Muscle-specific knockout of PKC-\(\lambda\) impairs glucose transport and induces metabolic and diabetic syndromes

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Obesity, the metabolic syndrome, and type 2 diabetes mellitus (T2DM) are major global health problems. Insulin resistance is frequently present in these disorders, but the causes and effects of such resistance are unknown. Here, we generated mice with muscle-specific knockout of the major murine atypical PKC (aPKC), PKC-\(\lambda\), a postulated mediator for insulin-stimulated glucose transport. Glucose transport and translocation of glucose transporter 4 (GLUT4) to the plasma membrane were diminished in muscles of both homozygous and heterozygous PKC-\(\lambda\)-knockout mice and were accompanied by systemic insulin resistance; impaired glucose tolerance or diabetes; islet \(\beta\) cell hyperplasia; abdominal adiposity; hepatosteatosis; elevated serum triglycerides, FFAs, and LDL-cholesterol; and diminished HDL-cholesterol. In contrast to the defective activation of muscle aPKC, insulin signaling and actions were intact in muscle, liver, and adipocytes. These findings demonstrate the importance of aPKC in insulin-stimulated glucose transport in muscles of intact mice and show that insulin resistance and resultant hyperinsulinemia owing to a specific defect in muscle aPKC is sufficient to induce abdominal obesity and other lipid abnormalities of the metabolic syndrome and T2DM. These findings are particularly relevant because humans who have obesity, impaired glucose tolerance, and T2DM reportedly have defective activation and/or diminished levels of muscle aPKC.

Introduction

Glucose transport is rate-limiting for glucose metabolism in most tissues. In muscle, the major tissue for whole-body glucose disposal, defects in insulin-stimulated glucose transport are observed in obesity, the metabolic syndrome, with associated abdominal obesity, hyperlipidemia, and impaired glucose tolerance (IGT); and type 2 diabetes mellitus (T2DM).

Factors that mediate insulin stimulation of glucose transport are still poorly defined. Studies using inhibitory agents in cultured myocytes and adipocytes suggest that insulin-stimulated glucose transport is regulated by atypical PKCs (aPKCs), PKC-\(\alpha\) and PKC-\(\lambda\) (1–7), and by PKB/Akt (8–12), operating downstream of PI3K. There is, however, little evidence from gene-targeting studies to implicate specific isoforms of aPKC and PKB that are required for insulin-stimulated glucose transport in intact muscle. Total-body KO of PKB-\(\alpha\)/Akt1 does not alter glucose homeostasis (13), and total-body KO of PKB-\(\beta\)/Akt2 produces a diabetic state, characterized by markedly defective hepatic glucose metabolism and a partial decrease in insulin-stimulated glucose transport in muscle that is evident only at submaximal insulin concentrations (14). Total-body KO of PKC-\(\lambda\), the major murine aPKC, is embryonic lethal, and, despite reports of liver-specific (15) and pancreatic \(\beta\) cell–specific (16) PKC-\(\lambda\)-KO, there are no reports to our knowledge of muscle-specific PKC-\(\lambda\)-KO. Information on effects of this deficiency in muscle aPKC is particularly relevant, because aPKC levels are diminished and/or poorly activated in muscles of humans who have obesity (17, 18), IGT (19, 20), and T2DM (17, 20, 21).

Here, we report on a muscle-specific PKC-\(\lambda\)-KO created by breeding mice harboring Cre recombinase transgene controlled by a muscle creatine kinase (MCK) promoter/enhancer with mice carrying a targeted PKC-\(\lambda\)-allele with loxP sites flanking an essential exon located at nucleotides 110–233; details of the floxed PKC-\(\lambda\)-construct were described previously (22). In these mice, we directly tested the hypothesis that aPKC is required for glucose transport effects of insulin; moreover, we examined the metabolic consequences of defects in aPKC availability/activation and glucose transport occurring specifically in muscle. We found that insulin-stimulated glucose transport was markedly impaired in heart muscle of KO mice in which aPKC levels were diminished. Moreover, this isolated defect in muscle glucose transport was accompanied by systemic insulin resistance, glucose intolerance, abdominal obesity, hyperlipidemia, and hepatosteatosis, i.e., features of metabolic syndrome.

Results

Glucose transport in muscles of homozygous KO mice. Total aPKC and PKC-\(\lambda\)-levels were markedly diminished (~80%) in vastus lateralis and heart muscles of mice with homoygous muscle-specific PKC-\(\lambda\)-KO (Cre\(^{loxP}\)\(^{+/+}\); Figure 1A and B, and Figure 2A). This aPKC deficiency was accompanied by marked decreases in muscle uptake of

Nonstandard abbreviations used: aPKC, atypical PKC; AS160, Akt substrate 160; 2-DG, 2-deoxyglucose; FAS, fatty acid synthase; GLUT, glucose transporter; GSK, glycogen synthase kinase; HGP, hepatic glucose production; IGT, impaired glucose tolerance; IRS, insulin receptor substrate; MCK, muscle creatine kinase; PIP2, phosphatidylinositol 3,4,5-triphosphate; T2DM, type 2 diabetes mellitus.

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glucose, both basally and during insulin treatment, in vivo (Figure 1, A and B). Small residual effects of insulin on glucose transport in muscles of KO mice may be mediated by PKC-ζ, a minor aPKC in mouse muscle (6), the levels of which were unaltered in muscles of KO mice (Figure 2A).

Levels of aPKC and insulin-stimulated [$^3$H]2-deoxyglucose ([H]2-DG) uptake in vitro were also diminished in slow-twitch soleus and fast-twitch extensor digitorum longus muscles of heterozygous KO mice (Figure 1E). Uptake of [H]2-DG was not influenced by the presence of MCK-controlled Cre recombinase in the absence of loxP sites in PKC-λ, i.e., in Cre$^{-}\text{loxP}^{\text{–/–}}$; HET KO, and homozygous KO (Cre$^{-}\text{loxP}^{\text{–/–}}$; Homo KO) groups. aPKC levels in muscles of heterozygous control (Cre$^{-}\text{loxP}^{\text{–/–}}$; HET con) and Cre control (Cre$^{-}\text{loxP}^{\text{–/–}}$; Cre con) mice were the same as in WT mice (not shown). *P < 0.05; **P < 0.01; ***P < 0.001.

**Figure 1**

Effects of homozygous and heterozygous muscle-specific KO of PKC-λ on basal and insulin-stimulated glucose transport in vivo (A–D), glucose transport in vitro (E), and plasma membrane (PM) GLUT4 levels (F). Equal numbers of 5-month-old male and female mice were grouped (results were indistinguishable and therefore pooled), and after an overnight fast, glucose transport and GLUT4 translocation were measured. Glucose uptake was measured in vastus lateralis (VL) and heart muscle during 10 minutes insulin or vehicle treatment (A–D). (E) Glucose transport was measured in isolated extensor digitorum longus (EDL) and soleus muscles obtained from male mice. (F) Vastus lateralis and gastrocnemius muscles were used to prepare plasma membranes. Values are mean ± SEM. n for each group is shown in parentheses. Insets show immunoreactive levels of total cellular aPKC (A, B, and E) or plasma membrane and internal membrane (IM) GLUT4 levels or IGF1 receptor (IGF1R) and insulin receptor (InsR) β subunit levels as markers to show equal plasma membrane loading (F) in WT (Cre$^{-}\text{loxP}^{\text{–/–}}$), heterozygous KO (Cre$^{-}\text{loxP}^{\text{–/–}}$; HET KO), and homozygous KO (Cre$^{-}\text{loxP}^{\text{–/–}}$; Homo KO) groups. aPKC levels in muscles of heterozygous control (Cre$^{-}\text{loxP}^{\text{–/–}}$; HET con) and Cre control (Cre$^{-}\text{loxP}^{\text{–/–}}$; Cre con) mice were the same as in WT mice (not shown). *P < 0.05; **P < 0.01; ***P < 0.001.
Effects of homozygous and heterozygous muscle-specific KO of PKC-λ on levels of insulin-sensitive signaling factors and glucose transporters in skeletal and heart muscle. Muscles were obtained from mice treated as described in Figure 1, A–D. Shown are representative immunoblots of muscle factors from basal and insulin-stimulated WT, heterozygous control, homozygous KO, and heterozygous KO mice. In A, VL and heart muscle of WT and homozygous KO mice, treated with or without insulin were compared. In B, VL and heart muscle of WT and heterozygous KO mice were compared. p-, phosphorylated. See Figure 1 for comparison of aPKC levels amongst groups.

Figure 2
Effects of homozygous and heterozygous muscle-specific KO of PKC-λ on levels of insulin-sensitive signaling factors and glucose transporters in skeletal and heart muscle. Muscles were obtained from mice treated as described in Figure 1, A–D. Shown are representative immunoblots of muscle factors from basal and insulin-stimulated WT, heterozygous control, homozygous KO, and heterozygous KO mice. In A, VL and heart muscle of WT and homozygous KO mice, treated with or without insulin were compared. In B, VL and heart muscle of WT and heterozygous KO mice were compared. p-, phosphorylated. See Figure 1 for comparison of aPKC levels amongst groups.

the low total aPKC activity presumably reflected the greater abundance of PKC-λ in mouse muscle (6). Lower-than-expected muscle aPKC activity in heterozygous KO mice may reflect poor aPKC responsiveness to phosphatidylinositol-3,4,5-(PO_4)_3 (PIP_3), which increased activity of aPKC immunoprecipitated from muscles of heterozygous control mice, but not heterozygous KO mice (Figure 3B). Such aPKC unresponsiveness to PIP_3 also occurs in some insulin-resistant humans and rodents (e.g., refs. 18, 20, 23).

Unlike aPKC activation, insulin-stimulated PKB activity and Ser473 phosphorylation in muscle increased substantially in homozygotes but was markedly enhanced in heterozygous KO mice, treated with or without insulin. In addition, fasting blood glucose levels in KO mice were increased (20%–25%) during insulin and glucose tolerance tests in heterozygotes, and serum insulin levels increased to a greater extent during glucose tolerance tests in heterozygotes (Figure 5). Also, modestly increasing dietary fat content from 5% to 10% for 2 months increased blood glucose levels in both WT and heterozygous KO mice, but differences remained apparent at all time points, including fasting levels, during glucose tolerance testing (Figure 6B). Moreover, food intake increased by 20%, a significant difference, in heterozygotes consuming standard chow. Dietary intake in homozygotes was not appreciably different from that of WT mice (data not shown). In summary, heterozygotes apparently developed an obesity/diabetes syndrome that was abetted by dietary excess.

Interestingly, serum glucose and insulin levels were higher in male KO mice relative to female KO mice (Figures 5 and 6), whereas abdominal obesity and serum lipid abnormalities were at least as prominent in female KO mice (see Serum glucose and insulin levels in KO mice). The reasons for these differences were unclear, as we did not discern sex-related differences in muscle glucose uptake or insulin signaling to PKB and aPKC in liver or to PKB in muscle.

Peripheral and hepatic insulin sensitivity and resistance in hyperinsulinemic euglycemic clamp studies. At the time of the clamp, mean fasting plasma glucose levels were not significantly different among the WT, heterozygous KO, and homozygous KO groups (Figure 7A). To evaluate the effects of muscle-specific deficiency of PKC-λ on insulin-stimulated glucose metabolism in vivo, 2-hour hyperinsulinemic-euglycemic clamps were conducted in conscious male mice. During the clamp, insulin was infused at a constant rate (18 pmol/kg/min) to raise plasma insulin within a physiological range, and plasma glucose was clamped at approximately 115 mg/dl (Figure 7A). Glucose infusion rates in heterozygous and homozygous KO mice needed to maintain euglycemia were identical, but consistently lower than that needed in WT mice (Figure 7B). This whole-body insulin resistance was predominantly accounted for by decreases in insulin-stimulated fasting (4–6 hours) blood and serum glucose levels in homozygotes were normal or only slightly increased (Figure 5). Nevertheless, serum glucose levels increased in ad libitum–fed male homozygotes (Figure 6A), and insulin tolerance and glucose tolerance were impaired in male and female homozygotes (Figure 5). Furthermore, serum insulin levels were increased in ad libitum–fed male homozygotes (Figure 6A) and were significantly higher or trended higher during glucose tolerance tests in male and female homozygotes (Figure 5B).
Alterations in abdominal fat and body weight in KO mice. Paralleling alterations in serum glucose and insulin levels, abdominal fat content and total body weight increased more in heterozygous KO mice than in homozygous KO mice (Figure 8A). In contrast, weights of vastus lateralis and heart muscles and livers were not significantly altered in KO mice (Figure 8A).

Alterations in serum lipid levels in KO mice. Following a 20-hour fast, serum FFA levels increased by about 40% in homozygous KO mice and about 90% in heterozygous KO mice, perhaps reflecting abdominal obesity. Fasting serum triglyceride levels increased in heterozygous KO mice, and there were reciprocal alterations in LDL-cholesterol and HDL-cholesterol (increase and decrease, respectively) that were comparable in heterozygotes and homozygotes (Figure 8A).

Discussion

The present findings provide compelling evidence that aPKCs are required for insulin-stimulated glucose transport in mouse muscle. This seems clear, not only from studies of glucose transport in vivo and in vitro, but also from phenotypic abnormalities of mice with homozygous muscle-specific PKC-λ KO. Abnormalities in glucose metabolism and expansion of islet β cell mass and resul-
Because PKC-λ can reasonably be attributed to muscle aPKC deficiency. Either total aPKC levels or insulin-stimulated glucose transport impaired actions of insulin in liver and adipocytes, and therefore inhibits insulin-stimulated glucose transport in rat-derived L6 myotubes, which are rich in PKC-ζ and have little or no PKC-λ (5). Also note that expression of PKC-λ rescues glucose transport in L6 myotubes in which PKC-ζ is depleted; conversely, expression of PKC-ζ rescues glucose transport in mouse-derived adipocytes in which PKC-λ is depleted (5). The fact that both aPKCs can function interchangeably in glucose transport presumably reflects evolutionary conservation of important functional domains in PKC-λ and PKC-ζ needed for glucose transport and other essential biological functions.

Despite marked losses of PKC-λ, there were no compensatory increases in PKC-ζ levels in the muscle of KO mice. Also, although we were unable to measure separate enzyme activities of PKC-ζ and PKC-λ, the marked loss of total aPKC activity in muscle of KO mice confirmed that the activity of PKC-ζ was not compensatorily increased.

The fact that PKB activation and AS160 phosphorylation were intact or enhanced in muscles of PKC-λ KO mice is important, as it indicates that aPKC, as well as PKB, is required for insulin-stimulated GLUT4 translocation and subsequent glucose transport. Given the complexity of the translocation process, it is not surprising that more than one insulin-sensitive protein kinase was operative in regulating this process.

It was surprising to find that phenotypic abnormalities were as prominent, or more so, in heterozygous KO mice than in homozygous KO mice. However, we found several factors that may have contributed importantly to phenotypic differences. First, insulin-stimulated hepatic PKB activity, which diminishes as hyperglycemia (diabetes) ensues in rodent models (24), trended downward in heterozygotes, in contrast to the marked increases in homozygotes. Second, as in liver, insulin-stimulated PKB activation was enhanced in muscles of homozygotes, but not heterozygotes, possibly enhancing muscle glucose metabolism. Third, feeding-dependent increases in expression and nuclear levels of SREBP-1c (which transactivates multiple lipid-synthesizing enzymes, including FAS) were greater in heterozygotes and may have abetted the development of obesity and lipid abnormalities. Fourth, levels of serum FFA—which adversely alter HGP, insulin sensitivity, and glucose tolerance—were higher in heterozygotes. Fifth, correlating with abdominal obesity and hyperlipidemia, hepatosteatosis was greater in heterozygotes, and these lipid factors may diminish insulin sensitivity. Sixth, remaining PKC-λ in heterozygote muscle was poorly responsive to PIP₃, presumably impairing glucose disposal beyond that attributable to partial aPKC loss. Seventh, dietary intake was increased in heterozygotes, but not in homozygotes; further studies are needed to determine whether hypothalamic centers controlling appetite and energy consumption are altered, particularly in heterozygotes. Eighth, serum adiponectin levels were increased in homozygotes, but not in heterozygotes, and this adipokine activates S₅'-AMP-dependent protein kinase and enhances insulin signaling (including PKB activation/phosphorylation) and actions in muscle and liver (25). However, whether adiponectin favorably influenced muscle and liver PKB activation and hepatic SREBP-1c expression in homozygotes, and why adiponectin secretion was increased in homozygotes but not in heterozygotes, are unclear.
In considering factors that influence phenotypes, hepatosteatosis and abdominal obesity as well as elevated serum FFA/triglyceride levels may have intensified insulin resistance and insulin-mediated phenotypic abnormalities. Indeed, a vicious cycle may exist in which alterations in lipid metabolism increase insulin resistance and, conversely, insulin resistance and resulting hyperinsulinemia increase obesity and serum lipid abnormalities, particularly in heterozygotes.

The reason for excessive increases in insulin-stimulated PKB activation and phosphorylation in liver and muscle of homozygotes is uncertain, but did not appear to reflect alterations in PKB levels or activation of signaling factors immediately upstream of PKB, namely IRS-1–dependent PI3K in muscle (26, 27) and both IRS-1–dependent and IRS-2–dependent PI3K in liver (26–28). In support of this idea, activation of hepatic aPKC, which, like PKB, is dependent on IRS-2–dependent PI3K (27, 28) and 3-phosphoinositide–dependent protein kinase 1 (PDK1), was not excessive in homozygous liver. On the other hand, Ser473-PKB phosphorylation was increased in liver and muscle, and factors that phosphorylate Ser473 include not only a putative PDK2 (29), but also other kinases such as MAPK2, p38 MAPK, mTOR, PKC-α, and PKC-β; unfortunately, the significance of these kinases is poorly understood (30). Interestingly, Ser473-PKB phosphorylation is improved in insulin-resistant mice by adiponectin (25), which was increased in homozygotes, but not in heterozygotes (the latter failure may reflect greater adiposity, which correlates inversely with adiponectin). The lack of increase in serum adiponectin and PKB activity and phosphorylation in liver and muscle may be particularly important for the greater-than-expected phenotypic abnormalities we observed in heterozygotes. Further studies are needed to examine these and other reasons for phenotypic differences.

Findings in KO mice, particularly homozygous, may be relevant to the question of whether features of the metabolic syndrome, most notably hyperlipidemia and obesity, reflect excessive or diminished insulin effects in liver and/or adipocytes. In homozygotes, insulin-stimulated glucose transport in adipocytes was intact and fat stores were increased; moreover, insulin signaling in liver was normal or enhanced, and HGP was not significantly altered. Thus, at least in homozygotes, alterations in serum triglycerides, FFAs, and LDL-cholesterol and HDL-cholesterol occurred in the

![Figure 5](image-url)

**Figure 5**
Effects of homozygous and heterozygous muscle-specific KO of PKC-λ on insulin tolerance (A) and glucose tolerance (B) in 5-month-old male and female mice fasted for 4–6 hours. (A) Insulin tolerance was determined by i.p. administration of 0.75 mU insulin per gram body weight. (B) Glucose tolerance was determined by i.p. administration of 2 mg glucose per gram body weight. Values of tail vein blood glucose and serum insulin are mean ± SEM. n for each group is shown in parentheses. *P < 0.05; **P < 0.01; ***P < 0.001 versus respective control (ANOVA).
absence of measurable defects in insulin signaling or action in adipocytes and liver. We therefore surmise that in homozygotes, (a) metabolic syndrome features including abdominal obesity and hyperlipidemia reflected enhanced, rather than diminished, insulin actions in liver and adipocytes, and (b) hyperinsulinemia caused by impaired muscle glucose transport is sufficient to cause or abet the development of abdominal obesity and hyperlipidemia.

It is interesting to compare the phenotype of mice with muscle-specific KO of PKC-λ to that of mice with muscle-specific KO of Glut4 or insulin receptor. As with PKC-λ KO, muscle-specific Glut4 KO mice display similar loss of insulin-stimulated glucose transport with associated insulin resistance and glucose intolerance; however, unlike PKC-λ KO, insulin action is defective in adipocytes and liver, hyperglycemia is more prominent, and obesity and hyperlipidemia are absent in muscle-specific Glut4 KO mice (31–33). On the other hand, mice with muscle-specific insulin receptor KO are obese and have elevated serum triglycerides and FFAs as did PKC-λ KO mice; however, unlike PKC-λ KO, these mice do not display hyperinsulinemia or glucose intolerance, at least initially, perhaps reflecting increases in adipose-dependent glucose disposal (33, 34).

Another interesting comparison is with mice with combined KO of Glut4 in muscle and adipocytes. These mice, like muscle-specific PKC-λ KO mice, are insulin resistant and glucose intolerant or diabetic, but are not obese and do not have elevated serum triglyceride or FFA levels, perhaps reflecting diminished adipogenesis and enhanced clearance and utilization of liver-derived lipids by muscle (37).

From these comparisons, the muscle-specific PKC-λ KO mouse model appears to be unique in mimicking — within the same mouse — most, if not all, metabolic syndrome features seen in humans. Moreover, blood glucose levels apparently can progress to at least mild diabetic levels with age and increased dietary fat and/or caloric content in this model.

Finally, it is important to consider findings in muscle-specific PKC-λ KO mice in the context of human obesity, IGT, and T2DM. First, glucose-tolerant first-degree relatives of humans with T2DM have defects in glucose disposal and muscle glucose transport, despite normal PKB activation and action (38); it will be interesting to examine αPKC levels and activation in these muscles. Second, insulin-stimulated glucose transport and αPKC activation are defective in cultured myocytes of obese IGT subjects (19); why these defects persist in passaged myocytes is intriguing. Third, humans with IGT and T2DM have 35%–40% lower αPKC levels and even more severe defects in αPKC activation in muscle (17, 20, 21), similar to what we observed in heterozygous PKC-λ KO mice. Accordingly, alterations in insulin sensitivity, glucose metabolism, serum lipids, and adiposity in human forms of IGT and T2DM may, at least in part, reflect the known abnormalities in muscle αPKC in these conditions in humans. On the other hand, non-αPKC insulin signaling defects have also been found in human T2DM muscle, including defective activation of IRS-1–dependent PI3K (39) and PKB (40–42), and these abnormalities undoubtedly contribute importantly to the phenotype of patients with T2DM.

In summary, our findings show that defects in αPKC availability and/or activation limited glucose transport in muscles of intact mice. Moreover, this tissue-specific limitation in transport was
accompanied by an insulin-resistant, glucose-intolerant, hyperlipidemic, abdominal obesity–prone, hepatosteatotic phenotype. Although the relevance of muscle-specific aPKC deficiency in mice to obesity, the metabolic syndrome, and T2DM in humans requires further scrutiny, the presently described mouse heterozygous and homozygous muscle-specific PKC-λ KO models seem well suited to provide readily available and effective means for devising and testing new therapies for these global health problems.

Methods

Muscle-specific KO of PKC-λ. We have previously reported on introduction of loxP sites flanking the exon at nucleotides 110–233 in genomic mouse PKC-λ, insertion of floxed PKC-λ into mouse ES cells, and deletion of floxed PKC-λ by expression of Cre recombinase in ES cells (22). ES cells containing the floxed PKC-λ allele were used to generate mice (C57BL/6 and 129P2/Sv background) with germline-transmitted floxed PKC-λ, and these mice were crossed with mice (FVB background) harboring an MCK-regulated Cre recombinase transgene to generate heterozygous and homozygous muscle-specific PKC-λ KO mice and various littermates.

Mouse care. Mice were maintained in light- and temperature-controlled environments (12-hour light/12-hour dark, 20–24°C) in Vivaria of the James A. Haley Veterans Administration Hospital and Yale University School of Medicine. Mice were fed standard chow containing 5% fat by weight, and some mice were fed a 10% fat diet for 2 months. Protocols were approved by the Institutional Animal Care Committees of the University of South Florida College of Medicine or of the Yale University School of Medicine. Studies were in accordance with guidelines of the National Institutes of Health and Principles of the Declaration of Helsinki.

Genotyping. DNA was isolated from tail clips with a DNAeasy Kit (QIAGEN). For detection of the MCK-controlled Cre recombinase transgene, PCR was performed with JumpStart ReadyMix REDTaq DNA polymerase (Sigma-Aldrich) using primers ATGTCCAATTTACTGACCG (forward) and CGCGCCTGAAGATATAGAAG (reverse). For detection of the loxP site, the primers were TTGTGAAAGCGACTGGATTG (forward) and CTTGGGTGGAGAGGCTATTC (reverse). For detection of WT, primers were TTGTGAAAGCGACTGTAC (forward) and AATGTTCATGTTCAACACTGCT (reverse). For detection of the deletion, primers were ACTAGCATTGCCTGGCATC (forward) and AATGTTCATGTTCAACACTGCT (reverse).

Glucose transport in vivo. As described previously (23, 43), following a 16- to 20-hour overnight fast, 0.2 ml physiologic saline containing the following was administered i.p. 10 minutes before killing: 0.05 μCi [3H]2-DG per gram body weight (NEN/Life Science Products Inc.); 0.005 μCi [14C]l-glucose (NEN/Life Science Products Inc.); and where indicated, 1 mU insulin (Sigma-Aldrich), a dosage just sufficient to elicit maximal increases in insulin signaling to PKB and aPKC in muscle and liver. Glucose uptake in muscle was measured by dividing the tissue [3H]-cpm (corrected for nonspecific trapping of extracellular water per [14C]l-glucose radioactivity) by the specific [3H]-radioactivity of serum 2-DG/glucose.

GLUT4 translocation in vivo. Mice fasted overnight were treated for 20 minutes with 1 mU insulin per gram body weight; vastus lateralis and gastrocnemius muscles were harvested, homogenized, and subjected to ultracentrifugation to purify plasma and internal membranes in a discontinuous sucrose gradient; and Glut4 was measured by Western blot analysis (4–6). Plasma membrane recovery was monitored by Western blot analysis of β subunits of insulin and IGF-1 receptors.

Glucose transport in isolated muscles. Extensor digitorum longus and soleus muscles were obtained from male mice, stretched to maintain resting length and tension, and incubated under 95% O2/5% CO2 in glucose-free Krebs-Ringer bicarbonate medium containing 1 mM sodium pyruvate.
Glucose transport in adipocytes. As described previously (43), pooled epididymal and intra-abdominal adipose tissues were digested with collagenase and isolated adipocytes (6% adipocrit) were incubated first for 30 minutes in glucose-free Krebs-Ringer phosphate medium without or with insulin and then for 1 minute with 50 nM \(^{3}H\)2-DG, after which uptake of label was measured by flotation of adipocytes through oil.

aPKC activation. aPKCs were immunoprecipitated with a rabbit polyclonal antiserum (Santa Cruz Biotechnology Inc.) that recognizes the C termini of PKC-\(\lambda\) and PKC-\(\zeta\); collected on Sepharose-AG beads; and incubated for 8 minutes at 30°C in 100 \(\mu\)l buffer containing 50 mmol/l Tris-HCl (pH 7.5), 100 \(\mu\)mol/l Na\(_3\)VO\(_4\), 100 \(\mu\)mol/l Na\(_4\)P\(_2\)O\(_7\), 1 mmol/l NaF, 1 mmol/l NaCl, 100 \(\mu\)mol/l PMSF, 4 \(\mu\)g phosphatidylserine (Sigma-Aldrich), 50 \(\mu\)mol/l \(\gamma\)-\(^{32}\)P]ATP (NEN/Life Science Products Inc.), 5 mmol/l MgCl\(_2\), and, as substrate, 40 \(\mu\)mol/l serine analog of the PKC-\(\epsilon\) pseudosubstrate (BioSource). After incubation, \(^{32}\)P-labeled substrate was trapped on P-81 filter paper and counted in a liquid scintillation counter.

PKB/Akt activation. PKB activation was assessed as described previously (4–6, 18, 20, 24, 44) by Western blot analysis and immunoblotting for PDK2-dependent phosphorylation of Ser473 in PKB, enzymatic activity of immunoprecipitable PKB using antibodies and assay reagents supplied by Upstate, and phosphorylation of PKB substrates GSK3\(\alpha\), GSK3\(\beta\), and AS160.

IRS-1–dependent and IRS-2–dependent PI3K activation. IRS-1–dependent and IRS-2–dependent PI3K activities were measured as previously described (4–6, 18, 20, 24, 44). Briefly, IRS-1 or IRS-2 was precipitated with rabbit polyclonal antiserum (Upstate) and incubated with phosphatidylinositol substrate to generate \(^{32}\)P-PI-3-PO\(_4\), which was purified by thin-layer chromatography and quantified in a Bio-Rad Phosphorimager.

Western blot analyses. As described (4–6, 18, 20, 23, 24, 44), muscle lysates were immunoblotted for PKC-\(\zeta/\lambda\) (rabbit polyclonal antiserum; Santa Cruz Biotechnology Inc.), which recognizes C termini of both aPKCs; phosphorylated Ser473-PKB (rabbit polyclonal antiserum; Upstate); IRS-1 (rabbit polyclonal antiserum; Upstate); phosphorylated AS160 (rabbit polyclonal antiserum; Cell Signaling Technology Inc.); mouse monoclonal anti-GLUT4 antibody (AbD Serotec Ltd.); rabbit polyclonal anti-GLUT1 antisera (Santa Cruz Biotechnology Inc.); rabbit polyclonal anti–PKC-\(\lambda\) and anti–PKC-\(\zeta\) antisera (prepared by M. Leitges); goat polyclonal anti-PKB-\(\alpha/\beta\) antisera (Santa Cruz Biotechnology Inc.); rabbit polyclonal

as an energy source, first for 30 minutes with or without 100 nM insulin, and then for 5 minutes with 50 \(\mu\)M \[^{3}H\]2-DG and trace amounts of \[^{14}\]C-glucose to correct for nonspecific trapping of extracellular water, as described previously (23, 43).

Figure 8 Effects of homozygous and heterozygous muscle-specific KO of PKC-\(\lambda\) on (A) total body weight and weight of abdominal fat, vastus lateralis muscle, heart ventricle muscle, and liver; (B) serum levels of FFAs and triglycerides; and (C) serum levels of total cholesterol, LDL-cholesterol, and HDL-cholesterol in 5-month-old mice. (A and C) The control group included pooled results of true WT and heterozygous control mice, which were indistinguishable. Ad libitum–fed mice were used in A. (B and C) Equal numbers of 20-hour-fasted male and female mice were used; results were indistinguishable and therefore pooled. (B) Mice were injected i.p. with saline or insulin (1 U/kg body wt) 10 minutes before killing. Values are mean ± SEM. n for each group is shown in parentheses. * \(P < 0.05\); ** \(P < 0.01\); *** \(P < 0.001\) versus WT or as indicated by brackets (ANOVA).
anti-PKB-α/β antiserum (Upstate); rabbit polyclonal anti-GSK3α and anti-GSK3β antisera (Cell Signaling Technology); mouse monoclonal anti-SREBP-1c antibody (Neomarkers Inc.); and rabbit polyclonal anti–insulin receptor β subunit and anti–IGF-1 receptor β subunit antisera (Santa Cruz Biotechnology Inc.).

Nuclear preparation. Liver nuclei were prepared with NE-PER Nuclear and Cytoplasmic Extraction Reagents obtained from Pierce Biotechnology.

mRNA analyses. Tissues were added to TRIzol reagent (Invitrogen), and RNA was extracted and purified with an RNA-Easy Mini-Kit and RNase-free DNAase kit (QIAGEN), quantified by measuring A_{260}/A_{280} and checked for purity by electrophoresis on 1.2% agarose gels; mRNA was quantified by quantitative real-time RT-PCR. Reverse transcription was accomplished with TaqMan reverse transcription reagent from Applied Biosystems. Mouse SREBP-1c mRNA was measured with a SYBR Green kit (Applied Biosystems) using primers at nucleotides ATC-GGCGCGGAAGCTTGTC-GGGTGACGGTC (forward) and ACTGTCTTGGTGATGAGCTGAGCAT (reverse). Mouse FAS was measured with primers GAGGACACT-CAATGGGGCTGA (forward) and GTGAGTGGCTTGTC-GTCTTG (reverse). The housekeeping gene hypoxanthine phosphoribosyl-transferase, used for recovery correction, was measured with primers TGAAAGACTTGCTCGAGATGTCA (forward) and AAAGAATATAGCCCTGTTGA (reverse).

Blood, serum, and tissue analyses. Serum glucose was measured using a OneTouch SureStep glucometer from LifeScan (Johnson & Johnson). Immunoreactive insulin was measured using a sensitive insulin RIA kit (catalog SRI-13K) from Linco Inc. Nonesterified FFAs were measured by NEFA C (catalog 994-75409) from Wako. Serum triglycerides were measured using the Serum Triglycerides Determination kit (catalog TR0100) from Sigma-Aldrich. Total cholesterol, LDL-cholesterol, and HDL-cholesterol were measured by an Advia 1650 Autoanalyzer (Bayer Instruments). Adiponectin, leptin, and resistin were measured by Quantikine kits (R&D Systems).

Hyperinsulinemic-euglycemic clamps. Mice used in clamp studies were allowed free access to water and food following transfer to the Yale University School of Medicine. Seven days prior to the hyperinsulinemic euglycemic clamp studies, indwelling catheters were placed into the right external jugular vein extending to the right atrium. After overnight fast, [3-3H]glucose (HPLC purified; Perkin Elmer Inc.) was infused at a rate of 0.05 μCi/min for 2 hours to assess the basal glucose turnover. Following the basal period, hyperinsulinemic euglycemic clamps were conducted for 130 minutes with a primed/continuous infusion of human insulin (126 pmol/kg prime, 18 pmol/kg/min infusion; Novo Nordisk Co.) to raise plasma insulin to within the physiological range. Blood samples (10 μl) were collected at 10- to 20-minute intervals for the immediate measurement of plasma glucose, and 20% dextrose was

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**Figure 9**

Effects of homozygous and heterozygous muscle-specific KO of PKC-λ on hepatic SREBP-1c and FAS expression, liver lipids, and islet β cell volume. (A) Expression of hepatic SREBP-1c and FAS by quantitative real-time RT-PCR in overnight fasted and ad libitum–fed conditions. Inset shows active nuclear SREBP-1c immunoreactivity. The control group included pooled results of true WT and heterozygous control male mice, which were comparable. (B) Livers of 5-month-old fed male WT and KO mice were examined by Oil Red O staining of lipids in frozen liver sections and chemical analysis of triglyceride levels in liver homogenates. (C) Livers of mice as in B were examined by islet anti-insulin immunohistological analysis of β cell volume, and representative insulin-stained areas are shown. After immunologic development of insulin-stained cells, mean cross-sectional areas of β cell regions of islets were determined from 6 random microscopic areas (each containing 2–5 islets) from each of 4 mice per group. Original magnification, ×10. Values are mean ± SEM. n for each group is shown in parentheses. *P < 0.05; **P < 0.01; ***P < 0.001 versus WT (ANOVA).
infused at variable rates to maintain plasma glucose at basal concentrations (~6.7 mM). To estimate insulin-stimulated whole-body glucose fluxes, [3-3H]glucose was infused at a rate of 0.1 μCi/min throughout the clamps, and 2-deoxy-D-[1-14C]glucose (2-[14C]DG, HPLC purified; PerkinElmer) was injected as a bolus 75 minutes into the clamp to estimate the rate of insulin-stimulated tissue glucose uptake, as described previously (45). Blood samples (10 μl) for the measurement of plasma [3H]glucose and [14C]glucose activities were taken at the end of the basal period and during the last 45 minutes of the clamp. Additional blood samples were obtained for the measurement of plasma insulin and FFA concentrations at the end of basal and clamp periods. At the end of the clamp, mice were anesthetized with pentobarbital sodium injection, and tissues were taken for biochemical measurements within 4 minutes. Each tissue, once exposed, was dissected out within 2 seconds, frozen immediately using liquid N2–cooled aluminum blocks, and stored at -80°C for subsequent analysis.

Glucose flux calculation. For the determination of plasma 3H-glucose, plasma was deproteinized with ZnSO4 and Ba(OH)2, dried to remove 3H2O, resuspended in water, and counted in scintillation fluid (Ultima Gold; PerkinElmer) in a Beckman Coulter scintillation counter. Rates of basal and insulin-stimulated whole-body glucose turnover were determined as the ratio of the [3-3H]glucose infusion rate (dpm/min) to the specific activity of plasma glucose (dpm/μmol) at the end of the basal period and during the final 30 minutes of the clamp experiment, respectively. HGP was determined by subtracting the glucose infusion rate from the total glucose appearance rate.

The plasma concentration of 3H2O was determined by the difference between 3H counts without and with drying. Whole-body glycolysis was calculated as the rate of increase in plasma 3H2O concentration divided by the specific activity of plasma 3H-glucose as described previously (45). Whole-body glycogen synthesis was estimated by subtracting whole-body glycolysis from whole-body glucose uptake, assuming that glycolysis and glycogen synthesis account for the majority of insulin-stimulated glucose uptake (46).

For the determination of individual tissue glucose uptake, tissue samples were homogenized, and the supernatants were subjected to an ion-exchange column to separate tissue 3H-glucose (2-DG-6-P) from 14C-DG. Tissue glucose uptake was calculated from the area under the curve of the plasma 14C-DG profile and muscle 14C-2-DG-6-P content, as described previously (47).

Immunohistochemical analysis of pancreatic islet β cell mass. Pancreata were fixed in Bouin’s solution at room temperature overnight and embedded in paraffin, and 4-μm sections were cut and mounted on glass slides. Sections were deparaffinized in xylene and rehydrated in graded ethanol solutions followed by water. Antigen retrieval was performed by heating the sections at 92–95°C for 15 minutes in 0.01 M citrate buffer (pH 6.0). Nonspecific binding sites were blocked with 8% horse serum in PBS. Sections were incubated with insulin monoclonal antibody (diluted

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**Figure 10**

Effects of homozygous and heterozygous muscle-specific KO of PKC-λ on basal and insulin-stimulated glucose uptake and transport in whole adipose tissue, isolated adipocytes, and serum adipokines. (A) Glucose transport was measured in adipose tissues obtained from WT, homozygous KO, and heterozygous KO mice treated with or without insulin, as described in Figure 1. Insets show aPKC levels in indicated groups with or without insulin. (B) Adipocytes isolated from male mice were incubated with increasing concentrations of insulin. (C) Results in ad libitum–fed male mice. Values are mean ± SEM. n for each group is shown in parentheses. *P < 0.05 versus non–insulin-stimulated group of the same genotype (ANOVA). **P < 0.01 (ANOVA).


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