Diacylglycerol Kinase δ Regulates AMPK Signaling, Lipid Metabolism and Skeletal Muscle Energetics

Running Title: Diacylglycerol Kinase δ Regulates Muscle Energetics

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Decrease of AMPK-related signal transduction and insufficient lipid oxidation contributes to the pathogenesis of obesity and type 2 diabetes. Previously, we identified that diacylglycerol kinase δ (DGKδ), an enzyme involved in triglyceride biosynthesis, is reduced in skeletal muscle from type 2 diabetic patients. Here we test the hypothesis that DGKδ plays a role in maintaining appropriate AMPK action in skeletal muscle and energetic aspects of contraction. Voluntary running activity was reduced in DGKδ−/− mice, but glycogen content and mitochondrial markers were unaltered, suggesting DGKδ deficiency affects skeletal muscle energetics, but not mitochondrial protein abundance. We next determined the role of DGKδ in AMPK-related signal transduction and lipid metabolism in isolated skeletal muscle. AMPK activation and signaling was reduced in DGKδ+/− mice, concomitant with impaired lipid oxidation and elevated incorporation of free fatty acids into triglycerides. Strikingly, DGKδ deficiency impaired work performance as evident by altered force-production and relaxation dynamics in response to repeated contractions. In conclusion, DGKδ deficiency impairs AMPK signaling and lipid metabolism, thereby highlighting the deleterious role of excessive lipid metabolites in the development of peripheral insulin resistance and type 2 diabetes pathogenesis. DGKδ deficiency also influences skeletal muscle energetics, which may lead to low physical activity levels in type 2 diabetes.
INTRODUCTION

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 haploinsufficient (DGKδ +/-) mice develop obesity later in life (11) and knock-down of DGKδ markedly suppresses triglyceride synthesis in 3T3-L1 pre-adipocytes (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 Ser485/491 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 (ACC α/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, while 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. While complete loss of DGKδ is lethal due to respiratory dysfunction (13), DGKδ⁺⁻ mice are viable, but develop obesity and skeletal muscle insulin resistance with age (11). We found DGKδ⁺⁻ mice have reduced physical activity and increased muscle fatigue, implicating a role in skeletal muscle energetics. Furthermore, lipid storage is favored over lipid oxidation, concomitant with reduced AMPK activity in skeletal muscle. Thus, reduced DGKδ impairs AMPK signaling and muscle function presumably via altering DAG content.
RESEARCH DESIGN AND METHODS

DGKδ haploinsufficient mice

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

Voluntary Exercise Training Protocol

Mice were housed for four weeks 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 day 2 to 4 of the voluntary exercise training protocol was analyzed.

Locomotor Activity

Locomotor activity was measured before and after four weeks of voluntary wheel running as described (36). Mice were housed in individual plexiglass 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 h light:12 h dark cycle and had free access to standard rodent chow and water.
**Glycogen Content**

Gastrocnemius muscle was removed from untrained anesthetized five month old male DGKδ+/- mice and wild-type littermates. Muscle 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 media was prepared from a stock solution of pre-gassed (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 pre-incubated 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 [³H]2-Deoxy-D-glucose (American Radiolabeled Chemicals) and 0.7 µCi/ml [¹⁴C]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 (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 which 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 15% PCA was injected into the media. 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 1M 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 1M 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 media 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 0.3s (3). Muscles were frozen in liquid nitrogen and media was analyzed for tritiated water (9). Raw data was 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 was calculated in R software (http://www.r-project.org).

**Palmitate Oxidation and Incorporation into Intramuscular Lipids**

EDL and soleus muscles were pre-incubated 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 (Perkin Elmer)
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 tritiated 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 chloroform: methanol (1:1), spotted on thin-layer chromatography (TLC) plates (Whatman) and separated in a hexane-diethyl ether-acetic acid (80:20:3) system. 1,2-Dioctanoyl [1-14C] rac-glycerol and 1,3-Dioleoyl-rac-glycerol [oleoyl-1-14C] (American Radio Labelled Chemicals), [U-14C]palmitate and [14C]triolein (Perkin Elmer) were used as standards. Lipid species (triglycerides, free fatty acids, and 1,3 DAG) were quantified by autoradiography.

Quantitation of Malonyl-CoA and Acetyl-CoA Content
Malonyl-CoA and Acetyl-CoA content was determined in skeletal muscle (5). Snap frozen muscle samples (~10 mg) were collected in 2 ml homogenization tubes and stored at -80°C. Samples underwent a simultaneous and automated tissue homogenization and analyte extraction in methanolic trifluoroacetic acid, performed at -20°C in 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 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 electro spray tandem mass spectrometry using an Ultima PT mass spectrometer (Micromass, England). The detection limits 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 AMPK-α1 and -α2 subunits (Prof. D. G. Hardie) and incubated for 30 min (30°C) in a total volume of 30 μl, containing 833 μM DTT, 200 μM AMP, 100 μM AMARA peptide (Upstate Biotechnologies), 5 mM MgCl₂, 200 μM ATP, and 2 μCi [γ-³²P]ATP. The reaction was terminated by spotting 25 μl of the mixture onto P81 filter paper and washing four times 15 min in 1% phosphoric acid. The dried filter paper was analyzed by liquid scintillation.

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 Inc.). An equal amount of protein (30 μg) was subjected to Western blot analysis as described (11). Protein phosphorylation and abundance was determined using the following antibodies: phospho-AMPKα Thr172, phospho-AMPKα1 Ser485/α2 Ser491, 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 α (Molecular Probes Inc), PDK4 (Abgent Inc), GAPDH and phospho-ACCβ Ser219/221 (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 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, 1X 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 delta Ct method (Applied Biosystems).

**Statistical Analysis**

Results are expressed as mean ± SEM. 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 (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 four weeks with unlimited access to running wheels. Locomotor activity was measured before and after four weeks of voluntary wheel running. Locomotor activity was reduced in DGKδ^+/− mice before, but not after the 4 weeks of voluntary wheel running (Fig. 1A-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 day 1-3; p<0.05). These findings indicate that DGKδ deficiency may alter skeletal muscle energetics. However, after one week, DGKδ^+/− and wild-type mice performed the same amount of daily exercise (Fig. 1D, average of day 8-10). To ascertain whether this difference in locomotor activity at the onset of training was reflected by alterations in fuel selection or availability, muscle glycogen content was measured. Intramuscular glycogen levels were similar between untrained wild-type and DGKδ^+/− mice (Fig. 1E).

**Proteins related to mitochondrial function are unaltered by DGKδ 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 (PDK4), ATP synthase, and cytochrome c oxidase subunit 1 (COX1), was unaltered between DGKδ^+/− and wild-type mice (Fig. 2A). Furthermore, carnitine palmitoyltransferase I (CPT1) mRNA was unaltered between DGKδ^+/− and wild-type mice (Fig. 2B). Collectively, these results provide evidence to suggest that the reduction in voluntary physical activity in DGKδ^+/− mice is not associated with altered mitochondria protein abundance.
AMPK activation is attenuated in skeletal muscle from DGKδ+/− mice

To test whether the reduced voluntary wheel running in DGKδ+/− 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 DGKδ+/− mice (Fig. 3A,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 DGKδ+/− mice. DGKδ deficiency did not alter protein abundance of AMPKα1 and AMPKα2 subunits (Fig. 3E,F). Protein abundance of LKB1, an upstream regulator of AMPK, was unaltered between wild-type and DGKδ+/− mice (0.83 ± 0.09 versus 0.88 ± 0.10 mean ± SEM arbitrary units, respectively). Strikingly, we found 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 unaltered by DGKδ deficiency.

Effect of DGK deficiency on AICAR-induced Malonyl-CoA and acetyl-CoA levels

Malonyl-CoA is an allosteric inhibitor of carnitine palmitoyl transferase, 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 DGKδ+/− mice (Fig. 4A). Basal acetyl-CoA levels tended to be reduced in DGKδ+/− mice (Fig. 4B), however AICAR-stimulated levels were similar between genotypes.

DGK deficiency alters AMPK-induced lipid metabolism in skeletal muscle

We next examined if the diminution in AICAR-mediated AMPK signaling in DGKδ+/− mice is associated with impaired lipid metabolism. Palmitate oxidation was measured in EDL...
AICAR-stimulated palmitate oxidation was reduced in EDL and soleus muscle from DGKδ⁺/⁻ versus wild-type mice (Fig. 5A,B). To determine the fate of lipid species, EDL muscle was incubated with ¹⁴C-palmitate, and subsequently intramuscular triglyceride, fatty acids, and DAG was separated by thin layer chromatography (Fig. 5C). Quantification of these lipid species under basal and AICAR-stimulated conditions reveals that a greater amount of ¹⁴C-palmitate was incorporated into triglyceride in EDL muscle from DGKδ⁺/⁻ mice (Fig. 5D). Palmitate incorporation into triglyceride 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 have previously reported that total DAG content is increased in this model (11). 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 6A,B), glucose oxidation (Fig. 6C,D), and glucose incorporation into glycogen (Fig. 6E,F) was measured in EDL and soleus muscle in vitro. In EDL, but not soleus AICAR-stimulated glucose metabolism was increased compared to baseline in wild-type and DGKδ⁺/⁻ mice (Fig 6A-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. 6A-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 was 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 influences contraction-induced signaling events, phosphorylation of AMPK and ACC were measured in EDL muscle given their involvement in lipid oxidation.

While basal and contraction-induced AMPK<sup>Thr172</sup> 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). Base-line muscle tension was similar between wild-type and DGKδ+/- mice at the start of the first contraction interval (Fig. 8B,C), and was transiently increased in both strains after five contraction sessions. Yet, the increase in baseline muscle tension with each contraction interval was markedly greater in DGKδ+/- mice versus wild-type mice (p<0.05; Fig. 8B,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 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 terminates 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, while 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 DGKδ +/- 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 DGKδ +/- mice (Fig. 9). Here we report that DGKδ +/- mice preferentially favor lipid storage over oxidation. Indeed, we note elevated levels of
intramuscular DAG (11), a precursor of triglycerides in skeletal muscle from DGKδ+/- mice. The triglycerides pool can then be hydrolyzed to long chain fatty acid CoA (LCFACoA) and act as a substrate for ceramides generation. Increased ceramide content is associated with activated protein phosphatase 2A (PP2A), dephosphorylation of AMPK Thr172 and decreased AMPK activity (45) Thus, elevated PKC or PP2A may suppress AMPK activity (Fig. 9).

DGKδ expression and DGK activity is reduced in skeletal muscle from type 2 diabetic patients, concomitant with elevated DAG content (11). Moreover, DGKδ haploinsufficiency increased diacylglycerol content, impaired peripheral insulin signaling and glucose transport, and led to age-dependent obesity. Metabolic flexibility, evident by the transition between lipid and carbohydrate utilization during fasted and fed conditions, was also impaired in DGKδ+/- mice (11). Thus, DGKδ deficiency causes peripheral insulin resistance and metabolic inflexibility, and contributes to mild obesity in later life. In this study, we demonstrated that DGKδ+/- mice exhibit reduced AMPK activity and related signal transduction in skeletal muscle, in parallel with a shift toward enhanced incorporation of fatty acid 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-induced insulin resistance (26). Nevertheless, the abundance of several mitochondrial proteins and regulators of mitochondrial biogenesis was unchanged. In cultured pre-adipocytes, DGKδ silencing caused a concomitant decrease of DAG, PA and triglycerides (28). DGK overexpression in pre-adipocytes promotes lipogenesis (37), consistent with a role for DGK in lipid storage in fat cells. Skeletal muscle contains substantially less total lipid than adipocytes, so the pool of DAG and PA regulated by DGKδ may comprise a major portion of total lipid available for storage and oxidation. Thus, the reduction in lipid oxidation in skeletal muscle from DGKδ+/- mice might be secondary to the increase in lipid storage.
We observed AICAR-induced AMPK Thr\(^{172}\) phosphorylation was decreased and AMPK inhibitory Ser\(^{485/491}\) phosphorylation was increased in DGK\(\delta^{+/−}\) 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 DGK\(\delta^{+/−}\) mice. Thus, DGK\(\delta\) deficiency leads to selective AMPK resistance on lipid, but not glucose metabolism. While the precise mechanism for the selective AMPK resistance on lipid versus glucose metabolism is unclear, DAG levels are constitutively elevated in DGK\(\delta^{+/−}\) mice (11), and this may have a persistent effect to restrain lipid oxidation. Here we report that palmitate incorporation into TG is increased in DGK\(\delta^{+/−}\) 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 \(\beta\)-oxidation (33). We found that AICAR stimulation tended to reduce malonyl-CoA levels in skeletal muscle from wild-type, but not AMPK-resistant DGK\(\delta^{+/−}\) mice. Thus, DGK\(\delta^{+/−}\) mice are resistant to the action of AICAR to reduce malonyl-CoA levels and relieve the inhibition of CPT1 on lipid oxidation. Unexpectedly, ACC phosphorylation was preserved in DGK\(\delta^{+/−}\) mice despite diminished AICAR-stimulated AMPK activation. Notably, phosphorylation of other direct
substrates of AMPK, AS160 and TSC2 were unaltered in DGKδ±/− mice. However, given the lack of AICAR-induced AMPK activation and lipid oxidation in DGKδ±/− mice, our finding that malonyl-CoA levels were unaltered by AICAR stimulation in DGKδ±/− 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 DGKδ±/− mice. We also found LKB1 and AMPK isoform abundance were unaltered in DGKδ±/− mice. Thus, our finding of impaired AICAR-induced AMPK phosphorylation, but normal ACC and other AMPK targets 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).

Some notable differences in AICAR- and contraction-induced signaling and metabolism were observed in DGKδ±/− mice. Under AICAR-stimulated conditions, AMPK phosphorylation and activity was severely blunted, but ACC phosphorylation was robustly increased in DGKδ±/− mice. Yet in response to muscle contraction, AMPK activity was similarly increased between wild-type versus DGKδ±/− mice, but ACC phosphorylation was attenuated in DGKδ±/− 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 DGKδ⁺/⁻ 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 DGKδ 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 DGKδ⁺/⁻ mice. Impaired lipid metabolism in the DGKδ⁺/⁻ 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 DGKδ⁺/⁻ 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 DGKδ⁺/⁻ 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 DGKδ⁺/⁻ mice. In the absence of muscle fatigue, this rapid relaxation process is mediated by calcium efflux from the cytosol, which depends on the intact function of sarco/endoplasmic reticulum calcium ATPase (SERCA) (43). SERCA expression in DGKδ⁺/⁻ mice was unaltered (data not shown). However, as an active transporter of calcium, SERCA requires the hydrolysis of ATP to supply energy. Since AMPK-induced lipid oxidation was impaired in DGKδ⁺/⁻ mice, we hypothesize that the restricted energy supply during muscle contraction might influence force production and relaxation. Phosphatidylethanolamine (PE) is a component of the lipid bilayer of the sarcoplasmic reticulum that promotes calcium binding to SERCA (17). PA, which is the product of DGK, is also the substrate for
phosphatidylethanolamine biosynthesis (23). Thus, DGK haploinsufficiency may decrease
SERCA activity by reducing PE levels. Indeed, muscle-specific ablation of fatty acid
synthase causes muscle weakness by reducing PE content and SERCA activity in
sarcoplasmic reticulum (16). Whether insufficient energy supply or reduced SERCA activity
causes functional defects in skeletal muscle from DGKδ−/− mice remains to be determined.

In conclusion, DGKδ abundance influences physical activity and muscle fatigue,
implicating a possible role in skeletal muscle bioenergetics. Collectively, our results
implicate a role for DGKδ in the regulation of lipid oxidation and storage via AMPK
signaling pathways. Reduced DGKδ 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 DGKδ may reveal new treatment
opportunities for metabolic disorders.

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Conflict of Interest Disclosure

L.L. is an employee of AstraZeneca R&D, SE-431 83 Mölndal, Sweden. AstraZeneca is a
global biopharmaceutical company specializing in the discovery, development,
manufacturing and marketing of prescription medicine.

potential conflicts of interest relevant to this article.


FIGURE LEGENDS

Figure 1. Locomotor activity, voluntary wheel running and skeletal muscle glycogen content. Locomotor activity was assessed by CLAMS in wild-type (WT) or DGKδ+/- mice before (A) and after (B) four weeks voluntary wheel running. Mice were given free access to running wheels for 4 weeks and average cycles run during day 1-3 (C) or day 8-10 (D) of the training period were analyzed. (E) Skeletal muscle glycogen content. Results are mean ± SEM for n = 9 for WT and n = 7 for DGKδ+/- mice. *p<0.05: DGKδ+/- on Dark vs WT on Dark cycle; #p<0.05: dark vs light cycle.

Figure 2. Abundance of proteins related to mitochondrial function. (A) Representative Western blot for SIRT1, SUO, PDK4, ATP synthase, COX1, and GAPDH protein abundance in skeletal muscle from wild-type (WT; n = 7) or DGKδ+/- (n = 7) mice. (B) CPT1 mRNA expression quantified by qPCR in skeletal muscle from WT or DGKδ+/- mice. Results are mean ± SEM.

Figure 3. Effects of AICAR on AMPK activity and signal transduction. EDL muscle from wild-type (WT) and DGKδ+/- mice were incubated in the absence (Basal; □) or presence of AICAR (■). AMPK α1 (A) and AMPK α2 (B) isoform-specific kinase activities were measured (n = 5 muscles per group). Phosphorylation of AMPK Thr172 (C) and AMPK Ser485/491 (D), abundance of AMPKα1 (E) and AMPKα2 (F), phosphorylation of ACC Ser79 (G), ACCβ Ser219/221 (H), AS160 Ser/Thr (I) and Tuberin Ser1387 (J) was determined (n = 7 muscles per group). (E) and (F) share the same loading control (GAPDH). Results are mean ± SEM. *p<0.05: DGKδ+/- AICAR vs WT AICAR; †p<0.05: AICAR vs Basal.

Figure 4. Malonyl-CoA and Acetyl-CoA Content. EDL muscle from wild-type (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 mean ± SEM. *p<0.05: AICAR vs Basal.

Figure 5. Effects of AICAR on lipid metabolism. In vitro palmitate oxidation was assessed in EDL (A) or Soleus (B) muscle from wild-type (WT; n = 7) and DGKδ+/− (n = 6) mice. Muscles were incubated in the absence (Basal; □) or presence of AICAR (■). (C) Separation of 14C-labelled lipid species by thin layer chromatography from muscle incubated in the absence (Basal; B) or presence of AICAR (A). FA: fatty acids; TG: triglyceride; DAG: diacylglycerol; Ori: origin of sample loading. Quantification of incorporation of palmitate into TG in EDL (D) and Soleus (E). Results are mean ± SEM. *p<0.05: DGKδ+/− AICAR vs WT AICAR; *p<0.05: DGKδ+/− Basal vs WT Basal.

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

Figure 7. In vitro contraction-stimulated signal transduction and lipid oxidation. (A) In vitro palmitate oxidation was measured in EDL muscle from wild-type (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 mean ± SEM. □ p<0.05: DGKδ+/− vs WT; + p<0.05: Contracted vs Basal.

Figure 8. Force generation during in vitro contraction. EDL muscle from wild-type (WT; n = 7) or DGKδ+/− mice (n = 7) were incubated under resting conditions (□) or subjected to in vitro electronic stimulation (■). (A) An in vitro contraction protocol was applied with alternating periods of stimulation (STIM) and rest (REST) following a 20 min recovery (REC)
period. Average muscle force generation in WT (B) or DGKδ+/− (C) mice. Quantification of basal force level at the start of each contraction interval (D) and area under the curve for each interval (E) is shown. Results are mean ± SEM. *p<0.05: DGKδ+/− vs WT.

**Figure 9. Hypothetical mechanisms by which DGKδ haploinsufficiency suppresses AMPK activity.** DGKδ haploinsufficiency leads to an accumulation of DAGs in skeletal muscle. The protein kinase C (PKC) family of serine/threonine kinases can be activated by both DAG and ceramides and induce AMPK phosphorylation on Ser^485/491 (an inhibitory site). DAG excess will be stored as triglycerides (TG). The triglycerides pool can then be hydrolyzed to long chain fatty acid CoA (LCFACoA) and act as a substrate for ceramides generation. Increase in ceramide levels activate protein phosphatase 2A (PP2A), which dephosphorylates AMPK on Thr^{172}, leading to the suppression of AMPK activity.
Figure 1

A. WT □ DGKδ−/−
Before wheel running

B. WT □ DGKδ−/−
After wheel running

C. WT □ DGKδ−/−
Day 1-3

D. WT □ DGKδ−/−
Day 8-10

E. Muscle glycogen (mmol/kg)

Before wheel running

After wheel running

Locomotion (counts/20mins)

Muscle glycogen (mmol/kg)

Locomotion (counts/20mins)

Muscle glycogen (mmol/kg)

Locomotion (counts/20mins)

Muscle glycogen (mmol/kg)
Figure 2

A

WT     DGKδ⁺⁻⁻

- SIRT1
- SUO
- PDK4
- ATP synthase
- COX 1
- GAPDH

B

mRNA of CPT1 (relative to 36B4)

WT     DGKδ⁺⁻⁻
Figure 5

A. EDL

Palmitate oxidation (pmol/mg/min)

WT  DGKδ+/−

B. Soleus

Palmitate oxidation (pmol/mg/min)

WT  DGKδ+/−

C. EDL

14C palmitate incorporation

TG  FA  1.3 DAG  Ori

EDL

D. EDL

14C incorporation into TG (AU)

WT  DGKδ+/−

E. Soleus

14C incorporation into TG (AU)

WT  DGKδ+/−

P=0.09
Figure 6

A. Glucose transport (nMol/mg/20 min) in EDL

B. Glucose transport (nMol/mg/20 min) in Soleus

C. Glucose oxidation (nmol/mg/hour) in EDL

D. Glucose oxidation (nmol/mg/hour) in Soleus

E. Glucose incorporation into Glycogen (nmol/mg/hour) in EDL

F. Glucose incorporation into Glycogen (nmol/mg/hour) in Soleus
Figure 7
Figure 8

**A**

*In-vitro* contraction protocol

- **Incubation with H\(^3\)-palmitate**

<table>
<thead>
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<th>5 min STIM</th>
<th>5 min REST</th>
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**B**

- **WT**

- **Force (N)**

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**C**

- **DGKδ\(^{+/−}\)**

- **Force (N)**

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**D**

- **WT**

- **DGKδ\(^{+/−}\)**

- **Baseline force (mN)**

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**E**

- **Area under curve (mN·min)**

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Increased lipid storage

Impaired lipid oxidation

DGK

↑ DAG

PA

↑ TG

FFA

LCACoA

Ceramides

PKC

↓ p-AMPK S485/491

↓ AMPK activity

↓ p-AMPK T172

↑ p-AMPK T172

IMPARED lipid oxidation

PP2A

?