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Citrus flavonoid, naringenin, increases locomotor activity and reduces diacylglycerol accumulation in skeletal muscle of obese ovariectomized mice

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Abstract

Scope—Estrogen deficiency has been associated with central obesity, muscle loss, and metabolic syndrome in postmenopausal women. This study assessed naringenin accumulation in tissues and investigated the hypothesis that naringenin reverses diet-induced metabolic disturbances in obese ovariectomized mice.

Methods and results—In study 1, we measured naringenin concentrations in plasma, liver, perigonadal and subcutaneous adipose tissues, and muscle of ovariectomized C57BL/6J female mice after 11 weeks of naringenin supplementation. Naringenin accumulated 5–12 times more in mice fed a 3% naringenin diet than in mice fed a 1% naringenin diet. In study 2, ovariectomized mice were fed a high-fat diet (60 kcal% fat) for 11 weeks and half of the mice were then supplemented with 3% naringenin for another 11 weeks. Dietary naringenin suppressed weight gain, lowered hyperglycemia, and decreased intra-abdominal adiposity evaluated by magnetic resonance imaging. Naringenin-fed mice exhibited elevated locomotor activity monitored by infrared beam breaks, maintained muscle mass, and reduced muscle diacylglycerol content. Real-time PCR analysis in muscle revealed decreased mRNA level for genes involved in de novo lipogenesis, lipolysis, and triglyceride synthesis/storage.

Conclusions—Long-term 3% naringenin supplementation resulted in significant naringenin accumulation in plasma and tissues, associated with attenuated metabolic dysregulation and muscle loss in obese ovariectomized mice.

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Keywords

Naringenin; Ovariectomy; Locomotor Activity; Muscle; Diacylglycerol

1 Introduction

Naringenin, a dietary flavonoid, displays a diverse range of biological functions, such as anti-carcinogenic, anti-inflammatory, and anti-atherogenic activities [1, 2]. Naringenin is abundant in citrus fruits and tomatoes [3, 4] and its bioavailability has been studied in human and murine models. Naringenin accumulated significantly in plasma [5, 6] and its metabolites were detected in liver, kidney, spleen, heart, and brain of rats fed naringenin [6, 7]. In human studies, naringenin readily accumulates in plasma after consumption of orange juice, grapefruit juice [8], and tomato paste [9]. Therefore, naringenin appears to be readily bioavailable from the diet, suggesting that beneficial health effects could be attributed to this flavonoid in individuals who consume naringenin food sources regularly.

Although naringenin accumulation in tissues was measured in rodents after short-term oral administration of naringenin, to our knowledge, few studies have examined naringenin concentrations after long-term feeding in tissues that heavily participate in the regulation of metabolic homeostasis, including liver, skeletal muscle, and adipose tissues. Thus, we assessed the accumulation of naringenin in mice supplemented with 1% or 3% dietary naringenin for 11 weeks.

Additionally, reports from the Centers for Disease Control and Prevention showed that the prevalence of obesity in women aged 60 years and older increased from 31% in 2003–2004 to 38% in 2011–2012 [10]. Eighty-five percent of women undergo menopause by age 55 and the loss of ovarian function at menopause has been associated with weight gain, central obesity, muscle loss and metabolic syndrome [11–13]. Therefore, weight management and health maintenance in postmenopausal women is a growing field in medical practice and scientific investigation. Naringenin appears to be a promising candidate for mitigating adverse metabolic changes in postmenopausal women. We recently showed that naringenin attenuated estrogen deficiency related to metabolic disturbances, including high blood glucose, body fat accumulation, and liver steatosis, in mice fed a normal-fat diet (10% calories from fat) [14]. Additionally, several studies demonstrated that dietary supplementation of naringenin to high-fat diets suppressed diet-induced obesity and attenuated metabolic disturbances in male rodents [15–17]. However, most of the studies supplemented high-fat diets with naringenin in the beginning of the study, when the mice were lean and healthy. Few studies have examined whether naringenin reverses diet-induced metabolic disturbances when mice are already obese, a scenario more reflective of postmenopausal status. Therefore, in the present study, we fed ovariectomized mice, a mouse model mimicking postmenopausal women, a very high-fat diet (60% calories from fat) to induce obesity followed by naringenin supplementation in order to examine if naringenin exerts similar beneficial effects on metabolism in obese ovariectomized mice.

2 Materials and methods

2.1 Animals and diets

C57BL/6J mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA) and acclimated to the new environment for 1–2 weeks. Mice were housed 5 per cage at 22 ± 0.5°C on a 12:12-hour light-dark cycle. Body weight and food intake were measured daily. All procedures were in accordance with institution guidelines and approved by the Institutional Animal Care and Use Committee at The Ohio State University.

2.2 Study 1: Naringenin accumulation

Five-week old female C57BL/6J mice, ovariectomized at 4 weeks of age, were fed a semi-purified high-fat diet (HFD, D12451, 45 % calories from fat, Research Diets Inc. New Brunswick, NJ, USA) for 6 weeks to generate high-fat diet induced obesity. Then mice were randomized by weight to one of three diet groups: CON group, continued on the high-fat diet, 1% NAR group, switched to the high-fat diet supplemented with 1 wt/wt % naringenin, and 3% NAR group, switched to the high-fat diet supplemented with 3 wt/wt % naringenin. Naringenin powder used in the diets was purchased through Sigma (W530098, St. Louis, MO, USA) and was custom prepared by Research Diets Inc. The dose of naringenin is based on a previously published study in C57BL/6J male mice showing amelioration of metabolic disturbances in mice fed a high-fat diet with 1% and 3% naringenin [15]. After 11 weeks of experimental diets, mice were fasted for 5 hours, anesthetized with isoflurane for blood collection via cardiac puncture, and then euthanized by cervical dislocation. Blood was collected in EDTA-coated collection tubes and plasma was obtained after centrifugation at 1,500 g for 20 min at 4 °C. Plasma (~100 μl) for total naringenin accumulation was transferred to another tube with 10% vol/vol of glacial acetic acid and stored at −80 °C. Tissues (~100 mg) for total naringenin accumulation, including subcutaneous adipose tissues (SCAT, thoracic and abdominal mammary fat pads), liver, perigonadal fat (PGAT), and quadriceps skeletal muscle, were excised, weighed, placed in a tube with 10% vol/wt of glacial acetic acid, frozen in liquid nitrogen, and stored at −80 °C.

2.3 Tissue sample extraction for total naringenin analysis

About 100 mg of tissue samples (4–5 mice / dietary group) were probe sonicated with 400μL water and 800μL of acetonitrile to precipitate proteins before centrifugation. Pellets were re-suspended in 1.2 mL 2:1 volume/ volume acetonitrile: water and probe sonicated again and then centrifuged. For plasma, 2 volume of acetonitrile were added to plasma for the first extraction and repeated as described above. The supernatants were pooled and dried by SpeedVac. Residues were resuspended in 1mL of 2M acetate buffer, pH 5.5, and glucuronidase/sulfatase was added to deconjugate naringenin metabolites for 2hr. Naringenin aglycone was extracted twice with 3 volumes diethyl ether and dried under nitrogen. Residues were redissolved in 300μL methanol for HPLC-MS/MS analysis.

2.4 HPLC-MS/MS

Samples were injected on an Agilent 1200SL HPLC and separated with a 4.6 x 75mm, 3μm ACE C18AR column (MacMod Analytical Inc., Chadds Ford, PA) using a 0.1% formic acid

in water and 0.1% formic acid in acetonitrile mobile phase gradient. HPLC eluent was interfaced with a QTrap 5500 mass spectrometer (ABSciex, Concord, Canada) via an electrospray probe in both negative and positive polarity mode.

2.5 Study 2: The effects of naringenin on ovariectomized mice with diet-induced obesity

Because we did not observe developed mammary glands in the tissue sections stained with hematoxylin and eosin (H&E) and the CON mice stopped gaining weight after 10 weeks of the high-fat diet in study 1, we used older mice to better mimic the postmenopausal state and fed the mice with higher fat diet to induce obesity in study 2. Twenty-week old female C57BL/6J mice (n=20) mice, ovariectomized at 19 weeks of age, were fed a semi-purified very high-fat diet (VHF diet, D12492, 60% calories from fat, Research Diets Inc.) for 11 weeks to induce obesity. The mice were then randomized by weight to one of two diet groups: VHF group, continued on the high-fat diet, and VHFN group, switched to the very high-fat diet supplemented with 3% naringenin. After 11 weeks on the randomized diets, mice were fasted for 5 hours, anesthetized with isoflurane for blood collection via cardiac puncture, and then euthanized by cervical dislocation. Blood was collected into EDTA-coated tubes and plasma was obtained after centrifugation at 1,500 g for 20 min at 4 °C. Tissues, as mentioned in Study 1, were excised, weighed, snap frozen in liquid nitrogen, and stored at −80 °C until further analysis. There were 10 mice assigned to each diet group; however, the number of mice for some experiments was smaller due to limitations of indirect calorimetry cages, blood and/or tissue obtained at necropsy. Due to differences of replicates, numbers of mice used for each endpoint is indicated in detailed methods below and in Figure legends or Table footnotes.

2.6 Fasting glucose analysis

After a 5 hour-fast, glucose was measured via tail vein puncture (OneTouch Ultra blood glucose meter, LifeScan Inc., Milpitas, CA, USA) at week 0 and 5.

2.7 Plasma analysis

Plasma insulin levels were measured by ELISA (Millipore, Billerica, MA, USA) according to the manufacturer's instructions. Plasma glucose and triglycerides were assayed using a Cholestech LDX analyzer (Cholestech Corporation, Hayward, CA, USA). Total plasma cholesterol (Pointe Scientific Inc., Canton, MI, USA) was determined by enzymatic colorimetric assays.

2.8 Indirect calorimetry and locomotor activity

During week 17, 6–8 mice in each group were housed individually in metabolic chambers at 22°C, allowed free access to food and water, and acclimated for 24-hour prior to metabolic assessments. Measurements were taken for a 24-hour period, including a 12-hour light cycle and a 12-hour dark cycle. Oxygen consumption (VO_2), carbon dioxide production (VCO_2), and ambulatory activity (by infrared beam breaks) were measured every 20 minutes using a computer-controlled, open-circuit Oxymax /CLAMS System (Columbus Instruments, Columbus, OH, USA). Respiratory exchange ratio (RER) was calculated as the ratio of VCO_2 to VO_2. Heat, representing the standard measure of energy expenditure, was

calculated by the formula, Heat (kcal/hr) = (3.815 + 1.232 x RER) x (VO_2) x (1L/1000 ml x 1kg/1000g) x (body weight)

2.9 Magnetic resonance imaging (MRI)

During week 19, total, intra-abdominal, and subcutaneous adiposity were analyzed by MRI using a Bruker Biospin 94/30 magnet (Billerica, MA, USA) and a 70 mm diameter linear volume coil, as previously described [14]. T1-weighted coronal images of the whole mouse torso were collected using a respiratory-gated RARE sequence (TR/TE=1570/7.5ms, RARE factor=4, FOV=70x45 mm^2, matrix size=256x192, slice thickness=1 mm, navg=2). The whole body and abdominal masks were then used to calculate the percentage of segmented voxels in the whole body and abdomen, respectively. Subcutaneous fat was calculated by subtracting intra-abdominal fat from total fat.

2.10 Triacylglycerol and diacylglycerol analysis

Total lipids were extracted from liver and muscle samples with 2:1 (v/v) chloroform: methanol and washed with 0.88% KCL [18]. The chloroform phase was transferred to a weighed test tube and dried under nitrogen gas at room temperature. The dried sample and test tube was weighed again to calculate total extracted lipid. Following the lipid extraction, triacylglycerol and diacylglycerol were obtained using solid-phase extraction [19] and solubilized in tert-butanol, methanol, Triton X-100 [20]. Analysis was performed using enzymatic colorimetric assay [21].

2.11 RNA extraction and RT-PCR analysis of gene expression

Frozen perigonadal adipose tissue was cut on dry ice to obtain samples of a suitable size and then total RNA was extracted by using RNeasy Lipid Tissue Mini Kit (Qiagen, Valencia, CA, USA) following manufacturer's instructions. Muscle total RNA was extracted with TriZol reagent (Invitrogen, Carlsbad, CA, USA) and precipitated with the addition of a high salt solution (1.2M NaCl, 0.8 M sodium citrate). RNA was then reversed transcribed to cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Quantitative RT-PCR analysis of individual cDNA was performed with ABI Prism 7300 sequence detection system (Applied Biosystems) using TaqMan Gene Expression. Target gene expression was normalized to 18S rRNA for perigonadal adipose tissue or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for muscle tissue. Endogenous Controls (VIC probes) were amplified in the same reaction and expressed as 2^(−ddCt) compared to the VHF group [22].

2.12 Western blotting

Frozen muscle was cut on dry ice to obtain samples of a suitable size and was homogenized in lysis buffer (20 mM Trizma base, 1% Triton-X100, 50 mM NaCl, 250 mM sucrose, 50 mM NaF, and 5 mM $Na_4P_2O_7 \cdot 10H_2O$) with the Complete Mini Protease Inhibitor Cocktail (Roche Diagnostics, Indianapolis, IN, USA) and PhosSTOP Phosphatase Inhibitor Cocktail Tablets (Roche Diagnostics). Homogenates were incubated for 1 hour and centrifuged at 16,000 g for 15 minutes at 4°C. Supernatant was collected and protein concentration was determined by BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA). Protein

(40 μg/sample) was separated on polyacrylamide gels and transferred to nitrocellulose membranes using transfer buffer containing 25 mM Tris,192 mM glycine, and 10% methanol. Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline with 0.1% Tween-20 (TBST) and incubated overnight at 4°C with primary antibodies (Cell Signaling Technology, Danvers, MA, USA). After washing, membranes were incubated for 1 hour at room temperature with HRP-linked anti-rabbit IgG, (Cell Signaling Technology). Bands were visualized with chemiluminescence (SuperSignal West Femto Maximum Sensitivity Substrate, Thermo Fisher Scientific, Rockford, IL, USA) using Carestream Image Station 4000GL PRO (Carestream Health, Inc., Rochester, NY, USA). Densitometric analysis was conducted using Carestream Molecular Imaging Software. GAPDH was used as a loading control.

2.13 Statistical analysis

All data are presented as mean ± standard error (SEM) with $P<0.05$ considered significantly different. Statistical analysis was performed using SPSS version 20.0 software (SPSS, Inc., Chicago, IL, USA). One outlier, defined as a value more than 1.5 times the interquartile range, was removed from the following data sets: adipose MCP-1, muscle diacylglycerol, muscle PGC1α and muscle FASN. Significance was determined using two-tailed unpaired Student's t test, and naringenin accumulation results were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. Heat (energy expenditure) was analyzed by ANCOVA with body weight as a covariate [23]. Pearson correlation coefficient was used for correlation analyses.

3 Results

3.1 Naringenin accumulation

To examine whether increasing doses of dietary naringenin results in increased naringenin accumulation in plasma and tissues, ovariectomized mice were fed high-fat diets containing 0%, 1%, and 3% of naringenin for 11 weeks. No adverse clinical signs, sudden death, and differences in spleen and kidney weights were observed (data not shown), suggesting that 3% naringenin was well tolerated in these mice. Body weights and cumulative food intake were not different between diet groups (data not shown). Total naringenin concentrations in plasma and tissues were 5–12 times higher in mice fed a diet with 3% naringenin compared to mice fed 1% naringenin (Fig. 1). Mice fed 3% naringenin accumulated 18.03 ± 4.72 μM total naringenin in plasma compared to 1.50 ± 0.46μM in mice fed a diet with 1% naringenin. Relative accumulation of naringenin in tissues was greatest in liver (1% NAR, 7.61 ± 1.14 μmol/kg tissue and 3% NAR, 37.04 ± 8.33 μmol/kg tissue) followed by perigonadal adipose tissue (PGAT, 1% NAR, 0.85 ± 0.30 μmol/kg tissue and 3% NAR, 5.11 ± 1.26 μmol/kg tissue), and lastly subcutaneous adipose tissues (SCAT) and muscle, which had similar magnitudes of naringenin accumulation (SCAT, 1% NAR, 0.47 ± 0.19 μmol/kg tissue and 3% NAR, 3.02 ± 1.99 μmol/kg tissue; muscle, 1% NAR, 0.30 ± 0.06 μmol/kg tissue and 3% NAR, 2.17 ± 1.04 μmol/kg tissue).

3.2 Body weight and caloric intake

In the second study, mice were fed a very high-fat diet (VHF) to induce an obese phenotype. After 11 weeks on a very high-fat diet, mice gained 19 g (Fig. 2A) and exhibited dysregulated glucose metabolism as manifested by elevated blood glucose levels (178.4 ± 4.3 mg/dL compared to 135.3 ± 4.5 mg/dL at week 0). Then, half of the mice were switched to the VHF supplemented with 3% naringenin (VHFN). Mice fed naringenin gained only 0.89 ± 1.52 g in 11 weeks of treatment, compared to VHF mice, which gained 4.09 ± 0.83 g (Fig. 2A). Cumulative caloric intake was not different between groups (Fig. 2B). Feed efficiency was calculated to measure the ability of the mice to transform ingested calories into body weight. A decrease in the feed efficiency ratio was observed in VHF group from weeks 0 – 11 to weeks 12 – 22 as body weight of the VHF group reached a plateau at week 16. Nonetheless, naringenin tended to lower feed efficiency ($p=0.06$, Table 1).

3.3 Locomotor activity, energy expenditure, and respiratory exchanging ratio

Indirect calorimetry was performed to assess metabolic changes after naringenin supplementation. Compared to ambulatory activity levels of ovariectomized mice fed a normal-fat diet from our previous study [14], the activity levels were reduced by 50% in VHF mice (156 ± 13 counts vs. 76 ± 11 counts in the light phase and 428 ± 52 counts vs.192 ± 42 counts in the dark phase). In the current study, mice with naringenin supplementation (VHFN) increased activity counts about 2-fold in both light and dark phases compared to VHF mice (VHFN: 134 ± 15 vs. VHF: 76 ± 11 in the light phase and VHFN: 455 ± 40 vs. VHF: 192 ± 42 in the dark phase, Fig. 3A & 3B). There were no differences in energy expenditure between groups after adjusting for body weight (Fig. 3C). RER was around 0.7 in both groups, indicating that fat was the predominant fuel source for energy need (Fig. 3D). Interestingly, mice supplemented with naringenin had higher RER at specific time points in the light phase, suggesting a decrease in reliance on fatty acid oxidation for energy needs.

3.4 Adiposity and tissue weights

After 19 weeks of VHF diet (at the time when the MRI analysis was performed), the mice accumulated 64% of body fat and 30% of the body fat accumulated in intra-abdominal area. Dietary naringenin significantly decreased total body fat by 11% and intra-abdominal adiposity by 20% compared to VHF mice (Fig. 4A & 4B). Subcutaneous adiposity was unaffected. At the end of the study, neither subcutaneous nor perigonadal adipose tissue mass were significantly different between the groups (Table 1). Monocyte chemoattractant protein-1 (MCP-1) is a key inflammatory mediator in adipose tissues. In our previous study with ovariectomized mice fed a normal-fat diet [14], naringenin significantly down-regulated mRNA levels of MCP-1 mRNA levels in perigonadal adipose tissue. When compared with our previous ovariectomized mice fed a normal-fat diet, MCP-1 expression was 400% increase in perigonadal adipose tissue of VHF mice (data not shown). In the current study, naringenin also significantly reduced MCP-1 mRNA expression by 40% in perigonadal adipose tissue of obese ovariectomized mice (Figure 4C).

Weight loss is often associated with a decrease in lean mass [24]. Here we found maintenance of quadriceps muscle mass in naringenin-fed mice (Table 1.) and negatively

correlated with total and intra-abdominal adiposity (Total, r = −0.808, p = 0.001; intra-abdominal, r= −0.924, p<0.001). The muscle mass was significantly higher in VHFN mice compared to VHF mice after normalized to body weight muscle mass (Table 1.).

3.5 Plasma analyses and hepatic and muscle lipid content

Naringenin supplementation resulted in decreased fasting plasma glucose levels (Fig. 5A), but did not affect fasting plasma insulin levels (Fig. 5B). Naringenin supplementation also failed to attenuate plasma cholesterol and triglyceride in obese ovariectomized mice (Fig. 5C & 5D). We quantified the levels of triacylglycerol and diacylglycerol in liver and muscle. No significant difference was observed in extracted triacylglycerol and diacylglycerol levels in liver between groups (Fig. 6A & 6B). On the contrary, extracted diacylglycerol levels in muscle were significantly lower in naringenin-fed mice without changes in extracted triacylglycerol levels (Fig. 6C & 6D).

3.6 Mitochondrial biogenesis in muscle

Exercise activates signaling cascades, induces changes in gene expression, and results in mitochondrial biogenesis [25]. Since VHFN mice showed elevated locomotor activity, we measured expression of mRNA for mitochondrial biogenesis (Fig. 7A). Peroxisome proliferator-activated receptor-γ coactivator 1 alpha (PGC1α), a transcriptional coactivator, acts as a central regulator of mitochondrial biogenesis and its expression has been shown to be increased in human muscle after exercise [26]. When compared with ovariectomized mice fed a normal-fat diet [14], the RNA levels of PGC1α were 6-fold lower in the muscle of VHF mice. The levels of PGC1α transcript were about 40% greater in the muscle of VHFN mice compared to VHF mice, but did not reach significance (p=0.17). Nuclear respiratory factor 1 (NRF1) interacts with PGC1α and induces expression of transcription factors essential for proper mitochondrial DNA transcription, including mitochondrial transcription factor A (TFAM), mitochondrial transcription factor B1 (TFB1M), and mitochondrial transcription factor B2 (TFB2M) [25]. No difference was observed in mRNA levels of these genes between the groups. A previous study showed that PGC1α was up-regulated 2 hours after a single bout of exercise in human muscle but down-regulated after 24 hours, while mRNA levels of COX IV (COX4I1), a nuclear encoded protein of the mitochondrial respiratory chain, and mitochondrial fusion proteins MFN1 and MFN2 were induced until 24-hour post-exercise [27]. Therefore, we measured the transcript levels of these late-responsive genes but still found no difference between two groups of mice. We also measured mitochondrial DNA (mtDNA) copy number, a surrogate marker of mitochondrial function [26], yet found no difference. Collectively, our data suggested that increased activity did not induce mitochondrial biogenesis in postmenopausal obese mice.

3.7 Markers of lipid metabolism in muscle

Since we observed a reduction in muscle diacylglycerol in the naringenin-fed mice, we measured muscle mRNA levels of genes involved in lipid metabolism. The expression of lipoprotein lipase (LPL), an enzyme that releases fatty acids from circulating triglycerides and facilitates uptake of fatty acids by muscle cells, and the expression of carnitine palmitoyltransferase I beta (CPT1β), a key regulator of mitochondrial fatty acid transport, were not different between groups (Fig. 7B). However, the expression of fatty acid synthase

(FASN) and stearoyl-CoA desaturase (SCD1), two genes encoding proteins involved in de novo lipogenesis, was significantly lower (by 70%) in the mice fed a naringenin diet. Transcript levels of diacylglycerol acyltransferase-2 (DGAT2), an enzyme that incorporates diacylglyceride to triacylglyceride, were down-regulated in naringenin-fed mice. We also measured the levels of genes involved in intramuscular lipolysis, e.g., adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL). The expression of ATGL was unaffected, while, mRNA levels of HSL were decreased by 40% in naringenin-fed mice.

3.8 AMPK and Akt signaling in muscle

Previous studies have shown that naringenin stimulates glucose uptake in L6 myotubes by activating 5′-AMP-activated protein kinase (AMPK) [28]. However, we did not detect a significant difference between groups in the levels of AMPK Thr172 phosphorylation, a measure of AMPK activation (Fig. 8A). It has been suspected that accumulation of intracellular fatty acid metabolites, diacylglycerol and ceramide, are the key intermediates that affect insulin signaling, leading to insulin resistance in skeletal muscle [29]. Since we observed significant decreases in both plasma glucose levels and muscle diacylglycerol content in the mice fed naringenin, we measured the activity of protein kinase Akt/PKB, a central mediator of insulin signaling. However, we did not observed increased phosphorylation levels of Akt at both phosphorylation sites, Ser 473 and Thr 308, in muscle of naringenin-fed mice (Fig. 8B).

4 Discussion

The present study reveals novel information on naringenin accumulation in tissues after long-term supplementation. Eleven weeks of 3% naringenin feeding resulted in naringenin accumulation in plasma, liver, perigonadal and subcutaneous adipose tissues, and muscle more than 3 times of the amount accumulated in mice fed 1% naringenin. Furthermore, the present study evaluated the effects of dietary naringenin on body weight and energy metabolism in a mouse model of postmenopausal obesity. Dietary naringenin suppressed body weight gain, decreased intra-abdominal fat, and reduced plasma glucose levels. Ovariectomized mice fed naringenin also exhibited a higher locomotor activity, the maintenance of muscle mass, a reduction in muscle diacylglycerol content, and changes in muscle lipid metabolism.

Naringenin bioavailability has gained much interest due to its potential beneficial effects on human health. Studies in humans and rodents suggest that bioavailability of naringenin could be affected by the form of naringenin consumed. In humans, after consumption of cooked tomato paste, the time needed to reach peak concentration (Tmax) of naringenin in plasma was 2 hours [9] compared to 4.8 hours and 5.5 hours after consuming orange and grapefruit juices, respectively [8]. The differences in Tmax could be due to the different forms of naringenin ingested (glycosidic forms such as naringenin-7- rhamnoglucoside or naringenin-7-glucoside in orange and grapefruit juices and aglyconic form in tomato skin) that may be absorbed via different routes in the digestive tract. Rodent studies have also demonstrated that naringenin, aglycone form, and naringenin-7-glucoside have faster absorption kinetics and higher 24-hour urinary excretion compared to naringenin-7-rhamnoglucoside (narirutin), one of the predominant forms in grapefruit [5, 6]. Therefore, in

the present study, we supplemented aglyconic form of naringenin into the diets to reduce inter-individual variances on hydrolysis of glycosidic linkage. At the end of the study, we estimated that the mice fed a 3% naringenin diet consumed about 1g/kg body weight of naringenin, which equates to 9 g of naringenin or 17L of grapefruit juice for a 60 kg person based on surface area [30].

Most studies examined bioavailability of naringenin by using plasma kinetics and urinary excretion as indicators and only give a single-dose administration. However, the response to naringenin in blood and urine could simply reflect the release of naringenin from the food matrix and absorption and not accurately indicate the degree of tissue uptake [31]. Therefore, the bioavailability of naringenin and its metabolites in target tissues is much more important than the knowledge of its concentration in plasma and urine. In the present study, we demonstrated that the concentrations of total naringenin in tissues of mice fed a diet with 3% naringenin were more than 3 times higher (5–12 times) than the accumulation in tissues of mice fed a 1% naringenin-containing diet, suggesting that higher naringenin levels in tissues from 3% naringenin supplementation may have resulted in the beneficial effects presently observed. Our pilot studies [32] and other studies [7] showed that the major forms of naringenin in blood and organs are sulfates and glucuronides. In the present study, we only measured total naringenin concentration and did not identify and quantify of naringenin and its metabolites in tissues. The release, expression, and function of enzymes responsible for glucuronide or sulfate conjugation have shown to be altered during inflammation or progression of nonalcoholic fatty liver disease [32, 33]. Therefore, it is intriguing to investigate the changes of naringenin metabolite profile and identify the potential biological activity of the metabolites during progression of the diseases.

Recently, we reported that dietary naringenin decreased body weight, reduced adiposity, and improved glucose metabolism in ovariectomized mice [14]. In the present study, we also observed reduction of weight gain and abdominal adiposity in the obese ovariectomized mice fed a naringenin-enriched diet. In accordance with our previous data, dietary naringenin reduced intra-abdominal adiposity as well as expression of MCP-1 in perigonadal adipose tissue of the obese mice. Although plasma glucose levels were lower in obese mice fed naringenin, we did not observe changes in plasma insulin. However, the mice began naringenin supplementation when they had already gained 179% of their baseline body weight. It is possible that naringenin supplementation may result in better outcomes if naringenin is given before the onset of severe obesity.

Bjursell et al (2008) demonstrated that reduced locomotor activity occurred acutely in mice 3–5 hour after switching to a high-fat diet and the reduction was sustained after 21 days of feeding, when energy intake, energy absorption, and energy expenditure were normalized [34]. In the present study, dietary naringenin did not affect caloric intake but increased locomotor activity levels in obese ovariectomized mice to the levels shown in our previous study with ovariectomized mice fed a normal-fat diet [14]. It is likely that recovery of locomotor activity by naringenin results in the suppression of body weight in obese mice. Additionally, Janssen et al [13] showed that a high prevalence of women over age 60 in the Third National Health and Nutrition Examination Survey (NHANES III) exhibited sarcopenia (loss of skeletal muscle mass with aging). Sarcopenic obesity (low muscle mass

with high body fat) increases with aging and is associated with functional impairment, disabilities, and falls [35]. We found that naringenin maintained quadriceps muscle mass in obese ovariectomized mice. Further investigation is required to determine the influence of naringenin in the maintenance of muscle health and prevention of sarcopenia.

Mulvihill et al (2009) demonstrated that dietary naringenin decreased fatty acid synthesis in muscle but did not affect fatty acid oxidation in muscle of low density lipoprotein receptor (LDLR) knockout mice fed a high-fat diet [15]. In line with their findings, dietary naringenin did not affect fatty acid oxidation in muscle of obese ovariectomized mice but down-regulated mRNA expression of genes involved in lipid metabolism, including de novo lipogenesis, lipolysis, and triglyceride synthesis/storage. Although fatty acid synthase expression in skeletal muscle is not abundant and the role of de novo lipogenesis was assumed negligible [36], skeletal muscle specific knockdown of fatty acid synthase increased whole body glucose tolerance and insulin sensitivity in mice fed a high-fat diet [37]. Concurrently, the levels of diacylglycerol but not triacylglycerol were lower in the obese mice fed a naringenin-enriched diet, suggesting little contribution of de novo lipogenesis on triacylglyceride content in muscle. Expression of two genes that directly regulate diacylglycerol metabolism, DGAT2 and HSL, were significantly decreased in naringenin-fed obese mice. Interestingly, HSL knockout mice exhibited impaired insulin signaling and diacylglycerol accumulation in muscle [38]. Furthermore, DGAT2 overexpression in muscle resulted in accumulation of triacylglycerol and ceramide but a reduction in diacylglycerol levels [39]. Thus, the reduction of mRNA expression of HSL and DGAT2 may be the result, rather than the cause of lower diacylglycerol content in muscle. Several lines of evidence suggest that diacylglycerol acts like a signaling molecule to activate protein kinase C (PKC) isoforms, resulting in impaired insulin signaling in liver and muscle [40]. Although we observed lower glucose levels in naringenin-fed mice, we did not observe changes in Akt activity in muscle, or changes in total body insulin sensitivity by performing an insulin tolerance test (data not shown). Future studies should use hyperinsulinemic-euglycemic clamp to further investigate the effect of naringenin on muscle insulin sensitivity.

As life expectancy increases, the postmenopausal period for women will likely increase as well. Considering the prevalence of obesity in women and the increasing rate of obesity after menopause [10], the need for approaches that reduce the rate of postmenopausal obesity is crucial. This present study demonstrates that naringenin supplementation maintains body weight, increases activity, reduces intra-abdominal adiposity, and decreases plasma glucose levels in obese ovariectomized mice. Additionally, in muscle, dietary naringenin maintains tissue mass, reduces diacylglycerol content, and alters lipid metabolism. The results suggest that naringenin may modulate energy metabolism in obese menopausal women and is a potential supplement to treat postmenopausal obesity.

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Abbreviations

VHF	very high-fat diet
VHFN	very high-fat diet containing 3% naringenin
OVX	ovariectomized
PGAT	perigonadal adipose tissue
SCAT	subcutaneous adipose tissue
RER	respiratory exchange ratio

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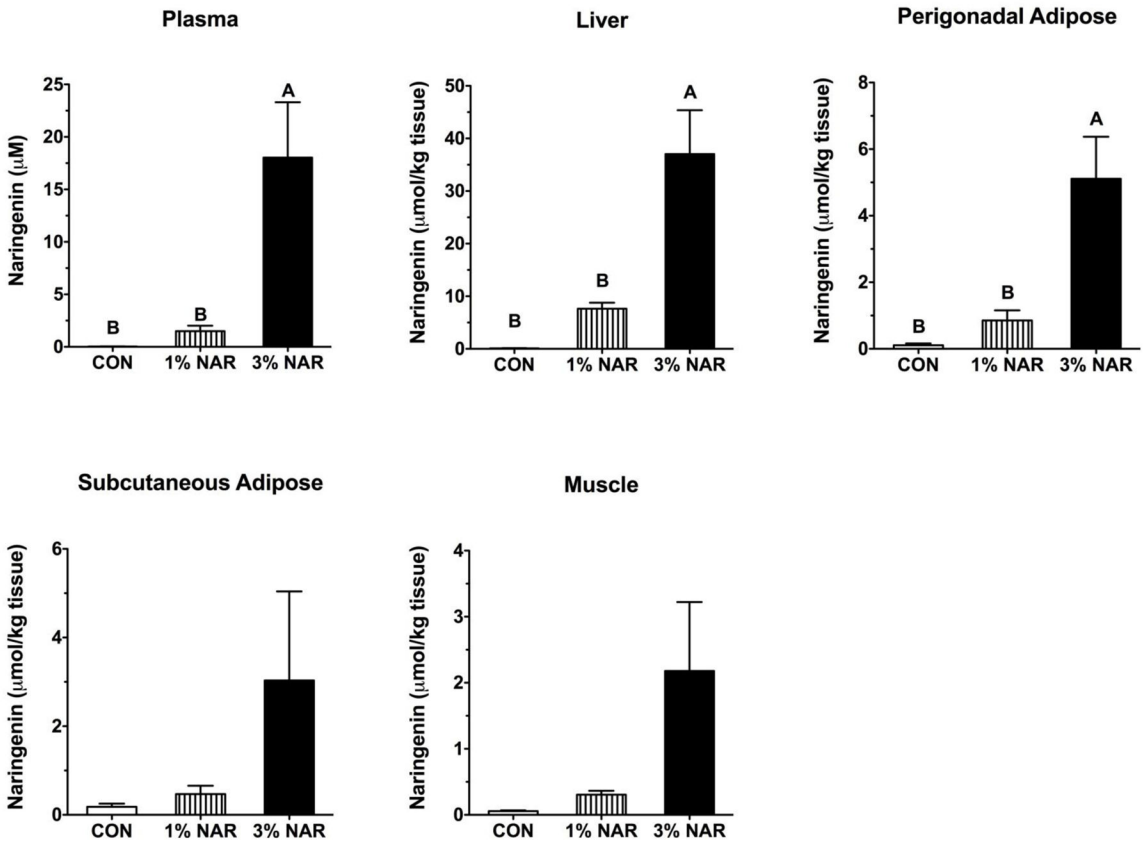

Figure 1. Naringenin accumulation in plasma and tissues after 11 weeks of supplementation

OVX C57BL/6J mice were fed a high-fat diet for 6 weeks and switched to the high-fat diet with 0%, 1%, and 3% wt/wt naringenin. After 11 weeks of the diets, total naringenin concentrations were 5–12 times higher in plasma (n=4/group) or tissue samples (n=5/group) of mice fed 3% naringenin than the levels in mice fed 1% naringenin. Values are presented as mean ± SEM. Data were analyzed using one-way ANOVA with post hoc Tukey's test. Significant difference ($P < 0.05$) between groups with different mean superscripts.

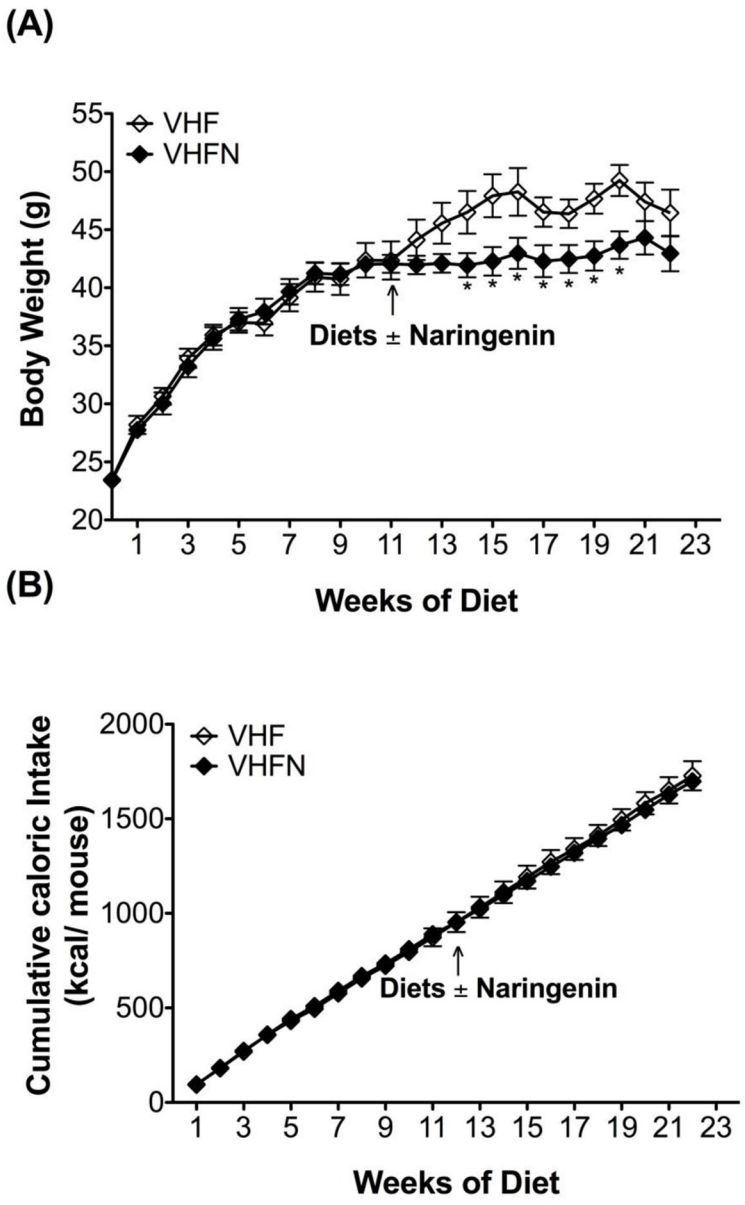

Figure 2. Effect of naringenin supplementation on body weight and caloric intake

OVX C57BL/6J mice were fed a very high-fat diet for 11 weeks. Then half of the mice (VHFN) were given a very high-fat diet supplemented with 3% naringenin for another 11 weeks. (A) Body weight (n=10/group). (B) Cumulative caloric intake of mice (n=2 cages/group). Values are presented as mean ± SEM. Significance between groups was determined by Student's t test. *P < 0.05 compared VHFN with VHF.

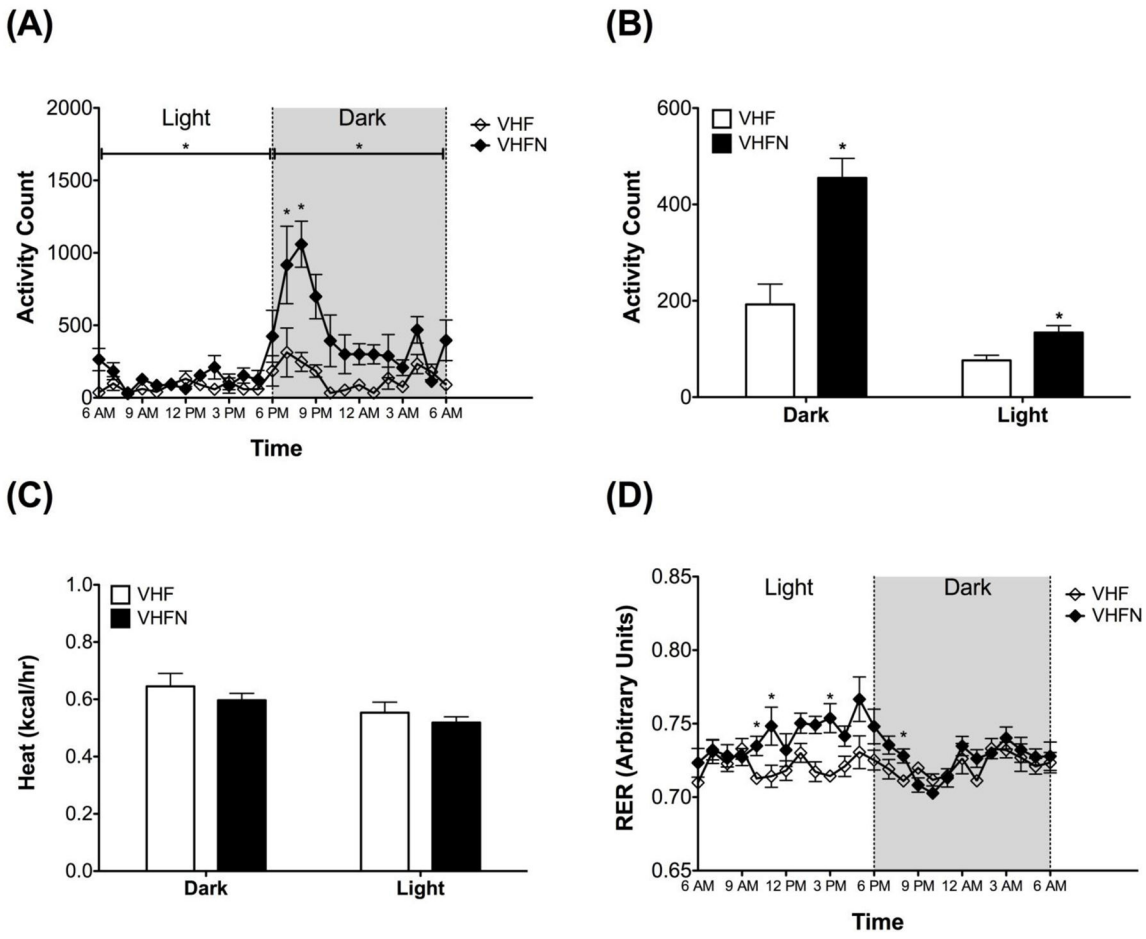

Figure 3. Effect of naringenin supplementation on indirect calorimetry and locomotor activity

At week 17, metabolic parameters were measured in the CLAMS chambers for 24 hours following a 24-hour acclimation (VHF: n=6, VHFN: n=8). (A) Ambulatory locomotor activity in the horizontal plane measured by infrared beam breaks. (B) Ambulatory counts in the dark and light phase. (C) Estimated energy expenditure (heat) after controlling for body weight in the dark and light phase. (D) Respiratory exchanging ratio (RER). Values are presented as mean ± SEM. Significance between groups was determined by Student's t test, and metabolic data of heat was analyzed by ANCOVA with body weight as a covariate. *P < 0.05 compared VHFN with VHF.

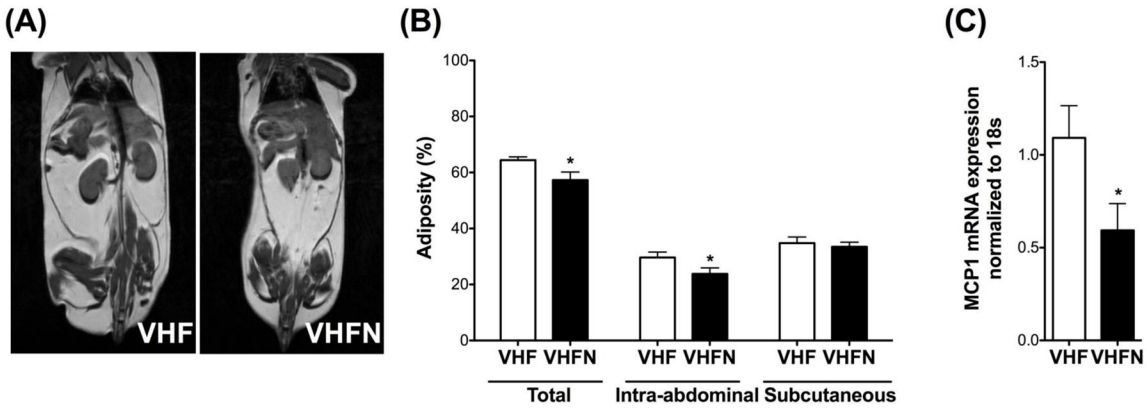

Figure 4. Effect of naringenin supplementation on adiposity, fat distribution, and MCP1/Ccl2 mRNA expression in perigonadal adipose tissue

(A) Representative coronal views of MRI from each group. (B) Percentage of total body fat and percentage of two adipose tissue depots, intra-abdominal and subcutaneous adipose tissues were determined by MRI (n=6/group). (C) mRNA expression of MCP1 in perigonadal adipose tissue (n=8–9/group). Values are presented as mean ± SEM. Significance between groups was determined by Student's t test. *P < 0.05 compared VHFN with VHF.

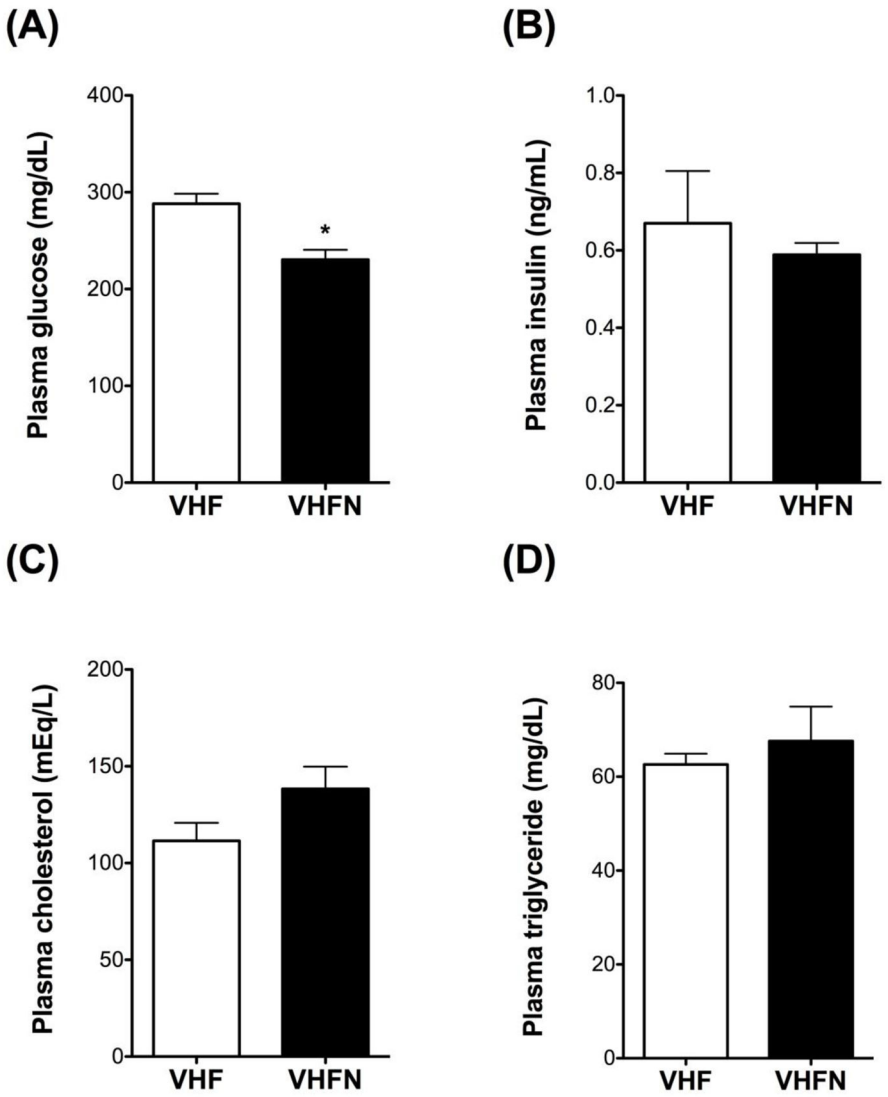

Figure 5. Effect of naringenin supplementation on plasma analysis

(A) Fasting plasma glucose levels (n=5/group), (B) Fasting plasma insulin levels (n=5/group), (C) Fasting plasma cholesterol levels (VHF: n=7, VHFN: n=8), and (D) Fasting plasma triglyceride levels (n=5/group). Values are presented as mean ± SEM. Significance between groups was determined by Student's t test. *P < 0.05 compared VHFN with VHF.

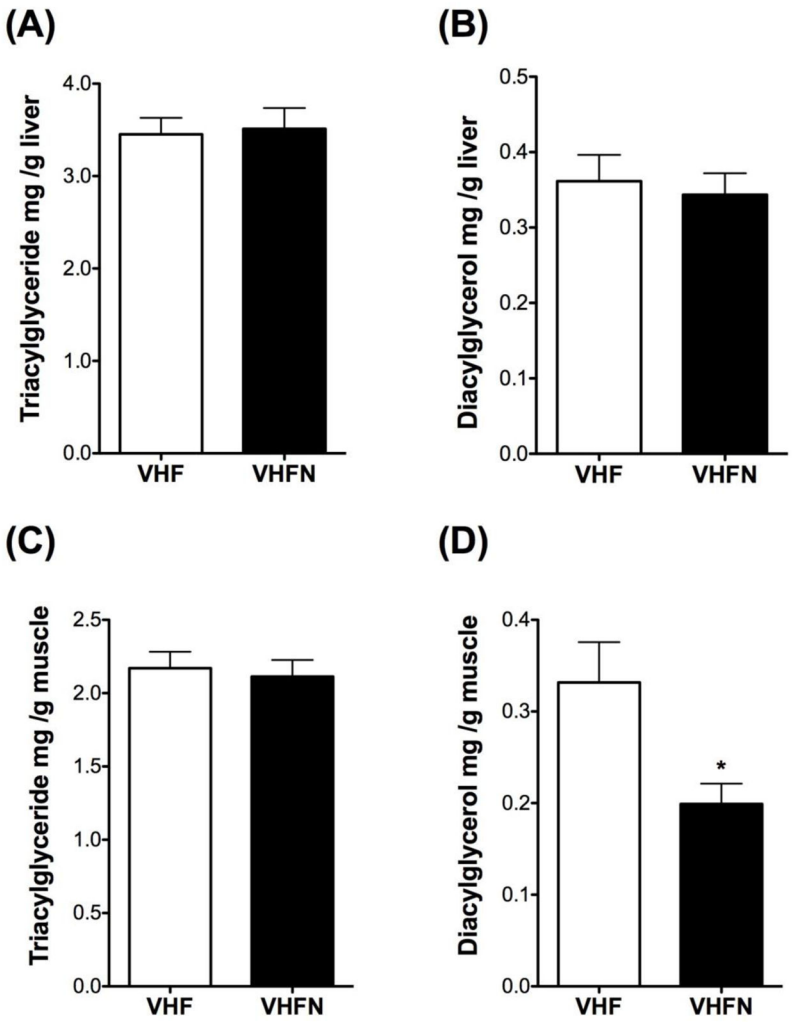

Figure 6. Effect of naringenin supplementation on hepatic and muscle lipid profiles

(A) Extracted triacylglycerol content per gram of liver section (n=10/group). (B) Extracted diacylglycerol content per gram of liver section (n=10/group). (C) Extracted triacylglycerol content per gram of muscle section (n=10/group). (D) Extracted diacylglycerol content per gram of muscle section (n=9–/group). Values are presented as mean ± SEM. Significance between groups was determined by Student's t test. *P < 0.05 compared VHFN with VHF.

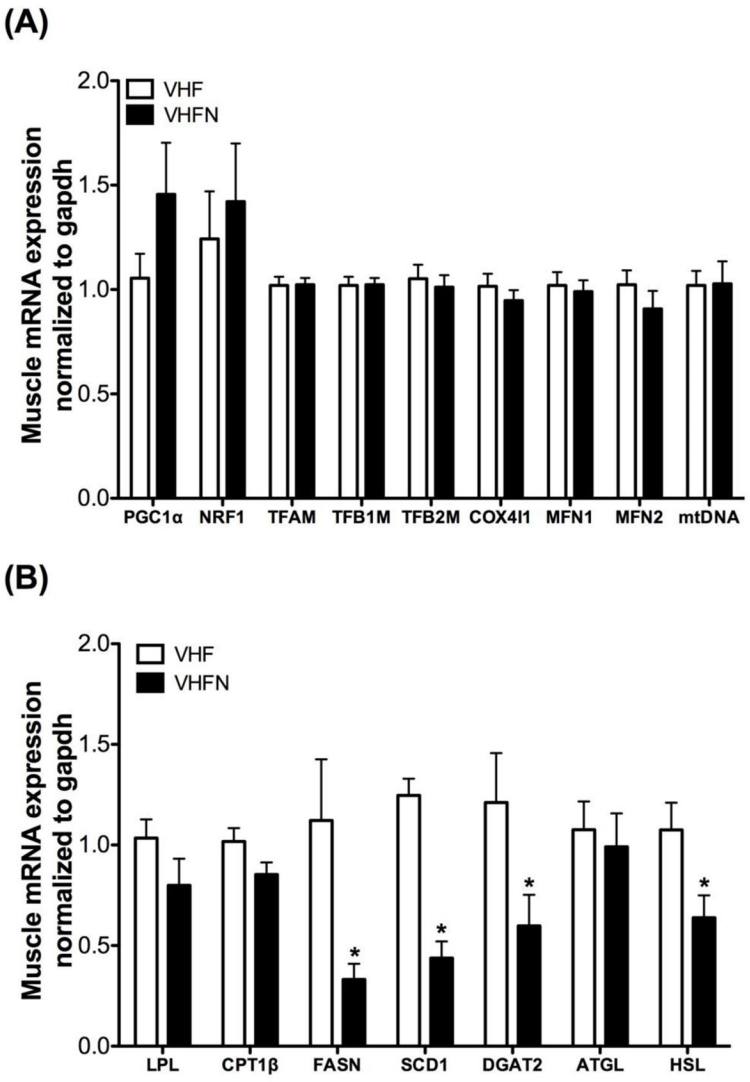

Figure 7. Effect of naringenin supplementation on mRNA expression in muscle

mRNA expression of genes for mitochondrial biogenesis (A) and genes involved in lipid metabolism (B). Values are presented as mean ± SEM (n=9–10/group). Significance between groups was determined by Student's t test. *P < 0.05 compared VHFN with VHF.

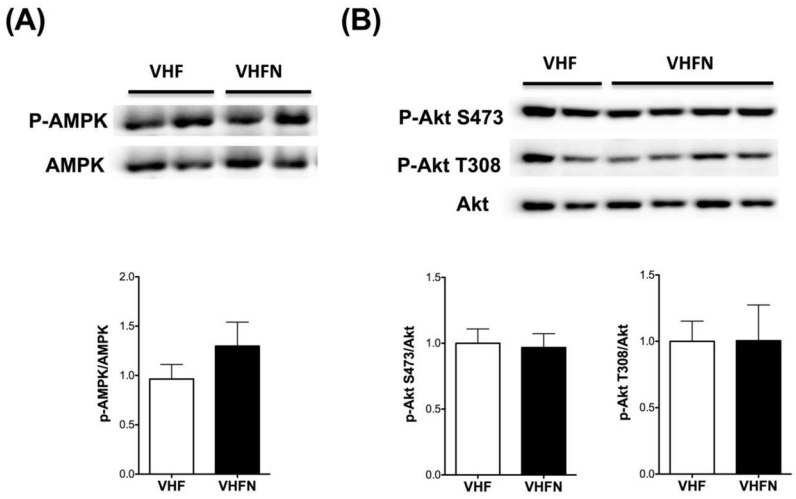

Figure 8. Effect of naringenin supplementation on AMPK and Akt activity in muscle

Ratio of phosphorylated AMPK Thr172 to total AMPK protein with representative blots (A) and ratio of phosphorylated Akt Ser473 and Thr308 to total Akt protein with representative blots (B) in muscle. Values are presented as mean ± SEM (AMPK: VHF: n=10, VHFN: n=8; Akt: VHF: n=5, VHFN: n=8). Significance between groups was determined by Student's t test. *P < 0.05 compared VHFN with VHF.

Table 1

Effect of dietary naringenin on feed efficiency and tissue mass in obese ovariectomized C57BL/6J female mice

	VHF	VHFN
Feed Efficiency (mg/kcal)		
Before Supplementation (0–11 week)	0.022 ± 0.002	0.021 ± 0.001
After Supplementation (12–22 week)	0.005 ± 0.001	0.001 ± 0.002
Tissue Weights (g)		
Subcutaneous Adipose Tissue	3.78 ± 0.23	3.43 ± 0.24
Liver	1.42 ± 0.07	1.35 ± 0.04
Perigonadal Adipose Tissue	3.79 ± 0.09	3.20 ± 0.18
Quadriceps Muscle	0.36 ± 0.01	0.39 ± 0.01
Tissue Weight Percentages (%)		
Subcutaneous Adipose Tissues	8.26 ± 0.28	8.06 ± 0.33
Liver	3.12 ± 0.09	3.22 ± 0.08
Perigonadal Adipose Tissue	7.81 ± 0.25	7.55 ± 0.23
Quadriceps Muscle	0.79 ± 0.02	0.93 ± 0.03*

Feed efficiency ratio = body weight gain (mg) /cumulative caloric intake (kcal)

Significance between groups was determined by Student's T-test (n=10/group). Data represent the mean ± SEM.

* P < 0.05 compared VHFN with VHF