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Diosgenin Supplementation Prevents Lipid Accumulation and Induces Skeletal Muscle-Fiber Hypertrophy in Rats

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Summary Diosgenin (Dio) is a steroid sapogenin found in plants such as *Dioscorea* species, and is recognized as a phytochemical against various disorders as well as a natural precursor of steroidal drugs. The present study used rats fed high-cholesterol (Chol) diets supplemented with or without 0.5% Dio for 6 wk to investigate the effects of dietary Dio on lipid metabolism. Dio supplementation significantly increased serum high-density lipoprotein Chol concentrations and fecal Chol content, and significantly decreased fecal bile acid content compared rats fed a high-Chol diet alone, showing that dietary Dio may facilitate excretion of Chol rather than bile acids. A reduction in the liver triglyceride content and intra-abdominal visceral fat was observed in Dio-supplemented rats. Interestingly, dietary Dio also significantly increased the skeletal muscle-fiber diameter and area in the thigh muscles of the rats. Mouse myoblast-derived C2C12 cells were used to examine whether Dio directly affected skeletal muscle. Dio promoted fusion of myoblasts into multinucleated cells or myotubes. Furthermore, in myotube C2C12 cells, protein levels of phosphorylated AMP-activated protein kinase (AMPK) increased with Dio treatment in a dose-dependent manner. These results indicate that Dio may not only induce myoblast fusion and enhance skeletal muscle as an energy expenditure organ, but may also activate the catabolic pathway via AMPK in skeletal muscle cells. Thus, these effects of Dio on skeletal muscles may contribute to inhibition of visceral fat accumulation.

Key Words dietary supplements, lipid metabolism, cholesterol, visceral fat, skeletal muscle, skeletal muscle cells, myoblast fusion, AMPK

Diosgenin (Dio; Pubchem ID 99474) is a steroid sapogenin found in plants such as *Dioscorea*, *Trigonella*, *Costus*, and *Smilax* species, and is a constituent of the saponin, dioscin, which is found in the rootstock of yam (*Dioscorea*) (*1*, *2*). Dio is a natural precursor of steroidal drugs, including sex hormones and corticosteroids, indicating its importance in the pharmaceutical industry. Additionally, Dio itself has pharmacological potential and behaves as a biologically active phytochemical that is used in the treatment of various disorders, including inflammation, hypercholesterolemia, cancer, and several types of infections. Tohda et al. identified $1,25D_3$-membrane-associated, rapid response steroid-binding protein ($1,25D_3$-MARRS) as a target protein of Dio and demonstrated that Dio induces axonal growth and regrowth of neurons as an exogenous stimulator of $1,25D_3$-MARRS, and may act on critical signaling pathways as a therapy for Alzheimer's disease (*3*). Another study reported that Dio caused ectodomain shedding of tumor necrosis factor (TNF) receptor 1 as an activator of $1,25D_3$-MARRS, attenuating the effects of TNF-alpha. This suggested that Dio may downregulate the inflammatory response in vascular endothelial cells (*4*).

Dio is a phytosterol and is detected in the plasma of rats and humans after oral administration (*5*–*7*), demonstrating that orally ingested Dio is absorbed and acts on tissues of the body. We examined whether dietary Dio ameliorated lipid metabolism and altered skeletal muscle structure in the rats chronically fed a high-cholesterol (Chol) diet. It was shown that supplementation with Dio for 4 wk resulted in a slight increase in serum high-density lipoprotein (HDL)-Chol and fecal bile acid levels, and in a decrease in the size of lipid droplets in the liver (*8*). While these results indicated that Dio slightly improved lipid metabolism in rats regularly ingesting a high-Chol diet, we were unable to clarify the role of dietary Dio.

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Table 1. Composition of the experimental diets.

(g/100 g diet)

	Ordinary diet	High-Chol diet	Dio-supplemented diet
Casein	25	25	25
Corn oil	9	9	9
Cellulose	4	4	4
Mineral mixture	4	4	4
Vitamine mixture	2	2	2
Corn starch	56	55.38	54.88
Dio	—	—	0.5
Chol	—	0.5	0.5
Sodium cholate	—	0.125	0.125

In the present study, in order to elucidate the points, we examined the effects of Dio supplementation on lipid metabolism and skeletal muscles.

MATERIALS AND METHODS

Animals and diets. Six-week-old male Wistar rats (n=18) weighing 170–190 g were purchased from Japan SLC, Inc. (Japan). Rats were housed in individual cages in an animal holding room with a 12-h light/12-h dark cycle at 22±2˚C. On arrival, rats were acclimatized for 1 wk and fed a standard rodent chow diet (CE-2, CLEA Japan, Inc., Japan) with access to water ad libitum. Following acclimatization, rats were randomly divided into three groups of six animals: control (CTL), high-Chol diet (Chol), and high-Chol Dio-supplemented diet (Chol+Dio) groups. Rats in the CTL group were fed standard rodent chow, while those in the Chol group were fed high-Chol diets containing 0.5% Chol and 0.125% sodium cholate for 6 wk (Table 1) as previously described (*8*). The Chol+Dio group was fed a high-Chol diet supplemented with 0.5% Dio (Tokyo Chemical Industry Co., Ltd., Japan) for 6 wk. The total energy content of each diet was 372 kcal/100 g.

Rats were weighed weekly and all uneaten food was weighed to calculate food intake. Rat feces were collected during the last 4 d of the treatment period and were dried and weighed. After fasting for 10 h, rats were sacrificed under pentobarbital anesthesia (Somnopentyl, Kyoritsu Seiyaku Corporation, Japan). Blood, liver, left quadriceps, and intra-abdominal visceral fat (fat around the kidneys, testicles, and the posterior wall of the stomach) were collected.

All animal experiments were performed in accordance with the guidelines for animal experimentation from the Ministry of Environment of Japan and were approved by the Animal Experimental Committee of Nagoya Women's University (authorization number: No. 24-5).

Measurements of lipid components in blood and tissues. Blood, feces, and liver tissues were processed as previously described (*8*). Briefly, blood was centrifuged at 1,700 ×g for 10 min (KUBOTA 5200, Japan) after sitting at room temperature for 30 min, and supernatants were collected. Total lipids were extracted from liver tissue using chloroform–methanol (2 : 1). Fecal samples were extracted with 99% ethanol. Total Chol (T-Chol), HDL-Chol, triglyceride (TG), and the bile acid content of tissues, feces, and blood samples were determined using Chol E Test Wako, HDL-Chol E Test Wako, TG E Test Wako, and Total Bile Acids Test Wako (FUJIFILM Wako Pure Chemical Corporation, Japan), respectively. The Friedewald formula was used to estimate low-density lipoprotein (LDL)-Chol levels as follows: LDL-Chol=T-Chol−HDL-chol−(TG/5).

Observations of skeletal muscle and liver tissues. Left quadriceps and liver tissues were fixed in a 4% paraformaldehyde phosphate buffer solution as previously described (*8*), and histological sections were examined and photographed under a microscope after staining with hematoxylin and eosin (H&E). Image analyses were performed using Image J software. The size of skeletal muscle fibers was analyzed by measuring the areas and the lesser diameter (minimal Feret's diameter) (*9*), and the number of muscle fibers per unit area were calculated.

Cell culture. C2C12 cells were cultured in growth medium [high glucose Dulbecco's modified Eagle's medium (DMEM; FUJIFILM Wako Pure Chemical Corporation) supplemented with 10% fetal calf serum and penicillin/streptomycin (P/S; FUJIFILM Wako Pure Chemical Corporation)], in 5% CO_2 at 37˚C. The culture medium was replaced every 2 d, and cells were passaged when they reached 80–90% confluence. To differentiate the C2C12 cells, cells were exposed to differentiation medium (DMEM supplemented with 2% horse serum and P/S) when they reached 80–90% confluence, replacing the culture medium every other day.

C2C12 myoblast proliferation and fusion. To assess the effects of Dio on myoblast proliferation, C2C12 cells were plated at a density of 3.3×10^3 cells per cm^2. After 48 h, cells were cultured in growth medium containing 20 μM Dio or control vehicle (methanol), and the media were replaced every day. At days 1 and 2, C2C12 myoblast proliferation was evaluated by directly measuring the number of cells and the results were presented as mean±SD of five independent experiments in triplicate. To assess myoblast fusion in the presence of Dio, C2C12 cells were plated at a density of 1×10^4 cells per cm^2 as previously described by Shafey et al., with modi-

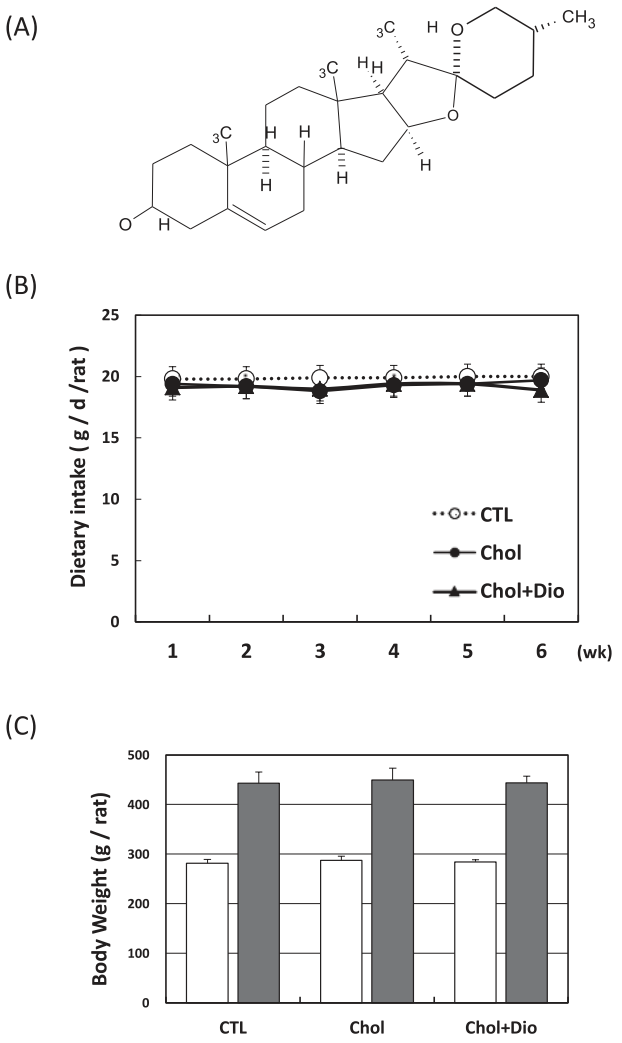

Fig. 1. Effects of Dio on body weight of rats fed a high-Chol diet. The constitutional formula of Dio (A). Changes in cumulative dietary intake (B) and rat body weight (C) during the 6-wk experiment. Rat body weights were measured at the beginning (open bars) and end (closed bars) of the 6-wk study. Data represent mean±standard deviation (SD) of six rats.

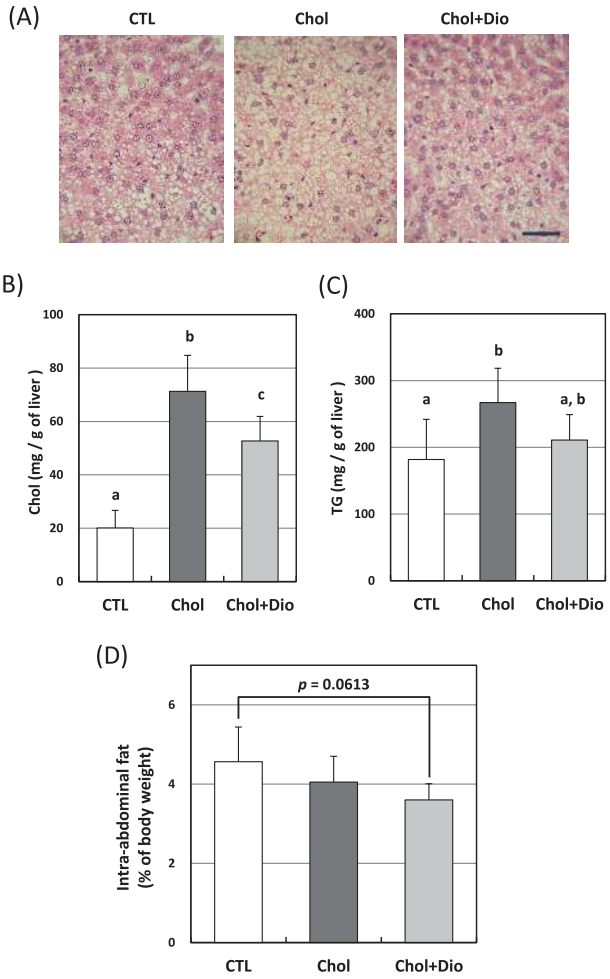

Fig. 2. Dio decreases lipid accumulation in liver and intra-abdominal fat in high-Chol diet-fed rats. Representative H&E-stained sections of rat livers after the 6-wk treatment period are shown (A). Scale bar represents 400 μm. Liver Chol content (B) and liver TG content (C) are presented as mg/g of liver. Intra-abdominal fat weight includes dissected kidney leaf, epididymal, and mesenteric fat, and is presented relative to body weight (D). Data are presented as mean±SD of six rats. Different letters indicate significant differences between groups; Turkey–Kramer test, $p<0.05$.

fications (*10*). After 24 h, cells were cultured in growth medium containing either 20 μM Dio or control vehicle for 24 h. Nuclei were stained using Hoechst 33342 and phase-contrast and fluorescent images were taken using a Nikon microscope (ECLIPSE T*i*). The number of multinuclear C2C12 cells was calculated as percentage of the total cell number and presented as mean±SD of five independent experiments performed in triplicate.

Western blot analysis. Cells were washed with ice-cold phosphate-buffered saline and lysed in RIPA lysis buffer [150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM TrisHCl, pH 7.2] containing protease inhibitor cocktail (Sigma) and phosphatase inhibitor cocktail 1 and 2 (Sigma). Cell debris was removed by centrifugation at 13,000 ×*g* for 10 min at 4°C, and the protein concentration in the supernatant was measured using a Pierce BCA Protein Assay Kit (Thermo Scientific).

Cell lysates were treated with SDS sample buffer and immediately boiled for 5 min. Proteins were separated by SDS-PAGE in the presence of 2-mercaptoethanol then electrotransferred onto polyvinylidene difluoride membranes (Hybond-P, GE Healthcare). Total AMP-activated protein kinase (AMPK) was estimated using an anti-AMPK-α antibody (Cell Signaling Technology) and phosphorylated AMPK-α was detected using an anti-phospho-AMPK-α (Thr172) antibody (40H9, Cell Signaling Technology). ECL blotting reagents (Thermo Scientific) were used to detect immunoreactive proteins, and densitometry was performed using NIH Image/Image J software.

Statistical analysis. Data are expressed as mean± standard error of the mean. All statistical analyses were performed using Ekuseru-Toukei 2012 (Social Survey

Table 2. Serum lipid concentrations.

	CTL	Chol	Chol+Dio
TG (mg/dL)	61.7±21.05[a]	34.4±15.86[b]	36.58±11.94[b]
T-Chol (mg/dL)	78.2±5.78[a]	105±15.56[b]	113±17.76[b]
HDL-Chol (mg/dL)	60.7±7.69[a]	66.0±17.00[a]	87.4±9.93[b]
LDL-Chol (mg/dL)	5.16±3.66[a]	31.6±24.91[b]	18.7±9.41[a,b]

Data are presented as means±SD of six rats. Different letters indicate significant differences between groups; Turkey–Kramer test, $p<0.05$.

Research Information Co., Ltd., Japan). Statistical differences between the groups were evaluated using one-way ANOVA with Tukey–Kramer multiple comparison tests. Independent sample *t* test was used to compare results between two conditions.

RESULTS

Dio-supplemented high-Chol diet led to decreased lipid accumulation in liver and intra-abdominal visceral fat

Dio (Fig. 1A) is absorbed from the gut when ingested orally (*5–7*). To determine the effects of dietary Dio on lipid metabolism and sleletal muscles, rats were fed either standard rodent chow (CTL group) or high-Chol diets supplemented with or without 0.5% Dio (Chol+Dio and Chol groups, respectively) for 6 wk (Table 1). The dietary intake throughout the experimental period did not significantly differ between the three groups (Fig. 1B). Rat body weight in the three groups also increased and did not significantly differ (Fig. 1C). These data showed that 0.5% Dio supplementation for 6 wk had little effect on body weight. To first elucidate whether dietary Dio altered lipid metabolism, we examined changes in hepatic lipid storage of these rats. H&E analysis of liver tissues showed large unstained vesicles, which were identified as lipid droplets, throughout the liver in the Chol group, showing that high-Chol diet feeding led to increased lipid storage in the liver (Fig. 2A). In the Chol+Dio group, smaller unstained vesicles were observed in the liver and the staining patterns were similar to those observed in the CTL group. These results suggested that Dio supplementation may inhibit lipid accumulation in the liver. To clarify this, we examined differences in lipid content of the liver between the three groups. Liver Chol and TG content in the Chol group were significantly higher than those in the CTL group ($p<0.001$ and $p=0.034$, respectively), demonstrating that the high-Chol diet resulted in accumulation of TG as well as Chol in the liver (Figs. 2B and C). The liver Chol content in the Chol+Dio group was significantly higher than that of the CTL group ($p<0.001$), but was significantly lower than that of the Chol group ($p=0.016$, Fig. 2B), suggesting that Dio may inhibit Chol absorption in the intestine and/or facilitate excretion of Chol from the liver. The liver TG content in the Chol+Dio group was lower than that of the Chol group ($p=0.164$), and remained at around the same level as that of the CTL group ($p=0.609$, Fig. 2C). Taken together, these findings indicate that Dio supplementation inhibited high-Chol diet-induced accumulation of lipid in the liver, with an accompanying decrease in liver TG and Chol content.

We then examined the effects of Dio on accumulation of intra-abdominal visceral fat around the kidneys, testicles, and posterior wall of the stomach. The intra-abdominal visceral fat content in the Chol+Dio group was decreased compared with that of the CTL group ($p=0.061$) and was lowest in the three groups (Fig. 2D), while intra-abdominal visceral fat contents did not differ significantly between CTL and Chol groups as our previous data (*8*). Furthermore, serum TG concentrations in the Chol and Chol+Dio groups were significantly decreased compared with that of the CTL group ($p=0.032$ and $p=0.049$, respectively, Table 2). These data indicated that dietary Dio reduced fat storage in visceral fat and blood as well as the liver.

Dio supplementation induces an increase in serum HDL-Chol concentrations without a marked increase in serum LDL-Chol concentration, despite high-Chol diet feeding

We then investigated whether Dio supplementation affected serum Chol levels. While serum T-Chol concentrations were significantly higher in the Chol and Chol+Dio groups compared with the CTL group ($p=0.014$ and $p=0.0015$, respectively, Table 2), serum HDL-Chol concentrations were significantly higher in the Chol+Dio group compared with the CTL and Chol groups ($p=0.0047$ and $p=0.021$, respectively). Serum LDL-Chol concentration were highest in the Chol group, but there were no significant differences between the Chol+Dio and CTL groups as well as between the Chol+Dio and Chol groups. We next compared fecal bile acid and Chol content between three groups. Although the fecal bile acid content in the Chol and Chol+Dio group was significantly higher than that of the CTL group ($p<0.001$ for both groups), it was significantly lower in the Chol+Dio group compared with the Chol group ($p=0.0048$, Fig. 3A). Fecal Chol content in the Chol+Dio group was highest among the three groups and was significantly higher than that of the Chol group ($p<0.001$, Fig. 3B). Taken together, these findings suggested that Dio supplementation resulted in inhibition of Chol absorption from the intestine and/or facilitation of Chol excretion from the liver to the feces.

Dio supplementation increases skeletal muscle-fiber size

To next determine whether Dio affected skeletal muscle tissue, we analyzed H&E-stained cross-sections of the quadriceps. The Chol+Dio group showed a decreased expression in connective tissue composed of

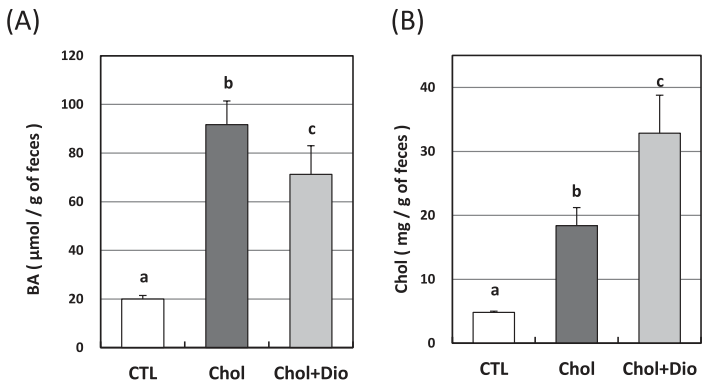

Fig. 3. Dio increases Chol content in feces in high-Chol diet-fed rats. Fecal bile acid content (A) or fecal Chol content (B) are presented as mg/g of dry feces. Data represent mean±SD of six rats. Different letters indicate significant differences between groups; Turkey–Kramer test, $p<0.005$.

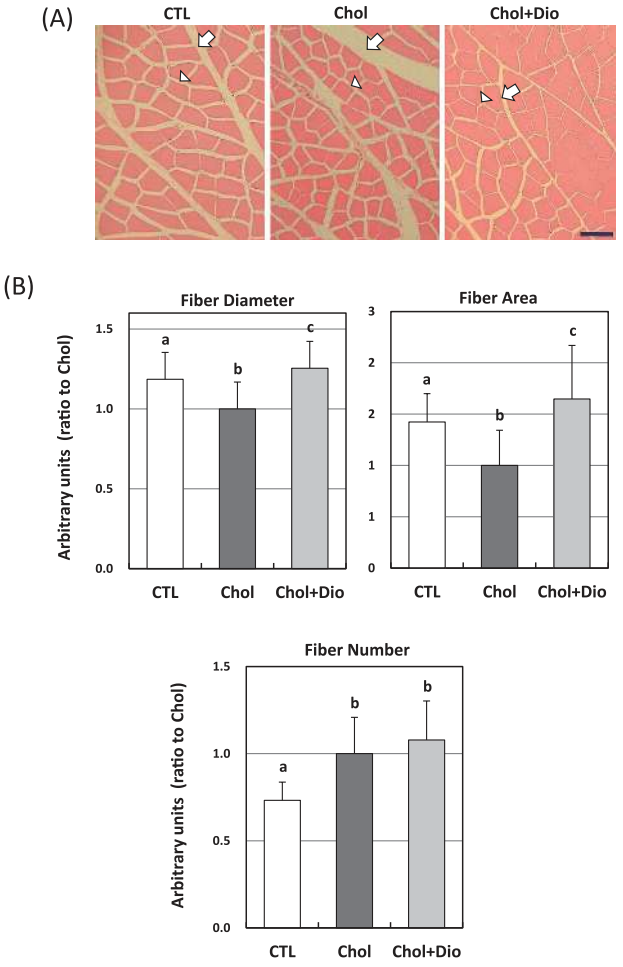

Fig. 4. Dio increases the density of skeletal muscle fibers in high-Chol diet-fed rats. Representative H&E-stained sections of rat femoral muscles are shown (A). Arrows indicate the perimysium that surrounds a group of muscle-fiber bundles, and arrow heads indicate the endomysium that surrounds and interconnects individual muscle fibers. Scale bar represents 100 μm. Minimal Feret's diameters (fiber diameter), areas (fiber area), and numbers (fiber number) were determined from >200 measurements of five sections per 4–5 femoral muscles from each group (B). Data are presented relative to Chol values. Different letters indicate significant differences between groups; Turkey–Kramer test, $p<0.01$.

perimysium, which surrounds a group of muscle-fiber bundles, and endomysium, which surrounds and interconnects individual muscle fibers (Fig. 4A), suggesting that Dio supplementation may enhance the density of skeletal muscle fibers. We then performed histomorphometric analysis using the H&E-stained quadriceps cross-sections to quantify the density of the skeletal muscle fibers. The diameters and areas of the skeletal muscle fiber were significantly increased in order corresponding to the Chol, CTL, and Chol+Dio groups ($p=0.0013$ for CTL versus Chol+Dio group fiber diameter; $p<0.001$ for all other comparisons), although there was no significant difference in the number of muscle fibers between the Chol and Chol+Dio groups (Fig. 4B). These results indicate that Dio supplementation thickened skeletal muscle fibers and reduced the connective tissue. Taken together, these findings suggest that enlargement of muscle fibers due to Dio intake may promote energy consumption in skeletal muscle, leading to reduced fat storage in rats.

Dio stimulates myoblast fusion and activation of AMPK in C2C12 cells

To test whether Dio acts directly on skeletal muscle cells, we used mouse myoblast-derived C2C12 cells, which are commonly used to study skeletal muscle cell differentiation and energy metabolism, and investigated whether Dio stimulated cell growth. Proliferating C2C12 cells were treated with 20 μM Dio for either 24 or 48 h, and cell numbers and morphology were analyzed. While there was little difference between treatment with and without Dio for 24 h, cell numbers after Dio treatment for 48 h were not increased compared with untreated cells ($p<0.001$, Fig. 5A). C2C12 cells exposed to Dio for 48 h showed a cell morphology resembling that of myotube cells (Fig. 5B), suggesting that Dio may not stimulate cell growth of C2C12 myoblasts but promote fusion of C2C12 myoblasts into myotubes. Next, we examined whether Dio promoted formation of multinuclear myotubes in C2C12 cells using a cell fusion assay (Fig. 5C). Exposure to Dio for 24 h was significantly associated with an eight-fold increase in the percentage of multinucleated cells ($p<0.001$, Fig. 5D). These results demonstrated that Dio induced C2C12 cell

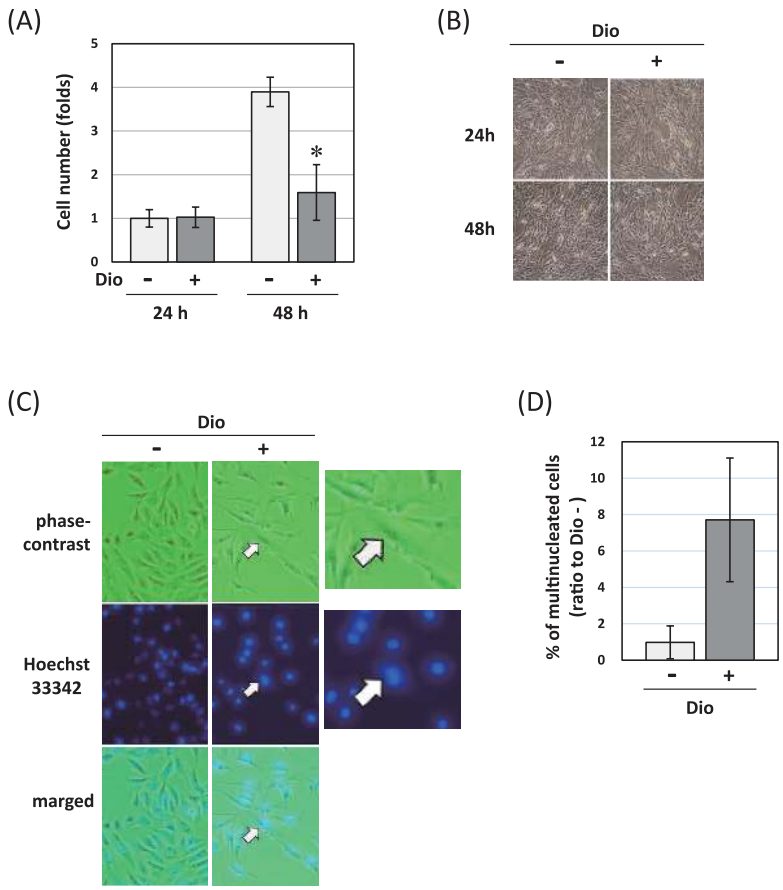

Fig. 5. Dio accelerates myoblast fusion in C2C12 cells. Proliferating C2C12 myoblasts were incubated with (+) or without (−) 20 μM Dio for 24 or 48 h. Cell numbers were counted (A) and phase-contrast micrographs of these cells are shown (B). Data represent the mean of four independent experiments ($n=3$)±SD. Proliferating C2C12 myoblasts were incubated with (+) or without (−) 20 μM Dio for 24 h. Nuclei were stained by Hoechst 33342 and phase-contrast and fluorescent images were photographed (C). Images inserted on the right show enlarged regions, indicated by the arrow. The number of multinuclear C2C12 cells was evaluated as a percentage of the total cell number and is shown as mean±SD of five independent experiments performed in triplicate (D). Asterisks indicate statistically significant differences between with and without Dio (Student's t test, $^*p<0.001$).

differentiation by promoting fusion of myoblasts into myotubes.

Finally, we investigated whether Dio triggered AMPK phosphorylation in skeletal muscle cells. AMPK, which senses cellular energy status and regulates cellular and whole-body energy balance, is activated by phosphorylation of Thr172, leading to the promotion of catabolic pathways that generate ATP and inhibition of anabolic pathways and processes that consume ATP (*11*). We measured the ratio of phosphorylated to total AMPK using Western blotting analysis to clarify whether Dio activates AMPK in skeletal muscle cells. Exposure to Dio for up to 3 h increased levels of phosphorylated AMPK in a dose-dependent manner, reaching a maximum level at 40 μM (Fig. 6). This indicated that Dio triggered activation of AMPK and switched on a catabolic pathway in skeletal muscle cells. Taken together, these effects of Dio on skeletal muscle may contribute to reduced visceral fat in Dio-supplemented rats.

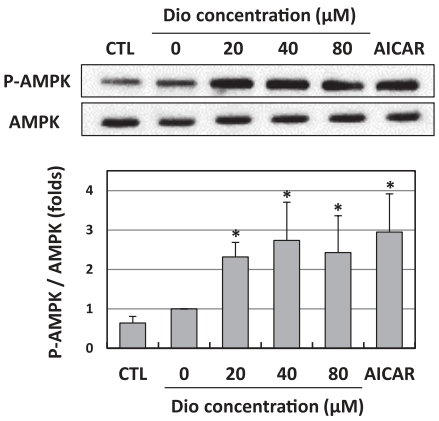

Fig. 6. Dio activates AMPK signaling in differentiated C2C12 cells. C2C12 myotubes were incubated with Dio (0, 20, 40, and 80 μM) or AICAR (1 mM) for 3 h prior to immunoblot analysis with the indicated antibodies. Upper panel shows representative immunoblots, lower panel shows quantification. Data are shown as mean±SD of five independent experiments. Asterisks indicate statistically significant differences from 0 μM Dio (Student's t test, $^*p<0.05$).

DISCUSSION

The present study demonstrated that supplementation with 0.5% Dio during high-Chol feeding for 6 wk led to elevation of both HDL-Chol concentration and fecal Chol content, inhibition of visceral fat accumulation, and expansion of skeletal muscle fibers. Furthermore, using C2C12 cells, it was demonstrated that Dio induced skeletal muscle cell differentiation by promoting fusion of myoblasts and triggered phosphorylation of AMPK in myotubes. These results suggest that the effects of Dio on skeletal muscle might regulate whole-body energy balance and contribute to an inhibition of visceral fat accumulation.

We previously report that supplementation of 0.5% Dio for 4 wk resulted in a slight elevation of serum HDL-Chol concentration, even in rats that already had low serum HDL-Chol concentrations, when fed a chronic high-Chol diet (*8*). Taken together, dietary Dio may elevate serum HDL-Chol concentrations, regardless of baseline HDL serum levels. Other studies also showed that Chol absorption was significantly decreased, and fecal Chol excretion was significantly increased in mice and rats treated with Dio (*12*, *13*). Using a mouse knockout model for Niemann-Pick C1-like 1, which was identified as an essential protein for intestinal Chol absorption, dietary Dio was shown to stimulate fecal sterol secretion independent of intestinal Chol adsorption (*14*). In addition, a study using mice deficient for ATP binding cassette half-transporter G8 (ABCG8), which mediates normal hepatobiliary secretion of Chol with ABCG5, Dio supplementation failed to increase biliary Chol secretion and had no effect on hepatic ABCG5/G8 expression at both the mRNA and protein levels (*15*). Therefore, it is assumed that Dio does not directly interact with ABCG5/G8, but increases Chol delivery to the heterodimer (*15*, *16*). Phytosterols compete with Chol during micelle formation and have been identified as inhibitors of Chol absorption, indicating that their intake is inversely correlated with serum LDL-Chol concentration (*17*). However, Dio was assumed to trigger an increase in biliary Chol secretion via ABCG5/G8 in the liver and result in inhibition of a high-Chol diet-induced increase in serum LDL-Chol and HDL-Chol concentrations.

We previously also reported that Dio supplementation of chronically high-Chol diet-fed rats only resulted in a reduction of lipid droplets in hepatocytes, with no effect on liver TG content (*8*). However, the present study showed that consumption of a Dio-supplemented diet for 6 wk led to a reduction in not only the size and number of hepatocyte lipid droplets, but also liver TG, visceral fat (including fat around the kidneys, testicles, and posterior stomach wall), and serum TG concentration (Figs. 2A, C and D, Table 2). Furthermore, we also showed that orally administered Dio resulted in enhanced skeletal muscle fibers in rats (Fig. 4), and that treatment with Dio led not only to myoblast fusion in proliferating C2C12 cells (Fig. 5), but also to phosphorylation of AMPK in differentiated C2C12 cells (Fig. 6). In addition, we have obtained preliminary results that Dio activates molecules downstream of AMPK in the C2C12 cells. Skeletal muscle accounts for nearly half the body weight of non-obese individuals and functions as the predominant organ for energy expenditure and locomotion (*18*). These functions require contractile muscle fibrils in the cytoplasm of muscle fibers, which are mitotically inactive large multinucleated cells, and are influenced by skeletal muscle fiber mass (*19*). A critical event in muscle growth is the fusion of myoblasts, either with each other or with existing myofibers, to generate and enlarge the multinucleated myofibers (*20*). In the present study, Dio supplementation accelerated fusion of myoblasts to generate new multinucleated myofibers at the cellular level (Fig. 5). This effect of Dio on skeletal muscle cells may reflect the significant increase in the diameter, area, and number of skeletal muscle fibers in rats fed a Dio-supplemented diet (Fig. 4). However, it remains unclear how Dio facilitates plasma membrane fusion involving complex processes, such as recognition, adhesion, cell signaling, cytoskeletal alterations, and membrane rearrangement.

AMPK is a serine/threonine heterotrimeric kinase in the cytoplasm and is an important regulator of cellular and whole-body energy homeostasis (*21*, *22*). It comprises an α-catalytic subunit in combination with β-scaffolding and γ-regulatory subunits, and is activated by Thr172 phosphorylation on the α-subunit via upstream kinases, such as liver kinase B1 and Ca^{2+}/calmodulin-dependent protein kinase kinase-β, with an accompanying conformational change upon binding of AMP to the γ-subunit. Although several natural AMPK activators exist, such as resveratrol (*23*, *24*), curcumin (*25*), epigallocatechin-3-gallate (*26*), and D-xylose (*27*), Dio may be one of those activators. Once activated, AMPK phosphorylates downstream substrates to inhibit anabolic pathways that consume ATP and activate catabolic pathways that generate ATP, and regulates transcription factors and their coactivators involved in gene expression and nuclear events that lead to metabolic reprogramming and cell survival (*28*, *29*). Dio may play roles in developing skeletal muscle as the predominant organ of energy metabolism via myoblast fusion, as well as upregulating catabolism in muscle fibers by activating the AMPK pathway. Furthermore, in this experiment, we found that higher blood concentrations of bile acids such as cholic acid, β-muricholic acid, and ω-muricholic acid, which are murine bile acids produced from chenodeoxycholic acid, in the Chol+Dio group compared with the Chol group (data not shown). Bile acids are primarily synthesized in the liver from Chol and play well-established roles in dietary lipid absorption and Chol homeostasis. Additionally, bile acids were recently revealed to also have systemic endocrine functions as signaling molecules, activating the G-protein-coupled receptor, TGR5, and nuclear hormone receptors, such as farnesoid X receptor α (*30*–*34*). These signaling pathways regulate diverse metabolic pathways such as glucose metabolism, lipid metabolism, and energy expenditure. Dio may have both direct and indirect effects on energy expenditure, and contribute to

the reduction of visceral fat.

Dio is a safe compound with an oral toxicity dose (LD_{50}) of >8,000 mg/kg in mice, rats, and human (5), and the present study concluded that the additive amount of Dio was safe. Furthermore, oil solvents lead to higher and more persistent levels of Dio in the plasma and brain compared with water solvents (5), showing that the oral bioavailability of Dio is influenced by dietary conditions. In view of these facts, further studies are required to establish effective methodology for dose and duration of Dio supplementation.

Disclosure of state of COI

There are no conflicts of interest to be declared.

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