

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

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Gao Z, Wang Z, Zhang X, Butler AA, Zuberi A, Gawronska-Kozak B, Lefevre M, York D, Ravussin E, Berthoud H-R, McGuinness O, Cefalu WT, Ye J. Inactivation of PKC θ leads to increased susceptibility to obesity and dietary insulin resistance in mice. *Am J Physiol Endocrinol Metab* 292: E84–E91, 2007. First published August 8, 2006; doi:10.1152/ajpendo.00178.2006.—In this study, we investigated the metabolic phenotype of PKC θ knockout mice (C57BL/6J) on chow diet and high-fat diet (HFD). The knockout (KO) mice are normal in growth and reproduction. On the chow diet, body weight and food intake were not changed in the KO mice; however, body fat content was increased with a corresponding decrease in body lean mass. Energy expenditure and spontaneous physical activity were decreased in the KO mice. On HFD, energy expenditure and physical activity remained low in the KO mice. The body weight and fat content were increased rapidly in the KO mice. At 8 wk on HFD, severe insulin resistance was detected in the KO mice with hyperinsulinemic euglycemic clamp and 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 on HFD. This study suggests that loss of PKC θ reduces energy expenditure and increases the risk of dietary obesity and insulin resistance in mice.

protein kinase C θ ; dietary obesity; insulin resistance

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 PKC α or PKC β 1 augments insulin signaling in muscle and fat (21, 36). However, the change in glucose homeostasis is subtle in these KO mice. Knockout of PKC β 1 increased insulin-stimulated glucose uptake in vivo during an insulin tolerance test, but it did not alter glucose tolerance. Knockout of PKC α does not alter basal insulin or glucose concentration. Systemic insulin sensitivity has not been reported in the PKC α -KO mice. Although PKC δ ^{-/-} and PKC ζ ^{-/-} mice have been made, insulin sensitivity has not been reported in these transgenic mice (22, 26, 27).

The role of PKC θ , the θ -isoform of PKC, in the regulation of insulin sensitivity is controversial. PKC θ was proposed to

mediate the FFA signal for insulin resistance in skeletal muscle (34). In obesity, PKC θ activation may be related to the elevation in DAG that was observed during lipid infusion that mimics an increase in FFA in obesity (39). PKC θ 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 PKC θ is required for the maintenance of insulin sensitivity, especially in skeletal muscle (2, 9, 16, 17, 32). A reduction in PKC θ expression was observed in skeletal muscle of rat and human with insulin resistance (2, 9, 16, 17, 32). Consistently, muscle-specific inhibition of PKC θ activity using DN-PKC θ (dominant negative mutant of PKC θ) led to insulin resistance in transgenic mice (33). The DN-PKC θ 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 PKC θ -KO (PKC θ ^{-/-}) 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, PKC θ is required for normal energy metabolism and serves to protect against HFD-induced insulin resistance.

RESEARCH DESIGN AND METHODS

Mice. Male PKC θ -KO (PKC θ ^{-/-}) mice in C57BL/6 gene background (40 total) were used in this study. WT C57BL/6 mice (40 mice) were used as controls. The PKC θ -KO mice were originally made by homologous recombination for analysis of PKC θ function in signal transduction of T cell receptor (37). The PKC θ -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

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(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 Bruker model mq10 NMR analyzer (Bruker, 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 μ Ci) continuous (0.05 μ Ci/min) infusion of HPLC-purified [3 H]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 mU \cdot kg $^{-1}\cdot$ min $^{-1}$) and red blood cells (3 μ l/min) was initiated. The constant [3 H]glucose infusion rate was increased (0.1 μ Ci/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 $t = 80, 90, 100, 120$ min, plasma glucose specific activity was assessed. At $t = 120$ min, a 12- μ Ci bolus of 2-deoxyglucose (DG) was administered (3). Blood (30 μ l per mouse) was sampled at different times ($t = 122, 125, 130, 135, 145$ min) to determine arterial blood glucose and plasma [14 C]DG concentrations. At $t = 145$ min, a blood sample (100 μ l) 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 $t = 0, 120, 145$ min with a double-antibody method (28). Radioactivity of [3 H]glucose and [14 C]DG in deproteinized plasma samples and radioactivity of [14 C]DG and [14 C]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 (R_a) was calculated as the ratio of the [3 H]glucose infusion rate (dpm \cdot kg $^{-1}\cdot$ min $^{-1}$) and plasma glucose specific activity (dpm/mg). The rate of endogenous glucose appearance during the hyperinsulinemic clamp was calculated as the difference between R_a and the exogenous glucose infusion rate.

Metabolic chamber. Energy expenditure, respiratory exchange ratio (RER), spontaneous physical movement, and food intake were simultaneously measured for each mouse by use of the Comprehensive Laboratory Animal Monitoring System (Columbus Instruments, Columbus, OH) as described previously (1). The temperature in the

metabolic chamber was 24°C . The mice (180 days old) were housed individually in the metabolic chamber. After 48 h of adaptation, the data in all parameters were recorded and used in analysis of the mouse phenotype. The mice were kept in the metabolic cage for 1 wk in this study.

In vitro muscle glucose uptake. Intact soleus muscles were isolated from the WT and PKC θ -KO mice for in vitro glucose uptake assay as described elsewhere (15). The muscle was preincubated at 37°C for 30 min in 2 ml of KHB buffer (Krebs-Henseleit buffer, oxygenated 95% O $_2$ -5%CO $_2$, supplemented with 5 mM glucose and 0.2% radioimmunoassay-grade BSA). The muscle was incubated with insulin (100 nM) in KHB for 30 min followed by a 30-min additional incubation in the presence of 1 mM 2-[3 H]DG (2-DG, 300 mCi/mmol; Amersham). The muscle was quickly washed three times with ice-cold PBS and dried by blotting on filter paper. The entire soleus muscle was dissolved in 0.5 ml of 0.5 N NaOH. After the muscle was completely solubilized, 5 ml of scintillation cocktail were added, and the specific intracellular accumulation of 2-DG was determined. The muscle glucose uptake was normalized by extracellular 2-DG of samples treated with cytochelasin B.

FFA assay. The FFA level in the plasma was measured using an assay kit for nonesterified fatty acids (NEFA C kit; Wako Chemicals, Richmond, VA). The test was conducted with 5 μ l of plasma from each mouse according to the instructions for the kit.

Adiponectin mRNA assay. Taqman quantitative real-time RT-PCR was used in mRNA measurement. Total RNA was extracted from epididymal fat pads and used for mRNA assay of adiponectin. A Taqman RT-PCR primer and probe set (Mm00456425_m1) from Applied Biosystems (Foster City, CA) was used in the determination of mRNA of adiponectin. Mouse ribosome 18S rRNA_s1 (without intron-exon junction) was used as an internal control. The forward primer (5'-GGGAATCAGGGTTCGATTCC-3'), reverse primer (5'-CTGCCTTCCTTGGATGTGGTA-3'), and probe (5'-AGCCTGAGAAACGG-3') were made for the ribosome 18S rRNA_s1 by Applied Biosystems.

Data analysis. The data for glucose, insulin, glucose uptake, cytokines, body weight, and body fat content are presented as means \pm SD of multiple samples ($n = 5-12$). Student's t -test was used to analyze the data with significance of $P < 0.05$.

RESULTS

Increased adiposity in KO mice. In muscle-specific DN-PKC θ mice (33), severe obesity was observed after 6 mo of age on a low-fat diet (chow), suggesting that PKC θ 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 PKC θ -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 PKC θ 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 ($P < 0.05$)

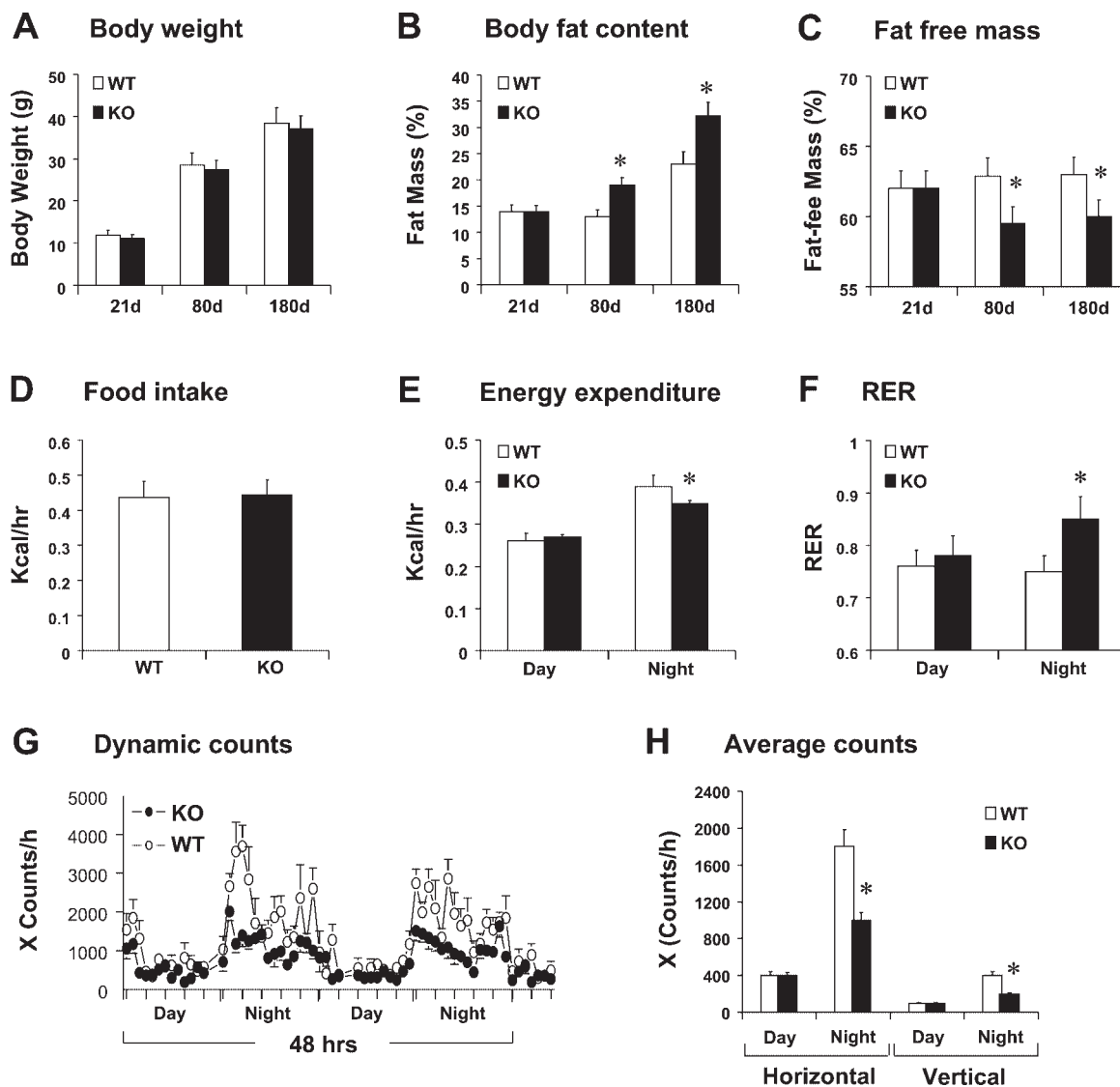


Fig. 1. Physiology of PKC θ -knockout (KO) mice on chow diet. Effect of age (21, 80, and 180 days) on body weight (A), percentage of fat (B), and fat-free mass (C) in wild-type (WT) and KO mice. Body composition was determined by NMR. D: food intake at 80 days of age. Food intake is expressed in kcal and normalized with time (h) for easy estimation of energy balance. E: energy expenditure determined by indirect calorimetry. Energy expenditure (kcal) is normalized to time (h). F: respiratory exchange ratio (RER). G: counts of spontaneous physical movements of mice in the metabolic chamber. Counts of movement in horizontal direction are presented. H: average movement counts in horizontal and vertical directions for day and night. Each bar represents mean \pm SD ($n = 10$). * $P < 0.05$. In this study, each data point represents a mean value of measurements from 10 mice.

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 PKC θ among all tissues/organs (4, 30). Inhibition of PKC θ in skeletal muscle leads to muscle insulin resistance in the DN-PKC θ mice (33). According to this observation, the PKC θ -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

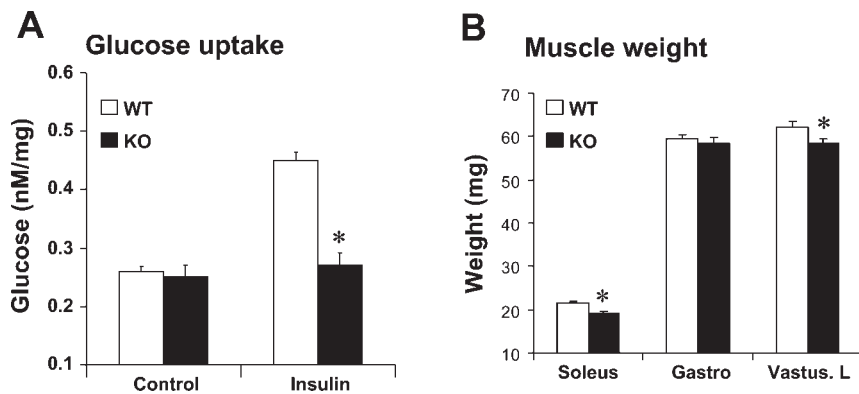


Fig. 2. Skeletal muscle. *A*: glucose uptake. Skeletal muscle was collected from mice at 5 wk age on standard chow diet. Insulin-induced glucose (nM) uptake was examined in soleus muscle from mice at equal muscle weight (mg). *B*: muscle mass. Intact skeletal muscles, including soleus, gastrocnemius (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 \pm SD ($n = 10$) unless otherwise indicated. *Significant difference, $P < 0.001$.

an 80% ($P < 0.05$) reduction in insulin-stimulated glucose uptake (Fig. 2A). The data suggest insulin resistance in the skeletal muscle of PKC θ -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 ($\uparrow 10\%$; $P < 0.05$) and the fat content ($\uparrow 78\%$; $P < 0.001$) 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-PKC θ 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-[14 C]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 R_a (Endo R_a) is an indicator of glucose production activity in the liver. Insulin suppresses the Endo R_a in consequence of inhibition of hepatic glucose production. In the KO mice, insulin suppression of Endo R_a 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 clamping. The result shows that FFA was significantly increased in the KO mice (Fig. 5). The increase suggests a decrease in fatty acid uptake by the peripheral tissues or an elevation in lipolysis in adipose tissue. These possibilities can be a result of insulin resistance and may contribute to insulin resistance. Insulin reduces plasma FFA level by stimulating FFA deposition in peripheral tissues and inhibiting lipolysis in adipocytes. Although the FFA result was obtained from the

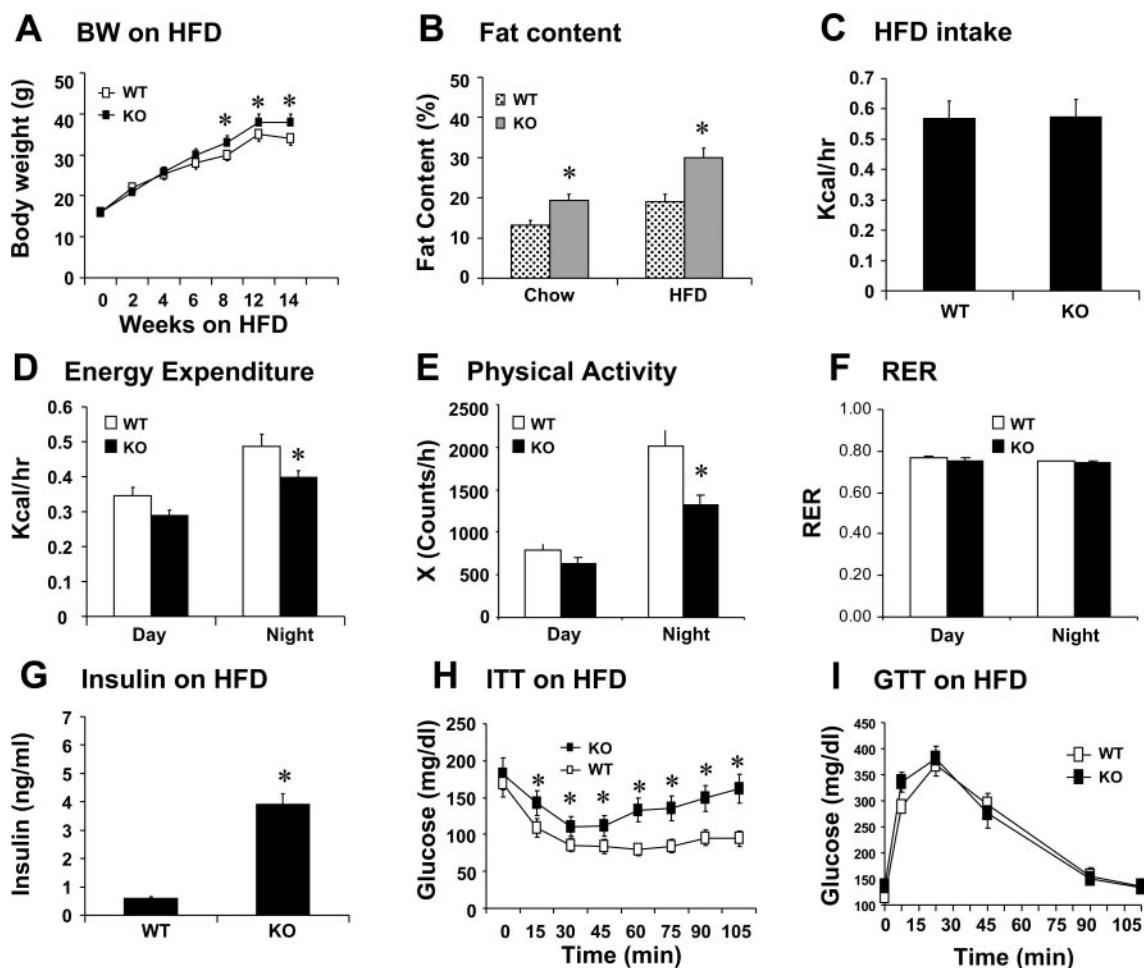


Fig. 3. Physiology of PKC θ -KO mice on high-fat diet (HFD). *A*: body weight (BW) was determined in mice over 14 wk on HFD in nonfasting condition. *B*: fat content at age of 12 wk (8 wk on HFD). *C*: food intake at the age of 12 wk. *D*: energy expenditure determined by indirect calorimetry. *E*: RER. *F*: blood insulin level was determined at 14 wk on HFD after 16-h fast. *G*: ip insulin tolerance test (ipITT) lasting for 4 h. The test was conducted with insulin at 0.75 U/kg body wt. *I*: ip glucose tolerance test (GTT). ITT and ip GTT tests were done at 8–10 wk on HFD. In this study, each data point represents mean value of measurements from 7–10 mice. *Significant difference ($P < 0.05$ – 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 PKC θ -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 PKC θ -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-PKC θ 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 PKC θ inactivation and in mouse strains might have contributed to the discrepancy. However, the two studies consistently support that inactivation of PKC θ in skeletal muscle leads to an increased risk for obesity and insulin resistance.

In a study by Kim et al. (19), the same PKC θ -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

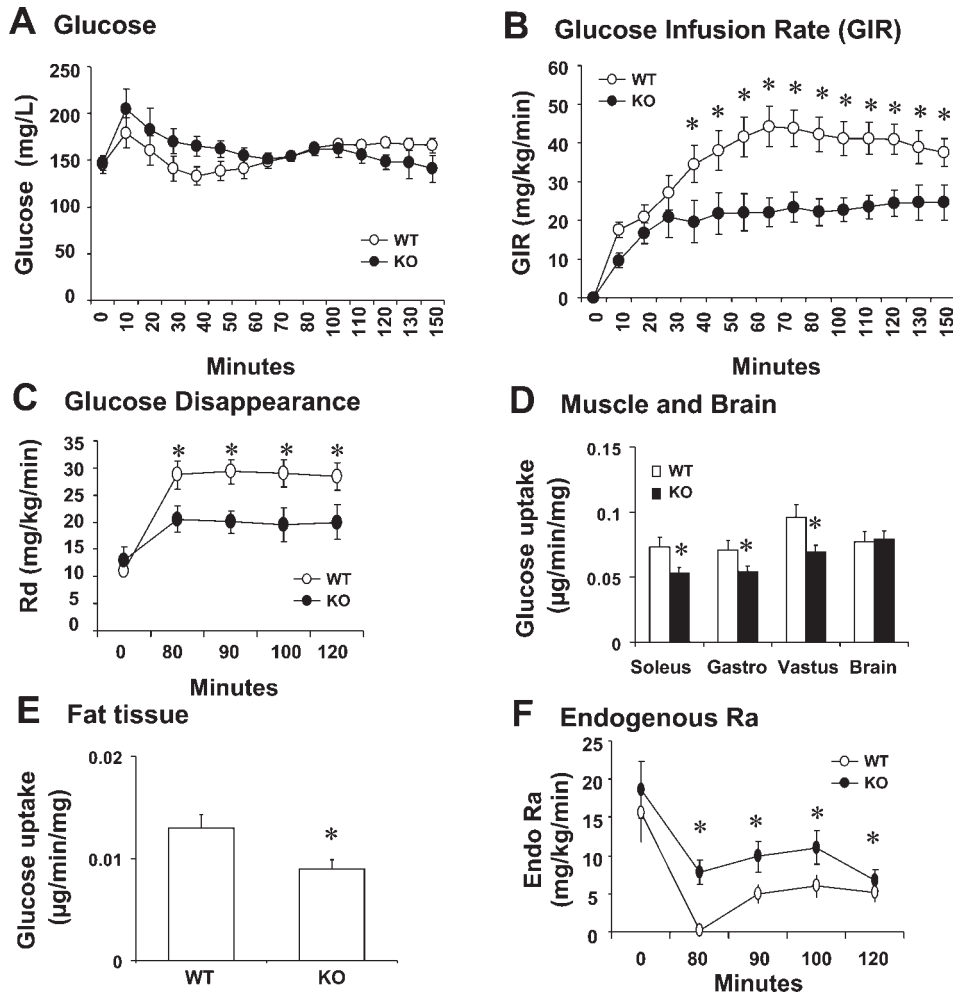


Fig. 4. Hyperinsulinemic euglycemic clamp in PKCθ-KO mice on HFD. Clamp test was performed in mice on HFD for 8 wk. *A*: blood glucose level. *B*: glucose infusion rate during the course of the clamp. *C*: glucose disappearance. *D*: glucose uptake by skeletal muscles and brain during clamp. Skeletal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

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 PKCθ 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/PKCθ-induced insulin resistance. Although this is a hypothesis, the possibility may contribute to the discrepancy in the

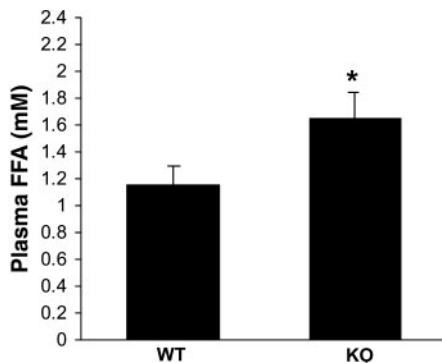


Fig. 5. FFA in KO mice. FFA was determined in plasma collected from mice after clamp. Assay was done with 5 µl of plasma in each mouse using a NEFA C kit (Wako Chemicals). Each bar represents mean value of 7 mice. *Significant difference, $P < 0.05$; $n = 7$.

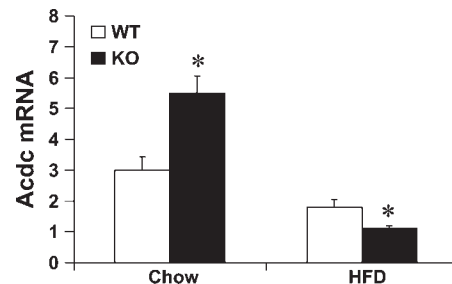


Fig. 6. Adiponectin expression in adipose tissue. Adiponectin (Acad) mRNA expression was examined in adipose tissue with Taqman quantitative RT-PCR. Sample was collected at 12 wk on chow diet and 53 wk on HFD, respectively. *Significant difference, $P < 0.05$.

conclusions of the current study and that of Kim et al. Additionally, the lack of knowledge about the physiological activity of PKC θ might limit the data interpretation in the two studies. The discrepancy may imply multiple faces of PKC θ activities. The two studies suggest that PKC θ might have more complicated functions than what was once thought.

Our study suggests that PKC θ plays an important role in the regulation of energy balance. The PKC θ -KO mice and DN-PKC θ mice consistently exhibited an increase in adiposity (33). In the DN-PKC θ 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-PKC θ mice exhibited no change in muscle histology (33).

The phenotype of PKC θ -KO mice and DN-PKC θ mice consistently supports that PKC θ is required for the maintenance of balance in energy and glucose metabolism (33). Both studies suggest muscle insulin resistance in the absence of PKC θ 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 PKC θ -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 PKC θ is required for the maintenance of energy expenditure and insulin sensitivity under physiological conditions. Loss of PKC θ activity may lead to an increased susceptibility to obesity and fat-induced insulin resistance. The PKC θ -KO mice developed severe insulin resistance on HFD. HFD might promote insulin resistance through obesity in the KO mice.

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REFERENCES

- Albarado DC, McClaine J, Stephens JM, Mynatt RL, Ye J, Bannon AW, Richards WG, Butler AA. Impaired coordination of nutrient intake and substrate oxidation in melanocortin-4 receptor knockout mice. *Endocrinology* 145: 243–252, 2004.
- Avignon A, Yamada K, Zhou X, Spencer B, Cardona O, Saba-Siddique S, Galloway L, Standaert ML, Farese RV. Chronic activation of protein kinase C in soleus muscles and other tissues of insulin-resistant type II diabetic Goto-Kakizaki (GK), obese/aged, and obese/Zucker rats. A mechanism for inhibiting glycogen synthesis. *Diabetes* 45: 1396–1404, 1996.
- Bagby GJ, Lang CH, Skrepnik N, Spitzer JJ. Attenuation of glucose metabolic changes resulting from TNF- α administration by adrenergic blockade. *Am J Physiol Regul Integr Comp Physiol* 262: R628–R635, 1992.
- Baier G, Telford D, Giampa L, Coggeshall KM, Baier-Bitterlich G, Isakov N, Altman A. Molecular cloning and characterization of PKC theta, a novel member of the protein kinase C (PKC) gene family expressed predominantly in hematopoietic cells. *J Biol Chem* 268: 4997–5004, 1993.
- Barthel A, Nakatani K, Dandekar AA, Roth RA. Protein kinase C modulates the insulin-stimulated increase in Akt1 and Akt3 activity in 3T3-L1 adipocytes. *Biochem Biophys Res Commun* 243: 509–513, 1998.
- Boden G. Role of fatty acids in the pathogenesis of insulin resistance and NIDDM. *Diabetes* 46: 3–10, 1997.
- Bruning JC, Michael MD, Winnay JN, Hayashi T, Horsch D, Accili D, Goodyear LJ, Kahn CR. A muscle-specific insulin receptor knockout exhibits features of the metabolic syndrome of NIDDM without altering glucose tolerance. *Mol Cell* 2: 559–569, 1998.
- Bruning JC, Winnay J, Bonner-Weir S, Taylor SI, Accili D, Kahn CR. Development of a novel polygenic model of NIDDM in mice heterozygous for IR and IRS-1 null alleles. *Cell* 88: 561–572, 1997.
- Chalfant CE, Ciaraldi TP, Watson JE, Nikoulina S, Henry RR, Cooper DR. Protein kinase C theta expression is increased upon differentiation of human skeletal muscle cells: dysregulation in type 2 diabetic patients and a possible role for protein kinase C theta in insulin-stimulated glycogen synthase activity. *Endocrinology* 141: 2773–2778, 2000.
- Chin JE, Liu F, Roth RA. Activation of protein kinase C alpha inhibits insulin-stimulated tyrosine phosphorylation of insulin receptor substrate-1. *Mol Endocrinol* 8: 51–58, 1994.
- Dresner A, Laurent D, Marcucci M, Griffin ME, Dufour S, Cline GW, Slezak LA, Andersen DK, Hundal RS, Rothman DL, Petersen KF, Shulman GI. Effects of free fatty acids on glucose transport and IRS-1-associated phosphatidylinositol 3-kinase activity. *J Clin Invest* 103: 253–259, 1999.
- Gao Z, Zhang X, Zuberi A, Hwang D, Quon MJ, Lefevre M, Ye J. Inhibition of insulin sensitivity by free fatty acids requires activation of multiple serine kinases in 3T3-L1 adipocytes. *Mol Endocrinol* 18: 2024–2034, 2004.
- Griffin ME, Marcucci MJ, Cline GW, Bell K, Barucci N, Lee D, Goodyear LJ, Kraegen EW, White MF, Shulman GI. Free fatty acid-induced insulin resistance is associated with activation of protein kinase C theta and alterations in the insulin signaling cascade. *Diabetes* 48: 1270–1274, 1999.
- Halseth AE, Bracy DP, Wasserman DH. Overexpression of hexokinase II increases insulin and exercise-stimulated muscle glucose uptake in vivo. *Am J Physiol Endocrinol Metab* 276: E70–E77, 1999.
- Henriksen EJ, Halseth AE. Early alterations in soleus GLUT-4, glucose transport, and glycogen in voluntary running rats. *J Appl Physiol* 76: 1862–1867, 1994.
- Itani SI, Ruderman NB, Schmedier F, Boden G. Lipid-induced insulin resistance in human muscle is associated with changes in diacylglycerol, protein kinase C, and I κ B α . *Diabetes* 51: 2005–2011, 2002.
- Itani SI, Zhou Q, Pories WJ, MacDonald KG, Dohm GL. Involvement of protein kinase C in human skeletal muscle insulin resistance and obesity. *Diabetes* 49: 1353–1358, 2000.
- Kellerer M, Mushack J, Seffer E, Mischak H, Ullrich A, Haring HU. Protein kinase C isoforms alpha, delta and theta require insulin receptor substrate-1 to inhibit the tyrosine kinase activity of the insulin receptor in human kidney embryonic cells (HEK 293 cells). *Diabetologia* 41: 833–838, 1998.
- Kim JK, Fillmore JJ, Sunshine MJ, Albrecht B, Higashimori T, Kim DW, Liu ZX, Soos TJ, Cline GW, O'Brien WR, Littman DR, Shulman

- GI. PKC-theta knockout mice are protected from fat-induced insulin resistance. *J Clin Invest* 114: 823–827, 2004.
20. **Lauro D, Kido Y, Castle AL, Zarnowski MJ, Hayashi H, Ebina Y, Accili D.** Impaired glucose tolerance in mice with a targeted impairment of insulin action in muscle and adipose tissue. *Nat Genet* 20: 294–298, 1998.
 21. **Leitges M, Plomann M, Standaert ML, Bandyopadhyay G, Sajan MP, Kanoh Y, Farese RV.** Knockout of PKC alpha enhances insulin signaling through PI3K. *Mol Endocrinol* 16: 847–858, 2002.
 22. **Leitges M, Sanz L, Martin P, Duran A, Braun U, Garcia JF, Camacho F, Diaz-Meco MT, Rennert PD, Moscat J.** Targeted disruption of the zetaPKC gene results in the impairment of the NF-kappaB pathway. *Mol Cell* 8: 771–780., 2001.
 23. **Li Y, Soos TJ, Li X, Wu J, DeGennaro M, Sun X, Littman DR, Birnbaum MJ, Polakiewicz RD.** Protein kinase C (theta) inhibits insulin signaling by phosphorylating IRS1 at Ser1101. *J Biol Chem* 279: 45304–45307, 2004.
 24. **Luiken JJ, Dyck DJ, Han XX, Tandon NN, Arumugam Y, Glatz JF, Bonen A.** Insulin induces the translocation of the fatty acid transporter FAT/CD36 to the plasma membrane. *Am J Physiol Endocrinol Metab* 282: E491–E495, 2002.
 25. **McGarry JD.** Banting lecture 2001: dysregulation of fatty acid metabolism in the etiology of type 2 diabetes. *Diabetes* 51: 7–18, 2002.
 26. **Mecklenbrauker I, Saijo K, Zheng NY, Leitges M, Tarakhovskiy A.** Protein kinase Cdelta controls self-antigen-induced B-cell tolerance. *Nature* 416: 860–865, 2002.
 27. **Miyamoto A, Nakayama K, Imaki H, Hirose S, Jiang Y, Abe M, Tsukiyama T, Nagahama H, Ohno S, Hatakeyama S, Nakayama KI.** Increased proliferation of B cells and auto-immunity in mice lacking protein kinase Cdelta. *Nature* 416: 860–865, 2002.
 28. **Morgan CR, Lazarow A.** Immunoassay of insulin using a two-antibody system. *Proc Soc Exp Biol Med* 110: 29–32, 1962.
 29. **Niswender KD, Shiota M, Postic C, Cherrington AD, Magnuson MA.** Effects of increased glucokinase gene copy number on glucose homeostasis and hepatic glucose metabolism. *J Biol Chem* 272: 22570–22575, 1997.
 30. **Osada S, Mizuno K, Saido TC, Suzuki K, Kuroki T, Ohno S.** A new member of the protein kinase C family, nPKC theta, predominantly expressed in skeletal muscle. *Mol Cell Biol* 12: 3930–3938, 1992.
 31. **Saltiel AR.** New perspectives into the molecular pathogenesis and treatment of type 2 diabetes. *Cell* 104: 517–529, 2001.
 32. **Schmitz-Peiffer C, Browne CL, Oakes ND, Watkinson A, Chisholm DJ, Kraegen EW, Biden TJ.** Alterations in the expression and cellular localization of protein kinase C isozymes epsilon and theta are associated with insulin resistance in skeletal muscle of the high-fat-fed rat. *Diabetes* 46: 169–178, 1997.
 33. **Serra C, Federici M, Buongiorno A, Senni MI, Morelli S, Segratella E, Pascuccio M, Tiveron C, Mattei E, Tatangelo L, Lauro R, Molinaro M, Giaccari A, Bouche M.** Transgenic mice with dominant negative PKC-theta in skeletal muscle: a new model of insulin resistance and obesity. *J Cell Physiol* 196: 89–97, 2003.
 34. **Shulman GI.** Cellular mechanisms of insulin resistance. *J Clin Invest* 106: 171–176, 2000.
 35. **Stahl A, Evans JG, Pattel S, Hirsch D, Lodish HF.** Insulin causes fatty acid transport protein translocation and enhanced fatty acid uptake in adipocytes. *Dev Cell* 2: 477–488, 2002.
 36. **Standaert ML, Bandyopadhyay G, Galloway L, Soto J, Ono Y, Kikkawa U, Farese RV, Leitges M.** Effects of knockout of the protein kinase C beta gene on glucose transport and glucose homeostasis. *Endocrinology* 140: 4470–4477, 1999.
 37. **Sun Z, Arendt CW, Ellmeier W, Schaeffer EM, Sunshine MJ, Gandhi L, Annes J, Petrzilka D, Kupfer A, Schwartzberg PL, Littman DR.** PKC-theta is required for TCR-induced NF-kappaB activation in mature but not immature T lymphocytes. *Nature* 404: 402–407, 2000.
 38. **Tinsley FC, Taicher GZ, Heiman ML.** Evaluation of a quantitative magnetic resonance method for mouse whole body composition analysis. *Obes Res* 12: 150–160, 2004.
 39. **Yu C, Chen Y, Zong H, Wang Y, Bergeron R, Kim JK, Cline GW, Cushman SW, Cooney GJ, Atcheson B, White MF, Kraegen EW, Shulman GI.** Mechanism by which fatty acids inhibit insulin activation of insulin receptor substrate-1 (IRS-1)-associated phosphatidylinositol 3-kinase activity in muscle. *J Biol Chem* 277: 50230–50236, 2002.