Diacylglycerol kinase-δ regulates AMPK signaling, lipid metabolism, and skeletal muscle energetics

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DIACYLGLYCEROL (DAG) KINASES (DGK) are a family of lipid kinases that catalyze the phosphorylation and conversion of DAG into phosphatidic acid (PA). Elevated DAG content is linked with the development of insulin resistance in type 2 diabetes (27, 36). Thus, modulating the level of distinct DGK isoforms may influence the level of DAG and consequently insulin sensitivity.

DGKs regulate signal transduction via protein kinase C, Ras and Rho family proteins, and phosphatidylinositol-5-kinases (42). Of the ten different isoforms of mammalian DGKs, each may have a different subcellular localization and function (31). Protein abundance of DGKδ and total DGK activity are reduced in skeletal muscle from type 2 diabetic patients and diabetic rodents and normalized upon correction of hyperglycemia (11). Given that DAG is a precursor for triglyceride biosynthesis, DGKs are potentially involved in regulating fat deposition. Indeed, DGKδ haplosufficient (DGKδ+/-) mice develop obesity later in life (11), and knockdown of DGKδ markedly suppresses triglyceride (TG) synthesis in 3T3-L1 preadipocytes (28). Yet, the mechanism by which DGKδ affects lipid synthesis and other aspects of lipid metabolism remains unclear.

AMP-activated protein kinase (AMPK) is a central regulator of energy metabolism. AMPK is heterotrimeric complex composed of a catalytic α-subunit and regulatory β- and γ-subunits. Phosphorylation on the Thr172 residue of α-subunit by liver kinase B (LKB1) (20) or calmodulin-dependent protein kinase kinase (CaMKK) (18, 19) is required for AMPK activation. Conversely, phosphorylation of the α-subunit on Ser218/221 by protein kinase A (15), protein kinase B (21), and protein kinase C (PKC) (12) blocks AMPK activation. Nutrient excess in type 2 diabetes or obesity elevates DAG levels and PKC activity and impairs insulin sensitivity (34). While AMPK activation reverses many diabetes-associated metabolic defects, AMPK activity is decreased in multiple tissues from insulin-resistant and obese animal models (35). Whether excessive DAG levels further impair AMPK signaling and contribute to diabetes-associated metabolic impairments is unknown.

In addition to AMPK signaling defects (34), impairments in lipid oxidation (4, 24) are also involved in the pathogenesis of obesity and type 2 diabetes. AMPK controls lipid metabolism through acetyl-CoA carboxylase (ACC) and malonyl-CoA decarboxylase (MCD) (33, 41). ACC has two isoforms (ACCα/β), and ACCβ is the dominant isoform expressed in skeletal muscle (39). Of the phosphorylation sites on ACC, Ser79 is the major site responsible for the inhibition of ACC activity, whereas Ser219/221 is associated with muscle contraction-induced fatty acid oxidation (39). ACC produces and MCD degrades malonyl-CoA, a factor which functions both as substrate for de novo fatty acid synthesis and as inhibitor of the fatty acid transporter carnitine palmitoyltransferase-1 (CPT-1) at the mitochondrial membrane (44). Whether DGKδ is coupled to AMPK activation and lipid metabolism remains unclear.

Here, we determined the effects of physical and chemical AMPK agonists to unravel the role of DGKδ in AMPK-related signal transduction and lipid metabolism. Whereas complete loss of DGKδ is lethal due to respiratory dysfunction (13), DGKδ+/- mice are viable but develop obesity and skeletal metabolism defects. Therefore, DGKδ+/- mice were used to investigate the effects of DGKδ deficiency on energy metabolism and insulin resistance.

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muscle insulin resistance with age (11). We found that DGKβ−/− mice have reduced physical activity and increased muscle fatigue, implying a role in skeletal muscle energetics. Furthermore, lipid storage is favored over lipid oxidation, concomitant with reduced AMPK activity in skeletal muscle. Thus, reduced DGKβ impairs AMPK signaling and muscle function presumably via altering DAG content.

**RESEARCH DESIGN AND METHODS**

**DGKβ haploinsufficient mice.** The method used to generate haploinsufficient DGKβ (DGKβ+/−) mice (13) and data regarding glucose and energy homeostasis have been reported (11). DGKβ mRNA expression, protein content, and kinase activity are reduced in DGKβ+/− mice (11). We (29) have previously reported that mRNA expression of DGKβ is similar between EDL and soleus muscle. Five month-old male DGKβ+/− mice and wild-type littermates were housed in a temperature-controlled 12:12-h light-dark environment and fed standard rodent chow and water ad libitum. Mice were fasted for 4 h and anesthetized with Avertin (2,2,2-tribromoethanol 99% and tertiary amyl alcohol, 1:1 wt/vol, 500 mg/kg) via intraperitoneal injection prior to all terminal experiments. The Regional Animal Ethics Committee (Stockholm, Sweden) approved the experimental procedures.

**Voluntary exercise training protocol.** Mice were housed for 4 wk in individual cages equipped with a running wheel. Running activity (wheel revolutions) was automatically recorded using VitalView Animal Monitoring software (Mini Mitter, Bend, OR). The average number of wheel revolutions per hour from days 2–4 of the voluntary exercise training protocol was analyzed.

**Locomotor activity.** Locomotor activity was measured before and after 4 wk of voluntary wheel running, as described (36). Mice were housed in individual Plexiglas cages of the Comprehensive Lab Animal Monitoring System (CLAMS; Columbus Instruments, Columbus, OH), and locomotor activity was continuously monitored. During the CLAMS session, mice were maintained on a 12:12-h light-dark cycle and had free access to standard rodent chow and water.

**Glycogen content.** Gastrocnemius muscle was removed from unretrained anesthetized 5-mo-old male DGKβ+/− mice and wild-type littermates. Muscles were cleaned of fat and blood and frozen in liquid nitrogen. Glycogen content was determined fluorometrically on HCl extracts (2).

**Skeletal muscle incubation and glucose transport.** Incubation medium was prepared from a stock solution of pregassed (95% O2-5% CO2) Krebs-Henseleit bicarbonate buffer (KHB) supplemented with 5 mM HEPES and 0.1% bovine serum albumin (BSA, RIA grade). Extensor digitorum longus (EDL) and soleus muscles were preincubated for 30 min in KHB supplemented with 5 mM glucose and 15 mM mannitol and incubated in the absence or presence of 2 mM 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR). Muscles were rinsed in glucose-free KHB containing 20 mM mannitol and transferred to KHB supplemented with 19 mM mannitol, 1 mM 2-deoxy-D-glucose, 2.5 μCi/ml 2-deoxy-D-[3H]glucose (American Radiolabeled Chemicals) and 0.7 μCi/ml [3H]mannitol (Moravek Biochemicals) and incubated in the absence or presence of AICAR for 20 min. All incubations were performed in individual vials placed in a shaking water bath (30°C) with a constant gas phase (95%O2-5%CO2).

**Glucose oxidation and glucose incorporation into glycogen.** The method was adapted from Ref. 1. Skeletal muscle was incubated at 30°C for 60 min in 2 ml of KHB supplemented with 5 mM [U-14C]glucose (0.3 μCi/ml) in a sealed flask that contained a center well. After incubation, the muscle sample was quickly removed for biochemical analysis, and the flask was resealed. Thereafter, 200 μl of protosol was injected into the center well, and 500 μl of 15% PCA was injected into the medium. The flask was incubated for 60 min, and liberated CO2 was collected into the protosol to assess glucose oxidation. The muscle was dissolved with 0.5 ml of 1 M NaOH at 70°C for 30 min to assess glucose incorporation into glycogen. After TCA deproteinization, glycogen was sedimented from the skeletal muscle lysate by adding 95% ethanol and dissolved in 200 μl of 1 M NaOH. The 14C collected in the protosol and glycogen was measured by liquid scintillation counting.

**In vitro muscle contraction.** In vitro contractions were performed as described (3). Paired EDL muscles were placed in contraction chambers (Multi Myograph System; Danish Myo Technology, Aarhus, Denmark) containing KHB supplemented with 5 mM glucose, 15 mM mannitol, 0.3 mM palmitate, and 4% fatty acid-free BSA and incubated for 30 min (3). Thereafter, the contraction chambers were emptied and refilled with the same medium containing 4 μCi/ml [9,10(n)-3H] palmitic acid (GE Healthcare). One muscle was incubated under resting conditions, while the contralateral muscle was subjected to a contraction protocol consisting of a 5-min contraction session followed by a 5-min resting session that was repeated for six cycles. During the contraction sessions, the isolated skeletal muscle was stimulated with 0.1-ms 25 Hz pulses for 0.3 s (3). Muscles were frozen in liquid nitrogen, and medium was analyzed for titrated water (9). Raw data were extracted from LabChart files containing recordings of the contraction experiments, and average trains were created in Python software (http://www.python.org). Baseline and area under the curve were calculated in R software (http://www.r-project.org).

**Palmitate oxidation and incorporation into intramuscular lipids.** EDL and soleus muscles were preincubated for 20 min in KHB containing 5 mM glucose, 15 mM mannitol, and 4% fatty acid-free BSA. Muscles were transferred to vials containing fresh medium supplemented with 300 μM of cold palmitate with 4 μCi/ml [9,10(n)-3H]palmitic acid for assessment of palmitate oxidation, or 2 μCi/ml [U-14C]palmitic acid (PerkinElmer) for assessment of palmitate incorporation into intramuscular lipids. Muscles were incubated for 2 h in the absence or presence of 2 mM AICAR. Muscles were immediately frozen in liquid nitrogen. Palmitate oxidation was quantified by measuring titrated water (9). The amount of palmitate metabolized into intramuscular lipids was determined (30). Intramuscular lipids were extracted in isopropanol-hexane-KCl (2:4:1). The lipid pellet was suspended in 50 μl of 15% PCA methanolic trifluoroacetic acid, performed at 80°C. Samples underwent simultaneous and automated tissue homogenization and analyte extraction in methanolic trifluoroacetic acid, performed at −20°C in the presence of BSA and an internal standard. Sample purification was achieved by a simple lipid extraction step using chloroform. After evaporation of the aqueous extract to dryness, analytes were dissolved in injection solvent to remove the trifluoroacetic acid. Samples were subsequently separated by ion-pair HPLC based on a di-isopropyl-ethylamine-ammonium acetate buffer in water-acetonitrile and a Hypersil GOLD reversed-phase C18 column with high pH stability. Malonyl-CoA and acetyl-CoA levels were determined quantitatively by negative electrospray tandem mass spectrometry using an Ultima Pm mass spectrometer (Micromass UK). The detection limit was <1 pmol. Calibration and quality control samples were included in each run.

**AMPK activity.** AMPK activity was determined as described (14). AMPK was immunoprecipitated from EDL and soleus muscle lysates (300 μg) using antibodies against AMPKα1 and α2 (Prof. D. G. Hardie) and incubated for 30 min (30°C) in a total volume of 30 μl,
western blot. Muscles were homogenized in ice-cold buffer (10% glycerol, 5 mM sodium pyrophosphate, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl2, 20 mM Tris, pH 7.8, 1% Triton X-100, 10 mM sodium fluoride, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 0.5 mM sodium vanadate, 1 mM benzamidine, 1 μM microcystin). Homogenates were rotated end over end for 1 h at 4°C and subjected to centrifugation (12,000 g for 10 min) at 4°C. Protein content in the supernatant was evaluated using a commercially available kit (Pierce, Thermo Fisher Scientific). An equal amount of protein (30 μg) was subjected to Western blot analysis as described (11). Protein phosphorylation and abundance were determined using the following antibodies: phospho-AMPKα Thr172; phospho-AMPKα1 Ser485/492; phospho-AMPKα2 Ser483/493; total AMPKα, phospho-ACCα/β Ser79, total ACCα/β, SIRT1, total LKB1, phospho-(Ser/Thr) Akt substrate (PAS160), phospho- tuberin/TSC2 Ser1387 (Cell Signaling Technology), AS160 (Rab-GAP) (Millipore), succinate-ubiquinol oxidoreductase (Complex II), and ATP synthase subunit-γ (Santa Cruz Biotechnology). GAPDH was used to confirm equal protein loading. Proteins were visualized by enhanced chemiluminescence (ECL; GE Healthcare) and quantified by densitometry using Quantity One Software (Bio-Rad).

Quantitative real-time PCR. Total RNA was purified from tibialis anterior muscle using TRIzol reagent (Invitrogen) and treated with DNase I (DNA-free kit, Ambion). cDNA synthesis was performed using the SuperScript First Strand Synthesis System (Invitrogen) and random hexamer primers. PCR was assessed in a final volume of 25 μl, consisting of diluted cDNA sample, 1× SYBR Green PCR Master Mix (Applied Biosystems), gene-specific primers, and nuclease-free water. CPT-1 mRNA was assessed using quantitative RT-PCR (ABI PRISM 7000 Sequence Detector System, Applied Biosystems). All samples were analyzed in duplicate. Relative quantities of CPT-1 were calculated after normalization by a reference gene (36B4, also known as RPLP0) using the ΔCt method (Applied Biosystems).

Statistical analysis. Results are expressed as means ± SE. Statistical analysis for voluntary wheel running activity was performed with general linear model and repeated measures using the Statistical Package for the Social Sciences (SPSS). Other statistical analyses were performed using two-way ANOVA followed by Bonferroni post hoc test or Student’s t-test when appropriate (Graph Prism v. 5.0). Results were considered statistically significant at P < 0.05.

RESULTS

Locomotor activity is reduced in DGKδ+/− mice. DGKδ+/− mice and wild-type littermates were housed individually for 4 wk with unlimited access to running wheels. Locomotor activity was measured before and after 4 wk of voluntary wheel running. Locomotor activity was reduced in DGKδ+/− mice before, but not after the 4 wk of voluntary wheel running (Fig. 1, A and B). DGKδ+/− mice ran fewer cycles than wild-type mice during the 12-h dark period at the onset of training (Fig. 1C; average of days 1–3, P < 0.05). These findings indicate that DGKδ deficiency may alter skeletal muscle energetics. However, after 1 wk, DGKδ+/−- and wild-type mice performed the same amount of daily exercise (Fig. 1D, average of days 8–10). To ascertain whether this difference in locomotor activity at the onset of training was reflected by alterations in fuel selection or availability,
CPT-1 mRNA was unaltered between DGK−/− mice. These results suggest that the reduction in voluntary physical activity in mice (Fig. 2B) is not associated with altered mitochondria protein abundance.

**AMPK activation is attenuated in skeletal muscle from DGK−/− mice.** To test whether the reduced voluntary wheel running in DGK−/− mice is related to alterations in energy sensing, EDL muscles were incubated in the absence or presence of the AMPK agonist (AICAR), and signal transduction was assessed. AICAR-induced AMPKα1 and AMPKα2 activity was abolished in skeletal muscle from DGK−/− mice (Fig. 3, A and B). Moreover, AMPK Thr172 phosphorylation was decreased (Fig. 3C) and AMPK inhibitory Ser485/491 phosphorylation was increased (Fig. 3D) in AICAR-treated skeletal muscle from DGK−/− mice. DGK−/− deficiency did not alter protein abundance of AMPKα1 and AMPKα2 subunits (Fig. 3,E and F). Protein abundance of LKB1, an upstream regulator of AMPK, was unaltered between wild-type and DGK−/− mice (0.83 ± 0.09 vs. 0.88 ± 0.10 mean ± SE arbitrary units, respectively). Strikingly, we found that AICAR-induced ACC phosphorylation at Ser79 (Fig. 3G) and Ser219/221 (Fig. 3H) was unaltered between genotypes. Phosphorylation of AS160 (Fig. 3I) and TSC2 (Fig. 3J), other AMPK targets, was not increased by AICAR stimulation and was unaltered by DGK−/− deficiency.

**Effect of DGK deficiency on AICAR-induced malonyl-CoA and acetyl-CoA levels.** Malonyl-CoA is an allosteric inhibitor of CPT, the enzyme that controls the transfer of long-chain fatty acyl CoA molecules into mitochondria where they are oxidized. We found a trend for reduced malonyl-CoA levels in AICAR-treated EDL muscle from wild-type (P = 0.11) but not DGK−/− mice (Fig. 4A). Basal acetyl-CoA levels tended to be reduced in DGK−/− mice (Fig. 4B); however, AICAR-stimulated levels were similar between genotypes.

**Proteins related to mitochondrial function are unaltered by DGK−/− deficiency.** Skeletal muscle abundance of Sirtuin 1 (SIRT1), an NAD-dependent deacetylase important for the regulation of mitochondrial biogenesis, as well as several mitochondrial proteins, including succinate dehydrogenase (SUO), pyruvate dehydrogenase kinase isoenzyme 4 (PD4K), ATP synthase, and cytochrome c oxidase subunit 1 (COX1), was unaltered between DGK−/− and wild-type mice (Fig. 2A). Furthermore, CPT-1 mRNA was unaltered between DGK−/− and wild-type mice (Fig. 2B). Collectively, these results provide evidence to suggest that the reduction in voluntary physical activity in DGK−/− mice is related to alterations in energy sensing.
DGK deficiency alters AMPK-induced lipid metabolism in skeletal muscle. We next examined whether the diminution in AICAR-mediated AMPK signaling in DGKδ+/− mice is associated with impaired lipid metabolism. Palmitate oxidation was measured in EDL (Fig. 5A) and soleus (Fig. 5B) muscle incubated in the absence or presence of AICAR. AICAR-stimulated palmitate oxidation was reduced in EDL and soleus muscle from DGKδ+/− vs. wild-type mice (Fig. 5, A and B). To determine the fate of lipid species, EDL muscle was incubated with [14C]palmitate, and subsequently intramuscular TG, fatty acids, and DAG were separated by TLC (Fig. 5C). Quantification of these lipid species under basal and AICAR-stimulated conditions reveals that a greater amount of [14C]palmitate was incorporated into TG in EDL muscle from DGKδ+/− mice (Fig. 5D). Palmitate incorporation into TG in soleus muscle was not altered by either AICAR treatment or DGKδ haploinsufficiency (Fig. 5E). The increase of [14C]palmitate incorporation into DAG did not reach statistical significance (quantification not shown); however, we (11) have previously reported that total DAG content is increased in this model. Thus, DGKδ deficiency may shift the balance of lipid metabolism by reducing fatty acid oxidation and increasing intramuscular lipid storage.

AICAR-stimulated glucose metabolism is unchanged in DGKδ+/− mice. To investigate whether DGKδ haploinsufficiency affects AMPK-mediated glucose metabolism, glucose uptake (Fig. 6, A and B), glucose oxidation (Fig. 6, C and D), and glucose incorporation into glycogen (Fig. 6, E and F) were measured in EDL and soleus muscle in vitro. In EDL, but not in soleus, AICAR-stimulated glucose metabolism was increased compared with baseline in wild-type and DGKδ+/− mice (Fig. 6, A–F). Rates of basal and AICAR-stimulated glucose uptake, glucose oxidation, and glucose incorporation into glycogen were similar between wild-type and DGKδ+/− mice (Fig. 6, A–F). Thus, DGK deficiency selectively impairs AMPK signal transduction on lipid, but not glucose metabolism.

DGKδ deficiency impairs contraction-induced lipid oxidation and signal transduction. Since AICAR-stimulated AMPK activation and fatty acid oxidation were attenuated in DGKδ+/− mice, we explored the response of other modes of AMPK activation that disturb energy homeostasis. Electrically stimulated contraction increased (15%) palmitate oxidation in EDL muscle from WT mice (Fig. 7A) but not in DGKδ+/− mice. To investigate whether the abundance of DGKδ influ-

Fig. 4. Malonyl-CoA and acetyl-CoA Content. EDL muscle from WT and DGKδ+/− mice were incubated in the absence (Basal; •) or presence of AICAR (●). Content of malonyl-CoA (A) and acetyl-CoA (B) was measured (n = 5 for WT and n = 7 for DGKδ+/− mice). Results are means ± SE. *P < 0.05: AICAR vs. Basal.

Fig. 5. Effects of AICAR on lipid metabolism. In vitro palmitate oxidation was assessed in EDL (A) or soleus (B) muscle from WT (n = 7) and DGKδ+/− (n = 6) mice. Muscles were incubated in the absence (Basal; •) or presence of AICAR (●). C: separation of [14C]-labeled lipid species by TLC from muscle incubated in the absence (Basal; •) or presence of AICAR (●). D: separation of TG, fatty acids; DAG, diacylglycerol; Ori, origin of sample loading. Quantification of incorporation of palmitate into TG in EDL (D) and soleus (E). Results are means ± SE. *P < 0.05: DGKδ+/− AICAR vs. WT AICAR; *P < 0.05: DGKδ+/− Basal vs. WT Basal.
ences contraction-induced signaling events, phosphorylation of AMPK and ACC was measured in EDL muscle given their involvement in lipid oxidation. While basal and contraction-induced AMPK Thr172 phosphorylation was not influenced by DGKδ deficiency (Fig. 7B), an overall reduction in ACC phosphorylation was observed in DGKδ−/− mice (Fig. 7C). These results further support a role for DGKδ in lipid metabolism in response to energy stress.

DGKδ abundance influences muscle force generation. To investigate whether the reduction in contraction-induced fatty acid oxidation in DGKδ−/− mice is associated with altered skeletal muscle energetics, we determined force generation during in vitro contraction (Fig. 8A). Baseline muscle tension was similar between wild-type and DGKδ−/− mice at the start of the first contraction interval (Fig. 8, B and C) and was transiently increased in both strains after five contraction ses-

Fig. 6. AICAR-stimulated glucose metabolism in skeletal muscle. In vitro glucose transport (A and B), glucose oxidation (C and D), and glucose incorporation into glycogen (E and F) was measured in EDL (A, C, E) and soleus (B, D, F) muscle from WT (n = 6) and DGKδ−/− (n = 6) mice. Muscles were incubated in the absence (Basal; □) or presence of AICAR (■). Results are means ± SE. *P < 0.05: AICAR vs. Basal.

Fig. 7. In vitro contraction-stimulated signal transduction and lipid oxidation. A: in vitro palmitate oxidation was measured in EDL muscle from WT (n = 7) or DGKδ−/− mice (n = 7) under resting (Basal; □) or contraction-stimulated (■) conditions. Abundance of AMPK Thr172 (B) and ACC Ser79 (C) was measured in EDL muscle. Results are means ± SE. *P < 0.05: DGKδ−/− vs. WT; †P < 0.05: Contracted vs. Basal.

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DGKδ abundance influences muscle force generation. To investigate whether the reduction in contraction-induced fatty acid oxidation in DGKδ−/− mice is associated with altered skeletal muscle energetics, we determined force generation during in vitro contraction (Fig. 8A). Baseline muscle tension was similar between wild-type and DGKδ−/− mice at the start of the first contraction interval (Fig. 8, B and C) and was transiently increased in both strains after five contraction ses-
sions. Yet, the increase in baseline muscle tension with each contraction interval was markedly greater in DGKδ−/− mice than in wild-type mice (P < 0.05; Fig. 8, B and C). Baseline muscle tension prior to the start of the sixth and final contraction interval was 2.6-fold higher in DGKδ−/− mice (Fig. 8D). However, net force generation, calculated as area under curve for each interval, was similar between wild-type and DGKδ−/− mice (Fig. 8E). This alteration in tension release indicates that DGKδ-deficient muscle is more prone to fatigue, possibly due to limited energy supply, as the transport of calcium into sarcoplasmic reticulum depends on the hydrolysis of ATP.

**DISCUSSION**

Dysregulation of AMPK signaling, arising from nutrient excess and elevated glucose and lipid levels in obesity and type 2 diabetes, has been linked to the development of insulin resistance (34). Nutrient excess decreases AMPK activity, concomitant with increased DAG content (12), highlighting a deleterious role for specific lipid metabolites such as DAGs and ceramides in the development of peripheral insulin resistance (27, 36). Here, we tested the hypothesis that the abundance of DGKδ is of relevance for maintaining appropriate AMPK action since they catalyze a reaction that removes DAG and thereby terminate PKC signaling and may subsequently influence AMPK signaling. The central findings of this study are that physical activity and skeletal muscle energetics are impaired in DGKδ−/− mice. Furthermore, lipid storage is favored over lipid oxidation, concomitant with reduced AMPK activity. AICAR-induced AMPK Thr172 phosphorylation was attenuated, whereas AMPK inhibitory Ser485/491 phosphorylation was increased. Thus, we provide evidence for a link between DGKδ and AMPK signaling. Reduced DGKδ impairs AMPK signaling and skeletal muscle function presumably via altering intramuscular DAG content and AMPK Ser485/491 phosphorylation.

The precise mechanism by which DGKδ haploinsufficiency suppresses AMPK activity remains unclear. However, AICAR-induced AMPK Thr172 phosphorylation is reduced and AMPK inhibitory Ser485/491 phosphorylation is increased in DGKδ−/− mice. Furthermore, we have previously reported that total DAG content is increased concomitant with PKC activation in...
DGKα<sup>+/−</sup> mice (11). PKC activation has been proposed as one mechanism for the inhibitory phosphorylation of AMPK Ser485/491 (12). Thus, excessive PKC activation concomitant with AMPK-inhibitory Ser485/491 phosphorylation may mechanistically suppress AMPK activity in the DGKα<sup>+/−</sup> mice (Fig. 9). Here, we report that DGKα<sup>+/−</sup> mice preferentially favor lipid storage over oxidation. Indeed, we note elevated levels of intramuscular DAG (11), a precursor of triglycerides in skeletal muscle from DGKα<sup>+/−</sup> mice. The TG pool can then be hydrolyzed to long-chain fatty acid CoA (LCFA-CoA) and act as a substrate for ceramide generation. Increased ceramide content is associated with activated protein phosphatase 2A (PP2A), which dephosphorylates AMPK on Thr172, leading to reduced AMPK activity and related signal transduction in skeletal muscle, in parallel with a shift toward enhanced incorporation of fatty acids into neutral lipid storage and diminished fatty acid oxidation. Partial degradation of fatty acids due to incomplete fatty acid oxidation can contribute to the development of lipid oxidation in skeletal muscle from DGKα<sup>+/−</sup> mice. FG, pools can then be hydrolyzed to long-chain fatty acid CoA (LCFA-CoA) and act as a substrate for ceramide generation. Increased ceramide content is associated with activated protein phosphatase 2A (PP2A), which dephosphorylates AMPK on Thr172, leading to reduced AMPK activity and related signal transduction in skeletal muscle, in parallel with a shift toward enhanced incorporation of fatty acids into neutral lipid storage and diminished fatty acid oxidation. Partial degradation of fatty acids due to incomplete fatty acid oxidation can contribute to the development of lipid oxidation in skeletal muscle from DGKα<sup>+/−</sup> mice. Therefore, we propose that elevated DAG content, impaired signal transduction in skeletal muscle, in parallel with a shift toward enhanced incorporation of fatty acids into neutral lipid storage and diminished fatty acid oxidation can contribute to the development of lipid metabolism. However, the abundance of several mitochondrial proteins and regulators of mitochondrial biogenesis was unchanged. In cultured preadipocytes, DGKα silencing caused a concomitant decrease of DAG, PA, and triglycerides (28). DGK overexpression in preadipocytes promotes lipogenesis (37), consistent with a role for DGKα in lipid storage in fat cells. Skeletal muscle contains substantially less total lipid than adipocytes, so the pool of DAG and PA regulated by DGKα may comprise a major portion of total lipid available for storage and oxidation. Thus, the reduction in lipid oxidation in skeletal muscle from DGKα<sup>+/−</sup> mice might be secondary to the increase in lipid storage.

We observed that AICAR-induced AMPK Thr<sup>172</sup> phosphorylation was decreased and AMPK inhibitory Ser<sup>485/491</sup> phosphorylation was increased in DGKα<sup>+/−</sup> mice, concomitant with impaired lipid oxidation. Nevertheless, AICAR-induced glucose uptake was intact, consistent with previous observations that AICAR increases glucose uptake in skeletal muscle from severely insulin-resistant rodents and type 2 diabetic patients (25, 38). Basal and AICAR-induced glucose oxidation and glucose incorporation into glycogen were also unaltered in skeletal muscle from DGKα<sup>+/−</sup> mice. Thus, DGKα deficiency leads to selective AMPK resistance on lipid, but not glucose, metabolism. Although the precise mechanism for the selective AMPK resistance on lipid versus glucose metabolism is unclear, DAG levels are constitutively elevated in DGKα<sup>+/−</sup> mice (11), and this may have a persistent effect to restrain lipid oxidation. Here, we report that palmitate incorporation into triglycerides is increased in DGKα<sup>+/−</sup> mice, suggesting a protective mechanism may limit the accumulation of lipid intermediates such as DAG. For example, in high-fat-fed mice, overexpression of a constitutively active mutant form of AMPK in skeletal muscle reduces DAG levels and restores lipid oxidation (2). Thus, the level of specific lipid metabolites may influence the efficacy of AMPK activators in promoting lipid oxidation. However, additional studies are warranted to elucidate the relationship between the accumulation of lipid intermediates and AMPK activity and to understand the impact these lipid metabolites on fatty acid oxidation and metabolism.

AMPK phosphorylates and inhibits ACC, which reduces malonyl-CoA, an inhibitor of CPT-1, the enzyme controlling transfer of lipids into the mitochondria for β-oxidation (33). We found that AICAR stimulation tended to reduce malonyl-CoA levels in skeletal muscle from wild-type but not AMPK-resistant DGKα<sup>+/−</sup> mice. Thus, DGKα<sup>+/−</sup> mice are resistant to the action of AICAR to reduce malonyl-CoA levels and relieve the inhibition of CPT-1 on lipid oxidation. Unexpectedly, ACC phosphorylation was preserved in DGKα<sup>+/−</sup> mice despite diminished AICAR-stimulated AMPK activation. Notably, phosphorylation of other direct substrates of AMPK, AS160 and TSC2, were unaltered in DGKα<sup>+/−</sup> mice. However, given the lack of AICAR-induced AMPK activation and lipid oxidation in DGKα<sup>+/−</sup> mice, our finding that malonyl-CoA levels were unaltered by AICAR stimulation in DGKα<sup>+/−</sup> mice was unsurprising and may provide a mechanism for the alteration in lipid fuel partitioning. Indeed, we found a reciprocal relationship between fatty acid synthesis and lipid oxidation in DGKα<sup>+/−</sup> mice. We also found that LKB1 and AMPK isoform abundance was unaltered in DGKα<sup>+/−</sup> mice. Thus, our finding of impaired AICAR-induced AMPK phosphorylation, but normal ACC and other AMPK targets of phosphorylation and acetyl-CoA content is consistent with the notion of redundant signaling, such that one or more kinases other than AMPK may impinge upon ACC (40).

Fig. 9. Hypothetical mechanisms by which DGKα haploinsufficiency suppresses AMPK activity. DGKα haploinsufficiency leads to accumulation of DAGs in skeletal muscle. The PKC family of Ser/Thr kinases can be activated by both DAG and ceramides and induce AMPK phosphorylation on Ser<sup>485/491</sup> (an inhibitory site). DAG excess will be stored as TG. The DGK pool can then be hydrolyzed to long-chain fatty acid CoA (LCFA-CoA) and act as a substrate for ceramide generation. Increased ceramide levels activate the protein phosphatase 2A (PP2A), which dephosphorylates AMPK on Thr<sup>172</sup>, leading to suppression of AMPK activity. PA, phosphatidic acid.
Some notable differences in AICAR- and contraction-induced signaling and metabolism were observed in DGK<sup>−/−</sup> mice. Under AICAR-stimulated conditions, AMPK phosphorylation and activity were severely blunted, but ACC phosphorylation was robustly increased in DGK<sup>−/−</sup> mice. Yet, in response to muscle contraction, AMPK activity was similarly increased between wild-type and DGK<sup>−/−</sup> mice, but ACC phosphorylation was attenuated in DGK<sup>−/−</sup> mice. This apparent disparity does not necessarily suggest that AMPK is dispensable for ACC activity; rather, our findings may reflect differential sensitivity to the integration of signals mediated via AMPK from diverse stimuli. For example, in response to electrically stimulated muscle contraction, ACC phosphorylation is detectable prior to measurable increases in AMPK activity (22). In humans, low-intensity exercise induces a profound increase of ACC phosphorylation with only a modest increase in AMPK phosphorylation (10). Furthermore, intravenous administration of AICAR in type 2 diabetic patients increases skeletal muscle ACC phosphorylation without any measurable change of AMPK phosphorylation (6). Thus, differences in the sensitivity of AMPK and ACC to the various stimuli, the time course of activation, or the involvement of other protein kinases may explain the divergent response of wild-type and DGK<sup>−/−</sup> mice between AICAR- or contraction-stimulated conditions.

Exercise capacity is often reduced in people with obesity or type 2 diabetes due to lack of physical activity (7, 46). Strikingly, we found that DGK<sub>6</sub> deficiency impairs overall work performance, as evident by reduced voluntary activity, and impaired skeletal muscle energetics, as evident by altered force production and relaxation in response to repeated contractions. Contrasting AMPK deficient “lazy” mouse models, skeletal muscle glycogen content was unaltered in DGK<sub>−/−</sub> mice. Impaired lipid metabolism in the DGK<sub>−/−</sub> mice may account for the reduced physical activity, since low- to moderate-intensity endurance exercise relies on lipid oxidation (8). Moreover, the reduction in voluntary running activity of untrained DGK<sub>−/−</sub> mice occurred in parallel with a reduction of AICAR- or contraction-induced lipid oxidation, which is likely due to intrinsic changes in skeletal muscle, consistent with earlier studies of AMPK-deficient mouse models (1, 32). Interestingly, voluntary activity of DGK<sub>−/−</sub> mice was normalized after 10 days of wheel running, which may reflect skeletal muscle remodeling and other exercise training-induced adaptations.

Following repeated contractions, resting tension was higher in skeletal muscle from DGK<sub>−/−</sub> mice. In the absence of muscle fatigue, this rapid relaxation process is mediated by calcium efflux from the cytosol, which depends on the intact sarcoplasmic reticulum (16). Whether insufficient energy supply or reduced SERCA activity causes functional defects in skeletal muscle from DGK<sub>−/−</sub> mice remains to be determined. In conclusion, DGK<sub>6</sub> abundance influences physical activity and muscle fatigue, implying a possible role in skeletal muscle bioenergetics. Collectively, our results imply a role for DGK<sub>6</sub> in the regulation of lipid oxidation and storage via AMPK signaling pathways. Reduced DGK<sub>6</sub> impairs AMPK signaling and muscle function presumably via altering intramuscular DAG content. Our results suggest that efforts to pharmacologically modulate the abundance or activity of DGK<sub>6</sub> may reveal new treatment opportunities for metabolic disorders.

REFERENCES


