Protein Kinase Cβ Deficiency Increases Fatty Acid Oxidation and Reduces Fat Storage

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Metabolic syndrome is common in the general population, but there is little information available on the underlying signaling mechanisms regulating triglyceride (TG) content in the body. In the current study, we have uncovered a role for protein kinase Cβ (PKCβ) in TG homeostasis by studying the consequences of a targeted disruption of this kinase. PKCβ−/− mutant mice were considerably leaner and the size of white fat depots was markedly decreased compared with wild-type littermates. TG content in the liver and skeletal muscle of PKCβ−/− mice was also significantly low. Interestingly, mutant animals were hyperphagic and exhibited higher food intake and reduced feed efficiency versus wild type. The protection from obesity involves elevated oxygen consumption/energy expenditure and increased fatty acid oxidation in adipose tissue with concurrent increased mitochondria genesis, up-regulation of PGC-1α and UCP-2, and down-regulation of perilipin. The ability of PKCβ deficiency to promote fat burning in adipocytes may suggest novel therapeutic strategies for obesity and obesity-related disorders.

Metabolic syndrome is an epidemic affecting a large percentage of the population of the world. Most of the enzymes that regulate triglyceride (TG) metabolism have been identified; however, signaling pathway(s) controlling this process is not known. Because of the high prevalence of dyslipidemia in the general population (1), it is important to identify signaling factors regulating metabolic and regulatory aspects of the TG metabolism. The greatest potential for arresting the current obesity epidemic is likely to come through an understanding of the regulatory pathways that link obesity to insulin resistance and other aspects of the metabolic syndrome.

The serine/threonine-specific protein kinase protein kinase C (PKC) is activated by lipids, especially diacylglycerol (an intermediate in TG biosynthesis), and has been particularly implicated in the pathogenesis of obesity and insulin resistance (2–4). PKC consists of several isoforms (5, 6), and evidence from knock-out studies has indicated that several PKC isoforms can modulate glucose or energy metabolism in an isoform-specific manner. For example, inactivation of PKCα did not alter basal glucose or energy metabolism (7), whereas inactivation of PKCθ slightly reduced liver TG contents, without affecting muscle TG levels, and caused increased overall insulin sensitivity (8). On the other hand, inactivation of PKCθ led to increased susceptibility to obesity and dietary insulin resistance in mice (9). Unlike many PKC isoforms, PKCβ is expressed as a major isoform in a variety of tissues, and knock out of PKCβ caused subtle changes in glucose homeostasis but significantly increased insulin-stimulated glucose uptake in adipocytes and muscle (10). We have previously shown involvement of PKCβ in regulating expression of low density lipoprotein receptor gene in cultured cells (11–13). Moreover, PKCβ has been linked with many vascular abnormalities in retinal, renal, and cardiovascular tissues (14, 15).

How does PKCβ deficiency affect glucose metabolism? As several manifestations of type 2 diabetes are associated with alterations in intracellular lipid partitioning, one plausible mechanism is the modulation of tissue TG metabolism. We hypothesized that PKCβ−/− mice would have reduced levels of tissue TG, causing increased sensitivity to insulin. To test this hypothesis, we studied the metabolism of mice with targeted disruption of the PKCβ gene (PKCβ−/−), with special attention to lipid metabolism. We found that mice lacking PKCβ show decreased fat in adipose tissue, liver, and muscle. These mice consumed 20–30% more food than did the wild type (WT), yet lost body weight. Finally, PKCβ−/− mice exhibit increased fatty acid oxidation with concurrent up-regulation of PGC-1α and UCP-2 genes. These results raise the possibility that pharmacological manipulation of PKCβ may lead to loss of body fat in the context of normal caloric intake.

EXPERIMENTAL PROCEDURES

Production and Genotyping of PKCβ−/− Mice in C57BL/6 Background—Generation of PKCβ−/− mice was described earlier (16). They were crossed back ten times to the C57BL/6 background. Mice were housed in a pathogen-free barrier facility, 25 °C, 12-h dark/12-h light cycle, and fed regular rodent chow food. All procedures on mice followed the guidelines established by The Ohio State University College of Medicine Animal Care Committee. We developed a PCR method for...
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genotyping using tail tips collected when the mice were weaned at 3 weeks of age. For genotyping, tail DNA was extracted, and polymerase chain reaction (PCR) test was performed using the following primers: PKCβ forward primer, 5’-TGTGCTTTTACGAGGGCTCTC-3’; PKCβ reverse primers, 5’-ATTACGGCTGGCAGCTG T-3’ and 5’-CTTCTCGAGATGGGTCATCT-3’. Polymerase chain reaction conditions were 35 cycles at 95 °C for 30 s, 55 °C for 45 s, and 72 °C for 50 sec. Products were separated by electrophoresis on a 5% polyacrylamide gel and visualized with ethidium bromide staining. Most of the experiments were performed on animals starved for 16 h.

Blood Chemistries—We determined plasma TG and cholesterol concentrations by colorimetric kit assays (Roche Diagnostics). Plasma glucose levels were determined with a kit assay (Sigma). Plasma leptin levels were determined by LINCO, Inc.

Tissue Lipids—We measured tissue TG with a TG 320A kit (Sigma) as described. For qualitative analysis of tissue lipids, lipids were extracted from tissues and separated on ALSILG Silica Gel TLC plates (Whatman) using hexane/ethyl acetate (83:16:1).

Histology—Tissues were fixed by immersion or perfusion in neutral buffered formalin, dehydrated in ethanol, transitioned into xylene, and embedded in paraffin. We stained sections with hematoxylin and eosin.

Diet, Feeding, and Weighing—At 32 weeks, the mice were housed individually and allowed to acclimatize for 2 weeks. At 34 weeks of age, the food was weighed three times weekly over a period of 10 weeks, and food intake was determined over the time period of 34–44 weeks of age (n = 6 of each genotype).

Gene Expression Analysis—Total RNA was isolated from white adipose tissue (WAT) and brown adipose tissue (BAT) of WT and PKCβ mutant mice, using the TRIzol method as described previously (17) (Invitrogen). All RNA samples were digested with DNase I to eliminate any contaminating DNA. Equal amounts of total RNA (1 μg/assay) were reverse-transcribed using the SuperScript First-Strand Synthesis Kit (Invitrogen, Life Technology, Inc.) and random hexamer primers. The resulting cDNA (reverse transcription mixture) was subjected to real-time PCR using SYBR Green PCR master mix kit (Applied Biosystems). To ensure the validity of the SYBR Green-based mRNA quantifications, most of the mRNA was also quantified using 32P-labeled dCTP. This alternative method and the SYBR Green method gave similar results. Forward and reverse primers for amplification of genes are described in supplemental Table 1. All data are expressed as the -fold induction relative to each control value.

Electron Microscopy—Epididymal fat pads and brown adipose tissue from WT and PKCβ mutant mice were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. The samples were postfixed with OsO4, dehydrated with alcohol, and embedded in epoxy embedding medium. Thin sections were cut at 70-nm thickness with a Leica EM UC6 ultramicrotome. Samples were stained with 2% uranyl acetate followed by Reynolds’s lead citrate. Electron microscope imaging of all the prepared materials was done with a Tencai G2 Spirit (FEI, Hillsboro, OR) operated at 80 kV.

Indirect Calorimetry—Oxygen consumption (VO2) and carbon dioxide production (VCO2) were determined in gender-matched PKCβ mutant and WT littermates using a single-chamber small animal VERSAMAX system (AccuScan Instruments, Inc., Columbus, OH). Mice were continuously assayed during 5-h dark (7:30 p.m.–12:30 a.m.) or 5-h light (9:30 a.m.–2:30 p.m.) cycles after acclimatization to the chambers for 2 h immediately prior to the collection period. The VERSMAX software includes an O2 and CO2 analyzer calibration program and data collection and analysis programs.

Production of 14CO2 from [1-14C]Palmitic Acid and [1-14C]Oleic Acid—Palmitate and oleate oxidation was measured by the production of 14CO2 from 1-[1-14C]palmitic acid (0.2 μCi/ml) and 1-[1-14C]oleic acid (0.2 μCi/ml) with unlabeled palmitate and oleate present in the medium. Cells were incubated for 1 h in 20-ml plastic scintillation flasks. The flasks had a centered isolated well containing a loosely folded piece of filter paper moistened with 0.2 ml of 2-phenylethanol/methanol (1:1, v/v). After the 1-h incubation period, the medium was acidified with 0.25 ml of H2SO4 (5N), and the flasks were maintained sealed at 37 °C for an additional 30 min. At the end of this period, filter papers were carefully removed and transferred into scintillation vials for radioactivity counting.

Statistical Analysis—All results are presented as means ± S.E. Statistical comparisons were by Student’s t tests or analysis of variance, as indicated in the text and figure legends. Statistical significance was set at p < 0.05, where NS indicates not significant.

RESULTS AND DISCUSSION

We first examined body weight of PKCβ mutant mice. Compared with WT mice of the same age and sex, PKCβ mutant mice have lower body weight. As shown in Fig. 1, this is especially pronounced in the older mice (33.34 ± 3.1 WT versus 27.61 ± 2.4 g PKCβ mutant, n = 12, p < 0.002) compared with younger mice (24.9 ± 2.3 WT versus 22.6 ± 1.9 g PKCβ mutant, n = 6, p < 0.05) (Fig. 1, A and B). The loss of PKCβ also led to changes in adipose tissue physiology (Fig. 1, C and D). The epididymal fat pads (WAT) in the PKCβ mutant mice were almost 3-fold smaller than controls (1.2 ± 0.27 WT versus 0.37 ± 0.07 g PKCβ mutant, p < 0.001, Fig. 1, D and E). Likewise, BAT of PKCβ mutant mice were slightly smaller than controls (140 ± 5.3 WT versus 98.5 ± 7.8 mg PKCβ mutant, p < 0.003). The liver (Fig. 1, D and F), kidney, heart, and skeletal muscle (results not shown) of WT and mutant mice appeared grossly normal and were of similar mass.

Histological analysis revealed greater numbers of larger adipocytes in WT than PKCβ mutant mice. This difference in frequency distribution was reflected in a ~2.5-fold reduction in mean surface area of adipocytes from PKCβ mutant mice, suggesting that decreased cell size partly contributed to fat mass decrease. The smaller adipocyte size in WAT of PKCβ mutant mice may reflect a certain population sensitive to lipid accumulation. Adipocytes of WT and PKCβ mutant pads were filled with multilocular lipid droplets that, overall, appeared larger in WT mice (Fig. 1G). Thickness of the adipose tissue beneath the dermis in PKCβ mutant mice was also clearly reduced compared with that in the WT mice (Fig. 1H). The mean thickness
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In addition to epididymal fat pads, total TG content in the liver (21.5 ± 1.3 WT versus 6.4 ± 0.35 mg/g PKCβ−/−, n = 12, p < 0.001) and muscle (13.04 ± 0.90 WT versus 3.29 ± 0.3 mg/g PKCβ−/−, p < 0.003) of PKCβ−/− mice was also dramatically decreased (Fig. 2A). Thin layer chromatography of total lipids extracted from liver and muscle of PKCβ−/− mice demonstrated markedly reduced TG levels compared with the lipids extracted from WT mice (Fig. 2B). The liver cholesterol contents were slightly reduced in mutant mice (1.05 ± 0.09 WT versus 0.90 ± 0.1 mg/g PKCβ−/−, p < 0.05, Fig. 2C). The differences in endogenous tissue TG between WT and PKCβ−/− mice may be secondary to differences in plasma TG levels, since there is a 33% reduction in plasma TG levels (64.5 ± 5.1 WT versus 43.8 ± 2.1 mg/dl PKCβ−/−, n = 8, p < 0.05; Fig. 2D). The plasma cholesterol levels, on the other hand, were similar in both groups of mice (71 ± 4.7 WT versus 73 ± 5.2 mg/dl, PKCβ−/−, n = 8, p = NS; Fig. 2E).

Although the body weight of PKCβ−/− mice was less than WT (Fig. 1B), these mice appeared to be mildly hyperphagic and consistently consumed a greater caloric load (21.6 ± 0.7 PKCβ−/− versus 17.5 ± 0.4 calories/day WT, a 20% increase for PKCβ−/− versus WT, n = 6, p < 0.05) (Fig. 3A). When energy intake was calculated relative to an increase in body weight (feed efficiency) over the 10-week period, the PKCβ−/− mice had a lower feed efficiency as compared with the WT mice (0.52 ± 0.02 WT versus 0.73 ± 0.1 calories/g of body weight gained/day PKCβ−/−, 40% increase, p < 0.001) (Fig. 3B). The plasma leptin levels were significantly reduced in PKCβ−/− mice compared with WT (3.7 ± 0.15 WT versus 0.85 ± 0.07 ng/ml, PKCβ−/−, n = 8, p < 0.001) (Fig. 3C). It is clear that the decreased body weight is not a result of decreased food intake and lower plasma leptin level may be sufficient to increase appetite. The interesting phenomenon that the PKCβ−/− mice ate more food than WT mouse daily, but gained less weight, suggests that

of adipose tissues of WT and PKCβ−/− mice was 53.1 ± 13.7 and 19.7 ± 3.1 μm (mean ± S.E.; n = 10 regions for each genotype of mice; p < 0.01), respectively.

FIGURE 1. Reduced adiposity in PKCβ−/− mutant mice. A, representative picture of a 10-month-old PKCβ−/− male mutant mouse compared with WT mouse. B, PKCβ−/− mice have reduced body weight compared with WT. Older (10 months) PKCβ−/− mice have >15% reduced body weight compared with older WT mice, whereas younger mutant mice (3 months) weigh ~10% less than younger WT mice (n = 6). C, abdominal view of the fat pad under the skin. D, pictures of WAT and livers of WT and PKCβ−/− mice. E, weight of WAT normalized by body weight (n = 12 each genotype of ages 8–11 months), *, p < 0.001. F, weights of liver normalized by body weight for the same group (n = 12). G, paraffin-embedded sections of WAT and BAT from WT and PKCβ−/− mice (n = 3) were stained with hematoxylin and eosin. H, lack of lipids in subcutaneous adipose tissue from PKCβ−/− mice. Caudal ventral skins from WT and PKCβ−/− mice stained with hematoxylin and eosin. The figure represents 15 fields of sections from three WT and three PKCβ−/− mice. Values represent mean and error bars indicate S.E.

FIGURE 2. PKCβ−/− mice show significant reduction in TG contents of plasma, liver, and muscle. A, relative amounts of liver and muscle TG contents in 8–11-month-old WT and PKCβ−/− mice (n = 12). *, p < 0.001; **, p < 0.003. B, TLC of total lipid extracts from livers and muscles of WT and PKCβ−/− mice. Each lane represents lipids from liver and muscle of three mice. C, relative amounts of cholesterol in the liver and muscle of the same group of WT and PKCβ−/− mice (n = 12). D, plasma TG levels in the plasma of the same group of WT and PKCβ−/− mice following fasting for 16 h (n = 8). Values represent mean ± S.E.; *, p < 0.05. E, plasma cholesterol levels in the same group of WT and PKCβ−/− mice (n = 8, p = NS). Values represent mean and error bars indicate S.E.
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there are important alterations in energy expenditure and disposition. To examine energy expenditure, we compared the weight loss of WT and PKCβ−/− mice after a 16-h fast. Because energy intake is eliminated, the weight loss under fasting conditions provides a simple observation of energy expenditure. Fig. 4A shows that fasting-induced weight loss was higher in PKCβ−/− mice than in the WT (11.7 ± 0.9 PKCβ−/− versus 7.8 ± 0.3% WT, n = 7, p < 0.003). These results suggested that the decreased adiposity of the PKCβ−/− was due to increased energy expenditure.

We next carried out indirect calorimetry to investigate whether the resistance to weight gain was caused by increased metabolic rate. Fig. 4B shows that fasting-induced weight loss was higher in PKCβ−/− mice than in the WT (11.7 ± 0.9 PKCβ−/− versus 7.8 ± 0.3% WT, n = 7, p < 0.003). The respiratory exchange ratio of 0.727 ± 0.004 for WT mice and 0.713 ± 0.003 for PKCβ−/− mice showed that both animals were largely using fatty acids as an energy source. Thus, the failure to accumulate fat with age in PKCβ−/− mice seems to stem from a sharp increase in metabolic rates.

To understand the molecular basis of fat loss in PKCβ−/− mice, we analyzed the expression levels of several key genes involved in adipose tissue energy homeostasis. Results are presented in Table 1. Neither peroxisome proliferator-activated receptor α (PPARα), which targets enzymes of fatty acid oxidation (18), nor PPARδ, recently shown to activate fat burning (19), were altered in PKCβ−/− mice. The expression of other adipogenic genes, such as CCAAT enhancer-binding protein α (C/EBPα) and CD36, were also unaltered in mutant mice. There was a significant increase in the expression of UCP-2 in BAT. UCP-2 is widely expressed (20) and was also increased in WAT. PPARγ coactivator α (PGC-1α), a key regulator of mitochondrial biogenesis and respiration (21), was strikingly elevated in both BAT and WAT. This is in agreement with a recent study showing an inverse relationship between PGC-1α protein expression and TG accumulation in rodent skeletal muscle (22). The increase in PGC-1α may also account for increased expression of UCP-2 (23). Consistent with an increase in PGC-1α, expression of medium chain acyl coenzyme A dehydrogenase, a nuclease encoded mitochondrial fatty acid β oxidation enzyme, regulated in parallel with fatty acid oxidation rates, was also increased. There was also an increase in carnitine palmitoyltransferase 2, which is involved in fatty acid transport to mitochondria. The increase in lipogenic SREBP-1c expression is possibly due to lower endogenous TG levels. The decreased expression of the lipid droplet protein perilipin can promote increased lipolysis in the PKCβ−/− adipocytes. Perilipin has recently been shown to play a key role in determining body habitus as its absence leads to a lean and obesity-resistant phenotype in mice (24).

To test whether PKCβ deficiency affected oxidation of fatty acids in BAT and WAT, production of 14CO2 from [1-14C]palmitate and [1-14C]oleate was measured. It is significantly increased in adipocytes of WAT (205 ± 15% palmitate and 190 ± 10% oleate) and BAT (187 ± 10% palmitate and 165 ± 9% oleate) from PKCβ−/− mice compared with WT littermates (Fig. 5). This appears to be accompanied by increase in number and size of mitochondria containing numerous cristae in both WAT and BAT of PKCβ−/− mice (Fig. 6). The above results are consistent with a recent demonstration that AMPK activation inhibits adipose fatty acid oxidation (25). Leptin is a known activator of AMPK, and low plasma leptin levels in PKCβ−/− mice (Fig. 3) are expected to promote adipose fatty acid oxidation in the PKCβ−/− mice.

It is interesting to note that disruption of PKCβ in mice achieves several outcomes that are typically associated with the inactivation of key components of adipose tissue storage capacity (26–29), resulting in enhanced lipolysis or a reduced ability to store TG. Importantly, this reduced energy storage consistently results in increased energy expenditure due to repartitioning of energy substrates, to the point where increased food intake cannot compensate. Increased expression of the mitochondrial genes (PGC-1α or UCPs) in PKCβ−/− mice could promote systemic energy expenditure because of thermogenic effects, i.e. generation of heat (reviewed in Ref. 30) and a concurrent decrease in oxidative phosphorylation (31). Potential
TABLE 1
Expression of various mRNAs in WAT and BAT of 8–11-month-old WT and PKCβ−/− mice

| Gene     | BAT Relative -fold change | WAT Relative -fold change |
|----------|--------------------------|---------------------------|
| UCP-1    | -0.1                     | 0.2                       |
| UCP-3    | -0.1                     | 1.0                       |
| PPARα    | 1.0                      | 1.0                       |
| PPARγ    | 1.0                      | 1.0                       |
| PGC-1α   | 8.7                      | 5.2                       |
| C/EBPα   | 1.0                      | 1.5                       |
| MCAD     | 2.6                      | ND                        |
| CPT-2    | 1.8                      | ND                        |
| Perilipin| -3.9                     | -2.7                      |
| SREBP-1c | 2.1                      | 1.5                       |
| CD36     | 1.0                      | 1.0                       |

Adipocytes from PKCβ−/− mice show increased production of 14CO2 from [1-14C]palmitic acid and from [1-14C]oleic acid. Data were compiled from WT and BAT were prepared, and palmitate and oleate oxidation was measured by the production of 14CO2 from labeled fatty acids. Data were compiled from three independent experiments with quadruplicates in each experiment. *, p < 0.05; **, p < 0.001. Values represent mean, and error bars indicate S.E.

FIGURE 5. Adipocytes from PKCβ−/− mice show increased production of 14CO2 from [1-14C]palmitic acid and from [1-14C]oleic acid.

FIGURE 6. Increased adipose mitochondrial hypergeneration in PKCβ−/− mice. Adipose tissues were fixed and prepared for electron microscopy (×11,000).

mechanisms for elevated fat loss in PKCβ−/− mice may be related to an increase in PGC-1α expression resulting in enhanced mitochondrial capacity and “internal combustion,” as forced expression of this coactivator in human fat cells enhances fatty acid oxidation (32). Increase in PGC-1α would thus expand the oxidative machinery required for enhanced oxidation, while increased UCP-2 protein would dissipate the unneeded energy as heat. Involvement of PKCβ in regulating mitochondrial biogenesis is supported by a recent observation that linked PKCβ with mitochondrial survival pathways (33). Taken together, it appears that one of the consequences of PKCβ deficiency is to partition fat toward increased oxidation and the elevation in fatty acid oxidation capacity is powerful enough to overcome increased food intake. It is not clear whether the increased fatty acid oxidation in adipose tissue is sufficient to significantly promote redistribution of fat from liver and muscle into the adipocytes, much as fat transplantation does in fat-deficient lipodystrophic mice (34). It is also conceivable that central regulatory actions of PKCβ in the brain, especially hypothalamus, may impact body energy expenditure and metabolic control. Various central nervous system-mediated gene targets result in a lean phenotype, often with increased metabolic rate (35). In any event, PKCβ-dependent increased oxidative capacity may have important therapeutic implications for the treatment of obesity and obesity-related disorders. PKCβ antagonists are currently undergoing clinical trials to reduce diabetes-linked complications (14, 15). Our data raise an interesting possibility that inhibition of PKCβ may also prevent or delay the development of obesity and obesity-related disorders.

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