PKCζ Phosphorylates SIRT6 to Mediate Fatty Acid β-Oxidation in Colon Cancer Cells

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Abstract
Protein kinase C (PKC) has critical roles in regulating lipid anabolism and catabolism. PKCζ, a member of atypical PKC family, has been reported to mediate glucose metabolism. However, whether and how PKCζ regulates tumor cells fatty acid β-oxidation are unknown. Here, we report that the phosphorylation of SIRT6 is significantly increased after palmitic acid (PA) treatment in colon cancer cells. PKCζ can physically interact with SIRT6 in vitro and in vivo, and this interaction enhances following PA treatment. Further experiments show that PKCζ is the phosphorylase of SIRT6 and phosphorylates SIRT6 at threonine 294 residue to promote SIRT6 enrichment on chromatin. In the functional study, we find that the expression of ACSL1, CPT1, CACT, and HADHB, the genes related to fatty acid β-oxidation, increases after PA stimulation. We further confirm that PKCζ mediates the binding of SIRT6 specifically to the promoters of fatty acid β-oxidation-related genes and elicits the expression of these genes through SIRT6 phosphorylation. Our findings demonstrate the mechanism of PKCζ as a new phosphorylase of SIRT6 in maintaining tumor fatty acid β-oxidation and define the new role of PKCζ in lipid homeostasis.

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Introduction
Protein kinase C (PKC) is a prototypical class of serine/threonine kinases and influences a variety of cellular events such as cell proliferation, cell cycle, differentiation, survival, migration, and polarity [1–3]. PKC was originally identified as a cellular receptor for the phorbol ester tumor promoters more than 30 years ago [4], and at least 10 isoforms were classified into 3 major groups: conventional (cPKCs: α, βI, βII and γ), novel (nPKCs: δ, ε, η, θ and μ), and atypical (aPKCζ and λ/ι) isoforms [5]. PKCζ belongs to aPKC isoymes which are structurally and functionally distinct from other PKCs, and its catalytic activity does not require diacylglycerol, pseudosubstrate, or calcium, and also it does not serve as cellular receptors for phorbol esters [6–8]. PKCζ is critical for cell survival signaling, presumably due to its role as a downstream effector of phosphoinositide 3-kinase (PI3K) [9]. The expression change of PKCζ has been reported in several human cancers [10–14]. PKCζ-deficient mice display increased Ras-induced lung carcinogenesis, showing the role of PKCζ as a tumor suppressor in vivo [13]. A proapoptotic function for PKCζ has been described in several cancer models including colorectal cancer. For example, PKCζ exhibited a proapoptotic function in ovarian cancer [15]. Also, PKCζ is reported to inhibit growth and promote differentiation and apoptosis in Caco-2 colon cancer cells. The inhibitory effect of PKCζ on the
transformed phenotype of these cells indicates that downregulation of PKCζ may contribute to colon tumorigenesis [16]. There are several reports, however, highlighting a prosurvival role for PKCζ [17–20]. For example, PKCζ mediates chemotaxis by regulating actin polymerization and cell adhesion, and downregulation of PKCζ expression inhibits chemotaxis signal transduction in human lung cancer cells [17]. Luna-Ulloa et al. show that the PKCζ-stably depleted cells exhibited diminished tumorigenic activity in grated mice. PKCζ activity regulates the nuclear localization of β-catenin and plays an important role in the positive regulation of canonical Wnt pathway [18]. PKCζ was reported to link multiple cellular processes of cancer, including cancer cells proliferation, cell cycle progression, tumorigenesis, promotion, invasion, and metastasis. However, the fuction of PKCζ on cancer metabolism especially in lipid metabolism is seldom mentioned and needs further exploration in detail.

SIRT6, a chromatin regulatory protein, is one member of the mammalian sirtuins family of NAD+-dependent deacetylases with multiple functions in aging, metabolism, and diseases [21]. It is a critical regulator of diverse cellular processes such as transcription, genome stability, DNA repair, telomere integrity, inflammation, and metabolism [22–25]. SIRT6 is also involved in regulating many aspects of cellular metabolism including lipid homeostasis. SIRT6-overexpressing mice fed a high-fat diet exhibit decreased visceral fat accumulation, improved blood lipid profile, glucose tolerance, and insulin secretion, indicating that SIRT6 can dramatically affect lipid homeostasis [26]. Kim et al. show that liver-specific deletion of SIRT6 in mice causes profound alterations in gene expression, leads to increased glycolysis and triglyceride synthesis, reduces fatty acid β-oxidation, and accelerates fatty liver formation [27]. SIRT6 and miR-122 negatively regulate each other’s expression to mediate fatty acid β-oxidation [28]. Also, a muscle-specific SIRT6 knockout mouse model shows the decreased expression of genes involved in glucose and lipid uptake, fatty acid β-oxidation, and mitochondrial oxidative phosphorylation in muscle cells through activation of AMP-activated protein kinase [29]. Despite these cumulative data, the challenge remains to explore the mechanism of SIRT6 on fatty acid β-oxidation.

Fatty acid β-oxidation plays a crucial role in maintaining body energy homeostasis mainly during catabolic states. It mainly occurs in mitochondria and involves a cyclical series of reactions that result in the shortening of fatty acids. Fatty acids comprise an efficient source of energy that results in the generation of a large quantity of ATP, and it is involved in the lipid metabolism of cancer cells [30–34]. In solid tumors, fatty acid β-oxidation activation links to the promotion of breast cancer cells survival triggered by loss of attachment to the matrix during metabolic stress [30]. Also, it is reported that cell survival of human LN18 glioblastoma and HeLa cells depended on fatty acid β-oxidation under conditions of complete nutrient deprivation [31]. Fatty acid β-oxidation connects with the malignant phenotype and regulates by the promyelocytic leukemia protein to active peroxisome proliferator-activated receptor signaling [32]. Recently, a study showed that SIRT6 overexpression induced increased expression of fatty acid β-oxidation–related genes such as carnitine palmitoyltransferase 1 (CPT1), hydroxacyl-CoA dehydrogenase trifunctional multienzyme complex subunit beta (HADHB), and carnitine O-octanoyltransferase [28], yet the effect of PKCζ on fatty acid β-oxidation is unknown. It is novelty if we can reveal a novel, cooperative model executed by PKCζ and SIRT6 to maintain fatty acid β-oxidation.

Here, we report that the phosphorylation of SIRT6 is significantly increased after palmitic acid (PA) treatment in variety of colon cancer cells. PKCζ can interact with SIRT6 directly and phosphorylates SIRT6 at threonine 294 residue in response to PA treatment. SIRT6 Thr294 phosphorylation is required for SIRT6 enrichment on chromatin to further bind to the promoters of fatty acid β-oxidation–related genes acyl-CoA synthetase long-chain family member 1 (ACSL1), CPT1, carnitine-acylcarnitine translocase (CACT), and HADHB and then induces the expression of these genes to mediate the fatty acid β-oxidation. Altogether, our data demonstrate that PKCζ plays a major role in regulating gene expression of fatty acid β-oxidation through SIRT6 phosphorylation. These findings further our understanding of the effect of PKCζ on lipid metabolism and may guide the design of new therapeutics to regulate lipid homeostasis.

Materials and Methods

Cell Culture and Palmitic Acid Treatment

Human colon cancer cell lines HCT116 and LoVo were purchased from American Type Culture Collection (ATCC, Manassas, VA). HCT116 cells were cultured in McCoy’s 5a medium supplemented with 10% heat-inactivated fetal bovine serum (FBS). LoVo cells were cultured in F-12K medium supplemented with 10% heat-inactivated FBS. All the cells were grown in the medium mentioned above with penicillin/streptomycin in a 37°C incubator with a humidified, 5% CO2 atmosphere. PA was purchased from Sigma (St Louis, MO) and prepared at a stock solution and stored at room temperature. For PA treatment, the PA stock solution was freshly added to the medium at various doses and then incubated at 37°C for the indicated time intervals. Control cells were treated with a control solution at equivalent doses and exposure times.

Protein Extraction and Western Blotting

Human colon cancer HCT116 and LoVo cells were harvested after treatment, the total or NP40 protein was extracted, and protein expression was detected by Western blotting as previously described with minor modifications [35]. Equal amounts of proteins were size fractionated by 9% to 15% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. anti-SIRT6 (2590S, Cell Signaling, Danvers, MA), anti-PKCζ (sc-17781, Santa Cruz, CA), anti-Pi3K (ab191606, abcam, Cambridge, MA), anti–serine/threonine kinase 1 (AKT) (9272, Cell Signaling, Danvers, MA), anti–glycogen synthase kinase-3 beta (GSK3β) (9315, Cell Signaling, Danvers, MA), anti–serine/threonine protein phosphatase 2A (PP2A) (ab3210, abcam, Cambridge, MA), anti–phospho-(Ser/Thr) (9631, Cell Signaling, Danvers, MA), anti-Flag (F1804, Sigma Aldrich), anti-His (PM032, MBL), anti–GST (sc-138, Santa Cruz, CA), anti-MyC (M047-3, MBL, Japan), anti–α-tubulin (BE0031, EASYBIO, Beijing, China), and anti–β-actin (4967, Cell Signaling, Danvers, MA) were used, and the blots were developed using an enhanced chemiluminescence kit (Amersham Corp.).

Co-Immunoprecipitation (Co-IP)

After treatment, HCT116 cells were harvested and lysed in different lysis buffers. Antibodies were then added to the supernatant on ice for 1 hour. Protein G- or A-Sepharose beads (GE Healthcare, Little Chalfont, UK) were then added, and the samples were mixed by rolling at 4°C for 1 hour. The beads were then washed three times with lysis buffer, and the pellets were dissolved into 2× SDS loading buffer after centrifugation. The protein was analyzed by Western blotting with different antibodies.

GST Pull-Down Assay
GST or GST fusion proteins were expressed in bacteria induced with isopropyl-β-D-thio-galactoside and purified with glutathione-Sepharose 4B beads (GE Healthcare, Little Chalfont, UK). Recombinant His-tagged proteins were purified from bacteria by Ni (ii)-Sepharose affinity (GE Healthcare, Little Chalfont, UK).

In Vitro Kinase Assay
To evaluate in vitro phosphorylation of SIRT6 by PKCζ, Myc-PKCζ-tagged recombinant proteins (4 mg) were incubated with purified GST-Vector, GST-SIRT6 (SIRT6-WT or SIRT6-T294A mutant construct) recombinant proteins (8 mg) in kinase buffer (20 mM HEPES at pH 7.4, 1 mM EGTA, 0.4 mM EDTA, 5 mM MgCl2, and 0.05 mM dithiothreitol, 10 μM cold ATP and 2 μCi [γ-32P]ATP) per reaction. Recombinant GST-SIRT6 and GST-SIRT6T294A proteins were bacterially purified. The kinase reaction was performed at 37°C for 30 minutes, the reaction products were separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and 32P-labeled proteins were detected by autoradiography.

RNA Extraction and RT-qPCR

Total RNA was isolated with TRIzol reagent (TianGen, Beijing, China). cDNA was synthesized from 2 μg of RNA using Quantscript RT Kit (Promega, Madison, WI) according to the manufacturer’s instructions. The primer sequences used for RT-PCR were as follows: SIRT6-F: 5’-ACGCCAAATCTTGGTGCTCT-3’, SIRT6-R: 5’-AG CACTAA CGCCTCTCCCTT-3’; PKCζ-F: 5’-CCCTCCGTGT TT G T G T C G A A - 3 ’ , P K C ζ - R : 5 ’ - A GACCATGACGTGGAATCAGA-3’; AC5L1-R: 5’-GTACCCAGTTCCAGCCTCA CA-3’; HADHB-F: 5’-ACCCGCCCGAGAGGAGATGTA-3’, Actin: 5’-ACCCGCCCGAGAGGAGATGTA-3’, Actin-R: 5’-ACCCGCCCGAGAGGAGATGTA-3’, PKCζ-F: 5’-CCCTCCGTGT TT G T G T C G A A - 3 ’ , P K C ζ - R : 5 ’ - A GACCATGACGTGGAATCAGA-3’; AC5L1-R: 5’-GTACCCAGTTCCAGCCTCA CA-3’; HADHB-F: 5’-ACCCGCCCGAGAGGAGATGTA-3’, Actin: 5’-ACCCGCCCGAGAGGAGATGTA-3’, Actin-R: 5’-ACCCGCCCGAGAGGAGATGTA-3’, PKCζ-F: 5’-CCCTCCGTGT TT G T G T C G A A - 3 ’ , P K C ζ - R : 5 ’ - A GACCATGACGTGGAATCAGA-3’; AC5L1-R: 5’-GTACCCAGTTCCAGCCTCA CA-3’; HADHB-F: 5’-ACCCGCCCGAGAGGAGATGTA-3’, Actin: 5’-ACCCGCCCGAGAGGAGATGTA-3’, Actin-R: 5’-ACCCGCCCGAGAGGAGATGTA-3’, PKCζ-F: 5’-CCCTCCGTGT TT G T G T C G A A - 3 ’ , P K C ζ - R : 5 ’ - A GACCATGACGTGGAATCAGA-3’; AC5L1-R: 5’-GTACCCAGTTCCAGCCTCA CA-3’; HADHB-F: 5’-ACCCGCCCGAGAGGAGATGTA-3’, Actin: 5’-ACCCGCCCGAGAGGAGATGTA-3’, Actin-R: 5’-ACCCGCCCGAGAGGAGATGTA-3’, PKCζ-F: 5’-CCCTCCGTGT TT G T G T C G A A - 3 ’ , P K C ζ - R : 5 ’ - A GACCATGACGTGGAATCAGA-3’; AC5L1-R: 5’-GTACCCAGTTCCAGCCTCA CA-3’; HADHB-F: 5’-ACCCGCCCGAGAGGAGATGTA-3’, Actin: 5’-ACCCGCCCGAGAGGAGATGTA-3’, Actin-R: 5’-ACCCGCCCGAGAGGAGATGTA-3’, PKCζ-F: 5’-CCCTCCGTGT TT G T G T C G A A - 3 ’ , P K C ζ - R : 5 ’ - A GACCATGACGTGGAATCAGA-3’; AC5L1-R: 5’-GTACCCAGTTCCAGCCTCA CA-3’; HADHB-F: 5’-ACCCGCCCGAGAGGAGATGTA-3’, Actin: 5’-ACCCGCCCGAGAGGAGATGTA-3’, Actin-R: 5’-ACCCGCCCGAGAGGAGATGTA-3’.

RNA Interference (RNAi)
RNA interference was performed as described[36]. The sequences of RNAi oligonucleotides for PKCζ and SIRT6 were as follows: PKCζ siRNA, 5’-GGCGGAGUCCUGUUTT-3’; SIRT6 siRNA, 5’-AAGAATGTGCCAAGTGTAAGA-3’. These RNAi oligonucleotides and controls (nonspecific siRNA) were transfected into HCT116 cells by using a Lipofectamine 2000 transfection kit (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Cells were harvested 48 hours after transfection and subjected to Western blotting, RT-qPCR, or a chromatin Immunoprecipitation (ChIP) assay, respectively.

Figure 1. The phosphorylation of SIRT6 dramatically increases in colon cancer cells after PA treatment. (A) HCT116 cells were transfected with Flag-SIRT6 for 24 hours and then exposed with or without PA (0.2 mM) for 18 hours. Cell lysates were extracted, and the same amount of SIRT6 protein was used for a Co-IP assay with an antiphosphorylation antibody followed by probing with anti-Flag to detect the level of phosphorylation of SIRT6. (B) HCT116 cells were treated with or without PA (0.2 mM) for 18 hours, cell lysates were then extracted, and the same amount of SIRT6 protein was used for a Co-IP assay using an antiphosphorylation antibody followed by anti-SIRT6 to detect the level of endogenous phosphorylation of SIRT6 in HCT116 cells. (C) LoVo cells were transfected with Flag-SIRT6 for 24 hours and then exposed with or without PA (0.2 mM) for 18 hours. Cell lysates were extracted, and the same amount of SIRT6 protein was used for a Co-IP assay using an antiphosphorylation antibody followed by probing with anti-Flag to detect the level of phosphorylation of SIRT6. (D) LoVo cells were treated with or without PA (0.2 mM) for 18 hours, cell lysates were then extracted, and the same amount of SIRT6 protein was used for Co-IP using an antiphosphorylation antibody followed by anti-SIRT6 to detect the level of endogenous phosphorylation of SIRT6 in LoVo cells.
**ChIP Assay**

HCT116 cells were cross-linked with 1% formaldehyde for 10 minutes at 37°C and then washed with cold PBS. The cell pellet was resuspended in lysis buffer followed by sonication to produce an average DNA length of 500 to 1000 base pairs. The indicated antibodies were added to each sample, and the samples were mixed by rotation at 4°C overnight. Protein A/G Sepharose beads were added to the complexes and incubated for 2 hours at 4°C. The agarose was washed sequentially with low-salt, high-salt LiCl and TE buffer and eluted with elution buffer (1% SDS and 0.1 M NaHCO₃). The cross-link was reversed at 65°C overnight, and the DNA was dissolved in Tris-EDTA buffer and analyzed by real-time PCR. The primers for all ChIPs are available upon request.

**Data Analysis**

![Figure 2.](image)

**PKCζ interacts with SIRT6 in vivo and in vitro following PA exposure.** (A) HCT116 cells were transfected with Flag-SIRT6 and then treated with or without PA at 0.2 mM for 18 hours; protein was then extracted for Co-IP assay with anti-PI3K, anti-AKT, anti-PP2A, anti-GSK3β, and anti-PKCζ antibodies followed by Western blotting using an anti-SIRT6 to detect the interaction between SIRT6 and these phosphorylases. (B) HCT116 cells were treated with or without PA for 18 hours, and then protein was extracted for Co-IP using an anti-Flag antibody followed by Western blotting using anti-SIRT6 or anti-PKCζ antibody to detect the endogenous interaction between PKCζ and SIRT6. (C) HCT116 cells were treated with or without PA for 18 hours, and then protein was extracted for Co-IP using an anti-PKCζ antibody followed by Western blotting using an anti-PKCζ or anti-SIRT6 antibody to detect the endogenous interaction between PKCζ and SIRT6. (D) Schematic of plasmids encoding full-length (FL) PKCζ, an N-terminal fragment (1-251 aa), a middle fragment (CD domain) (252-518 aa), and a C-terminal fragment (519-592 aa). (E) GST-PKCζ FL or fragments (purified from bacteria by using vector pGEX-4T3) were incubated with His-SIRT6 (cloned into pET28b(+)) and expressed in bacteria, and Western blotting with an anti-His antibody or Coomassie staining was performed to detect the direct binding of PKCζ and SIRT6 in vitro. *Indicates the specific bands. (F) Schematic of plasmids encoding FL SIRT6, an N-terminal fragment (1-34 aa), a middle fragment (CD domain) (35-276 aa), and a C-terminal fragment (277-355 aa). (G) GST-SIRT6 FL or fragments were incubated with His-PKCζ, and Western blotting with an anti-His antibody or Coomassie staining was performed to detect the interaction. *Indicates the specific bands.
Statistical analysis was performed to assess the difference between two groups under multiple conditions by one-way analysis of variance using PRISM statistical analysis software (GraphPad Software, Inc., San Diego, CA).

**Results**

*The Phosphorylation of SIRT6 Dramatically Increases in Colon Cancer Cells After PA Treatment*

Sirt6 was reported to be involved in many aspects of cell metabolic regulation [21,37]. However, our knowledge regarding its regulation remains limited. Here we investigate the effect of SIRT6 phosphorylation

![Figure 3](https://example.com/fig3.png)

**Figure 3.** PKCζ phosphorylates SIRT6 following PA exposure. (A) HCT116 cells were treated with various doses of PA (0, 0.1, and 0.2 mM) for 18 hours, and the total PKCζ protein expression was detected by Western blotting. β-Actin was used as a loading control. (B) HCT116 cells were treated with 0.2 mM PA for various intervals (0, 12, and 18 hours), and then the total PKCζ protein expression was detected by Western blotting. (C) HCT116 cells were treated with various doses of PA (0, 0.1, and 0.2 mM) for 18 hours, and then PKCζ mRNA expression was analyzed by real-time qPCR. mRNA levels of the control sample were set as 1, and relative mRNA levels of the experimental samples were normalized to this control. The data represent the means ± SD. *P < .05, **P < .01. (D) HCT116 cells were treated with 0.2 mM PA for various intervals (0, 12, and 18 hours), and PKCζ mRNA expression was analyzed by real-time qPCR. (E) HCT116 cells were transfected with PKCζ siRNA or nonspecific siRNA for 24 hours and then treated with or without PA (0.2 mM) for 18 hours. Cell lysates were extracted, and the same amount of SIRT6 protein was used for a Co-IP assay using an antiphosphorylation antibody followed by probing with anti-SIRT6 to detect the level of endogenous phosphorylation of SIRT6. The efficiency of PKCζ RNAi was detected by PKCζ antibody. (F) Myc-PKCζ, purified from HCT116 cells, was incubated with GST-Vector or bacterially purified GST-SIRT6 in the presence of [γ-32P] ATP and a kinase reaction buffer, and subsequently separated by SDS-PAGE, stained by CBB, or exposed by autoradiography to detect the effect of PKCζ on SIRT6 phosphorylation. The arrow indicates phosphorylation of SIRT6. The experiment was repeated three times.
on lipid metabolism. PA, a saturated long-chain fatty acid, was used to detect the expression of phosphorylation of SIRT6 in colon cancer HCT116 and LoVo cells. HCT116 cells were transfected with Flag-SIRT6 and then treated with or without PA at 0.2 mM for 18 hours; cell lysates were extracted and subjected to a Co-IP assay with anti-phosphorylation antibody followed by probing with anti-Flag antibody. As shown in Figure 1A, the same amount of SIRT6 protein was used before and after PA treatment, and the phosphorylation of SIRT6 increased in HCT116 cells after PA treatment compared with control cells. Also, the endogenous phosphorylation of SIRT6...
PKCζ Interacts with SIRT6 In Vivo and In Vitro Following PA Exposure

We performed a Co-IP assay to further detect the key phosphorylation of SIRT6 in response to PA stimulation. Flag-SIRT6 was transfected into HCT116 cells. The cells then treated with or without PA at 0.2 mM for 18 hours before lyses were collected for Co-IP with several phosphorylation antibodies followed by probing with anti-Flag. Among the numerous phosphorylases, we found that PI3K, AKT, GSK3β, PP2A, and PKCζ can interact with SIRT6. Remarkably, the interaction between the exogenous PKCζ and SIRT6 is significantly enhanced after PA stimulation (Figure 2A). Also, the endogenous interaction between PKCζ and SIRT6 was detected in HCT116 cells after PA treatment. HCT116 cells were treated with or without PA at 0.2 mM for 18 hours before cell lyses were collected for Co-IP with either anti-PKCζ or anti-SIRT6 antibody followed by probing with anti-SIRT6 or anti-PKCζ, respectively. Endogenous PKCζ and SIRT6 showed an enhanced interaction in HCT116 cells in response to PA treatment (Figure 2B, C). These data indicate that PKCζ can interact with SIRT6 in vivo and the interaction between PKCζ and SIRT6 is enhanced following PA exposure. We then performed a GST pull-down assay to investigate whether the interaction between PKCζ and SIRT6 is direct. To map the regions of PKCζ involved in SIRT6 binding, we constructed and purified the full length (FL, 1-592 aa) and several fragments of GST-PKCζ (N-terminus, 1-251 aa; core domain, 252-518 aa; and C-terminus, 519-592 aa) (Figure 2D). A His-tagged SIRT6 protein was expressed in bacteria, purified, and then incubated with GST (as a negative control) or several fragments of GST-PKCζ. The FL and the PKCζ N-domain fragments bound SIRT6 (Figure 2E), indicating that the PKCζ N-domain is responsible for this interaction. We then reciprocally mapped the regions of SIRT6 required for PKCζ binding by incubating FL or fragments of GST-SIRT6 (Figure 2F: FL, 1-355 aa; N-terminus, 1-34 aa; CD domain, 35-274 aa; C-terminus, 275-355 aa) with His-PKCζ. His-PKCζ specifically interacted with FL and the C-terminus fragment of GST-SIRT6 (Figure 2G), indicating that the SIRT6 C-domain is responsible for the interaction of PKCζ and SIRT6. These data indicate that PKCζ directly interacts with SIRT6 and that this interaction is enhanced in response to PA treatment.

PKCζ Phosphorylates SIRT6 Following PA Exposure

Based on the data above, we further explored the effect of phosphorylase PKCζ on SIRT6 phosphorylation in response to PA treatment. Firstly, the expression of PKCζ in HCT116 cells after PA treatment was detected by Western blotting. No significant difference was detected in the total level of PKCζ protein after PA treatment with various doses of PA (0, 0.1, and 0.2 mM) for 18 hours or by different times (0, 12, and 18 hours) (Figure 3A, B). Also, the mRNA level of PKCζ was detected by the same stimulation above, and there was no change to be found in the mRNA level of PKCζ after PA treatment (Figure 3C, D). These data imply that PA does not affect the expression of PKCζ in both protein and mRNA level. Remarkably, when we knocked down PKCζ by PKCζ siRNA, the phosphorylation level of SIRT6 after PA treatment was significantly reduced compared with nonspecific siRNA-treated cells, showing that PKCζ might be the phosphorylase of SIRT6 in response to PA treatment (Figure 3E). To confirm this result, in vitro phosphorylation assays were performed with purified PKCζ and SIRT6. We observed that PKCζ strongly transferred radiolabel (32P) from [γ-32P]-ATP to SIRT6 under standard kinase reaction conditions (Figure 3F). These data demonstrate that PKCζ is the phosphorylase of SIRT6 in response to PA treatment.

PKCζ Phosphorylates SIRT6 at Threonine 294 Residue, and SIRT6 Thr294 Phosphorylation Promotes SIRT6 Enrichment on Chromatin After PA Treatment

Next, we identified the specific PKCζ phosphorylation sites on SIRT6. Recombinant SIRT6 was incubated with functional PKCζ and then mass spectrometry was performed. These mass spectrometry data indicate that PKCζ may phosphorylate SIRT6 on threonine 294 residue after PA treatment (Figure 4A). To further confirm the effect of Thr294 residue on SIRT6 phosphorylation, a SIRT6 threonine 294 to alanine 294 mutant (SIRT6 (T294A)) plasmid was generated and transfected to HCT116 SIRT6 KO cells. As shown in Figure 4B, the levels of phosphorylation of SIRT6 were reduced significantly after PA stimulation in mutated SIRT6 T294A-transfected HCT116 KO cells compared with wild-type SIRT6-transfected cells. Also, an in vitro phosphorylation assay using 32P-labeled ATP revealed that SIRT6 (T294A) mutant blocked ortho-32P incorporation into SIRT6 (Figure 4C). PKCζ can phosphorylate WT SIRT6, yet it cannot phosphorylate SIRT6 T294A. These

Figure 4. PKCζ phosphorylates SIRT6 at threonine 294 residue, and SIRT6 Thr294 phosphorylation promotes SIRT6 enrichment on chromatin after PA treatment. (A) Bacterially purified SIRT6 FL (GST-tag was cut by HRV3C) was catalyzed by FLAG-PKCζ in vitro and subsequently separated by SDS-PAGE and stained with CBB. The SIRT6 band was excised from the gel and analyzed by mass spectrometry. (B) SIRT6 KO HCT116 cells were transfected with Flag-SIRT6 WT or Flag-SIRT6 T294A mutant plasmid for 24 hours and then treated with or without PA (0.2 mM) for 18 hours. Cell lyses were extracted, and the same amount of SIRT6 protein was used for a Co-IP assay using an antiphosphorylation antibody followed by probing with anti-Flag to detect the level of endogenous phosphorylation of SIRT6. (C) Myc-PKCζ, purified from HCT116 cells, was incubated with bacterially purified GST-Vector, GST-SIRT6 WT, or GST-SIRT6 T294A mutant in the presence of [γ-32P]ATP and a kinase reaction buffer, and subsequently separated by SDS-PAGE, stained by CBB, or exposed by autoradiography to detect the effect of threonine 294 site mutant on SIRT6 phosphorylation. The experiment was repeated three times. (D) HCT116 cells were treated with various doses of PA (0, 0.1, and 0.2 mM) for 18 hours. Dt and Chr proteins were then extracted for Western blotting and analyzed using a PKCζ or SIRT6 antibody. α-Tubulin and histone H3 antibodies were used as the loading controls for Dt and Chr proteins, respectively. (E) A PKCζ siRNA or a nonspecific siRNA NC was delivered into HCT116 cells and then treated with or without PA (0.2 mM) for 18 hours. Dt and Chr proteins were extracted and analyzed using antibodies against PKCζ and SIRT6. α-Tubulin and histone H3 antibodies were used as the loading controls for Dt and Chr proteins, respectively. (F) SIRT6 KO HCT116 cells were transfected with Flag-SIRT6 WT or Flag-SIRT6 T294A mutant plasmid for 24 hours and then treated with or without PA (0.2 mM) for 18 hours. Dt and Chr proteins were extracted and analyzed using antibodies against PKCζ and SIRT6. α-Tubulin and histone H3 antibodies were used as the loading controls for Dt and Chr proteins, respectively.
PKCζ regulates the expression of fatty acid β-oxidation–related genes after PA treatment. (A, C, E, and G) HCT116 cells were treated with various doses of PA (0, 0.1, and 0.2 mM) for 18 hours, and the mRNA expression of (A) ACSL1, (C) CPT1, (E) CACT, and (G) HADHB was analyzed by real-time PCR. mRNA levels of the control sample were set as 1, and relative mRNA levels of the other samples were normalized to this control. The data represent as the means ± SD. *P < .05, **P < .01. (B, D, F, and H) HCT116 cells were treated 0.2 mM PA for various intervals (0, 12, and 18 hours), and the mRNA expression of (B) ACSL1, (D) CPT1, (F) CACT, and (H) HADHB was analyzed by real-time PCR. (I) HCT116 cells were transfected with PKCζ siRNA or nonspecific siRNA for 24 hours and then treated with or without PA (0.2 mM) for 18 hours. The mRNA expression of ACSL1, CPT1, CACT, and HADHB was analyzed by real-time PCR.
findings suggest that SIRT6 is phosphorylated by PKCζ at Thr294 residue following PA exposure.

We further monitored the localization of PKCζ and SIRT6 to understand the biological function of the SIRT6 phosphorylation by PKCζ following PA exposure. The detergent extractable (Dt) and chromatin (Chr)-bound proteins were extracted separately and then analyzed for PKCζ and SIRT6 levels. Here, we found that the expression level of SIRT6 was significantly increased in Chr fractions following PA treatment in a PA dose-dependent manner (Figure 4D). These data suggest that SIRT6 localizes to and is enriched on chromatin after PA treatment. To further explore the effect of SIRT6 phosphorylation on SIRT6 enrichment on chromatin, double-stranded PKCζ siRNA or a nonspecific siRNA was firstly transfected into HCT116 cells, and the PA treatment was repeated. We found that enrichment of SIRT6 on chromatin was blocked after PA stimulation in PKCζ siRNA-treated cells compared with nonspecific siRNA-treated cells, showing that PKCζ might participate in the enrichment of SIRT6 in response to PA treatment (Figure 4E). Next, Flag-SIRT6 WT or Flag-SIRT6 T294A mutant plasmid was transfected into SIRT6 KO HCT116 cells, and then the cells were exposed to PA. The enrichment of SIRT6 on chromatin was significantly reduced in the Flag-SIRT6 T294A mutant transfected cells compared with Flag-SIRT6 WT transfected cells after PA stimulation (Figure 4F). These data demonstrate that SIRT6 Thr294 phosphorylation is required for SIRT6 enrichment on chromatin after PA treatment.

**PKCζ Regulates the Expression of Fatty Acid β-Oxidation Related Genes After PA Treatment**

SIRT6 plays an important role in glucose and lipid metabolism, and it was reported to be involved in mediating fatty acid β-oxidation recently [28]. However, it is unknown whether PKCζ is involved in the process of fatty acid β-oxidation. To solve this question, HCT116 cells were firstly treated with various doses (0, 0.1, and 0.2 mM) or different times (0, 12, and 18 hours) of PA, and then mRNA was extracted and real-time PCR was performed to analyze the expression of fatty acid β-oxidation–related genes. The expression level of ACSL1 gene increased in a PA dose- and time-dependent manner (Figure 5A, B). Also, the similar results were found in the expression of CPT1, CACT, and HADHB genes (Figure 5C-H). These data indicate that PA can induce the expression of some fatty acid β-oxidation–related genes. Next, we investigated the function of PKCζ in regulating mRNA expression of fatty acid β-oxidation–related genes. Increased ACSL1 expression in response to PA treatment was suppressed in PA-stimulated PKCζ siRNA-treated cells compared to nonspecific siRNA-treated cells, showing that ACSL1 expression is regulated by PKCζ (Figure 5I). Similar phenomena were found in the expression of CPT1, CACT, and HADHB genes (Figure 5J). Therefore, we conclude that PKCζ has an important role in regulating the expression of PA-induced fatty acid β-oxidation–related genes.

**PKCζ-Regulated SIRT6 Phosphorylation on Thr294 Promotes SIRT6 Binding to the Promoters of Fatty Acid β-Oxidation-Related Genes**

We finally assessed the mechanism underlying how PKCζ regulate the expression of fatty acid β-oxidation–related genes. In our study, we found that PKCζ is a response for SIRT6 enrichment on chromatin after PA treatment through SIRT6 phosphorylation at Thr294 residue. So, we firstly explored the role of SIRT6 on the expression of fatty acid β-oxidation–related genes. Increased ACSL1, CPT1, CACT, and HADHB gene expression in response to PA treatment was effectively suppressed in PA-stimulated SIRT6 siRNA-treated cells compared to nonspecific siRNA-treated cells, showing that ACSL1, CPT1, CACT, and HADHB expression was also regulated by SIRT6 (Figure 6A). Also, we transfected wild-type and T294A mutant SIRT6 plasmids into SIRT6 KO cells and then treated cells with or without PA at 0.2 mM for 18 hours, mRNA was extracted, and real-time PCR was performed to detect the expression of fatty acid β-oxidation–related genes. The gene expression of ACSL1, CPT1, CACT, and HADHB was increased significantly after PA treatment in wild-type SIRT6-transfected HCT116 cells, yet the expression of these genes was not increased any more in T294A mutant SIRT6-transfected SIRT6 KO cells, indicating that phosphorylation of SIRT6 at Thr294 residue is a key point in regulating PA-induced fatty acid β-oxidation–related gene expression and PKCζ might mediate the expression of these genes through SIRT6 phosphorylation (Figure 6B). To further explore the mechanism, we found that PA-stimulated SIRT6 binding to the ACSL1 promoter was significantly blocked in PKCζ siRNA-treated cells compared to nonspecific siRNA-treated HCT116 cells (Figure 6C). We then explored whether the phosphorylation of SIRT6, by PKCζ at Thr294 residue, may impact the binding activity of SIRT6 to the promoter of ACSL1. We found that the binding activity of SIRT6 to the promoter of ACSL1 was almost completely blocked in the T294A mutant SIRT6-transfected cells compared with the wild-type SIRT6-transfected cells after PA stimulation (Figure 6D). Similar results were found for the CPT1, CACT, and HADHB promoters (Figure 6E-J). These data indicate that SIRT6 does not drive ACSL1, CPT1, CACT, and HADHB genes expression when PKCζ is absent. Instead, in the presence of PKCζ, PKCζ-regulated SIRT6 phosphorylation on Thr294 promotes SIRT6 binding to the promoters of fatty acid β-oxidation–related genes to further mediate fatty acid β-oxidation.

**Discussion**

The present study has identified PKCζ as a new regulator of fatty acid β-oxidation through SIRT6 phosphorylation, thus adding a new layer of knowledge to PKCζ biology. In light of our data, we present a model to hypothesize how PKCζ regulates the expression of fatty acid β-oxidation (Figure 7). In response to PA treatment, PKCζ physically interacts with SIRT6 and phosphorylates SIRT6 at Thr294 residue. The phosphorylated SIRT6 binds to the promoters of fatty acid β-oxidation–related genes to activate the expression of these genes and regulates fatty acid β-oxidation. Our data demonstrate the mechanism of PKCζ on mediating fatty acid β-oxidation through SIRT6 phosphorylation. As we know, this is the first paper to define the role of PKCζ on fatty acid β-oxidation, and it will be beneficial for designing new therapeutic target in regulating lipid homeostasis.

The phenomenon of SIRT6 phosphorylation was first reported by Thirumurthi et al. They found that SIRT6 was phosphorylated at Ser (338) by the kinase AKT1, which induced the interaction and ubiquitination of SIRT6 by MDM2, targeting SIRT6 for protease-dependent degradation in various breast cancer cell lines [38]. Also, the survival of breast cancer patients was positively correlated with the abundance of SIRT6 and inversely correlated with the phosphorylation of SIRT6 at Ser (338) [38]. Furthermore, two recent studies showed that SIRT6 can phosphorylate by other phosphorylases. C-Jun N-terminal kinase was reported to
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β-SIRT6 binds to the promoters of fatty acid and phosphorylates SIRT6 at Thr294 residue. The phosphorylated genes to activate the expression of these genes and regulates fatty acid β-oxidation.

PKCζ phosphorylation assays were performed and showed that PKCζ was significantly enhanced in response to PA treatment (Figure 2). To interact with SIRT6 directly in vitro regulated SIRT6 phosphorylation on Thr294 promotes SIRT6 binding to the promoters of fatty acid β-oxidation. (A)

Enhanced after PA treatment in colon cancer cells in our study (Figure 3A). To identify the phosphorylation of SIRT6 after PA treatment, several phosphorylases were detected, and we found that PKCζ mediates phosphorylation of SIRT6 might be involved in the progression of breast carcinoma and predicted shorter survival of the diagnosed patients [40]. Similar with these studies, the semixogenous and endogenous phosphorylation of SIRT6 was found to be dramatically enhanced after PA treatment in colon cancer cells in our study (Figure 1). To identify the phosphorylation of SIRT6 after PA treatment, several phosphorylases were detected, and we found that PKCζ can interact with SIRT6 directly in vitro and in vivo and the interaction was significantly enhanced in response to PA treatment (Figure 2).

To further confirm the effect of PKCζ on SIRT6, in vitro phosphorylation assays were performed and showed that PKCζ is the phosphorylation of SIRT6 in response to PA treatment (Figure 3F). Also, threonine 294 residue was found to be the phosphorylation site of SIRT6 in vitro and in vivo (Figure 4A-C). These data indicate that PKCζ is a phosphorylation of SIRT6 and phosphorylates SIRT6 on threonine 294 residue after PA treatment.

Furthermore, we explored the effect of PKCζ on SIRT6 phosphorylation. Accumulating data showed that aPKC isoforms are involved in regulating lipid metabolism [41–47]. The aPKC activity was reported to play a dominant role in normal insulin signaling by activating PI3K activity and contributing to insulin-stimulated lipogenesis in the liver [42,43]. The activity of PKCζ stimulated by insulin was reduced 57% in obese and 65% in diabetic subjects. Importantly, weight loss in obese subjects normalized PKCζ activity and simultaneously increased PI3K activity, indicating the effect of PKCζ on obese and type 2 diabetic subjects [44]. In obesity, the critical role of aPKC in activating hepatic SREBP-1c and NF-κB, which are the major regulators of hepatic lipid synthesis and systemic insulin resistance, was shown in the fed state. Conserved hepatic aPKC-dependent activation of SREBP-1c and NF-κB contributed to hepatic lipogenesis, as well as the development of hyperlipidemia, and systemic insulin resistance [45]. Also, other studies reported a similar mechanism that aPKC mediated SREBP-1c to promote lipogenesis [43,46]. In addition, PKCζ was suggested to involve in mitogenic factor-stimulated preadipocytes proliferation and insulin-stimulated preadipocytes differentiation through the rapid increase of PKCζ in the cytosolic compartment and the translocation change into the nucleus [47]. In our study, we found that enrichment of SIRT6 on chromatin was blocked after PA stimulation in PKCζ siRNA-treated cells (Figure 4E). Also, the enrichment of SIRT6 on chromatin was significantly reduced in the Flag-SIRT6 T294A mutant transfected cells compared with Flag-SIRT6 WT transfected cells after PA stimulation, showing that PKCζ is a response for the SIRT6 enrichment on chromatin after PA treatment through SIRT6 phosphorylation at Thr 294 residues (Figure 4F). Phosphorylation can have diverse consequences on a protein, such as regulating its enzymatic activity or subcellular localization [48]. Our data showed that SIRT6 phosphorylation at Thr 294 residues is required for its enrichment on chromatin.

To further functional study, we found that the mRNA levels of fatty acid β-oxidation–related genes such as ACSL1, CPT1, CACT, and HADHB were increased after PA treatment and regulated by PKCζ (Figure 5). The SIRT6 RNAi and T294A mutant SIRT6 transfected study also showed that phosphorylation SIRT6 at Thr 294 residue is a key point on the regulation of PA-induced gene expression of fatty acid β-oxidation–related genes (Figure 6A, B). In further study of the mechanism, we found that the binding of SIRT6 to the ACSL1, CPT1, CACT, and HADHB promoters was increased after PA treatment and regulated by PKCζ through SIRT6 phosphorylation (Figure 6C–F). Recently, Khan et al. showed that SIRT6 transcriptionally regulated the expression of pyruvate dehydrogenase kinase 4 by binding to its promoter to further mediate glucose metabolism in heart [49]. SIRT6 transcriptional activation is less reported, but one study has shown that SIRT6 can interact with and recruit RNAP II to coactivate nuclear factor erythroid 2-related factor

Figure 7. A schematic showing a possible mechanism by which PKCζ regulates the expression of fatty acid β-oxidation–related genes. In the presence of PA, PKCζ physically interacts with SIRT6 and phosphorylates SIRT6 at Thr294 residue. The phosphorylated SIRT6 binds to the promoters of fatty acid β-oxidation–related genes to activate the expression of these genes and regulates fatty acid β-oxidation.

Figure 6. PKCζ regulated SIRT6 phosphorylation on Thr294 promotes SIRT6 binding to the promoters of fatty acid β-oxidation–related genes. (A) HCT116 cells were transfected with SIRT6 siRNA or nonspecific siRNA for 24 hours and then treated with or without PA (0.2 mM) for 18 hours. The mRNA expression of ACSL1, CPT1, CACT, and HADHB was analyzed by real-time PCR. mRNA levels of the control sample were set as 1, and relative mRNA levels of the other samples were normalized to this control. The data represent as the means ± SD. *P < .05, **P < .01. (B) SIRT6 KO HCT116 cells were transfected with Flag-SIRT6 WT or Flag-SIRT6 T294A mutant plasmid for 24 hours and then treated with or without PA (0.2 mM) for 18 hours. The mRNA expression of ACSL1, CPT1, CACT, and HADHB was analyzed by real-time PCR. (C, E, G, and I) HCT116 cells were transfected with PKCζ siRNA or nonspecific siRNA for 24 hours and then treated with or without PA (0.2 mM) for 18 hours. ChIP assay was performed to detect enrichment of SIRT6 at the (C) ACSL1, (E) CPT1, (G) CACT, and (I) HADHB promoters, respectively. The bands containing anti-immunoglobulin G (IgG) served as negative controls; (D, F, H, and J) SIRT6 KO HCT116 cells were transfected with Flag-SIRT6 WT or Flag-SIRT6 T294A mutant plasmid for 24 hours and then treated with or without PA (0.2 mM) for 18 hours. ChIP assay was performed to detect the enrichment of SIRT6 at the (D) ACSL1, (F) CPT1, (H) CACT, and (J) HADHB promoters, respectively. The bands containing anti-IgG served as negative controls. The data represent the means ± SD. *P < .05, **P < .01.
2 in human mesenchymal stem cells [50]. It needs to be further explored whether SIRT6 can recruit certain activator to the promoters of fatty acid β-oxidation–related genes to regulate fatty acid β-oxidation.

Taken together, we have identified a novel function of PKCζ on fatty acid β-oxidation. We found that PKCζ physically interacts with SIRT6 in vitro and in vivo, and phosphorylates SIRT6 at Thr294 residue after PA treatment. PKCζ mediated SIRT6 phosphorylation could recruit SIRT6 to the promoters of fatty acid β-oxidation–related genes and further regulated the expression of these genes. Understanding the new role of PKCζ on fatty acid β-oxidation will be useful for the future design of effective therapeutic targets to help regulate lipid homeostasis or treat metabolic diseases.

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