Role of PKCδ in Insulin Sensitivity and Skeletal Muscle Metabolism

Mengyao Li¹, Sara G.Vienberg¹², Olivier Bezy¹³, Brian T. O’Neill¹, C. Ronald Kahn¹

¹Section of Integrative Physiology and Metabolism, Joslin Diabetes Center, Harvard Medical School, Boston, Massachusetts 02215 USA
²Novo Nordisk Foundation Center for Basic Metabolic Research, Copenhagen University, Copenhagen, Denmark
³Current Address: Cardiovascular and Metabolic Diseases Research Unit, Pfizer Inc., Cambridge, Massachusetts, USA

Address correspondence to:
C. Ronald Kahn, M.D.
Joslin Diabetes Center
One Joslin Place, Boston, MA 02215
Phone: (617) 309-2635.
Fax: (617) 309-2487.
Email: C.Ronald.Kahn@joslin.harvard.edu

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PKCδ Regulates Glucose Tolerance and Muscle Insulin Signaling during Aging.

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ABSTRACT
Protein kinase C (PKC) δ has been shown to be increased in liver in obesity and plays an important role in the development of hepatic insulin resistance in both mice and humans. In the present study, we explored the role of PKCδ in skeletal muscle in the control of insulin sensitivity and glucose metabolism by generating mice in which PKCδ was deleted specifically in muscle using Cre-lox recombination. Deletion of PKCδ in muscle improved insulin signaling in young mice, especially at low insulin doses, however, this did not change glucose tolerance or insulin tolerance tests done with pharmacological levels of insulin. Likewise, in young mice, muscle-specific deletion of PKCδ did not rescue high-fat diet induced insulin resistance or glucose intolerance. However, with an increase in age, PKCδ levels in muscle increased, and by 6- to 7-months of age, muscle-specific deletion of PKCδ improved whole body insulin sensitivity and muscle insulin resistance, and by 15 months of age, improved the age-related decline in whole body glucose tolerance. At 15 months of age, M-PKCδKO mice also exhibited decreased metabolic rate and lower levels of some proteins of the OXPHOS complex suggesting a role for PKCδ in the regulation of mitochondrial mass at older age. These data indicate an important role of PKCδ in the regulation of insulin sensitivity and mitochondrial homeostasis in skeletal muscle with aging.
Introduction

Protein kinase C (PKC) is a family of serine/threonine kinases that play important roles in many cellular signaling events, including cell growth, differentiation, apoptosis and hormonal responses. PKCs are classified into three major categories: conventional PKCs (α, βI, βII, γ), novel PKCs (δ, ε, ν, θ) and atypical PKCs (ζ, ι, λ) (1-3). A number of PKC isoforms have been implicated in both insulin action (4;5) and insulin resistance (6;7). Activation of conventional and novel PKCs by insulin, hyperglycemia and lipids, especially diacylglycerol (DAG), have been shown to lead to insulin resistance (7-9). Protein kinase C δ (PKCδ) is a member of the novel family of PKC proteins and is involved in many pathological conditions, including ischemic heart disease (10;11) and cancer (12).

PKCδ has also been implicated in insulin action and insulin resistance (5;13-17). While in vitro studies have suggested that PKCδ plays a positive role in insulin-stimulated glucose uptake in muscle (4;5), animal studies, especially those focusing on liver, have indicated that PKCδ is a major contributor to hepatic insulin resistance (18). In previous studies using genome-wide scanning to compare the diabetes/obesity prone C57BL/6J (B6) mice and diabetes/obesity resistant 129S6/Sv (129) mice, we identified PKCδ as being strongly linked to the development of insulin resistance (15;16). Mice with liver-specific reduction in PKCδ gene expression display increased hepatic insulin sensitivity, improved glucose tolerance and reduced hepatic lipid accumulation, while mice with liver-specific over-expression of PKCδ develop hepatic insulin resistance, fatty liver and glucose intolerance (18). However, the contribution of muscle-derived PKCδ in the development of insulin resistance in vivo has not been explored.
Skeletal muscle is the predominant site of insulin-stimulated glucose uptake in the post-prandial state. Insulin resistance in muscle is one of the characteristic features of type 2 diabetes and has been shown to exist in genetically susceptible individuals, years prior to the onset of clinical diabetes (19). As noted above, *in vitro* studies have suggested a positive role of PKCδ in insulin-stimulated glucose uptake performed in skeletal muscle cells (2;13;20). Aging is associated with many metabolic changes including lipid accumulation and the development of insulin resistance (21;22). These changes, lead to increased prevalence of diabetes and metabolic syndrome beginning in middle-aged individuals and increasing thereafter. In the current study, we explored the role of PKCδ on muscle insulin sensitivity in relation to diet and age by generating mice in which the PKCδ gene has been specifically deleted in skeletal muscle using Cre-lox recombination. We found that while PKCδ does not appear to play a role in muscle insulin resistance associated with diet-induced obesity, PKCδ does play a role in the onset of insulin resistance in muscle as the mice enter middle-age. Thus, PKCδ levels in muscle increase with age, and muscle-specific deletion of PKCδ improves whole body insulin sensitivity, reverses whole body glucose intolerance and improves muscle insulin signaling in middle-aged mice. Hence, PKCδ is a component of insulin resistance as mice mature/age, suggesting its potential as a therapeutic target for type 2 diabetes.
Methods

Animals. PKCδ-floxed (control) and MCK-Cre transgenic mice have been previously described (18;23). All mice were housed in a 20-22°C temperature-controlled room on a 12 h-light/dark cycle, and were allowed ad libitum access to water and food. Animals were maintained on a standard chow diet containing 22% calories from fat (Mouse Diet 9F 5020; PharmaServ), or given a high-fat diet (HFD) containing 60% calories from fat (OpenSource Diet D 12492, Research Diet). All animal studies were performed according to protocols approved by the Institutional Animal Care and Use Committee (Joslin Diabetes Center, Boston).

Metabolic studies. For the glucose tolerance test, mice were fasted overnight and injected with glucose intraperitoneally (2 g/kg body weight). Intraperitoneal insulin tolerance tests were performed in the mice after 4 h fasting and insulin was given intraperitoneally (1 mU/g body weight). Glucose levels were measured in blood collected from the tail at the indicated times using Infinity Glucose Monitors and strips (US Diagnostics). Serum insulin levels were assessed by ELISA according to the manufacture’s recommendation (Crystal Chem).

Body composition and metabolic analysis. Body composition was measured using DEXA scanning (Lunar PIXIimus2 densitometer; GE Medical Systems) following anesthesia using Avertin (tribromoethanol:tert-amyl alcohol, 0.015ml/g i.p.). Mice were housed individually and assessed for their metabolic activities using an OPTO-M3 sensor system (Comprehensive Laboratory Animal Monitoring System, CLAMS; Columbus Instruments). Food intake, spontaneous activity, volume of oxygen consumption and volume of CO₂ production were measured over a 48 h light and dark cycle.
Mitochondrial isolation and oxygen consumption rate analysis. Mitochondria were isolated and purified from hindlimb muscle using a protocol modified from one previously described (24). Briefly, 0.5 g of hindlimb skeletal muscle was excised and digested in a buffer containing dispase (1 mg/g muscle weight) and trypsin (10 mg/g muscle weight) for 10 min at room temperature. The tissue pieces were homogenized using a Potter-Elvehjem homogenizer and centrifuged at 600 g for 10 min at 4°C. The supernatant containing the mitochondrial fraction was filtered through cheese cloth and centrifuged at 14,000 g for 10 min at 4°C. The pellet was resuspended and washed two more times in wash buffer (100 mM KCl, 50 mM Tris·HCl,1 mM MgCl₂, 0.2 mM EDTA, 0.2 mM ATP, pH 7.4) to get the final mitochondrial pellet. Isolated mitochondria were diluted in the Mitochondrial Assay Solution (70 mM sucrose, 220 mM mannitol, 10 mM KH₂PO₄, 5 mM MgCl₂, 2 mM HEPES, 1.0 mM EGTA and 0.2% fatty acid-free BSA, pH 7.2) supplied with 10 mM pyruvate/2 mM malate or 40 µM palmitoyl-carnitine/0.5 mM malate. Oxygen consumption rate (OCR) was measured using a Seahorse Bioscience XF24 analyzer in the absence (basal) or presence (state 3) of 400 µM adenosine diphosphate. ADP independent respiration activity (state 4) was measured in the presence of 2 µM oligomycin. Protein concentrations were measured using the Bradford method (Bio-Rad).

Hyperinsulinemic-euglycemic clamp. Insulin sensitivity was measured in the mice at 6-months of age by performing hyperinsulinemic-euglycemic clamp coupled with D-[3-²H]-glucose and ¹⁴C-deoxyglucose infusions as previously described (25). Whole body insulin sensitivity and muscle specific glucose uptake were measured using a continuous insulin infusion dose of 5 mU/kg/min and a constant level of blood glucose at 100 mg/dl
during the clamp. Whole body insulin sensitivity was expressed as glucose infusion rate (GIR), which was determined by the level of exogenous glucose infusion to maintain blood glucose levels as the initial levels during the clamp. Insulin-stimulated glucose uptake into the muscle was assessed during the final 45 minutes of the hyperinsulinemic-euglycemic clamp.

**In vivo insulin signaling, western blot and ROS/RNS assay.** After 6 hours of fasting, mice were anesthetized using Avertin (240 mg/kg body weight) and injected with regular human insulin (Novolin) at the doses of either 200 U/kg or 20 U/kg of body weight via the inferior vena cava. Muscles were dissected 10 minutes after the insulin bolus and frozen in liquid nitrogen. For western blot analysis, muscles were homogenized in RIPA buffer (Thermo Scientific) supplemented with phosphatase inhibitor and protease inhibitor cocktail (Sigma-Aldrich). Antibodies against phospho-Akt, phospho-ERK, phospho-IRβ, Akt, ERK and VDAC were purchased from Cell Signaling. Antibodies against PKCδ, IRβ and GAPDH were purchased from Santa Cruz. The phospho-IRS-1 antibody was purchased from Invitrogen, antibodies against IRS-1 were purchased from BD Bioscience, and antibodies against OXOPHOS proteins (mitochondrial complex I, II and IV) were from Abcam. For muscle fractionation, cytoplasmic and membrane protein were collected from skeletal muscle using a plasma membrane protein extraction kit (Abcam, ab65400). Muscle oxidative levels were determined by a spectrofluorimetric method using the OxiSelect in vitro ROS/RNS Assay kit (Cell Biolabs, STA-347).

**Ex vivo Glucose uptake.** Muscle glucose uptake was measured as described previously (26;27). Briefly, EDL and soleus muscles were dissected from 7-month old control and M-PKCδKO mice and incubated in Krebs-Ringer bicarbonate buffer (KRB) that contains
2mM of pyruvate for 1 hour in the presence or absence of 2.5mU/ml of insulin. Muscles were then transferred to transport solution in KRB buffer with 7mM $^{14}$C-D-mannitol and 1mM $^3$H-2-deoxy-glucose (2-DG), with or without insulin. Accumulation of $^3$H-2-DG was determined, and rates of uptake were calculated as described previously (26).

**Exercise capacity and muscle grip strength tests.** Maximal exercise tolerance was measured in 7-month old mice using a treadmill running protocol, modified from the previously described assay (28). In brief, mice were given 30 minutes to acclimate to the treadmill (Columbus Instruments, Columbus, OH). They then exercised at 5 m/min at $0^\circ$ incline for 5 min, and after each interval (5 min) the speed was increased by 5 m/min until reaching 20 m/min with $0^\circ$ incline. The slope was then increased 5 degrees every 5 min, while maintaining the speed at 20 m/min until the last mouse reached the exhaustion point. Maximal exercise tolerance was determined by the cumulative amount of work (kJ) that each mouse performed, calculated as body weight (kg) × vertical distance covered (m) × 9.81. Muscle strength of the forelimbs of each mouse was measured using a Grip Strength Meter (Columbus Instruments, Columbus, OH) as described previously (29). In brief, each mouse was held from the tip of its tail and the front paws grasped the grid. The grip was released when the mouse was pulled back gently. Hind limbs were kept free during the test. Each animal was tested 3 times with a 5 minute break between each measurement.

**Muscle fiber purification.** Mice were perfused with saline via the left ventricle. Single tibialis anterior (TA) muscle fibers were isolated with fine forceps under a bright-field microscope following perfusion. Isolated fibers were homogenized and processed as described above, and PKCδ protein expression was measured by western blot.
Gene expression analysis. Total RNA was extracted from tissues using Trizol. 1 µg of RNA was reverse-transcribed using a high-capacity complementary DNA reverse transcription kit (Applied Biosystems) according to the manufacture’s instructions. Quantitative realtime PCR was performed in 5 µl of the resulting cDNA after a 10-fold dilution in the presence of the SYBR Green PCR Master Mix (Applied Biosystems) and 300 nM primers. PCR reactions were run in duplicate in the ABI Prism 7700 Sequence Detection System, and Ct values were normalized to GAPDH gene levels. PKCδ primer sequence: F: 5’-CAGCCTTTCTGTGCTGTGAA-3’; R: 5’-CTGGATAACACCGCCTTCAT-3’. GAPDH primer sequence: F: 5’-TGTCGTGGAGTCTACTGGTGTCTT-3’; R: 5’-TCTCGTGGTTCACACCCATCACAA-3’.

Statistics. Data are expressed as mean ± SEM. Differences were analyzed using an unpaired Student’s t test, One-way ANOVA or two-way ANOVA. One-way ANOVA was followed by the Tukey's Multiple Comparison post-hoc test, and two-way ANOVA was performed using Bonferroni’s Multiple Comparison post-test. Statistical calculations were performed using the GraphPad Prism software (GraphPad, San Diego, CA). A probability value of < 0.05 was considered significantly different.
Results

Muscle PKCδ expression increases in B6 mice as they age but not in response to high-fat diet. Aging and obesity are two major causes of insulin resistance and diabetes (30). We first assessed if PKCδ expression was regulated in mice by dietary insult or during aging. PKCδ protein levels in muscle increased significantly in an age-dependent manner, increasing to 165% and 170% of 10-week old level at 8-months and 15-months of age respectively (Figure 1A). In contrast to our previous studies which showed increased hepatic PKCδ expression in response to HFD (18), muscles from mice challenged with a HFD for 10 weeks displayed a reduction in PKCδ protein levels by 50% (Figure 1B and C).

Mice with muscle-specific PKCδ deletion display improved insulin signaling at 10-weeks of age. Previous in vitro studies suggested an important role for PKCδ in insulin stimulated glucose uptake in muscle (4;5), while in vivo studies indicated a role for PKCδ in liver leading to insulin resistance (15-17). To assess the true metabolic role of PKCδ in muscle in vivo, we generated a mouse in which the PKCδ gene was deleted specifically in muscle by breeding mice in which exon 2 of PKCδ was flanked with loxP sites with mice carrying the Cre recombinase gene driven by the muscle creatine kinase (MCK) promoter (18;31). The resultant M-PKCδKO mice exhibited remarkable decreases of PKCδ mRNA levels in TA (74%), quadriceps (82.6%), gastrocnemius (79%), and extensor digitorum longus (EDL) (65%) muscles (all \( P < 0.05 \)) (Figure 2A). Likewise, a 70% reduction of PKCδ protein levels was observed in TA muscle fibers purified from M-PKCδKO mice (Figure 2B). At 10-weeks of age, M-PKCδKO mice did not show differences in body weight or body composition (i.e., fat and lean mass) as assessed by
DEXA when compared to control littermates (Figure S1A). Furthermore, at sacrifice, no differences in body weight or muscle weights were observed (data not shown). Muscle fiber size (data not shown) and gene expression of myosin - proteins which define fiber type - remained unchanged (Figure S1B). Intraperitoneal glucose tolerance tests (GTT) performed at 10-weeks of age revealed no difference between the M-PKCδKO and control mice with the exception of a small, but significant, decrease in glucose levels at 60 minutes in M-PKCδKO mice (Figure 2C and Figure S1C). Likewise, intraperitoneal insulin tolerance tests (ITT) demonstrated no difference in the insulin-mediated reduction of glucose levels between knockout and control mice (Figure S1D), and plasma insulin levels in both the fasted and fed states were unchanged (Figure S1E).

However, insulin signaling, especially at low insulin doses, was improved in the M-PKCδKO mice. Thus, following injection of insulin (20 U/kg body weight) into the vena cava of 10-week old mice, phosphorylation of IR in TA muscle was increased by 2.1-fold in M-PKCδKO mice compared to control (Figure 2D and E). Likewise, insulin stimulated phosphorylation of AKT and ERK was increased by 1.5-fold and 1.4-fold in M-PKCδKO mice when compared to controls. Interestingly, when insulin was injected at a higher dose (200 U/kg of body weight), no difference was observed between the control and M-PKCδKO mice, suggesting saturation of insulin receptor signaling (Figure S1F and G) at the high dose.

**High-fat diet-induced glucose intolerance and muscle insulin resistance are not prevented by PKCδ deletion in muscle.** Previous studies have shown that PKCδ is increased in the liver of HFD-induced obese mice and that PKCδ plays an important role in liver in HFD-induced insulin resistance (18). To assess the role of PKCδ in muscle on
HFD-induced insulin resistance, we placed 5-week old M-PKCδKO and control mice on either a HFD (60% fat by calories) or chow diet (23% fat by calories) and followed them for 10 weeks. During this time, control mice on chow diet gained 52% above their starting body weight, and those on HFD gained almost twice as much (Figure 3A). Body composition as assessed by DEXA at the end of the diet period revealed that the M-PKCδKO mice on CD had a tendency to have a greater lean mass than controls (Figure S2A), but this did not quite reach statistical significance. After 10 weeks of HFD, both control and M-PKCδKO mice had a similar 2-fold increase in fat mass (Figure S2B).

To determine whether this difference in body weight and composition was associated with a change in energy balance, we monitored the metabolic activities of the M-PKCδKO and control mice using metabolic cages. M-PKCδKO mice displayed similar food intake as control mice (Figure S2C), but showed a trend toward increased spontaneous activity during the light phase (Figure S2D). The oxygen consumption rate and respiratory exchange ratio (RER) were not different between M-PKCδKO and control mice (Figure S2E and F). Despite the higher spontaneous activity, M-PKCδKO mice showed no difference in the expression of genes that mark muscle fiber type (myosin IIa, myosin IIx and myosin IIb) or differentiation (myoD1 and myf5) on both CD and HFD (Figure S2G).

Fasting plasma glucose levels were unchanged between control and M-PKCδKO mice on either CD or HFD (Figure S3A). Control mice fed a HFD for 10 weeks showed a 2-fold increase in plasma insulin levels, and insulin levels were further increased by ~40% in M-PKCδKO mice on both chow and HFD diet compared to control (Figure 3B). Both control and M-PKCδKO mice showed impaired glucose tolerance on HFD (Figure
S3B) and higher levels of glucose throughout the insulin tolerance test (Figure S3C) with no differences between control and M-PKCδKO mice. As expected, HFD challenge in control mice resulted in reduced muscle insulin signaling with decreased levels of IRS1 tyrosine phosphorylation and AKT serine phosphorylation (Figure 3C), even when stimulated with an insulin at a dose of 200 U/kg of body weight. The reduction in insulin signaling, however, was not affected by PKCδ deletion in muscle. Consistent with the data shown in Figure 1, western blot analysis revealed that PKCδ expression in muscle was reduced by HFD treatment by 50% in control mice. Since PKCδ activation is associated with translocation to cell membranes (32), we determined the amount of PKCδ protein associated with the membrane fraction in muscle of control and M-PKCδKO mice on both diets. The ratio of membrane to cytosolic fraction of PKCδ was increased by 54% on HFD in control mice, consistent with the increased activation of PKCδ by HFD. Compared with control, M-PKCδKO mice demonstrated a 56% decrease in the ratio of membrane to cytosolic fraction of PKCδ on CD, and a 70% decrease in the ratio on HFD (Figure 3D). No differences were found in the levels of the reactive oxygen species (ROS) and reactive nitrogen species (RNS) between M-PKCδKO and control mice on CD or HFD (Figure S3D).

**Insulin sensitivity and glucose tolerance are improved in M-PKCδKO mice at 6- to 7-months of age.** This increase was associated with a trend towards increased uptake of $^{14}$C-deoxyglucose in the skeletal muscles (TA, EDL, soleus and quad) of the M-PKCδKO mice (Figure 4B). When insulin-stimulated glucose uptake was assessed *ex vivo* in soleus and EDL muscle strips, the M-PKCδKO mice also displayed a trend to increased glucose uptake, with 44-47% increases of insulin-stimulated $^3$H-2-deoxy-glucose uptake into
isolated EDL and soleus muscles (Figure S4A and Figure 4C). This was associated with significant 21-25% reductions in glucose levels at 60 and 90 minutes during intraperitoneal glucose tolerance testing (GTT) in 7-month old M-PKCδKO mice (Figure 4D and S4B). Insulin secretion at 0, 15 and 30 minutes during GTT was unchanged in M-PKCδKO mice (Figure 4E), and intraperitoneal insulin tolerance tests (ITT) demonstrated no difference in the insulin-mediated reduction of glucose levels between M-PKCδKO and control mice (Figure S4C). Assessment of maximal exercise capacity and forelimb grip strength revealed no differences between age-matched control and M-PKCδKO mice (Figure S5A and B).

Glucose intolerance and insulin resistance are alleviated by muscle-specific PKCδ deletion at 15-months of age. At 15-months of age, muscle-specific PKCδ deletion led to a marked improvement of glucose tolerance with a 28% decrease in AUC ($P < 0.05$) (Figure 5A). As in the younger animals, this improved glucose tolerance was not associated with changes in serum insulin levels in the fasted or fed state (Figure S7A). To determine whether the improvement of glucose tolerance was related to improved muscle insulin sensitivity in old M-PKCδKO mice, we measured insulin signaling in the TA muscle from older control and M-PKCδKO mice at 15-months of age. At this age, following high dose (200 U/kg body weight) intravenous insulin injection, phosphorylation of AKT on serine 473 was increased by 3.5-fold, with parallel changes in ERK phosphorylation in M-PKCδKO muscle when compared to controls (Figure 5B and C).

To determine whether deletion of PKCδ in muscle could prevent the age-related decline in insulin signaling, we compared insulin-medicated AKT and ERK
phosphorylation in muscle from control and M-PKCδKO mice at different ages. When compared to 10-week old mice, control mice at 15-months of age exhibited a 68% reduction in insulin-stimulated AKT serine 473 phosphorylation, which was prevented in M-PKCδKO mice (Figure 5D and E). This correlated with increased protein levels of PKCδ in muscle as the mice aged (Figure S6). Thus, when compared to control mice, M-PKCδKO mice exhibited age-dependent decreases in PKCδ protein levels, decreasing by 30% and 75% at 4- and 15-months of age respectively. The residual protein levels PKCδ may represent an incomplete rearrangement of the floxed alleles in a small subset of myofibers or, more likely, the presence of other cells types such as satellite cells, adipocytes, endothelial cells and fibroblasts (23). Indeed, isolated endothelial cells exhibited 15-fold higher levels of PKCδ mRNA expression compared to skeletal muscle (Figure S6B and C) when normalized to GAPDH. This high level of PKCδ expression in endothelial cells likely accounts for the residual expression of PKCδ observed in both the whole tissue lysates and the isolated muscle fibers.

**Impact of muscle-specific PKCδ deletion on body composition and energy expenditure at 15-months of age.** Metabolic assessment was performed in M-PKCδKO and control mice at 15-months of age. While the total body and muscle weight did not change (data not shown), DEXA analysis at this age revealed that M-PKCδKO mice had 10% less total fat and 9% less visceral fat when compared to control mice (Figure 6A and B). Furthermore, M-PKCδKO mice had a 16.5% increase in the ratio of lean mass to fat mass when compared to control mice (Figure 6C). Gene expression of muscle fiber type markers and markers of differentiation indicated that there was no change in the characteristics of muscle from older M-PKCδKO mice (Figure S7B). CLAMS metabolic
cage analysis revealed that while M-PKCδKO mice showed similar food intake and activity as control mice (Figure S7C and D), both oxygen consumption and CO₂ production normalized to lean body mass in older M-PKCδKO mice were reduced significantly by 10% and 11% respectively during the light cycle. A similar trend was also observed during the dark cycle (Figure 6D and E). RER was not different between the two genotypes (Figure S7E).

To investigate the mechanism by which muscle-specific PKCδ deletion leads to decreased oxygen consumption, we isolated mitochondria from 15-month old M-PKCδKO and control mice, and measured their metabolic capacity using different substrates in the absence (basal) or presence of ADP (state 3). In the presence of pyruvate/malate (PDH and complex I substrate), the addition of ADP increased the oxygen consumption rate (OCR) by 1.6-fold over basal in mitochondria isolated from older control mice, and mitochondria of age-matched M-PKCδKO mice displayed a similar change (Figure S7F). Likewise, in the presence of palmitoyl-carnitine/malate (β-oxidation substrate), both control and M-PKCδKO mice displayed a 2.2-fold increase in the OCR in state 3 when compared to basal state (Figure S7G). The respiratory control ratio (RCR: state 3/state 4), an index of mitochondrial coupling, was also similar between control and M-PKCδKO mice in the presence of either pyruvate/malate or palmitoyl-carnitine/malate (Figure S7H). Protein levels of the voltage dependent anion channel (VDAC) were decreased by 80% and levels of complex I subunit also trended to be decreased in 15-month old M-PKCδKO mice when compared to control littermates (Figure S8A and B). Compared to 10-week old mice, the ROS and RNS levels in muscle were significantly increased by 19% in 15-month old control mice (Figure 6F). Together
these data suggest a role for PKCδ in the regulation of mitochondrial homeostasis at older age.
Discussion

We have previously demonstrated an important role of PKCδ in the regulation of hepatic insulin sensitivity and hepatosteatosis in both mice and humans (18). Indeed, PKCδ levels in liver are higher in strains of mice that are susceptible to diet induced obesity and insulin resistance. Overexpression of PKCδ in liver of mice increases insulin resistance, and liver-specific knockout of PKCδ in mice challenged with HFD improves insulin sensitivity and glucose tolerance. On the other hand, previous studies with myoblasts and myotubes in vitro indicate that PKCδ may play a different role in muscle serving as a positive regulator of insulin signaling (5;13;33). Moreover, overexpression of PKCδ in primary muscle cells has been shown to increase GLUT4 translocation and glucose uptake in the basal state, and pharmacological inhibition of PKCδ caused down-regulation of insulin-induced GLUT4 translocation and glucose uptake (5). In cultured muscle cells, insulin stimulation has also been shown to lead to a direct association of PKCδ with the insulin receptor, with enhanced insulin-induced tyrosine phosphorylation and insulin receptor internalization (33). In Psammomys obesus, exercise also increases PKCδ levels in muscle and insulin receptor phosphorylation (34;35).

In the present study, we investigated the role of PKCδ in skeletal muscle insulin action and whole body insulin sensitivity by creating a muscle-specific PKCδ knockout (M-PKCδKO) mouse. We found that at a young age, muscle insulin signaling was improved in M-PKCδKO mice when stimulated with a low dose of insulin of 20 U/kg body weight, but was not changed at a maximal doses (200 U/kg), consistent with improved insulin sensitivity with no change in maximal response. Despite the increased signaling in M-PKCδKO muscle at lower dose, decreased PKCδ level in muscle had no
effect on whole body glucose tolerance in mice placed on either CD or HFD. However, at 6-months of age M-PKCδKO mice demonstrated an improvement in whole body insulin sensitivity as measured during a hyperinsulinemic-euglycemic clamp. At 15-months of age, muscle-specific deletion of PKCδ in mice improved muscle insulin signaling and resulted in a significant improvement in glucose tolerance. The improvement in insulin sensitivity and glucose tolerance was also accompanied by a shift towards a “leaner” body composition in the older M-PKCδKO mice with less fat, especially visceral fat, and a higher ratio of lean to fat mass. Since the accumulation of visceral fat is an important contributor to the development of diabetes and insulin resistance (19;36), it is possible that the decreased visceral fat observed in old M-PKCδKO mice plays a role in the improved insulin sensitivity and glucose tolerance. These data also suggest a crosstalk between muscle and fat, similar to what was previously observed when the insulin receptor was deleted from muscle (MIRKO mouse) but in the opposite direction, i.e., in MIRKO mice fat mass is increased (37). One reason for the different responses observed in M-PKCδKO mice at different ages is the increase in PKCδ expression in muscle as the mice progressed in age. Indeed, PKCδ protein levels in muscle increase by 70% as the mice mature from 10-weeks to 15-months of age. Many previous in vitro studies have shown that upon exposure to reactive oxygen species (ROS), PKCδ is up-regulated and activated to initiate important cellular responses (38;39). Indeed, at 15-months of age mice demonstrated a 19% increase of ROS/RNS levels in skeletal muscle when compared to younger cohorts, suggesting the accumulation of ROS during the aging process may contribute to the increased levels of PKCδ.
Previous studies have shown that PKCδ plays important roles in the regulation of mitochondrial mass and mitochondrial metabolism (40-42). Indeed, markers of mitochondrial mass, such as VDAC protein and mitochondrial complex I protein, were decreased in 15-month old M-PKCδKO mice. M-PKCδKO mice also displayed a 10% reduction in oxygen consumption at the whole body level when compared to controls. To see if loss of PKCδ impairs mitochondrial function, we determined oxygen consumption rates in isolated mitochondria from M-PKCδKO and control mice at 15-months of age. Surprisingly we observed no differences in basal or ADP-dependent mitochondrial oxidative capacity, when using two different substrates and normalized to mitochondrial protein. These data suggest PKCδ may play an important role in the maintenance of mitochondrial mass in skeletal muscle, but does not have as great an impact on mitochondrial function.

We previously showed that liver-specific reduction of PKCδ in HFD-treated obese mice improved glucose tolerance and insulin sensitivity by decreasing IRS-1 serine phosphorylation and increasing Akt phosphorylation (18). We hypothesized that similar mechanisms might contribute to HFD-induced insulin resistance in muscle and that muscle-specific PKCδ deletion may rescue HFD-induced insulin resistance. Indeed, several previous studies have shown that diacylglycerol (DAG) accumulation in muscle can activate novel PKCs, such as PKCθ, and impair muscle insulin action (43). However, our results show that, in contrast to liver-specific PKCδ deletion, PKCδ deletion in muscle is not sufficient to rescue the HFD-induced glucose intolerance and muscle insulin resistance. These opposite observations may be explained by the opposing expression patterns of PKCδ in the two tissues when exposed to HFD. Hepatic PKCδ
expression was increased by more than 50% in the HFD-treated B6 mice when compared to CD group. However, muscle PKCδ expression was decreased by 50% in mice placed on HFD when compared to those on CD, indicating differences in PKCδ regulation in these tissues. While the mechanism of the HFD-induced differences in PKCδ expression in liver and muscle are unclear, activation of other PKC isoforms in muscle, such as PKCθ and ε, may contribute to the impairment of insulin signaling in muscle and overcome the protective effect of PKCδ deletion in the muscle of diet-induced obese mice (44-46).

In summary, the role of PKCδ in muscle is different depending on the etiology of insulin resistance. While the role of PKCδ in elderly animals remains to be determined, PKCδ levels are increased in skeletal muscle of middle-aged mice, and muscle-specific deletion of PKCδ improved the middle-age-related decline in glucose tolerance and whole body insulin resistance. It also improved the ratio of lean to fat mass. In HFD-induced obesity, PKCδ levels were decreased in skeletal muscle, and deletion of PKCδ did not rescue HFD-induced insulin resistance and glucose intolerance. The distinct pattern of regulation in muscle and liver sheds new light on how PKCδ functions in different insulin-responsive tissues and the therapeutic potential of PKCδ as a target for the tissue-specific improvement in insulin resistance.
Figure Legends

**Figure 1. Effect of aging and HFD on PKCδ muscle expression in wild type B6 mice.**

(A) PKCδ protein content in triceps muscle of 10-week, 8-month and 15-month old wild type B6 mice as measured by western blot analysis (n = 8 per group). (B) PKCδ mRNA expression and (C) protein content in tibialis anterior (TA) muscle of 3-month old wild-type B6 mice fed either a CD or HFD for 10 weeks. A representative western blot is shown in Figure 4E. Data are mean ±SEM. n = 8. * P < 0.05 vs. 10 weeks by one-way ANOVA; ‡ P < 0.05 vs. CD by Student’s t-test.

**Figure 2. Glucose tolerance and muscle insulin signaling are normal in muscle-specific PKCδ knockout mice at 10-weeks of age.** (A) PKCδ mRNA expression in tibialis anterior (TA), quadriceps (Quad), gastrocnemius (Gastro), extensor digitorum longus (EDL) and soleus muscles from Lox and M-PKCδKO mice. (B) Representative western blots and densitometric quantification of PKCδ in the purified TA muscle fibers of Lox and M-PKCδKO mice with quantification below. (C) Intraperitoneal glucose tolerance tests were performed in Lox and M-PKCδKO mice. (D) Western blot analysis of insulin signaling pathway in skeletal muscle of Lox and M-PKCδKO mice after intravenous insulin injection (20 U/kg of body weight). (E) Densitometric analysis of IR, IRS-1, AKT and EKR phosphorylation from panel D after insulin stimulation. White bars represent control mice; black bars represent M-PKCδKO mice. Data are mean ±SEM. n=6-8 per group in panels (A) – (C), and n = 4 per group for panels (D) and (E). ‡ P < 0.05 by Student’s t-test.
Figure 3. Muscle-specific knockout of PKCδ does not alleviate HFD-induced muscle insulin resistance. (A) Body weight changes of Lox and M-PKCδKO mice fed with either a CD or HFD from 5- to 16- weeks of age (n = 10 per group). (B) Insulin levels were measured in 4-month old Lox and M-PKCδKO mice fed either a CD or HFD (n = 6-8 per group). (C) Western blot analysis of insulin signaling in TA muscle of Lox and M-PKCδKO after intravenous insulin injection (200 U/kg of body weight). (D) Western blot of PKCδ protein in cytoplasmic (cyto) and membrane (MEM) fractions of gastrocnemius muscle from Lox and M-PKCδKO mice fed either a CD or a HFD. White bars represent control mice; black bars represent M-PKCδKO mice. Data are mean ±SEM. * P < 0.05 by two-way ANOVA in figure 3A (CD-treated control vs. HFD-treated control mice) and 3B.

Figure 4. Muscle-specific knockout of PKCδ improves whole body insulin sensitivity and glucose tolerance at 6- to 7-months of age. (A) Glucose infusion rate and (B) insulin stimulated $^{14}$C-deoxyglucose uptake were measured during hyperinsulinemic-euglycemic clamps in 6-month old M-PKCδKO mice (n=3-7). (C) Rates of $^{3}$H-2-deoxyglucose uptake into soleus muscles in the presence or absence of 2.5 mU/ml of insulin (n = 4-5). (D) Intraperitoneal glucose tolerance tests (GTT), and (E) Insulin secretion during GTT at 0, 15 and 30 min (n = 5). White bars represent Lox mice; black bars represent M-PKCδKO mice. Data are mean ±SEM. ‡ P < 0.05 by Student’s t-test.

Figure 5. Glucose tolerance and muscle insulin signaling are improved in M-PKCδKO mice at 15-months of age. (A) Intraperitoneal glucose tolerance tests were performed in 15-month old Lox and M-PKCδKO mice (n=12 per group) and area under the curve was measured. (B) Insulin signaling was analyzed in TA muscle of 15-month
old Lox and M-PKCδKO mice by western blot after intravenous insulin injection (200 U/kg of body weight). (C) Insulin-stimulated phosphorylation of the insulin receptor, AKT and ERK after insulin stimulation were quantified by densitometric analysis. (D) Representative western blot of insulin signaling from TA muscles of Lox and M-PKCδKO mice at 10 week, 4 months and 15-months of age. (E) Densitometric analysis of insulin-stimulated AKT and ERK phosphorylation at 10-weeks and 15-months of age. White bars represent Lox mice; black bars represent M-PKCδKO mice. Data are mean ±SEM. ‡ P < 0.05 by Student’s t-test; * P < 0.05 by two-way ANOVA.

Figure 6. Impact of muscle-specific deletion of PKCδ on body composition and energy expenditure at 15-months of age. (A) Total fat and lean mass, (B) percentage of visceral fat and subcutaneous fat per body weight and (C) ratio of lean mass to fat mass were assessed by DEXA in 15-month old Lox and M-PKCδKO mice (n=12 per group). (D) Oxygen consumption and (E) CO₂ production measured in Lox and M-PKCδKO mice over 48 h in CLAMS metabolic cages (n=12 per group). (F) Reactive oxygen and reactive nitrogen species were measured in TA muscles of Lox and M-PKCδKO mice at 10 weeks and 15-months of age. White bars represent control mice; black bars represent M-PKCδKO mice. Data are mean ±SEM. ‡ P < 0.05 by Student’s t-test. * P < 0.05 by two-way ANOVA.
Figure Supplement Legends

Figure S1. Insulin tolerance, gene expression of myogenesis markers and muscle fiber type markers and muscle insulin signaling in 10-week old mice. (A) Body weight, total fat and total lean mass of Lox and M-PKCδKO mice fed a chow diet were measured by DEXA analysis at 10-weeks of age. (B) Gene expression of muscle markers for muscle fiber type and myogenesis were assessed by qPCR in TA muscle of 10-week old Lox and M-PKCδKO mice. (C) Area under the curve of intraperitoneal glucose tolerance tests and (D) insulin tolerance tests were performed in Lox and M-PKCδKO mice. (E) Fasted and fed serum insulin levels in Lox and M-PKCδKO mice. (F) Western blot analysis of insulin signaling pathway in skeletal muscle of Lox and M-PKCδKO mice after intravenous insulin injection (200 U/kg of body weight). (G) Densitometric analysis of insulin receptor phosphorylation and AKT phosphorylation from panel F after insulin stimulation. White bars represent control mice; black bars represent M-PKCδKO mice. Data are mean ±SEM. n=6-8 per group in panels (A) – (E), and n = 3 per group in panels (F) and (G).

Figure S2. Body composition and energy expenditure in M-PKCδKO mice on either a chow diet or high-fat diet. (A) Total lean and (B) total fat mass were assessed by DEXA in 4-month old Lox and M-PKCδKO mice fed with CD or HFD. (C) Food intake was measured by CLAMS in a 48 hr cycle after 10 weeks treatment with CD or HFD. (D) Spontaneous activity was measured in Lox and M-PKCδKO mice during 48 h cycle in metabolic cages. (E) Oxygen consumption (VO$_2$) and (F) respiratory exchange ratio (RER) were analyzed by metabolic cage assessment. (G) Gene expression of muscle markers for muscle fiber type and myogenesis were assessed by qPCR in TA muscle of
Lox and M-PKCδKO mice fed either a CD or a HFD (n = 4 per group). n = 5 per group in panels (A) - (F). White bars represent control mice; black bars represent M-PKCδKO mice. Data are mean ±SEM.

Figure S3. Muscle-specific knockout of PKCδ does not alleviate HFD-induced muscle insulin resistance. (A) Glucose levels were measured in 4-month old Lox and M-PKCδKO mice fed either a CD or HFD. (B) Intraperitoneal glucose tolerance tests and its area under the curve, and (C) insulin tolerance tests were performed in 4-month old Lox and M-PKCδKO mice fed either a CD or HFD from 6-weeks of age. (D) Reactive oxygen and reactive nitrogen species were measured in TA muscles of Lox and M-PKCδKO mice fed either a CD or HFD. White bars represent control mice; black bars represent M-PKCδKO mice. Data are mean ±SEM. n= 5 per group in panels (A) to (D).

Figure S4. Ex vivo glucose uptake in EDL and insulin tolerance of M-PKCδKO mice at 6- to 7-months of age. (A) Rates of $^3$H-2-deoxy-glucose uptake into muscles in the presence or absence of 2.5 mU/ml of insulin (n = 4-5). (B) Area under the curve of intraperitoneal glucose tolerance tests (GTT), and (C) insulin tolerance tests performed in Lox and M-PKCδKO mice at 7-months of age (n = 5). White bars represent Lox mice; black bars represent M-PKCδKO mice. Data are mean ±SEM.

Figure S5. Exercise capacity and muscle grip strength of mice at 7-months of age. (A) Maximal exercise capacity was measured in 7-month old Lox and M- PKCδKO mice that were exercised on a treadmill until exhaustion. (B) Forelimb grip strength was determined in Lox and M- PKCδKO mice. Data are mean ±SEM.
Figure S6. PKCδ protein are increased in muscle of 15-month old mice. (A) PKCδ protein content in TA muscle of 10-week, 4- and 15-month old Lox and M-PKCδKO mice by western blot analysis. PKCδ (B) and CD31 (C) mRNA expression in gastrocnemius (Gastro), tibialis anterior (TA) and liver endothelial cells (Endothelial) from control mice. Data are mean ±SEM. * P < 0.05 by two-way ANOVA.

Figure S7. Plasma insulin and energy expenditure of mice at 15-months of age. (A) Fast and fed insulin levels were measured in 15-month old control and M-PKCδKO mice (n=6 per group). (B) mRNA levels of muscle fiber type markers were measured in TA muscle of 15-month old Lox and M-PKCδKO mice by qPCR (n = 5 per group). (C) Food intake in 15-month old Lox and M-PKCδKO mice (n=12 per group). (D) Spontaneous activity was measured in Lox and M-PKCδKO mice over 48 h in CLAMS metabolic cages (n=12 per group). (E) respiratory exchange ratio (RER) from the same analysis. (F) Basal and ADP-dependent (State 3) respiratory rates of isolated mitochondria from hindlimb muscle were measured using a Seahorse X24 Flux Analyzer in the presence of pyruvate/malate (n=4 per group) as described in Materials and Methods. (G) Basal and State 3 respiratory rates of isolated mitochondria from hindlimb muscle were measured with palmitoyl-carnitine/malate (fatty acid) as substrate (n=4 per group). (H) Respiratory control ratio (RCR) of isolated mitochondria was measured with pyruvate/malate (PM) or palmitoyl-carnitine/malate (PC) as substrate (n=4 per group). OCR, oxygen consumption rate. White bars represent control mice; black bars represent M-PKCδKO mice. Data are mean ±SEM.

Figure S8. Impact of muscle-specific PKCδ deletion on mitochondrial protein content at 10-week, 4-months and 15-months of age. (A) Protein levels of
mitochondrial marker (VDAC) and muscle oxidative phosphorylation complex subunits (mitochondrial complex I, II and IV) were assessed by western blot in TA muscles of Lox and M- PKCδKO mice at 10-weeks, 4-months and 15-months of age (n = 3 per group).

(B) Densitometric quantification of western blots. White bars represent Lox mice; black bars represent M-PKCδKO mice. Data are mean ±SEM. * P < 0.05 by two-way ANOVA;

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M.L. generated the data and wrote the manuscript. O.B. initiated the project, reviewed and edited the manuscript. B.T.O. and S.G.V. reviewed the manuscript and generated the data. C.R.K. oversaw the project, contributed to discussion, and helped write the manuscript. C.R.K. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. There are no potential conflicts of interest relevant to this article by any of the authors.
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Sequence of age-associated changes to the mouse neuromuscular junction and the protective effects of voluntary exercise. PLOS One 8:e67970, 2013


Fig. 1

A. PKCδ protein content

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Relative mRNA expression of PKCδ

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PKCδ protein content (Arbitrary Units)

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Fig. 3

A. Body Weight (g) vs. Age (wks)

B. Plasma Insulin (ng/ml)

C. Western Blot Analysis

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Insulin, PKCδ, P-IR (T1150/1151), IR, P-IRS1 (Y612), IRS1, P-Akt (S473), Akt, P-ERK1/2, ERK1/2, GAPDH

D. Western Blot Analysis (Cyto-MEM)

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PKCδ (lighter exposure)
**Fig. 5**

**A.**

- **Glucose (mg/dl) vs. Time (min)**
  - Graph showing glucose levels over time with different insulin and MPKCδKO conditions.
  - Lox and M-PKCδKO conditions are indicated.
  - Area under the curve is also shown.

**B.**

- **Insulin Treatment**
  - Table showing Insulin treatment conditions for Lox and M-PKCδKO.
  - **P-IR (T1150/1151), IR, P-Akt (S473), Akt, P-ERK1/2, ERK1/2, GAPDH**

**C.**

- **P-IR/IR (% of Lox)**
  - Graph showing P-IR/IR levels after insulin stimulation for Lox and M-PKCδKO.
  - Significance indicated.

- **P-AKT/AKT (% of Lox)**
  - Graph showing P-AKT/AKT levels after insulin stimulation for Lox and M-PKCδKO.
  - Significance indicated.

- **P-ERK/ERK (% of Lox)**
  - Graph showing P-ERK/ERK levels after insulin stimulation for Lox and M-PKCδKO.
  - Significance indicated.

**D.**

- **Insulin and Time Conditions**
  - Table showing Insulin and Time conditions for Lox and M-PKCδKO.
  - **P-Akt (S473), Akt, P-ERK, ERK, GAPDH**

**E.**

- **P-ERK/ERK (% of Lox) after insulin stimulation**
  - Graph showing P-ERK/ERK levels after insulin stimulation for different ages and populations.
  - Significance indicated.

**Diabetes**
Suppl. 1

A.

![Graph showing Mass (grams) for BW, Total Fat, and Total Lean](image)

B.

![Graph showing mRNA expression (fold change) for Myosin 2a, myosin 2x, Myosin 2b, MyoD1, and Myf5](image)

C.

![Graph showing Area under the curve (mg/dl/hr) for BW](image)

D.

![Graph showing Glucose (mg/dl) over Time (min) for Lox and M-PKCδKO](image)

E.

![Graph showing Plasma insulin (mg/ml) for Fast and Fed, with Lox and M-PKCδKO](image)

F.

![Western blots for Insulin, PKCδ, P-IR (Y1150/1151), IR, P-IRS1 (Y612), IRS1, PAkt (S473), Akt, PERK1/2, ERK1/2, and GAPDH](image)

G.

![Graph showing P-IR/IR (% of Lox) and P-AKT/AKT (% of Lox) after insulin stimulation for Lox and M-PKCδKO](image)
Suppl. 3  Diabetes

A. 

Plasma glucose (mg/dl)

0 30 60 90 120

CD-fl/fl
CD-M-PKCδKO
HFD-fl/fl
HFD-M-PKCδKO

Time (min)

B. 

Glucose (mg/dl)

0 30 60 90 120

CD-fl/fl
CD-M-PKCδKO
HFD-fl/fl
HFD-M-PKCδKO

Time (min)

Area under the curve (mg/dl/hr)

0 200 400 600 800 1000

CD HFD

C. 

Glucose (mg/dl)

0 30 60 90 120

CD-fl/fl
CD-M-PKCδKO
HFD-fl/fl
HFD-M-PKCδKO

Time (min)

D. 

ROS & RNS levels (Arbitrary units)

0 1000 2000 3000 4000

CD HFD

Suppl. 3 Page 44 of 49  Diabetes
A. Total Work (J)

B. Grip strength (Newton)
A.

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VDAC
CIV- mtCo1
CII-SDHB
CI- NDUFB8
GAPDH

B.

**VDAC protein content (Arbitrary Units)**

![Graph showing VDAC protein content over time]

**Complex II and IV protein content (Arbitrary Units)**

![Graph showing Complex II and IV protein content over time]

**Complex I protein content (Arbitrary Units)**

![Graph showing Complex I protein content over time]