anti-rabbit immunoglobulin (Cell Signaling) as a secondary antibody. Blots were visualized with chemiluminescence reagent (LumiGLO, Cell Signaling) and a ChemiDoc XRS imaging system (Bio-Rad, Hercules, CA).

Reverse transcription-polymerase chain reaction. For real-time RT-PCR analyses, total RNA was
reverse-transcribed using the SuperScript first-strand synthesis system (Invitrogen, Carlsbad, CA) and amplified on a thermal cycler (7500 Fast Real-Time PCR System; Applied Biosystems, Foster City, CA) using a Power SYBR Green Master Mix (Applied Biosystems). The primers used in this study were: PGC-1α-F: ATGTGTCGCTTCTTGCTCT, PGC-1α-R: ATCTACTGCCTGGGGACCTT, cyclooxygenase (COX)-IV-F: AGCTGAGCCAAGCAGAGAAG, COX-IV-R: AATCACCAGAGCCGTGAATC, 36B4-F: CTGATCATCCAGCAGGTT, 36B4-R: CCAGGAAGGCCTTGACCTTT.

**Measurement of AMPK activity.** AMPK activity was measured as previously described with minor modifications (13,22). Briefly, cells or tissues were homogenized in ice-cold lysis buffer [20 mM Tris-HCl (pH 7.4), 1% Triton X-100, 50 mM NaCl, 250 mM sucrose, 2 mM DTT, 50 mM NaF, 5 mM sodium pyrophosphate, phosphatase inhibitor cocktail] by a pestle in a microcentrifuge tube. The homogenates were kept on ice for 30 min and then insoluble materials were removed by centrifugation at 14,000 x g for 20 min at 4°C, and the protein content of the supernatants was determined. Aliquots of protein (200 μg) were immunoprecipitated with anti-AMPKα1 or anti-AMPKα2 antibodies (Upstate Biotechnology, Lake Placid, NY) coupled with Dynabeads protein G (Dynal Biotech, Oslo, Norway). Immunoprecipitates were washed twice with ice-cold lysis buffer and twice with wash buffer (240 mM HEPES and 480 mM NaCl). AMPK activity was assessed in buffer containing 40 mM HEPES (pH 7.0), 80 mM NaCl, 2% glycerol, 0.8 mM DTT, 0.8 mM EDTA, 5 mM MgCl2, 0.2 mM ATP, 0.2 mM AMP, 0.2 mM SAMS peptide (Millipore, MA), and 2 μCi [γ-32P] ATP for 20 min at 30°C. Reaction products (15 μl) were spotted on Whatman P81 filter paper and the filters were washed 3 times in 1% phosphoric.
acid and once with acetone. Radioactivity was quantified with a scintillation counter (Beckman Coulter, Fullerton, CA).

**Measurement of adenosine nucleotides.** Cell extracts for the measurement of adenosine nucleotides were prepared by the method of Soga *et al.* (K. Kami and T. Soga, personal communication). C2C12 cells were incubated with nootkatone for 30 min, washed twice with 5% mannitol solution, and scraped off the plates with a cell scraper into 1 ml ice-cold methanol containing 25 μM 2-N-morpholinoethanesulfonate (Dojindo, Kumamoto, Japan) and 25 μM trimesate (Wako, Osaka, Japan). After adding 500 μl H2O, 1.2 ml of methanol solution was obtained and then the lipids were extracted by adding 800 μl chloroform, followed by centrifugation at 20,000 x g for 15 min at 4°C. Proteins in the methanol-water layer (800 μl) were centrifugally removed by Microcon UltraCel YM-3 (Millipore, Billerica, MA) and the filtrates were centrifugally concentrated at 150 x g for 4 h at 40°C (TOMY CC-105; Tomy Seiko Co. Ltd, Tokyo). The dried samples were dissolved in 50 μl H2O and nucleotides were analyzed by capillary electrophoresis- time of flight mass spectrometry (CE-TOFMS) as described previously (9). This system consists of Agilent CE (G1600A) and TOFMS (G1969A; Agilent Technologies, Waldbronn, Germany). Data acquisition and evaluation were performed using G2201A ChemStation software (Agilent). AMP and ATP concentrations were determined by comparing to standard curves made from solutions of each.

**Animal protocol for AMPK assay.** Six-week-old male C57BL/6J mice were obtained from CLEA Japan (Tokyo, Japan). The mice were maintained at 23 ± 2°C under a 12-h light-dark cycle (lights on from 07:00 to 19:00) and given free access to water and a standard diet (CE-2; CLEA Japan). After 2 h
without access to food, mice were given either nootkatone (200 mg/kg body weight [BW]) orally in 2% (w/v) solution or saline (5 mice in each group). At the indicated times, the mice were anesthetized by diethylether and the livers, and soleus and gastrocnemius muscles were quickly dissected, frozen in liquid nitrogen, and stored at -80°C until use. All animal experiments were approved by the Animal Care Committee of Kao Tochigi Institute. All animal experiments followed the guidelines put forth by the committee for the use and care of laboratory animals.

Peroxisome proliferator-activated receptor (PPAR) luciferase assay. The pBIND-GAL4-PPARαLBD and pBIND-GAL4-PPARδLBD chimeric expression plasmids were described previously (21). The pG5luc reporter plasmid containing the GAL4 binding site was obtained from Promega (Madison, WI). CV-1 cells were plated in 12-well plates in DMEM supplemented with 5% charcoal-treated FBS (Thermo Trace, Melbourne, Australia). After 1 day, transfections were performed using SuperFect transfection reagent (QIAGEN, Valencia, CA). Cells were incubated in transfection mixture containing 6.25 μL SuperFect, 0.375 μg pBIND-GAL4-PPAR-LBD expression plasmid, and 0.375 μg pG5luc reporter plasmid for 3 h at 37°C. The cells were then incubated for 4 h in fresh DMEM (+5% charcoal-treated FBS). After treatment with or without each sample for 20 h, cells were lysed, then firefly and Renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega). Wy14643 (SIGMA) was used as a positive control for PPARα, and GW501516 (Wako) for PPARδ.

Measurement of β-oxidation activity. 6-week-old male C57BL/6J mice were randomly divided into 3 groups (n=8) and allowed ad libitum access to water and a standard diet (CE-2). Nootkatone (100 or
200 mg/kg BW) or saline was orally administered for 10 days. On the final day of the experiment, the gastrocnemius muscles and livers were dissected from each animal and frozen in liquid nitrogen for subsequent analyses.

Fatty acid β-oxidation activity was measured as described previously (21) using [1-14C]-palmitic acid as the substrate. Mouse gastrocnemius muscles or livers were homogenized on ice using a Physcotron homogenizer (Microtech, Chiba, Japan) in 250 mM sucrose and 1 mM EDTA in 10 mM HEPES (pH 7.2), and insoluble materials were removed by centrifugation at 600 x g for 5 min. Aliquots of the resultant supernatant (50 μg protein) were used for assays. The reaction was started by adding the substrate (0.1 μCi [14C]-palmitic acid) and terminated after 20 min by adding 200 μl of 0.6 N perchloric acid. After centrifugation, supernatants were extracted three times with 1 ml n-hexane to remove residual substrate. The radioactivity remaining in the aqueous phases was measured in a liquid scintillation counter 2550TR/LL (Packard). Values are expressed as percentages, taking levels in control mice as 100%.

Indirect calorimetry. Whole-body energy metabolism was examined using a magnetic-type mass spectrometric calorimeter, the ARCO-2000 (ARCO System Inc., Chiba, Japan). Male 6-week-old C57BL/6J mice were allowed ad libitum access to water and a standard diet (CE-2) for 1 week to stabilize the metabolic conditions. To reduce the inherent variation in energy metabolism, oxygen consumption and respiratory quotient (RQ) were measured for 4 days, and mice whose mean values were significantly larger or smaller than the average values of all mice were excluded from the analysis. Thus, 8 of 16 mice were selected. After acclimation to a chamber for 2 days, mice were deprived of food
for 3 h, and then nootkatone (200 mg/kg BW) or saline was orally administered using a crossover treatment paradigm with a 7-day washout period between treatments. After administration, mice were transferred to a chamber, and oxygen consumption (Vo$_2$) and carbon dioxide production (Vco$_2$) were monitored for 3 h. RQ was defined as the ratio of the Vo$_2$ to Vco$_2$ values. Locomotor activity was measured in an automated motion analysis system (Actracer-2000; ARCO System Inc.), which detects the amount of centroid fluctuation using a weighted transducer.

*Evaluation of anti-obesity effect.* Six-week-old male C57BL/6J mice were randomly divided into 5 groups (5 mice/cage), and were allowed ad libitum access to water and one of three synthetic diets: 1) a low fat diet containing 5% (w/w) plant oil (Summit oil mill Co. Ltd., Chiba, Japan), 20% casein (Oriental Yeast Co., Ltd, Tokyo, Japan), 66.5% potato starch (Oriental), mineral mixture (Oriental), and a vitamin mixture (Oriental); 2) a high fat diet containing 25% (w/w) plant oil, 5% lard (Oriental), 20% casein, 28.5% potato starch, 13% sucrose (Wako), mineral mixture, and a vitamin mixture; or 3) a nootkatone diet, which consisted of the high-fat diet supplemented with 0.1% to 0.3% (w/w) nootkatone. Animals were maintained on these diets for 18 weeks. Body weight was measured weekly throughout the study. Food intake was measured every 2 to 3 days on a per-cage basis throughout the study using two Rodent Cafe (Oriental) food dispensers per cage to minimize the dispersion of the diet. Total food intake is shown as energy value consumed during the experimental period per mouse. Energy values for each diet were calculated from the macronutrient composition using values of 4, 4, and 9 kcal/g for carbohydrate, protein, and fat, respectively. Feed efficiency was calculated as follows: total body weight gain per cage (5 mice, g)/kcal of food consumed per cage.
Blood analysis. Blood was collected under fasting conditions by tail snip on week 17 of the experiment. Plasma triglyceride, total cholesterol, non-esterified fatty acid, glucose, glutamic-oxaloacetic transaminase, and glutamic-pyruvic transaminase levels were determined using commercially available kits (Wako). Plasma insulin and leptin levels were measured using mouse insulin and leptin enzyme immunoassay kits (Morinaga, Yokohama, Japan). Adiponectin levels were analyzed using a mouse adiponectin ELISA kit (Otsuka, Tokyo, Japan).

Swimming exercise and evaluation of endurance capacity. The endurance capacity for swimming was evaluated using an adjustable-current water pool as described previously (18). Briefly, 5-week-old male BALB/c mice (Charles River, Kanagawa, Japan) were divided into two groups (n = 16) according to the initial swimming endurance capacity. In preliminary training sessions, mice were accustomed to swimming for 30 min, 3 times a week, at a 6 l/min flow rate. After training, mice were fasted for 2 h before swimming, and then their maximum swim times (initial endurance capacity) were measured at a flow rate of 7 l/min twice a week. Mice were allowed unlimited access to water and a synthetic diet containing 10% (w/w) plant oil, 20% casein, 55.5% potato starch, 8.1% cellulose, 2.2% vitamins, 0.2% methionine, and 4% minerals; control animals were fed this diet alone and experimental animals were fed this diet supplemented with 0.2% nootkatone for 10 weeks. During this period, mice were exercised in a pool at a flow rate of 6 l/min once a week to accustom them to swimming. We measured the endurance capacity of mice swimming at a flow rate of 7 l/min, every 1 to 2 weeks for the 10-week experimental period.

Statistical analysis.
All values are presented as the means ± SE. Comparisons of data were made using a one-way ANOVA. When the ANOVA indicated significant differences, each group was compared with the others by Dunnett’s test (StatView; SAS Institute Inc., Cary, NC). Statistical analysis on adenosine phosphates and endurance was conducted using Student’s t-test. Two-way repeated measures ANOVA was used to analyze the results of indirect calorimetric measurements. A $P$ value of less than 0.05 was considered significant.
Results

Nootkatone activates AMPK in C2C12 and Hepa 1-6 cells. The structure of nootkatone is shown in Fig. 1A. Because phosphorylation of AMPK-Thr\(^{172}\) is essential for AMPK activation, the activating effect of nootkatone was assessed by determining AMPK\(\alpha\) phosphorylation using an anti-phospho-AMPK\(\alpha\) antibody. Nootkatone increased the phosphorylation of AMPK\(\alpha\) in a concentration-dependent manner in both C2C12 and Hepa 1-6 cells (Fig. 1B). AMPK activation was confirmed by the increased phosphorylation of ACC, a downstream substrate of AMPK. Phosphorylation of LKB1-Ser\(^{428}\), which is considered an AMPK-kinase, was also enhanced by nootkatone treatment. We then examined the effect of nootkatone on isoform-specific AMPK activity in C2C12 cells using SAMS peptide as a substrate. Nootkatone induced a concentration-dependent increase in AMPK\(\alpha_1\) and AMPK\(\alpha_2\) activity (Fig. 1C). These findings indicate that nootkatone induces phosphorylation of both AMPK\(\alpha_1\) and AMPK\(\alpha_2\), which leads to the phosphorylation and inactivation of ACC.

Nootkatone activates AMPK in skeletal muscle and liver. To further determine the activating effect of nootkatone on AMPK, we examined AMPK\(\alpha\), ACC, and LKB1 phosphorylation, and AMPK activity in response to nootkatone treatment in the liver and muscle of C57BL/6J mice. Oral administration of nootkatone (200 mg/kg BW) increased AMPK\(\alpha\) and ACC, as well as LKB1 phosphorylation, in the gastrocnemius muscle (Fig. 2A) and liver (Fig. 2B). Nootkatone treatment produced a rapid and significant increase in AMPK\(\alpha_1\) and AMPK\(\alpha_2\) activity, which peaked at 60 min in the gastrocnemius muscle (Fig. 3A). In the soleus muscle, AMPK\(\alpha_1\) activity peaked at 30 min and
then quickly decreased to basal levels (Fig. 3B). In the liver, nootkatone also increased AMPKα2 activity and, to a lesser extent, AMPKα1 (Fig. 3C). These findings indicate that nootkatone activates the AMPK signaling pathway in vivo as well as in cells in culture.

*Nootkatone increases cellular AMP/ATP ratio.* To investigate the underlying mechanism of the effects of nootkatone on AMPK activation, we measured AMP, ADP, and ATP concentrations in C2C12 cells by CE-TOFMS to determine the cellular AMP/ATP ratio. Nootkatone treatment of C2C12 cells for 30 min significantly increased the AMP concentration and AMP/ATP ratio (Table 1), suggesting that the modulation of intracellular energy status by nootkatone triggers the activation of AMPK.

*Nootkatone-induced activation of AMPK is also mediated by CaMKK.* CaMKK also mediates AMPK activation (2,33); therefore, we examined the involvement of CaMKK in nootkatone-induced AMPK or ACC phosphorylation. Pretreatment of C2C12 cells with the CaMKK inhibitor STO-609 diminished the phosphorylation of AMPK and ACC by nootkatone without affecting LKB1 phosphorylation (Fig. 4A). Nootkatone induced the phosphorylation of AMPK and ACC in Hela cells, which lack endogenous LKB1 (Fig. 4B). Further, pretreatment of Hela cells with STO-609 completely suppressed AMPK phosphorylation (Fig. 4C). These results suggest that CaMKK is also involved in the nootkatone-induced activation of AMPK.

*Nootkatone stimulates PGC-1α expression.* Activation of AMPK increases PGC-1α expression and promotes mitochondrial biogenesis (25,37). Treatment of C2C12 cells with nootkatone increased PGC-1α and COX-IV mRNA, markers of mitochondrial biogenesis (Fig. 4D). Nootkatone-induced
PGC-1α and COX-IV mRNA expression was diminished by compound C (AMPK inhibitor) and STO-609 (Fig. 4D), indicating that nootkatone-induced upregulation of PGC-1α is mediated by AMPK. An 18-week nootkatone feeding period also increased the amount of PGC-1α protein in mouse soleus muscle (Fig. 4E).

**ROS or RNS are not involved in nootkatone-induced AMPK activation**

Recent studies indicate that reactive oxygen species (ROS) and reactive nitrogen species (RNS) have important roles in the activation of AMPK (3,11,17,26,27,38); therefore, we examined whether nootkatone-induced AMPK activation is mediated by ROS or RNS in C2C12 cells. Membrane-permeable catalases, which eliminate hydrogen peroxide; SOD, which catalyzes the dismutation of superoxide into oxygen and hydrogen; and L-N^G^-nitroarginine methyl ester (L-NAME) a nitric oxide synthase inhibitor, did not alter AMPK phosphorylation (Fig. 5), suggesting that ROS or RNS are not involved in nootkatone-induced AMPK activation.

**Nootkatone stimulates whole-body energy metabolism.** The acute effects of nootkatone on energy metabolism were examined by monitoring oxygen consumption and the RQ. A single dose of nootkatone significantly enhanced oxygen consumption relative to vehicle for the first 3 h after treatment (Fig. 6A). There were no significant differences in RQ (Fig. 6B) and locomotor activity (data not shown) between groups.

**Nootkatone stimulates fatty acid β-oxidation in skeletal muscle and liver.** To examine the effect of nootkatone on fatty acid metabolism, nootkatone was orally administered daily for 10 days. Nootkatone significantly increased fatty acid β-oxidation activity, assessed by the degradation of ^14^C-palmitate, in
both the gastrocnemius muscle and liver (Fig. 7).

**Effect of nootkatone on PPAR activation.** We tested the possibility that nootkatone acts as a ligand for PPARα and PPARδ, using the luciferase assay system. Although Wy14643, a PPARα agonist, and GW501516, a PPARδ agonist, significantly enhanced PPAR-dependent luciferase activities, nootkatone (50-100 μM) did not have a marked effect, indicating that nootkatone does not act as a direct ligand, or is only a weak ligand for PPARα and PPARδ (data not shown).

**Nootkatone suppresses diet-induced obesity.** The anti-obesity effect of nootkatone was examined in obesity- and diabetes-prone C57BL/6J mice. Consistent with previous reports (23,28), feeding C57BL/6J mice with the high-fat and high-sucrose diet for 18 weeks significantly increased body weight compared with mice fed the low-fat diet. The high-fat diet-induced body weight gain was markedly attenuated by feeding the mice diets containing 0.1 to 0.3% (w/w) nootkatone (Fig. 8A). Compared with that of mice fed the high-fat diet, cumulative energy intake tended to decrease in nootkatone-fed mice (Fig. 8B, Table 2). The body weight of mice fed 0.1, 0.2, or 0.3% nootkatone was significantly lower after 5, 6, and 1 weeks of feeding, respectively, and during that period the energy intake values between the high-fat and nootkatone groups were nearly identical (Fig. 8B). Supplementation with nootkatone significantly suppressed epididymal, retroperitoneal, and perirenal fat accumulation compared with the high-fat control group (Table 2). The feed efficiency of nootkatone-fed mice also decreased dose-dependently. The liver weight in the high-fat control group was significantly greater, and the increase was attenuated by nootkatone intake. These findings indicate that nootkatone intake effectively suppressed body fat accumulation.
In parallel with body fat reduction, nootkatone administration had a significant effect on plasma components. Plasma insulin and leptin levels were 1.9- and 1.8-fold higher, respectively, in the high-fat control group than in the low-fat diet group, and the increase was significantly attenuated in mice fed nootkatone (Table 2). The high-fat diet-induced increase in the plasma glucose concentration was also suppressed by nootkatone intake. Plasma adiponectin levels were not influenced by a high-fat diet, but were significantly decreased in mice fed nootkatone.

**Nootkatone improves endurance capacity.** The effect of nootkatone on endurance capacity was examined in BALB/c mice because this strain is considered to be a suitable model for evaluating swimming endurance (19,21). The swimming times to exhaustion at the 10th week are shown in Fig. 8C. Compared to control mice, mice fed 0.2% nootkatone had a 21% longer swimming time, indicating that nootkatone intake improved the endurance capacity for exercise.
The findings of the present study demonstrated that nootkatone potently activates the AMPK signaling pathway. Nootkatone stimulated the phosphorylation of AMPK-Thr\textsuperscript{172}, a crucial step for AMPK activation, and downstream ACC in C2C12 and Hepa 1-6 cells. Consistent with the increase in AMPK/ACC phosphorylation, nootkatone also induced phosphorylation of LKB1-Ser\textsuperscript{428}, a major upstream AMPK-kinase (2,8). LKB1 is a constitutively active kinase; the physiologic relevance of its phosphorylation, however, remains controversial (6). Xie et al. recently reported that Ser\textsuperscript{428} phosphorylation of LKB1 by PKC\textgreek{z} regulates the nuclear transport of LKB1 into the cytosol and is required for metformin-enhanced AMPK activation in endothelial cells (34). Therefore, nootkatone-induced phosphorylation of LKB1 may have a role in the cellular localization and subsequent AMPK activation of LKB1. AMPK can be activated in response to an increase in the AMP/ATP ratio and is proposed to occur through inhibition of the dephosphorylation reaction, catalyzed by protein phosphatases (2). Therefore, increases in the cellular AMP concentration might be a predominant mechanism for LKB1-dependent AMPK activation by nootkatone. The finding that nootkatone significantly increased cellular AMP concentration without affecting ATP levels indicates that the nootkatone-induced change in the AMP/ATP ratio might be a result of increased ATP utilization rather than decreased ATP production.

Nootkatone also induced phosphorylation of AMPK and ACC in Hela cells that do not express LKB1, and nootkatone-induced AMPK activation in Hela and C2C12 cells was diminished by treatment with the CaMKK inhibitor STO-609, indicating that nootkatone-induced activation of AMPK...
was mediated not only by LKB1, but also by the CaMKK signaling pathway.

AMPK is activated by cellular stress/stimuli, such as reactive oxygen species (ROS and RNS), as well as by events that increase the AMP/ATP ratio (3,11,17,26,27,38). AMPK activation by statins, a class of hypolipidemic drugs, and metformin, an anti-diabetic drug, is mediated by RNS (3,38).

Epigallocatechin gallate, which is abundant in green tea, activates AMPK in a ROS-mediated manner (11,22). In contrast to these compounds, however, nootkatone-induced activation of the AMPK pathway is likely due to a modification of the cellular energy status rather than ROS or RNS-mediated signals, because the cellular AMP/ATP ratio was increased in nootkatone-treated cells and the pretreatment with a catalase, SOD, or a nitric oxide synthase inhibitor, L-NAME, had no effect.

To further clarify the effects of nootkatone, we next investigated the in vivo effects. A single oral administration of nootkatone induced LKB1, AMPKα, and ACC phosphorylation in the gastrocnemius muscle and liver in mice. Nootkatone also increased AMPKα1 and AMPKα2 activity. These findings indicate that nootkatone functions as a potent activator of AMPK in vivo as well as in vitro.

Nootkatone markedly suppressed the high-fat and high-sucrose diet-induced development of obesity. Energy intake in nootkatone-fed mice tended to be decreased; however, it was difficult to determine whether the decrease in energy intake was a cause of the reduced body fat accumulation.

Body weight in nootkatone-fed mice was significantly decreased in as little as 6 weeks after feeding the diet supplemented with nootkatone, with no significant decrease in energy intake, indicating that the anti-obesity effect of nootkatone could not be attributed solely to the decrease in energy intake. Overall findings in this study suggest that activation of AMPK and subsequent induction of PGC-1α, which
possibly enhances oxidative energy metabolism, and the stimulation of energy expenditure is an underlying mechanism of the anti-obesity effects of nootkatone. In addition, although toxicologic evaluation is needed, there were no significant changes in glutamic-oxaloacetic transaminase and glutamic-pyruvic transaminase, markers of liver, heart, and skeletal muscle injury; therefore, anti-obesity effects of nootkatone do not seem to be due to its toxicity. The high-fat diet-induced increase in plasma insulin and leptin levels was attenuated by nootkatone. On the other hand, although there was no significant difference in plasma adiponectin levels between the high-fat diet and low-fat diet groups, the addition of nootkatone to the diet significantly decreased plasma adiponectin levels. The decrease in adiponectin might be due to the lower body fat mass in nootkatone-fed groups, because fat mass was significantly smaller than in the control low-fat and high-fat diet groups and relative adiponectin levels normalized to total fat mass was rather increased in nootkatone-fed groups (data not shown).

It is reasonable to expect that the stimulation of energy metabolism is associated with the anti-obesity effect. On the other hand, our results showed a close link between AMPK-associated stimulation of energy metabolism and endurance capacity. Skeletal muscle mainly utilizes fatty acids and glucose as sources of energy during exercise (14,15); however, the amount of glycogen as a form of glucose storage is limited. Therefore, preferential usage of fatty acids as an energy source by enhancing oxidative energy metabolism in the muscle is considered to lead to glycogen sparing and an increase in endurance. Nootkatone treatment of C2C12 cells upregulated the PGC-1α and COX-IV mRNA expression concomitant with AMPK activation, and feeding mice nootkatone increased PGC-1α
protein in soleus muscle. Considering the crucial role of AMPK and PGC-1α in mitochondrial biogenesis (25,37), chronic feeding of nootkatone may stimulate mitochondrial biogenesis and energy metabolism by inducing PGC-1α through AMPK activation. Our previous comparative study using high-endurance and low-endurance mice showed that muscle energy metabolism makes a crucial contribution to endurance capacity (7). In addition, muscle-specific overexpression of PGC-1α improves exercise performance, accompanied by enhanced mitochondrial function and oxidative enzymes (1). Based on these findings, activation of muscular AMPK and subsequent induction of PGC-1α by nootkatone not only decreases obesity, but also improves endurance. On the other hand, Wang et al. reported the important role of PPARδ in endurance capacity, where they showed that overexpression of PPARδ in skeletal muscle led to a greater endurance capacity together with the upregulation of mitochondrial biogenesis and oxidative enzymes (30). Further, PPARs play an important role in the transcriptional regulation of lipid metabolism-related molecules in the liver and skeletal muscle (16). Because nootkatone did not affect PPAR-dependent reporter activity, however, it is unlikely that the effects of nootkatone are mediated directly by PPAR.

In summary, nootkatone, a component of grapefruit, is a naturally occurring AMPK activator. Nootkatone induced the phosphorylation of AMPK and ACC, and enhanced AMPK activities in vitro and in vivo. The AMPK-activating effect of nootkatone is likely to be mediated by LKB1 and CaMKK, and was induced, at least in part, by changes in the cellular energy status as reflected by an increase in the AMP/ATP ratio. Administration of nootkatone was beneficial toward the prevention of obesity and hyperglycemia, as well as for improving endurance capacity. These results support the idea that AMPK
activators are useful as exercise mimetics or exercise-supporting drugs in treating obesity, and for improving physical performance. The administration of nootkatone is a potential candidate treatment for metabolic syndrome and may be a novel, useful tool for studying the role of AMPK.

Acknowledgments. We thank Atsuko Otsuka, Keiko Kawasaki, Yumiko Komine, and Eri Shimizu for experimental support.


22. Murase T, Misawa K, Haramizu S, and Hase T. Catechin-induced activation of the


37. Zong H, Ren JM, Young LH, Pypaert M, Mu J, Birnbaum MJ, and Shulman GI. AMP kinase

Figure Legends

Figure 1. Nootkatone activates AMPK in C2C12 and Hepa 1-6 cells.

(A) Structure of nootkatone. (B) C2C12 cells and Hepa 1-6 cells were treated with the indicated concentrations of nootkatone for 30 min. Phosphorylation of AMPKα, ACC, and LKB1 was determined by Western blotting using anti-phospho-AMPKα-, anti-phospho-ACC-, and anti-phospho-LKB1-specific antibodies. The corresponding non-phosphorylated form of each molecule was used as the control. A representative result from 3 independent experiments is shown. (C) C2C12 cells treated with nootkatone for 30 min were homogenized in ice-cold lysis buffer and immunoprecipitation was performed using anti-AMPKα1 or anti-AMPKα2 antibodies. AMPK activity was measured using SAMS peptide as the substrate. Enzyme activity is expressed as a percentage, taking the mean levels of the controls as 100%. Values are the means ± SE (n=5). *P<0.05, vs. control (Dunnett’s test).

Figure 2. Nootkatone stimulates the phosphorylation of AMPK, ACC, and LKB1 in the muscle and liver.

C57BL/6J mice were given nootkatone (200 mg/kg BW) orally as a 2% solution. At the indicated time points, mice were anesthetized and gastrocnemius muscles and livers were quickly dissected. Tissues were homogenized in ice-cold homogenization buffer, followed by centrifugation. The supernatants were removed and equal amounts of protein were analyzed by SDS-PAGE and blotted with specific antibodies. The non-phosphorylated form of each molecule was used as the control. (A) gastrocnemius muscle, (B) liver. A representative result from 3 independent experiments is shown.
Figure 3. Nootkatone increases AMPK activity in the muscle and liver.

C57BL/6J mice were given nootkatone (200 mg/kg BW) orally in 2% solution. At the indicated time points, mice were anesthetized and the gastrocnemius muscles and livers were quickly dissected. Tissues were homogenized in ice-cold homogenization buffer, followed by centrifugation. The supernatants were removed and immunoprecipitation was performed using anti-AMPKα1 or anti-AMPKα2 antibodies. AMPK activity was measured using SAMS peptide as the substrate. Enzyme activity is expressed as a percentage, taking the mean levels of the controls as 100%. (A) gastrocnemius muscle, (B) soleus muscle, (C) liver. Values are the means ± SE (n=5). *P<0.05, vs. 0 min (Dunnett’s test).

Figure 4. Role of LKB1 and CaMKK in nootkatone-induced AMPK activation and subsequent PGC-1α induction.

(A) C2C12 cells were preincubated with the CaMKK inhibitor STO-609 (10 μg/ml) for 60 min and subsequently incubated with 100 μM nootkatone for 30 min. Cell lysates were then prepared and subjected to Western blot analysis. A representative result from 2 independent experiments is shown. (B) Hela cells were treated with the indicated concentrations of nootkatone for 30 min and subjected to Western blot analysis. (C) Hela cells were preincubated with STO-609 (10 μg/ml) for 60 min and subsequently incubated with 100 μM nootkatone for 30 min, and then subjected to Western blot analysis. (D) C2C12 cells were preincubated with a LKB1 inhibitor Compound C (20 μM) and/or STO-609 (10 μg/ml) for 60 min and subsequently incubated with nootkatone (100 μM) for 6 h. Total RNA was subjected to real-time RT-PCR and the amount of each mRNA was normalized to 36B4
mRNA levels. Data are expressed as a percentage of the corresponding amount in the control group. Values are the means ± SE (n=3). *P<0.05, vs control (Dunnett’s test). (E) Soleus muscle from C57BL/6J mice fed high-fat +0.3% nootkatone diet for 18 weeks was homogenized and equal amounts of tissue lysates were then subjected to Western blot analysis. The amount of PGC-1α protein was normalized to that of α-tubulin and is expressed as a percentage of the corresponding amount in the high-fat control group. Values are the means ± SE (n=9). *P<0.05, vs high-fat control group (t-test).

Figure 5. Role of ROS in nootkatone-induced AMPK activation.

C2C12 cells were preincubated with a membrane-permeable catalase (500 U/ml), SOD (5 U/ml), or L-NAME (5 mM) for 30 min and subsequently incubated with the indicated concentrations of nootkatone for 30 min. Cell lysates were then prepared and subjected to Western blot analysis. A representative result from 2 independent experiments is shown.

Figure 6. Effect of nootkatone on whole-body energy metabolism.

C57BL/6J mice were deprived of food for 3 h, and then nootkatone (200 mg/kg BW) or saline was orally administered using a crossover treatment paradigm with a 7-day washout period between treatments. After administration, mice were transferred to a chamber, and oxygen consumption (A) and respiratory quotient (B) were measured for 180 min. Values are the means ± SE of 8 mice. Statistical analyses were performed using two-way repeated measures ANOVA.

Figure 7. Effect of nootkatone on fatty acid β-oxidation activity in the muscle and liver.

Gastrocnemius muscles or livers of mice orally administered nootkatone for 10 days were homogenized, and [1-14C]-palmitic acid oxidation activity was measured as described in the Materials
and Methods. Enzyme activities in gastrocnemius muscle (A) and liver (B) are expressed as a percentage, taking the mean levels of the controls as 100%. Values are the mean ± SE of 8 mice. *P<0.05, vs. control (Dunnett’s test).

Figure 8. Effect of nootkatone on the development of obesity, cumulative energy intake, and endurance capacity.

C57BL/6J mice were fed the indicated diets for 18 weeks. (A) Mean body weight of 10 mice at each time point. (B) Food intake was measured on a per-cage basis throughout the study and is represented as cumulative energy intake. (C) Swimming time to fatigue was measured at a flow rate of 7 l/min. Values are the mean swimming time of 15 to 16 BALB/c mice. Bars represent the SE. *P<0.05, vs control (t-test). LF, Low fat; HF, High fat.
Table 1. Nootkatone increases the cellular AMP/ATP ratio.

<table>
<thead>
<tr>
<th>Nootkatone</th>
<th>AMP</th>
<th>ADP</th>
<th>ATP</th>
<th>AMP/ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>0μM</td>
<td>0.65 ± 0.03</td>
<td>2.41 ± 0.53</td>
<td>106.12 ± 6.89</td>
<td>0.0062 ± 0.0005</td>
</tr>
<tr>
<td>100μM</td>
<td>0.90 ± 0.04*</td>
<td>4.17 ± 0.82</td>
<td>102.71 ± 9.70</td>
<td>0.0092 ± 0.0010*</td>
</tr>
</tbody>
</table>

C2C12 cells were incubated with nootkatone for 30 min. AMP and ATP concentrations were determined by CE-TOFMS and are presented as the AMP/ATP ratio. Values are the means ± SE (n=5).

*P<0.05 vs control (t-test).
Table 2. Effects of nootkatone on body weight, body fat accumulation, and serum components in C57BL/6J mice.

<table>
<thead>
<tr>
<th></th>
<th>Low fat</th>
<th>High fat</th>
<th>0.1</th>
<th>0.2</th>
<th>0.3</th>
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<tbody>
<tr>
<td>Initial body weight (g)</td>
<td>22.8 ± 1.3</td>
<td>23.0 ± 1.0</td>
<td>22.6 ± 0.7</td>
<td>22.7 ± 0.9</td>
<td>22.4 ± 1.2</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>35.8 ± 2.8*</td>
<td>43.2 ± 3.7</td>
<td>36.8 ± 3.9*</td>
<td>36.4 ± 2.7*</td>
<td>33.0 ± 2.6*</td>
</tr>
<tr>
<td>Total food intake (kcal)</td>
<td>1576</td>
<td>1711</td>
<td>1553</td>
<td>1602</td>
<td>1619</td>
</tr>
<tr>
<td>Feed efficiency (g/kcal)</td>
<td>0.00829</td>
<td>0.0118</td>
<td>0.00903</td>
<td>0.00856</td>
<td>0.00657</td>
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<tr>
<td>Liver weight (g)</td>
<td>1.40 ± 0.06*</td>
<td>1.86 ± 0.13</td>
<td>1.60 ± 0.06</td>
<td>1.66 ± 0.05</td>
<td>1.54 ± 0.04*</td>
</tr>
<tr>
<td>Relative liver weight (g/BW)</td>
<td>0.039 ± 0.001</td>
<td>0.043 ± 0.003</td>
<td>0.044 ± 0.001</td>
<td>0.046 ± 0.001</td>
<td>0.047 ± 0.001</td>
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<td>Epididymal fat (g)</td>
<td>1.23 ± 0.13*</td>
<td>1.85 ± 0.08</td>
<td>1.74 ± 0.12</td>
<td>1.49 ± 0.16</td>
<td>0.81 ± 0.13*</td>
</tr>
<tr>
<td>Retroperitoneal fat (g)</td>
<td>0.36 ± 0.04*</td>
<td>0.60 ± 0.03</td>
<td>0.49 ± 0.03</td>
<td>0.41 ± 0.04*</td>
<td>0.23 ± 0.05*</td>
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<tr>
<td>Perirenal fat (g)</td>
<td>0.20 ± 0.02*</td>
<td>0.42 ± 0.04</td>
<td>0.27 ± 0.02*</td>
<td>0.22 ± 0.02*</td>
<td>0.12 ± 0.02*</td>
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<tr>
<td>Total fat (g)</td>
<td>1.79 ± 0.18*</td>
<td>2.90 ± 0.09</td>
<td>2.49 ± 0.17</td>
<td>2.11 ± 0.22*</td>
<td>1.16 ± 0.20*</td>
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<tr>
<td>Triglyceride (mg/dl)</td>
<td>85.4 ± 4.7</td>
<td>78.3 ± 3.7</td>
<td>55.9 ± 4.5*</td>
<td>46.5 ± 3.3*</td>
<td>44.5 ± 2.8*</td>
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<tr>
<td>Total cholesterol (mg/dl)</td>
<td>114.2 ± 10.1*</td>
<td>171.1 ± 7.2</td>
<td>140.8 ± 4.5*</td>
<td>136.4 ± 3.2*</td>
<td>115.8 ± 9.0*</td>
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<tr>
<td>NEFA (mEq/l)</td>
<td>1.19 ± 0.08*</td>
<td>0.97 ± 0.06</td>
<td>0.87 ± 0.04</td>
<td>0.89 ± 0.06</td>
<td>0.81 ± 0.04</td>
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<tr>
<td>Glucose (mg/dl)</td>
<td>208.2 ± 13.2*</td>
<td>243.2 ± 5.4</td>
<td>222.4 ± 7.6</td>
<td>207.6 ± 6.7*</td>
<td>196.1 ± 12.7*</td>
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<tr>
<td>Insulin (ng/ml)</td>
<td>2.51 ± 0.60*</td>
<td>4.70 ± 0.77</td>
<td>2.56 ± 0.33*</td>
<td>2.19 ± 0.27*</td>
<td>1.21 ± 0.19*</td>
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<tr>
<td>Leptin (ng/ml)</td>
<td>19.8 ± 3.0*</td>
<td>34.9 ± 2.3</td>
<td>25.5 ± 3.0</td>
<td>20.4 ± 2.9**</td>
<td>7.5 ± 2.3*</td>
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<tr>
<td>Adiponectin (μg/ml)</td>
<td>4.22 ± 0.17</td>
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<td>4.51 ± 0.25</td>
<td>3.57 ± 0.18*</td>
<td>2.73 ± 0.16*</td>
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<td>GPT (Karmen U)</td>
<td>16.4 ± 0.8</td>
<td>19.6 ± 3.8</td>
<td>16.8 ± 1.8</td>
<td>20.8 ± 4.8</td>
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<td>GOT (Karmen U)</td>
<td>57.1 ± 7.5</td>
<td>47.1 ± 2.2</td>
<td>45.3 ± 5.5</td>
<td>46.3 ± 3.9</td>
<td>51.0 ± 2.7</td>
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NEFA, non-esterified fatty acids; GPT, glutamic pyruvate transaminase; GOT, glutamic oxaloacetate transaminase

Food intake was measured every 2–3 days on a per-cage basis (5 mice per cage) throughout the study and the energy value is represented as kcal consumed during the experimental period per mouse.
Feed efficiency was calculated as follows: \[ \text{body weight gain per cage (g)} / \text{[kcal of food consumed per cage]} \]. # Total fat: sum of epididymal, retroperitoneal, and perirenal fat. Values are the mean ± SE of 10 mice. * \( P < 0.05 \), vs the High-fat group (Dunnett’s test).
Figure 1

A

B

C2C12 cells

Hepa 1-6 cells

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Figure 1
Figure 2

A  Gastrocnemius muscle

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B  Liver

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</table>
A

Gastronemius muscle

AMPK activity (% of control)

Time (min)

B

Soleus muscle

AMPK activity (% of control)

Time (min)

C

Liver

AMPK activity (% of control)

Time (min)

Figure 3
Figure 4
Figure 5

- - - + + - - - + - -
- - - - + + - - + -
- - - - - - + + - +

p-AMPKα
β-actin
Nootkatone (µM)
PEG-CAT
SOD
L-NAME

0 30 100 30 100 30 100 30 100 0 0
A

Oxygen consumption (ml/kgBW/min)

P<0.05

Cont
Nootkatone

Time (min)

B

Respiratory quotient

Cont
Nootkatone

Time (min)

Figure 6
Figure 7
Figure 8