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RESEARCH ARTICLE

Diacylglycerol kinase δ is required for skeletal muscle development and regeneration

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Abstract

Diacylglycerol kinase δ (DGKδ) phosphorylates diacylglycerol to produce phosphatidic acid. Previously, we demonstrated that down-regulation of DGKδ suppresses the myogenic differentiation of C2C12 myoblasts. However, the myogenic roles of DGKδ in vivo remain unclear. In the present study, we generated DGKδ-conditional knockout mice under the control of the myogenic factor 5 (Myf5) gene promoter, which regulates myogenesis and brown adipogenesis. The knockout mice showed a significant body weight reduction and apparent mass decrease in skeletal muscle, including the tibialis anterior (TA) muscle. Moreover, the thickness of a portion of the myofibers was reduced in DGKδ-deficient TA muscles. However, DGKδ deficiency did not substantially affect brown adipogenesis, suggesting that Myf5-driven DGKδ deficiency mainly affects muscle development. Notably, skeletal muscle injury induced by a cardiotoxin highly up-regulated DGKδ protein expression, and the DGKδ deficiency significantly reduced the thickness of myofibers, the expression levels of myogenic differentiation markers such as embryonic myosin heavy chain and myogenin, and the number of newly formed myofibers containing multiple central nuclei during muscle regeneration. DGKδ was strongly expressed in myogenin-positive satellite cells around the injured myofibers and centronucleated myofibers. These results indicate that DGKδ has important roles in muscle regeneration in activated satellite cells. Moreover, the conditional knockout mice fed with a high-fat diet showed increased fat mass and glucose intolerance. Taken together, these results demonstrate that DGKδ plays crucial roles in skeletal muscle development, regeneration, and function.

KEYWORDS

diacylglycerol kinase, glucose tolerance, muscle development, muscle regeneration, satellite cell, type II diabetes

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1 | INTRODUCTION

Diacylglycerol kinase (DGK) is a lipid-metabolizing enzyme that phosphorylates diacylglycerol (DG) to produce phosphatidic acid (PA). Both DG and PA are known as lipid second messengers. For example, DG activates several signaling proteins, such as conventional protein kinase Cs (PKCs), novel PKCs, Ras guanyl nucleotide-releasing proteins, and chimaerins.[1–3] Moreover, recent studies have reported that PA regulates many functional proteins (e.g., Praja-1, the E3 ubiquitin ligase acting on the serotonin transporter in the brain, and α-synuclein, a protein associated with Parkinson's disease)[4–6] in addition to previously known signaling proteins such as phosphatidylinositol-4-phosphate 5-kinase and mammalian targets of rapamycin.[7–9] Therefore, DGK is thought to regulate a wide variety of biological processes in mammalian cells by controlling the balance of these signaling lipids.[10–13]

To date, 10 DGK isozymes (α, β, γ, δ, η, κ, ε, ζ, ι, and θ) have been identified in mammals, which are grouped into five subtypes (type I–V) based on their structural features.[10–13] DGKδ[14] belongs to the type II DGK family (δ, η, and κ isoforms),[15] and alternatively spliced variants (δ1 and δ2) of DGKδ have been identified.[16] DGKδ2 is especially abundant in the brain and testis[17,18] and is highly expressed in skeletal muscle myoblasts.[19] In contrast, DGKδ1 expression levels are generally low in various tissues and cell lines.[16]

DGKδ plays an important role in the skeletal muscle, which is a major insulin-target organ for glucose disposal.[20] Chibalin et al. reported that a decrease in DGKδ protein level suppresses insulin-mediated glucose uptake in the skeletal muscle, exacerbating the severity of type II diabetes.[21] Moreover, Miele et al. showed that DGKδ is activated in response to high glucose levels and controls glucose uptake into muscle cells by reducing PKCα activity.[22] Interestingly, exercise training increases expression levels of DGKδ, in addition to increasing muscle mass and improving insulin sensitivity.[23,24] Moreover, we have demonstrated that DGKδ consumes DG species containing palmitic acid (16:0), which was reported to be involved in type II diabetes pathogenesis through PKC activation[25] in C2C12 myotubes.[26] These studies indicate that DGKδ plays a key role in glucose uptake in skeletal muscles.

Recently, we have demonstrated a new function of DGKδ in skeletal muscle cells. DGKδ is highly expressed in myoblasts, and suppression of DGKδ expression inhibited myogenic differentiation.[19] In addition, myristic acid (14:0), a saturated fatty acid that up-regulates DGKδ protein levels in myoblasts/myotubes[27–29] and skeletal muscles,[30] increases β-tubulin levels to promote muscle development in a DGKδ-dependent manner.[31] These findings imply that DGKδ is associated with the differentiation and development of skeletal muscles. However, the functions of DGKδ in vivo remain unclear.

In the present study, to reveal the functions of skeletal muscle-related DGKδ in vivo, we generated conditional DGKδ-knockout mice under the control of the myogenic factor 5 (Myf5) gene promoter, which regulates myogenesis and brown adipogenesis because conventional (whole-body) DGKδ-knockout mice die within 24 h of birth.[32] We found that the deficiency of DGKδ, which was localized to muscle satellite cells, caused a reduction in muscle mass and attenuated the recovery of muscle injury.

2 | MATERIALS AND METHODS

2.1 | Mice

This study was approved by the Ethical Committee for Animal Research of Shimane University (permission number: IZ5-13-1). *Dgkd*-deleted mice (*Myf5-Cre*; *Dgkd*$^{flox/flox}$ mice) were generated by crossing *Dgkd*$^{flox/flox}$ mice[33] with *Myf5-Cre* mice (B6.129S4-*Myf5*$^{tm3(cre)Sor}$/J, Jackson Laboratory, Bar Harbor, ME, USA). The mice between 5 and 29 weeks after birth were used in this study and had age-matched littermate controls. PCR for genotyping was carried out using the following primers: *Myf5-Cre* (5'-CGTAGACGCCTGAAGAAGGTCAACCA -3′ and 5'-ACGAAGTTATTAAGGTCCCTCGAC-3′) and *Dgkd-loxP* (5'-TCCTACCTCTCTCTCCATTCCC-3′ and 5' -AAGGTGTTGAATAATACTCTGTGAC-3′). Mice were housed under a 12-h light/dark cycle and fed a standard diet (NMF; Oriental Yeast, Tokyo, Japan). To induce obesity, several 5-week-old mice were fed a high-fat diet (HFD, D12492; Research Diets, New Brunswick, NJ, USA) for 20 weeks. All procedures were performed according to the animal care guidelines of the committee. For measurement of tissue weights, all tissues were dissected after euthanasia and were immediately weighted using an analytical scale.

2.2 | Western blotting

Mice were euthanized with isoflurane, and tibialis anterior (TA) muscles and brown adipose tissues (BATs) were isolated. The samples were lysed in ice-cold lysis buffer (7 M urea, 0.1% Nonidet P-40, 500 mM triethylammonium bicarbonate buffer (Sigma–Aldrich, St. Louis, MO, USA), and Complete™ protease inhibitor (Roche Diagnostics, Mannheim, Germany)). The homogenates were centrifuged and the supernatants (50 μg lysate protein) were separated on SDS – PAGE gels for western blotting with anti-DGKδ,[14] anti-DGKζ (ab105195, Abcam, Cambridge,

UK), anti-Glut4 (ab33780, Abcam), anti-β-tubulin (2146, Cell Signaling Technology, Danvers, MA, USA), anti-β-actin (4970, Cell Signaling Technology), anti-GAPDH (010–25521, Wako Pure Chemicals, Osaka, Japan), anti-fatty acid synthase (FASN; sc-48357, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-peroxisome proliferator-activated receptor γ (PPARγ; 2443, Cell Signaling Technology), anti-uncoupling protein-1 (UCP1; U6382, Sigma–Aldrich), anti-embryonic myosin heavy chain (eMyHC; F1.652, Developmental Studies Hybridoma Bank, Iowa City, IA, USA), anti-Cyclin D3 (sc-182, Santa Cruz Biotechnology), anti-MyoD (sc-304, Santa Cruz Biotechnology), and anti-myogenin (ab124800, Abcam) antibodies. The immunoreactive bands were visualized using horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antibodies (Cell Signaling Technology) and Pierce™ ECL Western Blotting Substrate (Thermo Scientific, Waltham, MA, USA). The intensity of each band was measured using Amersham™ ImageQuant™ 800 (GE Healthcare, Chicago, IL, USA).

2.3 | Skeletal muscle injury and regeneration

For muscle regeneration studies, 50 μL of 10 μM cardiotoxin (CTX, Latoxan, Valence, France, diluted in phosphate-buffered saline) was injected into the right TA muscles. The mice were maintained for 5 or 7 days after injection to assess the effect of DGKδ-deficiency during muscle regeneration.

2.4 | Histological analyses

Mice were euthanized with isoflurane for histological analyses, and TA muscles and BATs were isolated. The samples were fixed with 10% formalin, embedded in paraffin, and sectioned at 4 μm. The sections were stained with hematoxylin and eosin (HE) and observed using a light microscope, Promostar3 (Carl Zeiss, Oberkochen, Germany). For measurement of sizes of immature myofibers with centrally located nuclei, images (one section from one mouse) were captured with 10× magnification using ZEN lite software (Carl Zeiss). The number of central nuclei and sizes of 69 myofibers from one section were measured using ZEN lite software (Carl Zeiss).

2.5 | Immunofluorescence

Mice were also euthanized with isoflurane and TA muscles were isolated. The samples were fixed with 10% formalin, immersed in 30% sucrose, and embedded in an OCT compound (Sakura Finetek, Torrance, CA, USA). Cryostat cross-sections were cut (7 μm thick), permeabilized with 0.2% Triton X-100 in phosphate-buffered saline, and blocked with blocking buffer (3% bovine serum albumin in phosphate-buffered saline). The sections were incubated with anti-DGKδ antibodies in the blocking buffer overnight at 4°C, and fluorescence signals were visualized using Alexa 594-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA). For wheat germ agglutinin (WGA) or myogenin staining, the samples were labeled with Alexa 647-conjugated WGA (W32466, Invitrogen, Carlsbad, CA, USA) or Alexa 488-conjugated anti-myogenin antibody (eBioscience, Thermo Fisher Scientific). These sections were mounted with ProLong® Diamond Antifade Mountant containing 4′,6-diamidino-2-phenylindole (Thermo Fisher Scientific). The slides were examined using a confocal laser scanning microscope (FLUOVIEW FV3000, Olympus, Tokyo, Japan). For measurement of myofiber sizes by WGA staining, images (one section from one mouse) were captured with 10× magnification using FV31S-SW software (Olympus). The sizes of 78 myofibers from one section were measured using ZEN lite software (Carl Zeiss).

2.6 | Glucose tolerance test

To assess glucose tolerance, mice were fasted for 15 h and then received an intraperitoneal injection of glucose (1 g/kg body weight). Blood was collected at consecutive time points (30, 60, 90, and 120 min) and blood glucose levels were measured using an animal glucometer (LAB Gluco, Tokyo, Japan).

2.7 | Statistical analysis

The data are presented as means ± SE. Statistical analysis was performed using a two-tailed *t*-test to compare two groups, and a *p*-value <0.05 was considered significant.

3 | RESULTS

3.1 | DGKδ knockout under the control of the Myf5 gene promoter results in a reduction of body weight and skeletal muscle mass

Myf5 is expressed specifically in the progenitor cells that differentiate into skeletal muscle cells and brown adipocytes.[34] Therefore, to elucidate the in vivo function of

DGKδ in skeletal muscles, the DGKδ gene was disrupted in skeletal muscles by crossing *Dgkd*$^{flox/flox}$ mice with *Myf5-Cre* mice. The generated mice were born at expected Mendelian ratios. The male mice exhibited a 4.5% reduction in body weight at the age of 5 weeks and showed further body weight losses at the age of 16 and 24 weeks (7.9% and 7.8% decreases, respectively) (Figure 1A). The change in the body weight of female mice was relatively small compared to that of male mice. Therefore, in this study, male mice were used in subsequent experiments.

We next measured organ and tissue weights in DGKδ-deficient mice. At 5 weeks of age, the weights of TA and extensor digitorum longus (EDL) muscles of DGKδ-deficient mice were slightly but significantly decreased (Figure 1B). The weights of gastrocnemius (Gas) and soleus (So) muscles also decreased. As a result, the total weight of the four muscles decreased by 6.7% (Figure 1B). However, the weights of other tissues, such as the brain and liver, in which DGKδ is expressed,[17] were not changed (Figure 1B). In addition, at 24 weeks of age, we clearly observed a weight loss in four muscles (TA: 10.7% decrease, EDL: 10.5% decrease, Gas: 12.3% decrease, and So: 13.8% decrease, respectively) as well as a reduction in the total muscle weight (11.9% decrease) (Figure 1C). These results indicate that DGKδ deficiency causes the reduction of muscle weight in an age-dependent manner. Moreover, the weights of interscapular BATs (iBATs) at 5 and 24 weeks of age also showed a decreasing tendency (Figure 1B,C).

3.2 | DGKδ deficiency affects the development of myofibers but not BAT

We performed western blotting to elucidate the effects of DGKδ knockout on TA muscles and iBATs. Although DGKδ protein levels were low in TA muscles in 5-week-old control mice, the protein levels were significantly decreased by 40.0% in TA muscles in the DGKδ-deficient mice (Figure 2A). We were not able to detect DGKδ protein in 24-week-old mice (Figure 2B), suggesting that DGKδ protein levels are reduced in mature myofibers. The expression levels of Glut4, which is a critical factor for glucose uptake, and β-tubulin, which is associated with muscle development, were also low and not affected by DGKδ deficiency at both 5 and 24 weeks of age (Figure 2A,B). We failed to detect the expression of differentiation markers such as myogenin and cyclin D3 at both 5 and 24 weeks of age in control mice because their expression levels were also low (data not shown).

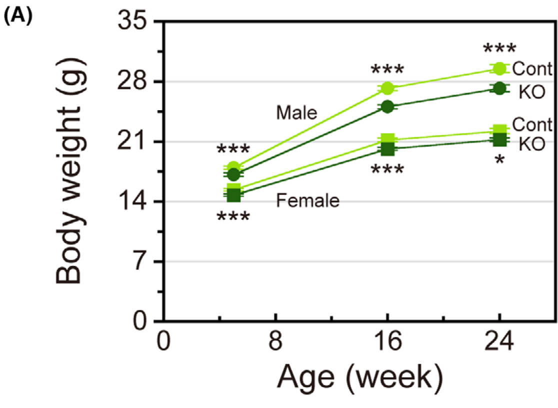
Therefore, we assessed the morphological changes of TA muscles in DGKδ-deficient mice. As shown in Figure 2C, the average minimum Feret diameters of whole TA muscle fibers of the DGKδ-deficient mice was the same as that of control mice. However, the number of medium-size myofibers (35–40 μm diameter) was decreased, and the number of smaller myofibers (30–35 μm diameter) was conversely increased (Figure 2C). On the other hand, the number of myofibers of 25–30 μm diameter was decreased in the DGKδ-deficient mice but that of larger myofibers (more than 40 μm diameter) was not affected (Figure 2C). These results imply that DGKδ deficiency disturbs and delays the development of myofibers in TA muscles.

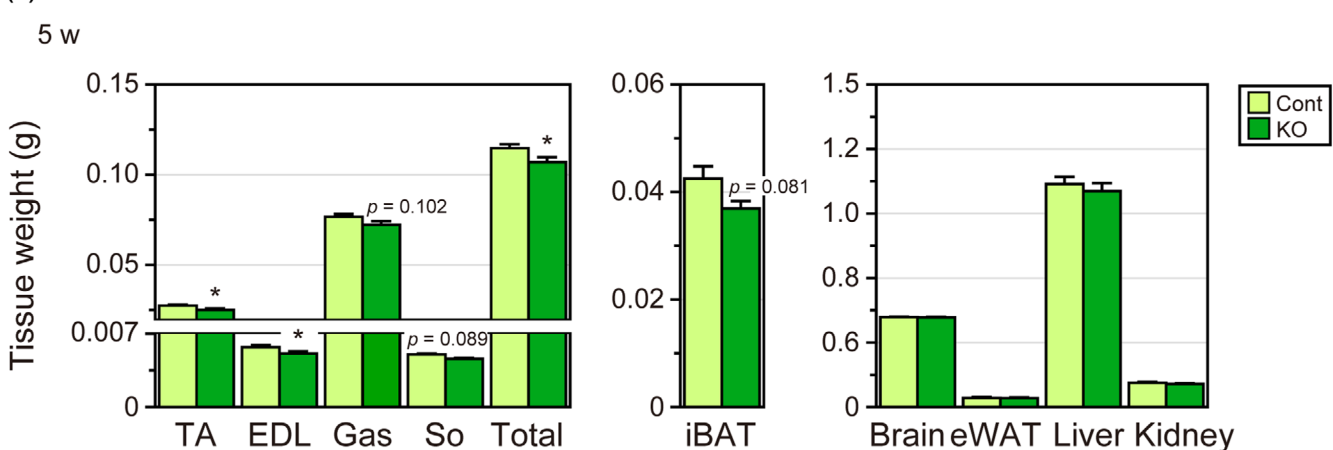
Among other DGK isozymes, DGKζ is abundantly expressed in skeletal muscle and induces muscle fiber hypertrophy,[35,36] Thus, we investigated the expression of DGKζ at 5 weeks of age in the DGKδ-deficient mice. The expression levels of DGKζ in DGKδ-KO TA tended to increase without statistical significance (Figure 2D), suggesting that DGKδ-KO has moderate compensatory effects of on the expression of skeletal muscle-related DGKζ.

DGKδ protein levels in iBATs of the DGKδ-deficient mice at 5 and 24 weeks of age were decreased by 41.6% (Figure 3A) and 59.4% (Figure 3B), respectively. However, we failed to detect changes in protein levels for FASN, PPARγ, and UCP1 (Figure 3A,B), which are an essential factor for lipid synthesis, a maker of adipocyte differentiation and a thermogenic marker, respectively. The levels of Glut4, β-tubulin, and β-actin were also unchanged (Figure 3A,B). Moreover, HE staining for iBAT did not show obvious differences between the DGKδ-deficient and control mice (Figure 3C).

3.3 | DGKδ deficiency attenuates muscle regeneration after injury

DGKδ protein levels were low in mature TA muscles in adult mice (Figure 2B). In contrast, DGKδ is highly expressed in C2C12 myoblasts (immature skeletal muscle cells).[19] Therefore, to investigate whether DGKδ plays a role in the function of skeletal muscle precursor cells (satellite cells), the myonecrotic agent CTX was injected into TA muscles of 29-week-old DGKδ-deficient and control mice and then muscle regeneration was analyzed. Both the control and DGKδ-deficient mice already displayed a large number of newly formed immature myofibers with centrally located nuclei during the regeneration 7 days after CTX injection. However, in TA muscles of the DGKδ-deficient mice, the ratio of myofibers containing two or more central nuclei was significantly decreased (Figure 4A,B). The average minimum Feret diameters of whole TA muscle fibers was also decreased in DGKδ-deficient mice (Figure 4A,C). In addition, the number of myofibers of 25–30 μm diameter was decreased, whereas that of smaller myofibers





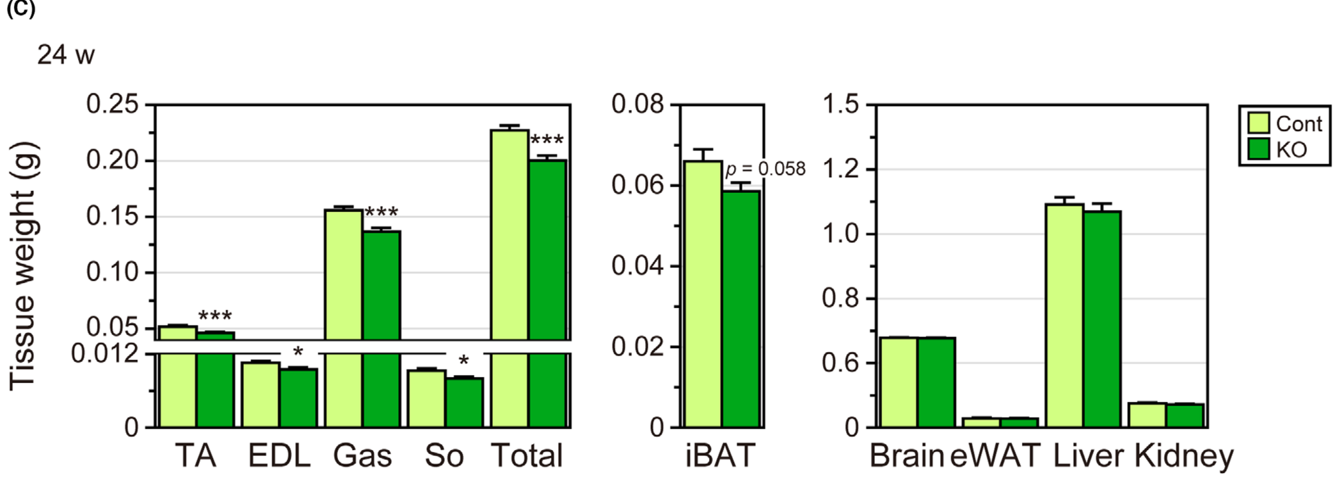
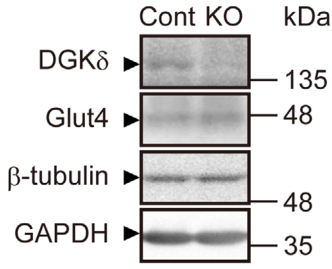
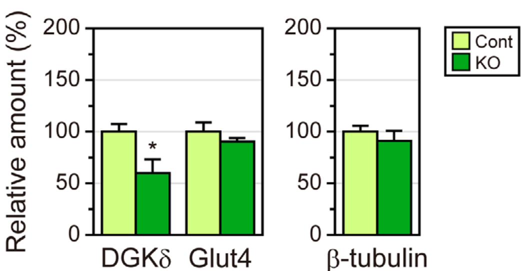


FIGURE 1 Body and tissue weights of DGKδ-deficient mice. (A) Body weights of mice from 5 to 24 weeks of age (male control mice: $N = 55$ (at 5 week age), 39 (at 16 week age), and 19 (at 24 week age), female control mice: $N = 52$ (at 5 week age), 36 (at 16 week age), and 31 (at 24 week age), male DGKδ-deficient mice: $N = 58$ (at 5 week age), 44 (at 16 week age), and 24 (at 24 week age), female DGKδ-deficient mice: $N = 57$ (at 5 week age), 31 (at 16 week age), and 26 (at 24 week age). (B, C) The weights of TA, EDL, Gas, and So from right hindlimb, iBAT, brain, right eWAT, liver, and right kidney at 5 (B, control mice: $N = 13$, DGKδ-deficient mice: $N = 11$) and 24 weeks of age (C, control mice: $N = 8$, DGKδ-deficient mice: $N = 10$). Values are presented as means ± SE. *$p < 0.05$ and ***$p < 0.005$.

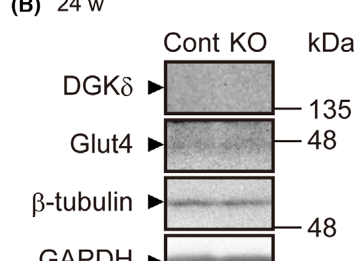
of 15–20 μm diameter increased (Figure 4A,C). These results indicate that DGKδ deficiency attenuates muscle regeneration after injury. Interestingly, DGKδ protein levels were markedly augmented and became clearly detectable 5 days after the injury in the TA muscles of control mice (Figure 4D). Moreover, the protein levels

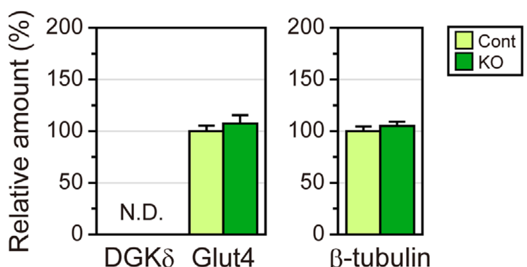
(A) 5 w



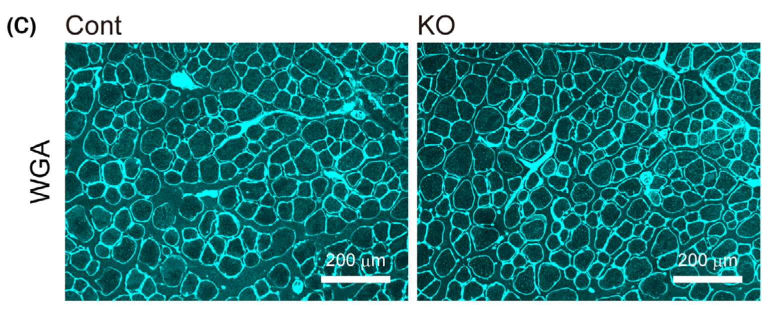


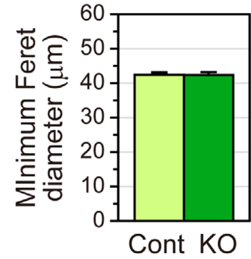
(B) 24 w

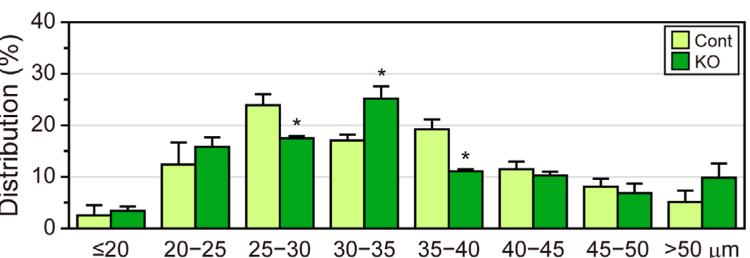




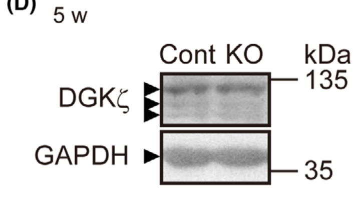
(C)







(D) 5 w



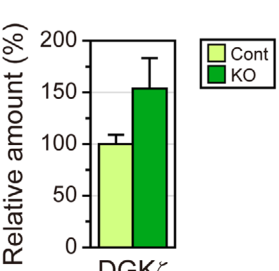


FIGURE 2 Expression of DGKδ, Glut4, β-tubulin, DGKζ, and GAPDH and diameter distribution of myofibers in TA muscles of DGKδ-deficient mice. (A, B) The expression of each protein in TA muscles at 5 (A, control mice: $N=4$, DGKδ-deficient mice: $N=4$) and 24 weeks of age (B, control mice: $N=8$, DGKδ-deficient mice: $N=7$) was detected by western blotting. Each protein level was normalized to the GAPDH level. The control protein levels were set to 100%. Values are presented as means ± SE. (C) Myofibers in TA muscle at 20 weeks of age were immunostained using WGA, and the representative images are shown. The minimum Feret diameters of 234 myofibers from three control and DGKδ-deficient mice were measured and the distribution is shown. (D) The expression of DGKζ in TA muscles at 5 weeks of age (control mice: $N=4$, DGKδ-deficient mice: $N=4$) was detected by western blotting. DGKζ protein level was normalized to the GAPDH level. The control protein levels were set to 100%. Values are presented as means ± SE. $^*p<0.05$.

in the DGKδ-deficient mice were decreased by 25.3% (Figure 4E). Furthermore, the expression levels of eMyHC, myogennin, and β-actin, which are markers of muscle regeneration, were significantly decreased by 31.3%, 17.2%, and 21.1%, respectively, in TA muscles with DGKδ deficiency 5 days after injection (Figure 4E). These results indicate that DGKδ deficiency attenuates differentiation and development during muscle regeneration after injury.

In addition, we performed immunostaining of DGKδ in control mice. The centronucleated myofiber is known to be a hallmark of muscle regeneration, indicating newly formed myofibers. As shown in Figure 4F, it was confirmed that although our anti-DGKδ antibody showed only weak staining in injured DGKδ-deficient mouse skeletal muscles, the antibody more strongly stained the injured skeletal muscles in control mice. The immunostaining showed that DGKδ was localized to and highly expressed in myogenin-positive satellite cells around injured mature myofibers and in newly formed immature myofibers with central nuclei but not in mature myofibers (Figure 4F). The activated satellite cells (myogenic cells) form the new myofibers due to their fusion and repair of the injured myofibers.[37,38] Therefore, these results indicate that DGKδ, strongly expressed in satellite cells, plays an important role in muscle regeneration.

3.4 | DGKδ deficiency in skeletal muscle and BAT reduces glucose tolerance

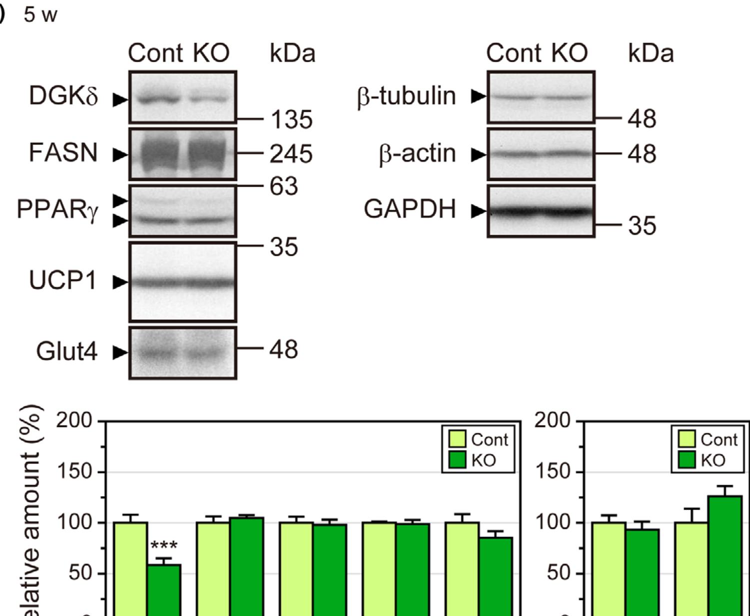
Previously, it has been reported that whole-body DGKδ-hetero knockout mice show induction of obesity and reduction of glucose tolerance.[21] To confirm whether skeletal muscle- and BAT-specific conditional DGKδ-knockout mice induce obesity and reduce glucose tolerance, the mice were fed with HFD containing 60 kcal% fat and 20 kcal% carbohydrates from 5 to 20 weeks of age. We first checked the body and skeletal muscle weights. The DGKδ-deficient mice showed body weight loss of around 10% from 5 to 11 weeks of age (Figure 5A). However, no significant change in body weights was observed from 13 to 24 weeks of age (Figure 5A). The total weight of the four muscles in the HFD-fed DGKδ-deficient mice at 25 weeks of age decreased by 5.1% (Figure 5B) as observed with the normal diet-fed knockout mice (Figure 1C). Although the weight losses of the TA and EDL muscles were small, those of Gas and So muscles were significant (Figure 5B). In addition, the weight of iBATs also showed a decreasing tendency (Figure 5B).

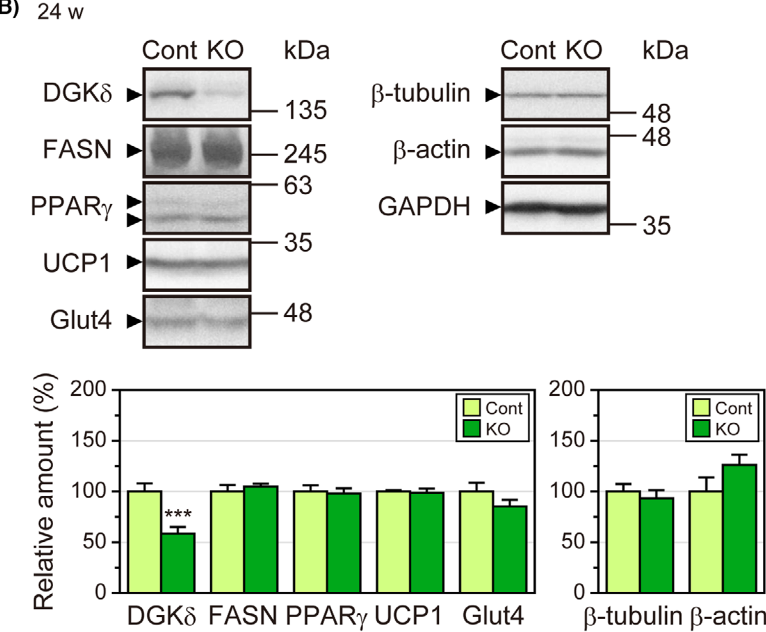
Next, we measured the weight of epididymal white adipose tissues (eWATs), an indicator of obesity. The eWAT weight showed an increasing tendency (Figure 5B). Moreover, the ratio of eWAT weight/body weight was significantly increased (Figure 5C). These results suggest that DGKδ deficiency is associated with obesity in HFD-fed mice.

Moreover, the glucose tolerance test showed that skeletal muscle- and BAT-specific DGKδ deficiency significantly increased blood glucose levels, indicating that the DGKδ deficiency reduced glucose tolerance (Figure 5D). Moreover, the calculated area under the curve (AUC) was markedly increased by 40.9% by DGKδ-deficiency (Figure 5D). These results indicate that skeletal muscle- and BAT-specific conditional DGKδ-knockout mice also show the induction of obesity and reduction of glucose tolerance, like whole-body DGKδ-hetero knockout mice.[21]

4 | DISCUSSION

We generated DGKδ-deficient mice by disrupting the DGKδ gene in skeletal muscle and BAT using a Myf5-Cre/LoxP system. The generated mice showed age-dependent reductions in body weight and skeletal muscle mass (Figure 1). Moreover, we observed that DGKδ-deficiency affected myofiber diameter (Figure 2C), suggesting that DGKδ deficiency disturbs the development of myofibers. Further analyses demonstrated inhibition of muscle regeneration after injury in the knockout mice (Figure 4A–C,E). In addition, DGKδ, whose expression was induced by muscle injury (Figure 4D), was strongly expressed in myogenin-positive satellite cells (skeletal muscle precursor cells) around injured myofibers and newly formed immature myofibers with central nuclei but not in mature myofibers (Figure 4F). Therefore, in the present study, we concluded that DGKδ plays an essential role





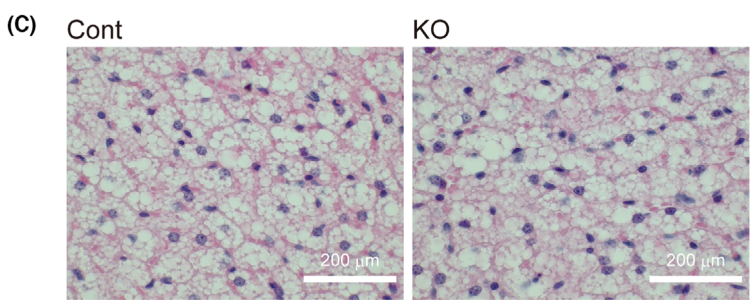


FIGURE 3 Expression of DGKδ, FASN, PPARγ, UCP1, Glut4, β-tubulin, β-actin, and GAPDH in iBAT of DGKδ-deficient mice. A and B, The expression of each protein in iBATs at 5 (A, control mice: $N = 6$, DGKδ-deficient mice: $N = 6$) and 24 weeks of age (B, control mice: $N = 7$, DGKδ-deficient mice: $N = 9$) were detected by western blotting. Each protein level was normalized to the GAPDH level. The control protein levels were set to 100%. Values are presented as means ± SE. (C) iBATs at 24 weeks of age were stained with HE, and the representative images are shown. ***$p < 0.005$ versus control mice.

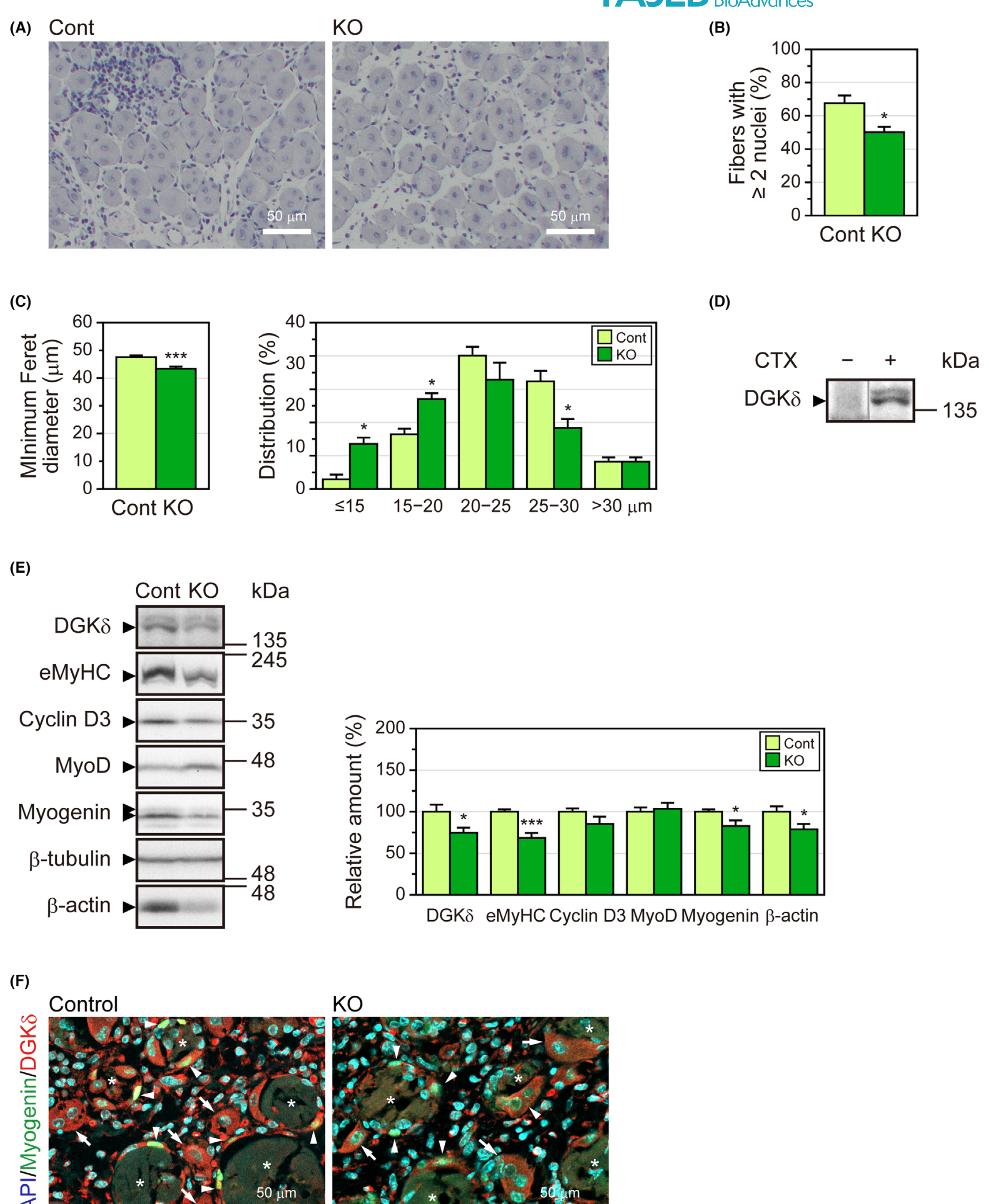


FIGURE 4 Legend on next page

in skeletal muscle development and maintenance in addition to glucose uptake, which is a previously known DGKδ function.[21,28]

In our present study, although we detected the expression of DGKδ protein in the TA muscles of control mice at 5 weeks of age (Figure 2A), the protein was not detectable

FIGURE 4 Muscle regeneration after injury in DGKδ-deficient mice. (A) CTX was injected into TA muscles of 29-week-old mice, and the sections of TA muscles at seven days after injection were stained with HE. The centronucleated myofiber is a hallmark of muscle regeneration, indicating newly formed myofibers. The representative images are shown. (B) The percentage of myofibers with ≥2 central nuclei from three control and DGKδ-deficient mice was calculated. (C) Minimum Feret diameters of 207 myofibers were measured, and their distribution is shown. Values are presented as means ± SE. $*p < 0.05$ and $***p < 0.005$. (D, E) CTX was injected into TA muscles of 13 to 24-week-old mice (control mice: $N = 9$, DGKδ-deficient mice: $N = 11$). (D) DGKδ and myogenin in TA muscles of control and DGKδ-deficient mice at five days after injection was detected by western blotting. The representative images are shown. The DGKδ protein level with CTX treatment was set to 100%. (E) DGKδ, eMyHC, cyclin D3, MyoD, Myogenin, β-tubulin, and β-actin in TA muscles five days after injection were detected by western blotting. Each protein level was normalized to the β-tubulin level. The control protein levels were set to 100%. Values are presented as means ± SE. $*p < 0.05$ and $***p < 0.005$. (F) CTX was injected into TA muscles at 20-week-old control mice. TA muscles five days after injection were immunostained with anti-DGKδ antibody. DGKδ was expressed in myogenin-positive satellite cells (arrowhead) around injured mature myofibers (asterisk) and newly formed myofibers with central nuclei (arrow). The representative image is shown.

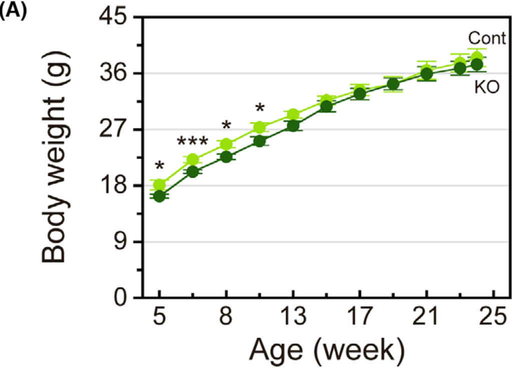
in 24-week-old mice (Figure 2B). Previously, we demonstrated that DGKδ is highly expressed in C2C12 myoblasts, which are immature skeletal muscle cells, and that DGKδ is necessary for differentiation into myotubes.[19] This suggests that DGKδ play a role in activated myosatellite cells, which are precursors of skeletal muscle cells. In the present study, we observed that DGKδ protein expression was augmented in muscles injured by CTX (Figure 4D) and that the protein was strongly expressed in satellite cells (Figure 4F). These results imply that DGKδ protein expression is muscle injury-dependent and is induced in activated satellite cells, but not in quiescent satellite cells, for muscle regeneration. In fact, in injured TA muscles, the expression levels of eMyHC, myogenin, and β-actin, which are markers of muscle differentiation and development, were suppressed in DGKδ-deficient mice (Figure 2E), strongly suggesting that DGKδ is associated with the differentiation of satellite cells to myotubes during muscle regeneration. Previously, we demonstrated that DGKδ regulates the myogenesis of C2C12 myoblasts by suppressing cyclin D1,[19] a key regulator of cell proliferation.[39] However, we did not detect cyclin D1 in TA muscle at even 5 days after CTX injection and, thus, could not analyze the involvement of cyclin D1 in muscle regeneration. In the near future, we will reveal in detail the molecular mechanism of DGKδ-dependent muscle regeneration.

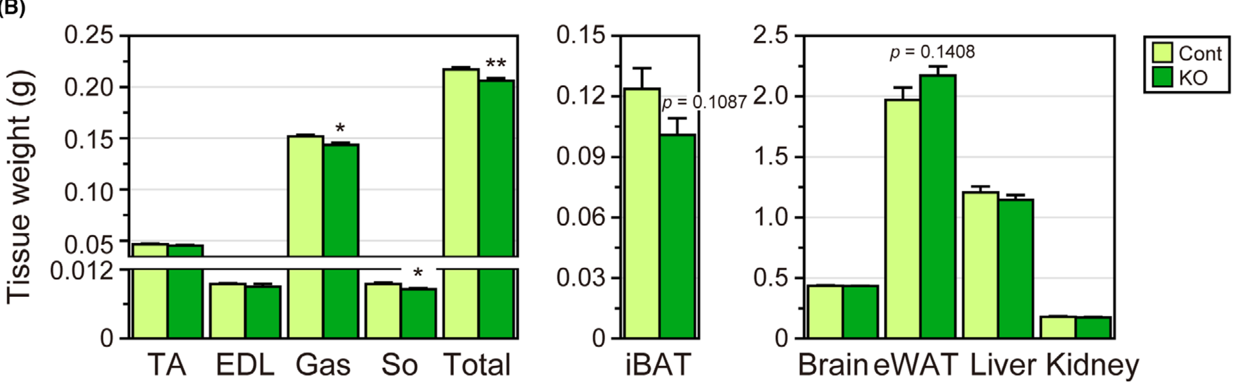
In addition to skeletal muscles, we observed the reduction of DGKδ in iBATs of DGKδ-deficient mice (Figure 3A,B). A previous study reported that DGKδ is associated with the lipogenesis of white adipose tissues, including eWATs.[40,41] However, in iBAT of DGKδ-deficient mice, we did not observe the alteration of FASN and PPARγ, which are markers of lipid synthesis and adipogenesis, respectively (Figure 3A,B). In addition, the expression levels of UCP1, a thermogenic marker, were not changed in DGKδ-deficient iBAT (Figure 3A,B). Furthermore, in iBATs of mice fed with HFD, DGKδ deficiency did not affect the expression of FASN, PPARγ, and UCP1 (data not shown). These results suggest that DGKδ does not affect the adipogenesis and thermogenesis in BAT. However, further research is needed to clarify the DGKδ function in BAT.

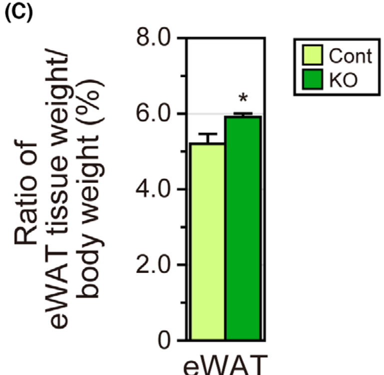
In the present study, the DGKδ-KO mice showed that the DGKδ expression in TA muscles and iBATs is not completely deleted (Figures 2A, 3A and 4E). As shown in Figure 4F, our anti-DGKδ antibody showed weak but clear staining in DGKδ-KO satellite cells and new myofibers, indicating that DGKδ expression in TA muscles and iBATs was not completely deleted by a Myf5-Cre/LoxP system. Moreover, scRNA-seq database for skeletal muscle (http://scmuscle.bme.cornell.edu/) indicates that DGKδ is expressed in endothelial cells and immune cells including macrophages during muscle regeneration.[42] These results imply that DGKδ expression in the DGKδ-KO muscles and iBATs is at least in part explained by contamination of other tissues and cells such as blood vessels and macrophages.

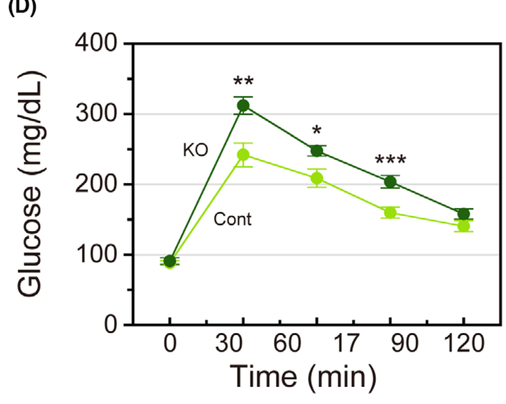
The expression levels of DGKζ in DGKδ-KO TA tended to be increased without statistical significance (Figure 2D). These results suggest that DGKδ-KO has moderate compensatory effects of on the expression of DGKζ. Because DGKζ is abundantly expressed in skeletal muscle and induces muscle fiber hypertrophy,[35,36] increased DGKζ may compensatory attenuate the effects of DGKδ-KO on skeletal muscle.

Chibalin et al. reported that whole-body DGKδ-hetero knockout mice showed an increase in body weight, induction of obesity and a reduction in glucose tolerance.[21] Our skeletal muscle- and BAT-specific conditional DGKδ-knockout mice fed with HFD exhibited an increase in the ratio of eWAT weight/body weight and a reduction in glucose tolerance (Figure 5B–D), in accordance with those of whole-body DGKδ-hetero knockout mice. For the experiments using whole-body DGKδ-knockout mice, we cannot deny the possibilities of the contributions of other tissues/organs instead of the muscle/BAT including









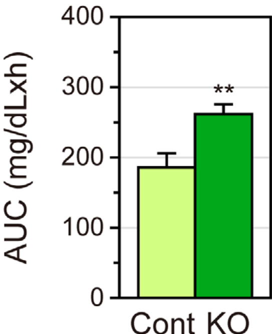


FIGURE 5 Legend on next page

FIGURE 5 Body and tissue weights and glucose tolerance of DGKδ-deficient mice fed with HFD. (A) Body weight at 5–24-weeks of ages (control mice: $N=6$, DGKδ-deficient mice: $N=7$). (B) Weights of TA, EDL, Gas, and So from the right hindlimb, iBAT, brain, eWAT, liver, and right kidney of 25-week-old mice (control mice: $N=6$, DGKδ-deficient mice: $N=7$). (C) The ratio of eWAT tissue weight/body weight. (D) Blood glucose concentrations at 24 weeks of age (control mice: $N=6$, DGKδ-deficient mice: $N=7$) during glucose tolerance test and the glucose areas under the curve (AUC). Values are presented as means ± SE. $*p<0.05$, $**p<0.01$ and $***p<0.005$.

the central nervous system to the induction of obesity and glucose tolerance reduction. However, in the present study, we demonstrated that DGKδ in skeletal muscle and BAT control obesity and glucose tolerance (Figure 5). In contrast to obesity and glucose tolerance, the conditional DGKδ-knockout mice showed a reduction of body weight (Figure 1A), unlike the whole-body DGKδ-hetero knockout mice. DGKδ is expressed in various tissues, such as the brain, liver, kidney, and white adipose tissue.[16,17,40] Therefore, the increase in body weight of whole-body DGKδ-hetero knockout mice may be due to decreased DGKδ expression in other tissues/organs, but not skeletal muscle or BAT.

In the present study, we could not reveal that the reduced glucose tolerance in the DGKδ-deficient mice depends on either skeletal muscle or BAT mass loss/dysfunction because glucose intake activities of TA muscles and iBATs were not examined. However, skeletal muscles are the main organ of glucose uptake.[43] Moreover, a previous report showed the reduction of glucose uptake in EDL and So muscles, but not BAT, under insulin stimulation in whole-body DGKδ-hetero knockout mice.[21] Therefore, the specific loss of DGKδ in skeletal muscles is likely to mainly contribute to hyperglycemia.

Skeletal muscles are the primary organ of glucose intake,[43] and insulin resistance reduces glucose uptake per myofiber and causes type II diabetes. In addition, a reduction in skeletal muscle mass is recognized as a critical factor that leads to insulin resistance. In fact, sarcopenia, which is the loss of skeletal muscle mass caused by aging and/or immobility, is strongly associated with insulin resistance.[44] Consequently, to ameliorate type II diabetes, improvements in both glucose uptake activity per myofiber (quality) and skeletal muscle mass (quantity) are essential. Previously, we demonstrated that myristic acid (14:0), which is a saturated fatty acid, increases the expression levels of DGKδ protein in myotubes and skeletal muscles.[27–29] In addition, myristic acid up-regulates glucose uptake in C2C12 myotubes in a DGKδ-dependent manner[28] and increases glucose tolerance in Nagoya-Shibata-Yasuda mice, which are model mouse for type II diabetes.[30] In the present study, DGKδ-deficient mice showed a reduction in skeletal muscle mass (Figure 1B,C). Therefore, myristic acid is expected to recover both glucose uptake activity per myofiber (quality) and skeletal muscle mass (quantity) through augmentation of DGKδ protein levels. It would be interesting to reveal whether myristic acid improves skeletal muscle loss.

Up-regulation of glucose uptake per myofiber and increased skeletal muscle mass are crucial to improving insulin resistance. In the present study, we demonstrated that DGKδ, which is expressed in activated satellite cells, has an essential role in muscle development and regeneration, in addition to glucose uptake. Therefore, the development of novel drugs and nutrients, including myristic acid, which has dual effects on the improvement of the quality and quantity of skeletal muscles via increasing DGKδ expression, could be useful for the treatment of skeletal muscle-related diseases, including type II diabetes and sarcopenia.

AUTHOR CONTRIBUTIONS

H. Sakai designed and performed the experiments and analyzed the data. C. Murakami and M. Takechi performed the experiments. H. Sakai and F. Sakane conceptualized the study, supervised all aspects of data collection, and wrote the manuscript. T. Urano provided advice on the experimental design and critically revised the manuscript for intellectual content. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

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