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

Takatoshi Murase, Koichi Misawa, Satoshi Haramizu, Yoshihiko Minegishi, and Tadashi Hase

Biological Science Laboratories, Kao Corporation, Tochigi, Japan

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Nootkatone, a characteristic constituent of grapefruit, stimulates energy metabolism and prevents diet-induced obesity by activating AMPK. Nootkatone induced an increase in AMPK tone, a constituent of grapefruit, as a naturally occurring AMPK metabolic syndrome. Here, we identified and characterized nootkatone, a constituent of grapefruit, as a naturally occurring AMPK

Address for reprint requests and other correspondence: T. Murase, Biological Science Laboratories, Kao Corporation, Tochigi, Japan (e-mail: murase.takatoshi@kao.co.jp).
( 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 nootkatoine (>98%; Alfa Aesar, Ward Hill, MA) for the indicated period.

Western blot analysis. Cells were treated with nootkatoine, and the lysates were prepared as described previously (22). The livers and muscles were homogenized in ice-cold lysis buffer with a motor-driven pestle in a microcentrifuge tube. The homogenates were kept on ice for 30 min and then centrifuged at 16,000 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, Beverly, 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).

RT-PCR. 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 Power SYBR Green Master Mix (Applied Biosystems). The primers used in this study were (where F, forward; R, reverse): PGC-1α F: ATGTTGTCGCCTTCTGTCT, PGC-1α R: ATCTAGCTCTGGG-GACCTT; cytochrome c oxidase (COX)-IV F: AGCTTAGGCAAGCAGAGAAG, COX-IV R: AATCACCAGAGCCGTGAATC; 36B4 F: GACCTT...
variation in energy metabolism, oxygen consumption (VO2) 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 body wt) 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 VO2 and carbon dioxide production (VCO2) were monitored for 3 h. RQ was defined as the ratio of the VO2-to-VCO2 values. Locomotor activity was measured in an automated motion analysis system (Actracer-2000, ARCO System), which detects the amount of centroid fluctuation using a weighted transducer.

Evaluation of antiobesity effect. Six-week-old male C57BL/6J mice were randomly divided into five 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% (wt/wt) plant oil (Summit Oil Mill, Chiba, Japan), 20% casein (Oriental Yeast, Tokyo, Japan), 66.5% potato starch (Oriental), mineral mixture (Oriental), and a vitamin mixture (Oriental); 2) a high-fat diet, containing 25% (wt/wt) 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, consisting of the high-fat diet supplemented with 0.1 to 0.3% (wt/wt) nootkatone. Mice were maintained on these diets for 18 wk. Body weight was measured weekly throughout the study. Food intake was measured every 2–3 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 snap on week 17 of the experiment. Plasma triglyceride, total cholesterol, nonesterified 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-wk-old male BALB/c mice (Charles River, Kanagawa, Japan) were divided into two groups (n = 16) according to their initial swimming endurance capacity. In preliminary training sessions, mice were accustomed to swimming for 30 min three 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% (wt/wt) 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 wk. During that 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 wk for the 10-wk experimental period.

Statistical analysis. All values are presented as 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 Insti-

**RESULTS**

Nootkatone activates AMPK in C2C12 and Hepa 1–6 cells. The structure of nootkatone is shown in Fig. 1A. Because phosphorylation of AMPK-Thr172 is essential for AMPK activation, the activating effect of nootkatone was assessed by determining AMPKα phosphorylation using an anti-phospho-AMPKα antibody. Nootkatone increased the phosphorylation of AMPKα 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-Ser428, 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 by using SAMS peptide as a substrate. Nootkatone induced a concentration-dependent increase in AMPKα1 and AMPKα2 activity (Fig. 1C). These findings indicate that nootkatone induces phosphorylation of both AMPKα1 and AMPKα2, 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 AMPKα, 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 body wt) increased AMPKα 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 AMPKα1 and AMPKα2 activity, which peaked at 60 min in the gastrocnemius muscle (Fig. 3A). In the soleus muscle, AMPKα1 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 Ca2+/calmodulin-dependent protein kinase kinase. Ca2+/calmodulin-dependent protein kinase kinase (CaM KK) also mediates AMPK activation (2, 33); therefore, we examined the involvement of CaM KK in nootkatone-induced AMPK or ACC phosphorylation. Pretreatment of C2C12 cells with the CaM KK 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...
Furthermore, 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-wk 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-NAME, nitroarginine methyl ester (l-NNAME) a nitric oxide synthase inhibitor, did not alter AMPK phosphorylation (Fig. 5), suggesting that ROS and 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 VO2 and the RQ. A single dose of nootkatone significantly enhanced VO2 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-oxidation activity, assessed by the degradation of [14C]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 by use of the luciferase assay system. Although Wy-14643, a PPARα agonist, and GW-501516, a PPARδ agonist, significantly enhanced PPAR-dependent luciferase activity, nootkatone did not stimulate luciferase activity (Fig. 8).

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**Fig. 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 nonphosphorylated 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-AMPK1 or anti-AMPK2 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 means ± SE (n = 5). *P < 0.05 vs. control (Dunnett’s test).
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 wk 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% (wt/wt) 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 and Table 2). The body weight of mice fed 0.1, 0.2, or 0.3% nootkatone was significantly lower after 5, 6, and 1 wk 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. Liver weight in the high-fat control group was significantly greater, and the increase was

<table>
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<th>Value 2</th>
</tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>AMPKα</td>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-ACC</td>
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<tr>
<td>LKB1</td>
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</table>

**Fig. 2.** Nootkatone stimulates phosphorylation of AMPK, ACC, and LKB1 in muscle and liver. C57BL/6J mice were given nootkatone (200 mg/kg body wt) orally as a 2% solution. At 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. Supernatants were removed, and equal amounts of protein were analyzed by SDS-PAGE and blotted with specific antibodies. The nonphosphorylated form of each molecule was used as the control. A: gastrocnemius muscle. B: liver. A representative result from 3 independent experiments is shown.

**Fig. 3.** Nootkatone increases AMPK activity in muscle and liver. C57BL/6J mice were given nootkatone [200 mg/kg BW (body wt)] orally in 2% solution. At 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. Supernatants were removed, and immunoprecipitation was performed using anti-AMPKα1 or anti-AMPKα2 antibodies. AMPK activity was measured using SAMS peptide as 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 means ± SE (n = 5). *P < 0.05 vs. 0 min (Dunnett’s test).
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). The swimming times to exhaustion at the 10th wk are shown in Fig. 8C. Compared with control mice, mice fed 0.2% nootkatone had a 21% longer

<table>
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<tr>
<th>Nootkatone</th>
<th>AMP</th>
<th>ADP</th>
<th>ATP</th>
<th>AMP/ATP</th>
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<tr>
<td>0 μM</td>
<td>0.65 ± 0.03</td>
<td>2.41 ± 0.53</td>
<td>106.12 ± 6.89</td>
<td>0.0962 ± 0.0005</td>
</tr>
<tr>
<td>100 μM</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>

Values are means ± SE (n = 5). C2C12 cells were incubated with nootkatone for 30 min. AMP and ATP concentrations were determined by CE-TOFMS and are presented as AMP/ATP ratio. *P < 0.05 vs. control (t-test).

Fig. 4. Role of LKB1 and CaMKK in nootkatone-induced AMPK activation and subsequent PGC-1α induction. A: C2C12 cells were preincubated with 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 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, incubated with 100 μM nootkatone for 30 min, and subjected to Western blot analysis. D: C2C12 cells were preincubated with 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 means ± SE (n = 3). *P < 0.05, vs control (Dunnett’s test). E: soleus muscle from C57BL/6J mice fed high-fat (HF) + 0.3% nootkatone diet for 18 wk 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 HF control group. Values are means ± SE (n = 9). *P < 0.05 vs. HF control group (t-test).
swimming time, indicating that nootkatone intake improved the endurance capacity for exercise.

DISCUSSION

The findings of the present study demonstrated that nootkatone potently activates the AMPK signaling pathway. Nootkatone stimulated the phosphorylation of AMPK-Thr172, 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-Ser428, a major upstream AMPK kinase (2, 8). LKB1 is a constitutively active kinase; the physiological relevance of its phosphorylation, however, remains controversial (6). Xie et al. (34) recently reported that Ser428 phosphorylation of LKB1 by PKCε regulates the nuclear transport of LKB1 into the cytosol.
and is required for metformin-enhanced AMPK activation in endothelial cells. 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 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 antidiabetic 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 the catalase SOD or the 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, AMPKε, and ACC phosphorylation in the gastrocnemius muscle and liver in mice. Nootkatone also increased AMPKε1 and AMPKε2 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 wk after feeding the diet supplemented with nootkatone, with no significant decrease in energy intake, indicating that the antiobesity 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 antiobesity effects of nootkatone. In addition, although toxicological 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, antiobesity effects of nootkatone do not seem to be due to its
Considering the crucial role of AMPK and PGC-1α, mice nootkatone increased PGC-1α expression concomitant with AMPK activation, and feeding addition, muscle-specific overexpression of PGC-1α makes a crucial contribution to endurance capacity (7). In our previous comparative study using high-endurance and metabolism by inducing PGC-1α proves exercise performance, accompanied by enhanced metabolism in the muscle is considered to lead to glycogen acids as an energy source by enhancing oxidative energy capacity. Skeletal muscle mainly utilizes fatty acids and other hand, our results showed a close link between AMPK-activity and insulin resistance. The high-fat diet-induced increase in plasma insulin and leptin levels were attenuated by nootkatone. On the other hand, although there was no significant difference in plasma adiponectin levels between the high-fat and low-fat diet groups, and relative adiponectin levels normalized to body weight were rather increased in nootkatone-fed diet groups, and relative adiponectin levels normalized to body weight were rather increased in nootkatone-fed groups (data not shown).

It is reasonable to expect that the stimulation of energy metabolism is associated with the antiobesity 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 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). On the basis of 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. (30) 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. Furthermore, 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.

Table 2. Effects of nootkatone on body weight, body fat accumulation, and serum components in C57BL/6J mice

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<th>Low Fat</th>
<th>High Fat</th>
<th>High Fat + Nootakone (%)</th>
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<tr>
<td></td>
<td>0.1</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>Initial body weight, g</td>
<td>22.8 ± 1.3</td>
<td>23.0 ± 1.0</td>
<td>22.6 ± 0.7 (22.7 ± 0.9)</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* (36.4 ± 2.7*</td>
</tr>
<tr>
<td>Total food intake, kcal</td>
<td>1576</td>
<td>1711</td>
<td>1553 (1602)</td>
</tr>
<tr>
<td>Feed efficiency, kcal/g</td>
<td>0.00829</td>
<td>0.0118</td>
<td>0.00903 (0.00856)</td>
</tr>
<tr>
<td>Liver weight, g</td>
<td>1.40 ± 0.06*</td>
<td>1.86 ± 0.13</td>
<td>1.60 ± 0.06 (1.66 ± 0.05)</td>
</tr>
<tr>
<td>Relative liver weight, g/BW</td>
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<td>0.043 ± 0.003</td>
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<tr>
<td>Epididymal fat, g</td>
<td>1.23 ± 0.13*</td>
<td>1.85 ± 0.08</td>
<td>1.74 ± 0.12 (1.49 ± 0.16)</td>
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<td>Retroperitoneal fat, g</td>
<td>0.36 ± 0.04*</td>
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<td>Perirenal fat, g</td>
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<td>Total fat, g</td>
<td>1.79 ± 0.18*</td>
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<td>Triglyceride, mg/dl</td>
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<td>78.3 ± 3.7</td>
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<td>Total cholesterol, mg/dl</td>
<td>114.2 ± 10.1*</td>
<td>171.1 ± 7.2</td>
<td>140.8 ± 4.5* (136.4 ± 3.2*)</td>
</tr>
<tr>
<td>NEFA, mEq/l</td>
<td>1.19 ± 0.08*</td>
<td>0.97 ± 0.06</td>
<td>0.87 ± 0.04 (0.89 ± 0.06)</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>208.2 ± 13.2*</td>
<td>243.2 ± 5.4</td>
<td>222.4 ± 7.6 (207.6 ± 6.7*)</td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>2.51 ± 0.60*</td>
<td>4.70 ± 0.77</td>
<td>2.56 ± 0.33* (2.19 ± 0.27*)</td>
</tr>
<tr>
<td>Leptin, ng/ml</td>
<td>19.8 ± 3.0*</td>
<td>34.9 ± 2.3</td>
<td>25.5 ± 3.0 (20.4 ± 2.9**)</td>
</tr>
<tr>
<td>Adiponectin, μg/ml</td>
<td>4.22 ± 0.17</td>
<td>4.46 ± 0.26</td>
<td>4.51 ± 0.25 (3.57 ± 0.18*)</td>
</tr>
<tr>
<td>GPT, Karmen U</td>
<td>16.4 ± 0.8</td>
<td>19.6 ± 3.8</td>
<td>16.8 ± 1.8 (20.8 ± 4.8)</td>
</tr>
<tr>
<td>GOT, Karmen U</td>
<td>57.1 ± 7.5</td>
<td>47.1 ± 2.2</td>
<td>45.3 ± 5.5 (46.3 ± 3.9)</td>
</tr>
</tbody>
</table>

Values are means ± SE of 10 mice. NEFA, nonesterified 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 energy value is represented as kcal consumed during the experimental period per mouse. Feed efficiency was calculated as follows: [body weight gain per cage (g)] / [kcal of food consumed per cage]. #Total fat: sum of epididymal, retroperitoneal, and perirenal fat. *P < 0.05 vs. high-fat group (Dunnett’s test).
REFERENCES


