Inactivation of PKCθ leads to increased susceptibility to obesity and dietary insulin resistance in mice

Zhanguo Gao,1 Zhong Wang,1 Xiaoying Zhang,1 Andrew A. Butler,1 Aamir Zuberi,1 Barbara Gawronska-Kozak,1 Michael Lefevre,1 David York,2 Eric Ravussin,1 Hans-Rudolf Berthoud,1 Owen McGuinness,3 William T. Cefalu,1 and Jianping Ye1

1Pennington Biomedical Research Center, Louisiana State University System, Baton Rouge, Louisiana; 2Center for Advanced Nutrition, Utah State University, Logan, Utah; and 3Department of Molecular Physiology and Biophysics, Vanderbilt University Medical Center, Nashville, Tennessee

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ELEVATED FREE FATTY ACID (FFA) is a risk factor for obesity-associated insulin resistance (6, 25, 31, 34). PKC is thought to play an important role in transducing the elevated FFA signal into insulin resistance. In cells, PKCs are activated by fatty acid derivatives, diacylglycerol (DAG), and their activation is associated with inhibition of insulin signal transduction in a variety of cellular models (5, 10, 11, 13, 18). In mice, knockout (KO) of either PKCα or PKCβ1 augments insulin signaling in muscle and fat (21, 36). However, the change in glucose homeostasis is subtle in these KO mice. Knockout of PKCβ1 increases insulin-stimulated glucose uptake in vivo during an insulin tolerance test. Insulin action in both hepatic and peripheral tissues was reduced in the KO mice. Plasma free fatty acid was increased, and expression of adiponectin in the adipose tissue was decreased, in the KO mice. Plasmas free fatty acid was increased, and expression of adiponectin in the adipose tissue was decreased, in the KO mice on HFD. This study suggests that loss of PKCθ reduces energy expenditure and increases the risk of dietary obesity and insulin resistance in mice.

The role of PKCθ, the θ-isofrom of PKC, in the regulation of insulin sensitivity is controversial. PKCθ was proposed to mediate the FFA signal for insulin resistance in skeletal muscle (34). In obesity, PKCθ activation may be related to the elevation in DAG that was observed during lipid infusion that mimics an increase in FFA in obesity (39). PKCθ may inhibit insulin signal transduction by increasing IRS-1 (insulin receptor substrate 1) phosphorylation at Ser307 or Ser1101 (12, 23, 39). On the other hand, multiple studies suggest that PKCθ is required for the maintenance of insulin sensitivity, especially in skeletal muscle (2, 9, 16, 17, 32). A reduction in PKCθ expression was observed in skeletal muscle of rat and human with insulin resistance (2, 9, 16, 17, 32). Consistently, muscle-specific inhibition of PKCθ activity using DN-PKCθ (dominant negative mutant of PKCθ) led to insulin resistance in transgenic mice (33). The DN-PKCθ mice developed systemic insulin resistance on chow diet at 4 mo of age. Although the transgenic mice exhibit obesity after 6 mo of age, the insulin resistance was observed prior to the development of obesity.

In this study, we investigated energy and glucose metabolism in the PKCθ-KO (PKCθ−/−) mice. Energy metabolism was examined using the metabolic chamber and quantitative nuclear magnetic resonance. Glucose metabolism was investigated using the hyperinsulinemic euglycemic clamp. Compared with wild-type (WT) mice, the KO mice exhibited a significant reduction in energy expenditure and developed severe insulin resistance on a high-fat diet (HFD). Our data suggest that, under physiological conditions, PKCθ is required for normal energy metabolism and serves to protect against HFD-induced insulin resistance.

RESEARCH DESIGN AND METHODS

Mice. Male PKCθ-KO (PKCθ−/−) mice in C57BL/6 background (40 total) were used in this study. WT C57BL/6 mice (40 mice) were used as controls. The PKCθ-KO mice were originally made by homologous recombination for analysis of PKCθ function in signal transduction of T cell receptor (37). The PKCθ-null mice were back-crossed with C57BL/6 mice in 12 generations for a clean C57BL/6 gene background. The mutant mice were normal in growth and fertilization. The control C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All of the mice were housed in the animal facility at the Pennington Biomedical Research Center with a 12:12-h light-dark cycle and constant temperature (22–24°C). The mice had free access to water and diet. The HFD (D12331; Research Diets, New Brunswick, NJ) contains 58% kcal in fat. The Chow diet...
(12.8% kcal in fat) was used as the low-fat diet. All procedures were performed in accordance with National Institute of Health guidelines for the care and use of animals and approved by the Institute Animal Care and Use Committee at the Pennington Biomedical Research Center. Intraperitoneal insulin tolerance test and glucose tolerance test. These two tests were performed according to methods described elsewhere (8, 20). Insulin tolerance was conducted using insulin at 0.75 U/kg body wt in mice after a 4-h fast. The intraperitoneal glucose tolerance test was conducted with glucose at 2 g/kg body wt after an overnight (16-h) fast. Blood glucose was monitored in the blood obtained from the tail vein using the FreeStyle blood glucose monitoring system (TheraSense, Phoenix, AZ).

Insulin and glucose assay. Insulin was determined using a Mouse Serum Adipokine LINCoplex Kit (Linco Research, cat. no. MADPK-71K) according to the manufacturer’s instructions. The kit included beads for insulin (no. 05-anti-insulin). Fasting glucose was measured using the FreeStyle blood glucose monitoring system (TheraSense) as described previously (12).

Nuclear magnetic resonance. Body composition was measured using quantitative nuclear magnetic resonance (NMR) (38). Live, conscious, unrestrained mice were placed in small tubes and inserted into a Brucker model mq10 NMR analyzer (Brucker, Canada, Milton ON, Canada). Total fat and lean mass were recorded after less than 1 min. Measurements were made in triplicate.

Hyperinsulinemic euglycemic clamp. The clamp was conducted in WT and KO mice at 8 wk on HFD at the Mouse Metabolic Phenotyping Center at Vanderbilt University. The surgical procedures utilized for implanting a chronic jugular vein catheter have been previously described (14, 29). Mice were allowed to recover from surgery for 5 days. Mice were studied only when body weight was restored to within 10% of presurgery body weight. The clamp was performed in 5-h-fasted mice. A primed (5 μCi) continuous (0.05 μCi/min) infusion of HPLC-purified [3H]glucose was initiated 120 before the clamp (−120 min). At t = 0 min, basal glucose concentration and specific activity were determined, after which a constant infusion of regular human insulin (4 μIU·kg−1·min−1) and red blood cells (3 μl/min) was initiated. The constant [3H]glucose infusion rate was increased (0.1 μCi/min) to minimize changes in glucose specific activity. Glucose (20%) was infused at a variable rate to maintain euglycemia. Glucose levels were tested every 10 min in the tail vein blood. At t = 80, 90, 100, and 120 min, plasma glucose specific activity was assessed. At t = 120 min, a 12-μCi bolus of 2-deoxy-glucose (DG) was administered (3). Blood (30 μl per mouse) was sampled at different times (t = 122, 125, 130, 135, and 145 min) to determine arterial blood glucose and plasma [14C]DG concentrations. At t = 145 min, a blood sample (100 μl) was obtained, and the mice were anesthetized with an infusion of pentobarbital sodium. Tissues were removed (gastrocnemius, superficial vastus lateralis, and soleus muscles, as well as the heart, brain, epididymal fat, and liver), immediately frozen in liquid nitrogen, and stored at −70°C until further analysis. Immunoreactive insulin was assayed in plasma at t = 0, 120, and 145 min with a double-antibody method (28). Radioactivity of [3H]glucose and [14C]DG in deproteinized plasma samples and radioactivity of [14C]DG and [3H]DG-6-phosphate in frozen muscle samples were determined by liquid scintillation counter (TRI-CARB 2900TR; Packard, Meriden, CT), as previously described (14). The rate of whole body glucose appearance (Ra) was calculated as the ratio of the [3H]glucose infusion rate (dpm·kg−1·min−1) and plasma glucose specific activity (dpm/mg).

Increased adiposity in KO mice. In muscle-specific DN-PKCθ mice (33), severe obesity was observed after 6 mo of age on a low-fat diet (chow), suggesting that PKCθ is involved in the regulation of energy metabolism. However, the mechanism of obesity was not investigated. In this study, energy metabolism was investigated systematically in the PKCθ-KO mice. On the chow diet, the body weight was not changed in KO mice (Fig. 1A). However, the body composition was altered. Compared with the WT mice, the KO mice exhibited a 40% (P < 0.05) increase in adiposity at day 80 and day 180 of age (Fig. 1B; n = 10). The epididymal fat pads were increased by 60% in weight in the KO mice (KO:WT = 266:168 in mg). This was associated with a 5% (P < 0.05) reduction in fat-free content in the KO mice (Fig. 1C). These data suggest that inactivation of PKCθ leads to an increase in the body fat content.

Energy balance was investigated in the KO mice by measuring food intake, energy expenditure, and substrate oxidation in the metabolic chamber. Compared with the WT mice, the KO mice did not exhibit an increase in food intake at age of 80 or 180 days. They consumed identical amounts of food on the chow diet (12% kcal in fat) to that of the WT mice (Fig. 1D). However, the KO mice exhibited a 10% reduction (P < 0.05)
in energy expenditure rate determined by indirect calorimetry (Fig. 1E). The reduction was observed at night, but not in the daytime. The reduction in energy expenditure was associated with a decrease in fatty acid oxidation as indicated by an increase in RER (Fig. 1F), suggesting that the KO mice might have a reduced capacity in oxidation of fatty acids.

Physical activity was monitored by frequency of laser beam break (horizontal) in the metabolic chamber over a period of 48 h (Fig. 1, G and H). The frequency represents the counts of physical movement of a mouse in the chamber. The KO mice exhibited a significant reduction in physical movement in both horizontal and vertical directions, and the reduction was observed only at nighttime. The reduction corresponded to the decrease in energy expenditure at nighttime, suggesting that in the KO mice the reduction in the spontaneous physical activity might contribute to the reduced energy expenditure. Resting energy expenditure rate was not examined specifically in this study. Because mice are inert in the spontaneous physical activity in daytime, the energy expenditure rate at the daytime may be close to the resting energy expenditure rate. The daytime data suggest that resting energy expenditure rate may not be changed in the KO mice. Therefore, the decrease in energy expenditure likely contributed to the increased adiposity in the KO mice whose food intake was not increased.

Skeletal muscle in KO mice. Skeletal muscle has the highest level of PKCα among all tissues/organs (4, 30). Inhibition of PKCα in skeletal muscle leads to muscle insulin resistance in the DN-PKCα mice (33). According to this observation, the PKCα-KO mice should exhibit insulin resistance in skeletal muscle. To test this possibility, insulin-induced glucose uptake was determined in isolated muscle of the KO mice at the age of 35 days. Compared with the WT muscle, the KO mice had
an 80% (P < 0.05) reduction in insulin-stimulated glucose uptake (Fig. 2A). The data suggest insulin resistance in the skeletal muscle of PKCθ-KO mice.

The reduction in lean mass suggested that the KO mice might have less skeletal muscle in whole body content. To test this possibility, several pieces of skeletal muscle, including soleus, gastrocnemius, and superficial vastus lateralis, were isolated from the rear legs of mice, and their weight was determined in grams using a balance. Of the three muscles, soleus and superficial vastus lateralis were significantly reduced in the KO mice (Fig. 2B). Gastrocnemius did not show a significant difference. These data support that muscle mass is reduced in the KO mice.

**Obesity and insulin resistance on HFD.** To examine the response of the KO mice to dietary fat, the KO mice were fed an HFD and subjected to energy balance analysis. At 8 wk on HFD (at age 13 wk), both the body weight († 10%; P < 0.05) and the fat content († 78%; P < 0.001) were significantly increased in the KO mice (Fig. 3, A and B). Food intake remained identical in the KO and WT mice on HFD (Fig. 3C). The energy expenditure was lower in the KO mice (Fig. 3D). This difference was observed at nighttime (P < 0.05) but not in daytime (P > 0.05). This pattern of reduction in energy expenditure correlated with a decrease in spontaneous physical activity at nighttime (P < 0.05; Fig. 3E). Although the physical activity seemed to be reduced in daytime, the change was not significant (Fig. 3E). Substrate utilization, as indicated by RER, became identical in the KO and WT mice on HFD (Fig. 3F). These data consistently suggest that the reduction in physical activity may have contributed to the lesser energy expenditure in the KO mice. The HFD made the KO mice gain more weight.

Regarding glucose metabolism in the KO mice on HFD, the plasma insulin was increased fourfold (P < 0.001), and insulin tolerance was impaired (Fig. 3, G and H). These data suggest that the KO mice developed obesity-associated insulin resistance. The glucose tolerance was not changed in the KO mice compared with the WT mice (Fig. 3I). The disassociation of insulin tolerance with glucose tolerance in the KO mice is likely due to pancreatic compensation for insulin resistance in the skeletal muscle. The disassociation was reported previously in transgenic mice with muscle-specific insulin resistance, such as DN-PKCθ mice and MIRKO (muscle-specific insulin receptor KO) mice (7, 33). In these two transgenic models, the impaired insulin tolerance was observed together with unchanged glucose tolerance.

**Insulin resistance in muscle, fat, and liver in KO mice.** To characterize insulin resistance further, a hyperinsulinemic euglycemic clamp was conducted in the KO and WT mice. The test was conducted after 8 wk on HFD. The blood glucose was clamped at 170 mg/dl through glucose administration (Fig. 4A). The glucose infusion rate (GIR) was monitored over 2 h. The KO mice exhibited a 50% reduction in GIR on average in the course of clamping (Fig. 4B). The difference between the KO and WT mice started at 10 min, became significant at 30 min, and remained throughout the clamp period (150 min).

**Reduced glucose deposition in muscle and adipose tissue.** The reduction in GIR suggests a severe peripheral insulin resistance. Consistent with this, the rate of glucose disappearance was decreased in the KO mice (Fig. 4C). Peripheral glucose deposition was examined in skeletal muscle, epididymal fat, and brain by use of 2-[14C]DG during the clamp. In the KO mice, glucose deposition was significantly decreased in all of the muscles examined (soleus, white vastus, and gastrocnemius; Fig. 4D). A reduction was also observed in adipose tissues (Fig. 4E) but not in the brain (Fig. 4D) during the hyperinsulinemic euglycemic clamp. These data confirm systemic insulin resistance in the KO mice on HFD.

**Increased hepatic glucose production.** In addition to insulin resistance in the skeletal muscle and adipose tissue, insulin resistance was also detected in the liver in the KO mice in the clamp test. The endogenous glucose Ra (Endo Ra) is an indicator of glucose production activity in the liver. Insulin suppresses the Endo Ra in consequence of inhibition of hepatic glucose production. In the KO mice, insulin suppression of Endo Ra was significantly decreased (Fig. 4F), suggesting hepatic insulin resistance. Thus, on HFD, the KO mice exhibited more severe insulin resistance in liver, muscle, and adipose tissue.

**FFA in KO mice.** An increase in plasma FFA is a risk factor for insulin resistance, especially in the obese condition. To understand the mechanism of insulin resistance in the KO mice, we compared FFA levels in the KO and WT mice by using the plasma samples that were collected immediately after complete isolation from the mice. Each bar represents mean ± SD (n = 10) unless otherwise indicated. *Significant difference, P < 0.001.

**Fig. 2.** Skeletal muscle. A: glucose uptake. Skeletal muscle was collected from mice at 5 wk age on standard chow diet. Insulin-induced glucose (nM) uptake was examined in soleus muscle from mice at equal muscle weight (mg). B: muscle mass. Intact skeletal muscles, including soleus, gastrocnemius (Gastro), and superficial vastus lateralis (Vastus L) were collected in mice after the clamp test. Weight (g) of each piece of muscle was determined immediately after complete isolation from the mice. Each bar represents mean ± SD (n = 10) unless otherwise indicated. *Significant difference, P < 0.001.
serum after clamping, it suggests that FFA may be increased in the KO mice before the clamping. The high level of insulin and glucose in the clamping should not increase plasma FFA.

**Adiponectin in the KO mice.** Expression of adiponectin in the adipose tissue was compared between the KO and WT mice (Fig. 6). On the chow diet, adiponectin expression was increased in the KO mice at age of 12 wk. On HFD, adiponectin was dramatically reduced in both WT and KO mice. However, the reduction in the KO mice was greater, and the final level of adiponectin was lower, in the KO mice. The data further suggest that, on HFD, insulin resistance is more severe in the KO mice. The high level of adiponectin in the KO mice before HFD may contribute to the homeostasis in glucose metabolism on chow diet.

**DISCUSSION**

Our data suggest that the PKCα-KO mouse represents a new animal model of dietary insulin resistance. The WT C57BL/6 mouse is often used in a model of dietary obesity. On HFD (58% kcal in fat), the obese mice develop hyperinsulinemia in 6–8 wk and hyperglycemia in 10–12 wk (12). The PKCα-KO mice on a C57BL/6 genetic background were used in this study. They exhibited a strong response to HFD. Compared with the WT mice, the KO mice gained more body weight and suffered more severe insulin resistance at 8 wk on HFD, suggesting that the KO mice have a higher risk for obesity and insulin resistance than the WT mice do. The KO mice did not suffer insulin resistance on the chow diet (data not shown). They exhibited impaired systemic insulin sensitivity only on HFD. This observation is different from that in the DN-PKCα mice, in which systemic insulin resistance was detected on the chow diet (33). The molecular basis of the discrepancy is not clear. A difference in the approaches for PKCα inactivation and in mouse strains might have contributed to the discrepancy. However, the two studies consistently support that inactivation of PKCα in skeletal muscle leads to an increased risk for obesity and insulin resistance.

In a study by Kim et al. (19), the same PKCα-KO mice were used in the analysis of insulin sensitivity under lipid infusion. Instead of HFD feeding, Kim et al. increased the plasma FFA by intravenous infusion of lipid-heparin for 5 h. The experimental setting revealed an acute or pharmacological effect of FFA on the KO mice. The high level of FFA in combination with a high level of insulin in their clamp test may have
induced an acute increase in intracellular DAG in the target tissue of insulin. It is known that insulin stimulates FFA uptake through membrane translocation of fatty acid transporter proteins, such as FATP-1 and CD36 (24, 35). DAG is an intermediate product of FFA during synthesis of triglycerides, whose formation is increased by insulin. When uptake of FFA is promoted by insulin, the DAG level may be increased accordingly inside the cells. This possibility was proved in skeletal muscle cells in an earlier study by the same group (39). In their experiment system, higher insulin sensitivity might have been associated with a faster DAG accumulation in the presence of FFA plus insulin. An acute increase in DAG concentration may lead to insulin resistance through overactivation of PKC\textsuperscript{\beta}\textsubscript{2} in muscle cells. In contrast, the tissue with lower insulin sensitivity should have slower DAG accumulation in the same condition and thus be protected from DAG/PKC\textsuperscript{\beta}\textsubscript{2}-induced insulin resistance. Although this is a hypothesis, the possibility may contribute to the discrepancy in the

**Fig. 4. Hyperinsulinemic euglycemic clamp in PKC\textsuperscript{\beta}\textsubscript{2}-KO mice on HFD. Clamp test was performed in mice on HFD for 8 wk. A: blood glucose level. B: glucose infusion rate during the course of the clamp. C: glucose disappearance. D: glucose uptake by skeletal muscles and brain during clamp. Skeletal muscles include soleus, gastrocnemius (Gastro), and superficial vastus lateralis (Vastus). E: glucose uptake by white (epididymal) adipose tissue. F: endogenous glucose appearance rate (Ra) during clamp. Each data point represents mean value of 8–12 mice. *Significant difference, \(P < 0.05\).**

**Fig. 5. FFA in KO mice. FFA was determined in plasma collected from mice after clamp. Assay was done with 5 \(\mu\)l of plasma in each mouse using a NEFA C kit (Wako Chemicals). Each bar represents mean value of 7 mice. *Significant difference, \(P < 0.05\); \(n = 7\).**

**Fig. 6. Adiponectin expression in adipose tissue. Adiponectin (Acdd) mRNA expression was examined in adipose tissue with Taqman quantitative RT-PCR. Sample was collected at 12 wk on chow diet and 53 wk on HFD, respectively. *Significant difference, \(P < 0.05\).**
conclusions of the current study and that of Kim et al. Additionally, the lack of knowledge about the physiological activity of PKCθ might limit the data interpretation in the two studies. The discrepancy may imply multiple faces of PKCθ activities. The two studies suggest that PKCθ might have more complicated functions than what was once thought.

Our study suggests that PKCθ plays an important role in the regulation of energy balance. The PKCθ-KO mice and DN-PKCθ mice consistently exhibited an increase in adiposity (33). In the DN-PKCθ mice, obesity was observed on the chow diet after 6 mo of age. However, the mechanism of obesity was not investigated. In the current study, the mechanism of obesity was investigated in the KO mice by use of the metabolic chamber. A decrease in energy expenditure was found in the KO mice. No change was found in food intake on either the chow or HFD diet. The decrease in energy expenditure was associated with a reduction in spontaneous physical activity in the KO mice. Although the reason for less physical activity remains to be investigated in the KO mice, a reduction in the mass of skeletal muscle might have contributed to this phenotype. Muscle histology remains to be examined in the KO mice, although the DN-PKCθ mice exhibited no change in muscle histology (33).

The phenotype of PKCθ-KO mice and DN-PKCθ mice consistently supports that PKCθ is required for the maintenance of balance in energy and glucose metabolism (33). Both studies suggest muscle insulin resistance in the absence of PKCθ activity in the mouse models. Although muscle suffered insulin resistance in the glucose uptake assay in vitro, glucose and insulin tolerance tests failed to demonstrate systemic insulin resistance in the KO mice on the chow diet (data not shown). This might be a result of compensation by adipose tissues that produce more adiponectin in the KO mice. A compensation in fat tissues was reported in mice with muscle-specific insulin resistance (7, 33). In the obese state, a reduction in the compensation may have contributed to the elevation of plasma FFA and insulin resistance in the PKCθ-KO mice. FFA may have contributed to systemic insulin resistance in the KO mice by targeting skeletal muscle and liver.

In summary, our data suggest that PKCθ is required for the maintenance of energy expenditure and insulin sensitivity under physiological conditions. Loss of PKCθ activity may lead to an increased susceptibility to obesity and fat-induced insulin resistance. The PKCθ-KO mice developed severe insulin resistance on HFD. HFD might promote insulin resistance through obesity in the KO mice.

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