Protein Kinase C θ (PKCθ)-dependent Phosphorylation of PDK1 at Ser\textsuperscript{504} and Ser\textsuperscript{532} Contributes to Palmitate-induced Insulin Resistance*

Received for publication, August 15, 2008, and in revised form, November 21, 2008. Published, JBC Papers in Press, December 1, 2008, DOI 10.1074/jbc.M806336200

Changhua Wang\textsuperscript{1}, Meilian Liu\textsuperscript{1}, Ramon A. Rojas\textsuperscript{3}, Xiaoban Xin\textsuperscript{1}, Zhanguo Gao\textsuperscript{1}, Rong Zeng\textsuperscript{2,11}, Jiarui Wu\textsuperscript{2,11}, Lily Q. Dong\textsuperscript{1,2,11}, and Feng Liu\textsuperscript{1,3,4,11}

From the Departments of \textsuperscript{1}Pharmacology, \textsuperscript{2}Biochemistry, and \textsuperscript{3}Cellular and Structural Biology and the \textsuperscript{4}Barshop Center for Longevity and Aging Studies, University of Texas Health Science Center, San Antonio, Texas 78229, the \textsuperscript{5}Pennington Biomedical Research Center, Louisiana State University, Baton Rouge, Louisiana, 70808, and the \textsuperscript{6}Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Science, Beijing 100864

Clinical, epidemiological, and biochemical studies have highlighted the role of obesity-induced insulin resistance in various metabolic diseases. However, the underlying molecular mechanisms remain to be established. In the present study, we show that palmitate-induced serine phosphorylation of phosphoinositide-dependent protein kinase-1 (PDK1) negatively regulates insulin signaling. PDK1-mediated Akt phosphorylation at Thr\textsuperscript{308} in the activation loop is reduced in C2C12 myotubes treated with palmitate or overexpressing Ser\textsuperscript{504} and Ser\textsuperscript{532} of PDK1 with alanine disrupted PKC-phosphorylation, suggesting that the inhibition could occur at a site independent of IRS1/2. The inhibitory effect of palmitate on PDK1 and Akt was diminished in PKC-deficient mouse embryonic fibroblasts (MEFs) by treating C2C12 myotubes with PKC\textsuperscript{0} pseudosubstrates. In vivo labeling studies revealed that PDK1 undergoes palmitate-induced phosphorylation at two novel sites, Ser\textsuperscript{504} and Ser\textsuperscript{532}. Replacing Ser\textsuperscript{504} and Ser\textsuperscript{532} with alanine disrupted PKC\textsuperscript{0}-catalyzed PDK1 phosphorylation in vitro and palmitate-induced PDK1 phosphorylation in cells. PDK1-deficient MEFs transiently expressing PDK1\textsuperscript{S504A/S532A} but not PDK1\textsuperscript{S504E/S532D} showed increased basal and insulin-stimulated Akt phosphorylation at Thr\textsuperscript{308} when compared with MEFs expressing wild-type PDK1. Taken together, our results identify PDK1 as a novel target in free fatty acid-induced insulin resistance and PKC\textsuperscript{0} as the kinase mediating the negative regulation.

A reduced capacity for insulin to stimulate glucose uptake and metabolism in target tissues such as skeletal muscle and adipose tissues is a common feature of obesity and diabetes.

Chronic elevation of free fatty acid (FFA\textsuperscript{2}) levels in plasma has been found to be closely associated with impaired insulin-mediated glucose uptake (1, 2) and often coexists with obesity and type 2 diabetes (3). However, although the association between hyperlipidemia and insulin resistance is well established, the molecular mechanisms remain to be established.

Insulin regulates circulating blood glucose levels and whole body energy homeostasis by interacting with its membrane receptor in cells of target tissues such as liver, skeletal muscle, and adipose. The binding of insulin to its receptor activates the phosphatidylinositol (PI) 3-kinase signaling pathway, which is essential for insulin-mediated biological events such as glucose uptake in insulin target cells. Components of the PI 3-kinase pathway include members of the protein kinase A/protein kinase G/protein kinase C family such as protein kinase B (PKB/Akt) and some protein kinase C (PKC) isoforms. Activation of these kinases requires phosphorylation in their activation loop, which is mediated by 3’ phosphoinositide-dependent kinase-1 (PDK1). Thus, PDK1 functions as a master kinase to regulate various downstream biological events such as insulin-stimulated glucose uptake and protein synthesis.

PDK1 is a 63-kDa Ser/Thr kinase with a catalytic domain near its N terminus and a pleckstrin homology domain at its C terminus. The pleckstrin homology domain is necessary for targeting PDK1 to the plasma membrane to interact with and phosphorylate its substrates such as Akt (4, 5). We and others have shown that autophosphorylation of PDK1 in the activation loop (Ser\textsuperscript{241}/Ser\textsuperscript{244}) for human and mouse PDK1, respectively) is essential for PDK1 activity (6, 7). In addition, we found that phosphorylation of endogenous PDK1 at this site could be stimulated by insulin in certain cell types (8), suggesting that the activity and function of endogenous PDK1 is regulated.

Recent studies suggest that PDK1 may be subject to phosphorylation-mediated negative regulation. Kondo and Kahn (8) showed that insulin-stimulated PDK1 phosphorylation at

\textsuperscript{8} This study was supported by a research award from the American Diabetes Association (to F. L.) and Grant 0355076Y from the American Heart Association, Texas Affiliate (to L. Q. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

\textsuperscript{1} To whom correspondence should be addressed: Dept. of Pharmacology, University of Texas Health Science Center, 7703 Floyd Curl Dr., San Antonio, TX 78229; E-mail: liuf@uthscsa.edu.

\textsuperscript{2} The abbreviations used are: FFA, free fatty acid; MEFs, mouse embryonic fibroblasts; PDK1, phosphoinositide-dependent protein kinase-1; PKC, protein kinase C; IRS, insulin receptor substrate; PDGF, platelet-derived growth factor; PI, phosphatidylinositol; JNK, c-Jun NH\textsubscript{2}-terminal kinase; YFP, yellow fluorescent protein; CHO, Chinese hamster ovary; IR, insulin receptor; DMEM, Dulbecco’s modified Eagle’s medium; BSA, bovine serum albumin.
Ser\textsuperscript{244} in the activation loop is greatly reduced in the liver of ob/ob mice. Reduced PDK1 phosphorylation in the activation loop was also found in cells treated with the proinflammatory cytokine tumor necrosis factor \( \alpha \) (9). These findings suggest a novel mechanism by which hyperlipidemia induces insulin resistance in vivo. However, how obesity leads to down-regulation of PDK1 activity and function remains unknown. Obesity has been shown to activate several serine kinases such as the c-Jun N-terminal kinase (JNK) (10, 11), inhibitor \( \kappa \)B kinase (12), and PKC\( \theta \) (13, 14) that act on IRS-1. Thus, the reduced PDK1 activity could be due to reduced tyrosine phosphorylation of IRS-1 and its downstream events. On the other hand, hyperlipidemia-induced negative regulation may target directly at PDK1.

In the present study, we investigated the molecular mechanism underlying FFA-induced insulin resistance. Our data show that PDK1 undergoes palmitate stimulation and PKC\( \theta \)-dependent phosphorylation at Ser\textsuperscript{504} and Ser\textsuperscript{532}. Replacing Ser\textsuperscript{504/S32} with alanine alleviates the inhibitory effect of palmitate on Akt phosphorylation, suggesting that phosphorylation of PDK1 at this site plays a negative role in PDK1 activity and function. The identification of PDK1 as a novel target in FFA-induced inhibition of insulin signaling should provide a better understanding of the molecular mechanism of insulin resistance, which is one of the major causes of type 2 diabetes.

MATERIALS AND METHODS

Plasmids and Reagents—Biochemical reagents were obtained from the following sources: anti-Myc monoclonal antibody, Covance; glutathione-Sepharose beads, insulin, and palmitic acid, Sigma; recombinant PKC isoforms, G\textsuperscript{O}6983, SP600125, and PKC\( \theta \) and PKC\( \varepsilon \) pseudosubstrates, Calbiochem; secondary antibodies conjugated to alkaline phosphatase and horseradish peroxidase, Promega; protein G-Sepharose beads, Amersham Biosciences; C\textsubscript{18} ZipTips, Millipore; L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin, Worthington Biochemical Corp.; and PDGF, antibodies, and phosphaetides to PDK1 and Akt, Cell Signaling Technology. Mammalian expression vectors encoding Myc-tagged mPDK1\textsuperscript{K114G} and PDK1\textsuperscript{K114G/S244A} were described previously (8). [\( \gamma \)-\textsuperscript{32}P]ATP was purchased from PerkinElmer Life Sciences. pcDNA plasmids encoding PDK1 point mutants and different regions of PDK1\textsuperscript{S244A} fused to YFP (YFP-PDK1\textsuperscript{S244A}, YFP-PDK1\textsuperscript{S244A/ΔC1(1–401)}, and YFP-PDK1\textsuperscript{S244A/ΔC2(1–200)}) were generated by standard molecular cloning techniques.

Preparing Palmitate Solution—BSA-bound palmitate was made according to the procedure as described previously with some modifications (13, 15). In brief, palmitate was dissolved in ethanol to a concentration of 0.75 \( \text{mM} \), which was then diluted with 20% FFA-free BSA to a working concentration of 7.5 \( \text{mM} \) (the molar ratio of palmitate to BSA is \( \approx 2.5 \)). This stock solution was filtered and stored at \(-20^\circ \text{C} \) and was used within 2 weeks. The same concentration of ethanol mixed with 20% of BSA was used as control.

PKC\( \theta \) Knock-out Mice (PKC\( \theta \)-/-)—PKC\( \theta \)-/- mice in C57BL/6 background were a generous gift of Dr. Jianping Ye (Pennington Biomedical Research Center) and were described previously (16).

Cell Culture, Immunoprecipitation, and Western Blot—Chinese hamster ovary cells stably expressing the insulin receptor (CHO/IR) cells were grown in Ham’s F-12 medium (Invitrogen) supplemented with 10% newborn calf serum and 1% penicillin/streptomycin. C212 cells (ATCC) were cultured in DMEM (ATCC catalog number 30-2002) supplemented with 10% newborn calf serum and 1% penicillin/streptomycin. Differentiation of C212 was performed by incubating cells in fresh medium containing DMEM, 1% penicillin/streptomycin, 0.1% fetal bovine serum, and 50 \( \text{nM} \) insulin for 4 days. Differentiation was observed when 80–100% of the cells formed myotubes. Wild-type and PDK1-deficient mouse embryonic fibroblasts (MEFs) (gifts of Dr. Wataru Ogawa, Kobe University, Kobe, Japan) were cultured in DMEM (Invitrogen) supplemented with 10% newborn calf serum and 1% penicillin/streptomycin. The PKC\( \theta \)-deficient MEFs were prepared from 13-day embryos of PKC\( \theta \)-/- knock-out mice (16). The embryo carcasses were minced and digested with trypsin after removal of the limbs, internal organs, and brain. After digestion at 37 \(^\circ \text{C} \) for 10 min, the cell suspension was collected as MEFs and washed with DMEM supplemented with 10% newborn calf serum. The cells were plated in a 100-\( \text{mm} \) cell culture plate in serum-containing medium, and the medium was changed 24 h later. After one passage, the cells were collected as MEFs. Transfections were performed using Lipofectamine reagent according to the manufacturer’s protocol (Invitrogen). For immunoprecipitation, cells were lysed in 300 \( \mu \text{l} \) of buffer A (50 \( \text{mM} \) Hepes, pH 7.6, 150 \( \text{mM} \) NaCl, 1% Triton X-100, 10 \( \mu \text{M} \) NaF, 20 \( \mu \text{M} \) sodium pyrophosphate, 20 \( \mu \text{M} \) \( \beta \)-glycerol phosphate, 1 \( \mu \text{M} \) sodium orthovanadate, 10 \( \mu \text{g} \)/ml leupeptin, 10 \( \mu \text{g} \)/ml aprotinin, and 1

FIGURE 1. Palmitate (PA) inhibits PDK1 and Akt phosphorylation in the activation loop. Serum-starved C212 myotubes were treated with or without 0.75 \( \text{mM} \) palmitate for the indicated times and then with or without insulin (10 \( \mu \text{M} \)) for 10 min. Phosphorylation of endogenous Akt (A) or PDK1 (B) was determined by Western blot using a phosphospecific antibody to Akt\textsuperscript{Thr308} (P-Akt\textsuperscript{Thr308}) or PDK1\textsuperscript{S244A} (P-PDK1\textsuperscript{S244A}), respectively. Results are representative of three independent experiments with similar findings.
Negative Regulation of PDK1 by Serine Phosphorylation

In Vitro Phosphorylation of PDK1 by PKCα—Wild-type PDK1 and its mutants were purified by immunoprecipitation from CHO/IR cells transiently expressing these proteins. Immunoprecipitated PDK1 proteins were incubated in 30 μl of buffer C (50 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 1 mM Na₃VO₄, 1 mM sodium pyrophosphate, 1 mM NaF, and 1 mM phenylmethylsulfonyl fluoride) containing purified PKCα and 2 μCi of [γ-³²P]ATP. In vitro phosphorylation reaction was carried out for 30 min at 30 °C, and in vitro phosphorylated proteins were separated by SDS-PAGE and visualized by autoradiography.

In Vivo ³²P Labeling, Phosphoamino Acid Analysis, and Phosphopeptide Mapping of PDK1—The experiments were carried out as described in our previous studies (17–19).

2-Deoxyglucose Uptake—Glucose uptake assays were performed as described previously (20).

Statistical Analysis—Quantification of the relative increase in insulin-stimulated protein phosphorylation was performed by analyzing Western blots using the NIH Scion Image software and was normalized with the amount of protein expression in each experiment. Results are expressed as the mean ± S.E. Differences between the groups were examined for statistical significance using analysis of variance.

RESULTS

PKCα Mediates the Inhibitory Effect of Palmitate—Serine-phosphorylated PDK1 and Akt in the Activation Loop

To study the molecular mechanism underlying hyperlipidemia-induced insulin resistance, we investigated PDK1 phosphorylation in C2C12 myotubes treated with or without palmitate, which has been shown to cause insulin resistance in these cells (13). Insulin greatly stimulated Akt phosphorylation at Thr³⁰⁸, a site targeted by PKD1 (Fig. 1A). The insulin-stimulated Akt phosphorylation in C2C12 myotubes was greatly inhibited by palmitate treatment in a time-dependent manner (Fig. 1A). Palmitate treatment also markedly inhibited PDK1 phosphorylation at Ser²⁴⁴ in the activation loop (Fig. 1B), which is consistent with previous findings that insulin-stimulated PDK1

mm phenylmethylsulfonyl fluoride). Cell lysates were centrifuged at 14,000 g for 10 min at 4 °C, and the supernatants were incubated with specific antibodies bound to protein G beads overnight at 4 °C. After incubation, immunoprecipitates were washed extensively with ice-cold buffer B (50 mM Heps, pH 7.6, 150 mM NaCl, and 0.1% Triton X-100). Proteins bound to the beads were eluted by heating at 95 °C for 4 min in SDS-PAGE sample loading buffer. The eluted proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and detected with specific antibodies.
phosphorylation at Ser\textsuperscript{244} is greatly reduced in the liver of ob/ob mice (8).

_Palmitate Inhibits Akt Phosphorylation Independent of IRS1/2—_FFAs have been shown to inhibit insulin-stimulated IRS-1 tyrosine phosphorylation, leading to reduced PI 3-kinase signaling (10, 12, 14). To test whether palmitate could inhibit PI 3-kinase signaling in an IRS-1/2-independent manner, we examined the effect of palmitate on PDGF-stimulated phosphorylation of Akt and PDK1. Unlike insulin, PDGF activates PI 3-kinase by promoting the interaction between PDGF receptor and p85 subunit of PI 3-kinase, independent of IRS1/2 (21). Treatment of C2C12 myotubes with either insulin or PDGF led to a marked increase in Akt phosphorylation at Thr\textsuperscript{308}, and this phosphorylation was markedly inhibited by palmitate (Fig. 2, A and B). These results suggest that palmitate-induced inhibition of Akt could occur at a step(s) other than IRS-1/2 along the PI 3-kinase signaling pathway. Consistent with the idea that PDK1 may be the site of negative regulation, palmitate treatment inhibited PDGF-stimulated PDK1 phosphorylation at Ser\textsuperscript{244} (Fig. 2, A and B).

_Palmitate-induced PDK1 Phosphorylation Is Mediated by PKC\textsuperscript{\theta}—_Activation of PKC\textsuperscript{\theta} has been implicated in obesity-induced insulin resistance (22, 23). To determine whether PKC\textsuperscript{\theta} is involved in palmitate-induced PDK1 phosphorylation, we examined the effect of palmitate on insulin-stimulated phosphorylation of PDK1 at Ser\textsuperscript{244} in wild-type and PKC\textsuperscript{\theta} \textsuperscript{-/-} MEFs. Insulin stimulated PDK1 phosphorylation at Ser\textsuperscript{244} and Akt phosphorylation at Thr\textsuperscript{308} in wild-type MEFs, and these phosphorylation events were markedly inhibited by palmitate (Fig. 2C). The basal and insulin-stimulated PDK1 Ser\textsuperscript{244} phosphorylation was greatly increased in the PKC\textsuperscript{\theta}-deficient MEFs, suggesting a negative role of PKC\textsuperscript{\theta} in PDK1 activity. Insulin-stimulated Akt phosphorylation at Thr\textsuperscript{308} was also greatly increased in the PKC\textsuperscript{\theta}-deficient MEFs, and knocking out PKC\textsuperscript{\theta} disrupted the inhibitory effect of palmitate on insulin-stimulated PDK1 and Akt phosphorylation in the activation loop (Fig. 2C). Consistent with a negative role of PKC\textsuperscript{\theta} in PDK1 activation, Akt phosphorylation of p85 subunit of PI 3-kinase was markedly inhibited by palmitate (Fig. 2D), and this phosphorylation was markedly inhibited by palmitate (Fig. 2, A and B). These results suggest that palmitate-induced inhibition of Akt could occur at a step(s) other than IRS-1/2 along the PI 3-kinase signaling pathway. Consistent with the idea that PDK1 may be the site of negative regulation, palmitate treatment inhibited PDGF-stimulated PDK1 phosphorylation at Ser\textsuperscript{244} (Fig. 2, A and B).

Identification of Ser\textsuperscript{260} and Ser\textsuperscript{232} as the PKC\textsuperscript{\theta}-stimulated Phosphorylation Site in PDK1—_To map the palmitate-stimulated phosphorylation site of PDK1, we generated plasmids encoding full-length and truncation mutants of PDK1 fused to YFP (Fig. 4A). In vivo labeling experiments revealed that deletion of the C terminus of PDK1 (amino acids 402–559) greatly reduced palmitate-stimulated PDK1 phosphorylation (Fig. 4B), suggesting that the phosphorylation site(s) is/are localized at or near the C

![Figure 3. PCK\textsuperscript{\theta} phosphorylates PDK1 at a novel site(s). A, C2C12 myoblasts overexpressing Myc-tagged PDK1\textsuperscript{K114G/S244A} were in vivo labeled with \textsuperscript{32P}orthophosphate. Cells were treated with or without indicated inhibitors for 1 h and then with or without 0.75 mM palmitate (PA) for 18 h. PDK1 was immunoprecipitated with an antibody to the Myc tag, and its phosphorylation was detected by autoradiograph (upper panel). The expression of PDK1 was determined by Western blot using an antibody to the Myc tag (lower panel). Pseudo., pseudosubstrates; P-PDK1, phosphorylated PDK1. B, Myc-tagged PDK1\textsuperscript{K114G} or PDK1\textsuperscript{K114G/S244A} were transiently expressed in CHO/IR cells and purified by immunoprecipitation using an antibody to the Myc tag. The purified PDK1 proteins were incubated with \textsuperscript{32P} and treated with palmitate in the presence of several protein kinase inhibitors. As shown in Fig. 3A, treating cells with palmitate greatly increased PDK1 phosphorylation. The palmitate-induced PDK1\textsuperscript{K114G/S244A} phosphorylation was suppressed by the PKC\textsuperscript{\theta} pseudosubstrate or the broad spectrum PKC inhibitor G\textsubscript{6983}, but not by the PKCe pseudosubstrate or the JNK inhibitor SP\textsubscript{600125}. To determine whether PKC\textsuperscript{\theta} is able to directly phosphorylate PDK1, we carried out an in vitro kinase assay using purified PDK1 mutants and PKC\textsuperscript{\theta}. We found that both PDK1\textsuperscript{K114G} and PDK1\textsuperscript{K114G/S244A} mutants were directly phosphorylated by PKC\textsuperscript{\theta} (Fig. 3B). These results reveal that PKC\textsuperscript{\theta} is a direct target of PKC\textsuperscript{\theta} and that the phosphorylation occurred at a site independent of Ser\textsuperscript{244} in PDK1.

To determine whether PKC\textsuperscript{\theta} negatively regulates PDK1/Akt by directly phosphorylating PDK1, we generated a double mutant of PDK1 in which the critical ATP binding site (Lys\textsuperscript{114}) and the auto-phosphorylation site (Ser\textsuperscript{244}) were mutated to glycine and alanine, respectively (PDK1\textsuperscript{K114G/S244A}). PDK1\textsuperscript{K114G/S244A} is not auto-phosphorylated in cells, which facilitates our study of heterogeneous kinase-mediated phosphorylation of PDK1. C2C12 myoblasts transiently expressing PDK1\textsuperscript{K114G/S244A} were in vivo labeled with \textsuperscript{32P} and treated with palmitate in the presence of several protein kinase inhibitors. As shown in Fig. 3A, treating cells with palmitate greatly increased PDK1 phosphorylation. The palmitate-induced PDK1\textsuperscript{K114G/S244A} phosphorylation was suppressed by the PKC\textsuperscript{\theta} pseudosubstrate or the broad spectrum PKC inhibitor G\textsubscript{6983}, but not by the PKCe pseudosubstrate or the JNK inhibitor SP\textsubscript{600125}. To determine whether PKC\textsuperscript{\theta} is able to directly phosphorylate PDK1, we carried out an in vitro kinase assay using purified PDK1 mutants and PKC\textsuperscript{\theta}. We found that both PDK1\textsuperscript{K114G} and PDK1\textsuperscript{K114G/S244A} mutants were directly phosphorylated by PKC\textsuperscript{\theta} (Fig. 3B). These results reveal that PKC\textsuperscript{\theta} is a direct target of PKC\textsuperscript{\theta} and that the phosphorylation occurred at a site independent of Ser\textsuperscript{244} in PDK1.
Negative Regulation of PDK1 by Serine Phosphorylation

To determine whether PKCθ directly phosphorylates PDK1 at the C terminus, we performed in vitro kinase assays using autophosphorylation-defective (S244A) full-length and truncation mutants of PDK1 at the C terminus. We found that deletion of the C terminus of PDK1 greatly reduced PKCθ-catalyzed PDK1 phosphorylation in vitro (Fig. 4C), confirming that the PKCθ-mediated phosphorylation sites are located at the C terminus.

To determine the nature of palmitate-stimulated PDK1 phosphorylation, we analyzed in vivo labeled PDK1S244A by phosphoamino acid analysis. We found that palmitate-stimulated PDK1S244A phosphorylation occurred almost exclusively at serine residues (Fig. 4D). There are three serine residues at the C terminus of mouse PDK1, including Ser504, Ser532, and Ser552 (17). To map the site(s) of phosphorylation, we generated PDK1K114G/S244A/S504A, PDK1K114G/S244A/S532A, and PDK1K114G/S244A/S552A triple mutants. In vivo labeling experiments showed that replacing Ser504 with alanine had no significant effect on palmitate-stimulated PDK1 phosphorylation (data not shown). However, replacing Ser504 or Ser532 with alanine greatly diminished palmitate-induced PDK1 phosphorylation (Fig. 4E). In vitro kinase assays showed that PKCθ efficiently phosphorylated PDK1K114G/S244A and that the phosphorylation was greatly reduced when Ser504 or Ser532 was mutated to alanine (Fig. 4F), further confirming that Ser504 and Ser532 are the major PKCθ-mediated phosphorylation sites on PDK1.

Phosphorylation at Ser532/Ser504 Negatively Regulates the Ability of PDK1 to Phosphorylate Akt—To investigate the functional role of PDK1 phosphorylation, we replaced Ser504 and Ser532 of PDK1 with negatively charged amino acid residues (PDK1K114G/S504E/S532D) to mimic phosphorylation. We also mutated Ser504 and Ser532 of PDK1 to alanine (PDK1K114G/S504A/S532A) to disrupt the potential phosphorylation sites. To avoid the potential masking effect of endogenous PDK1 on PDK1K114G/S504E/S532D and PDK1K114G/S504A/S532A, wild type or mutants of PDK1 were transiently expressed in PDK1-deficient MEFs that are defective in insulin-stimulated Akt phosphorylation (25). Cells expressing wild type or mutants of PDK1 were serum-starved and treated with palmitate followed by insulin. As shown in Fig. 5, A and B, overexpression of wild-type PDK1 rescued insulin-stimulated Akt phosphorylation in the PDK1−/− MEFs, and the stimulatory effect of insulin was inhibited by palmitate treatment. The stimulatory effect of insulin was notably increased in cells expressing PDK1K504A/S532A, probably due to removal of a feedback inhibition caused by Ser504/S532 phosphorylation. However, the insulin-stimulated Akt phosphorylation was still partially suppressed by palmitate, suggesting that in addition to negative regulation of PDK1, palmitate could induce insulin resistance by targeting other step(s) along the PI 3-kinase signaling pathway.

The stimulatory effect of insulin on Akt phosphorylation was reduced in PDK1K504E/S532D, further confirming that the negatively charged amino acid residues mimics palmitate-induced phosphorylation to negatively regulate PDK1 function.
Negative Regulation of PDK1 by Serine Phosphorylation

The role of obesity in insulin resistance has been well recognized, but the underlying molecular mechanisms remain to be fully elucidated. Obesity has been shown to activate several serine kinases such as the JNK (10, 11), inhibitor kB kinase (12), and PKCθ (13, 14) that negatively regulates insulin signaling by serine phosphorylation of IRS-1/2. However, it is unclear whether hyperlipidemia-induced insulin resistance could occur at other sites along the insulin-signaling pathway.

In the present study, we show that palmitate inhibited both insulin-stimulated and PDGF-stimulated PDK1 and Akt phosphorylation in the activation loop (Fig. 2A). Because PDGF activates Akt independent of IRS-1/2 (21), these results suggest that FFA-activated protein kinases could target components in the PI 3-kinase signaling pathway other than IRS-1/2. In agreement with this view, we found that PDK1 undergoes palmitate-stimulated and PKCθ-dependent phosphorylation at Ser504 and Ser532 in C2C12 cells, and replacing these residues with alanine improves PDK1 kinase activity toward Akt in intact cells (Fig. 5). These results provide evidence for the first time that FFA could induce insulin resistance by targeting multiple components downstream of the insulin receptor. Our result is consistent with previous findings that insulin-stimulated PDK1 phosphorylation at Ser544 is greatly reduced in the liver of ob/ob mice (8) and in cells treated with tumor necrosis factor α (9). Thus, serine phosphorylation of PDK1 at Ser504 and Ser532 may provide a mechanism underlying obesity-induced insulin resistance in vivo.

Our results showed that treating cells with inhibitors of PKCε or JNK, which are two kinases that have been implicated in obesity-induced insulin resistance (11, 26), had only a minor effect on palmitate-induced PDK1 phosphorylation. On the other hand, suppressing the expression levels or activity of PKCθ greatly reduced palmitate-stimulated PDK1 phosphorylation (Fig. 3A). These results indicate that PKCθ is the major kinase that mediates palmitate-induced PDK1 phosphorylation. Our findings are consistent with numerous recent studies showing that PKCθ plays a critical role in hyperlipidemia-induced insulin resistance both in insulin-sensitive cells (13, 14, 26–30) and in the skeletal muscle of diabetic humans and animals (26, 31–33). However, a recent study showed that PKCθ-knock-out mice gained more body weight and suffered more severe insulin resistance on high fat diet than wild-type littermates (16). This result suggests that under normal physiological conditions, basal PKCθ activity is important for regulation of energy homeostasis.

A question that remains to be established is how PKCθ-mediated phosphorylation at Ser504/532 negatively regulates PDK1 function. We previously found that PDK1 activity is induced by dimerization and trans-phosphorylation (19). Thus, serine phosphorylation of PDK1 at Ser504/532 may reduce PDK1 activity by inhibiting its dimerization and trans-phosphorylation. However, we found that palmitate treatment had little effect on PDK1 dimerization (data not shown). Another possibility is that palmitate-stimulated PDK1 Ser504/532 phosphorylation may induce a conformational change that inhibits PDK1 autophosphorylation in the activation loop. Serine phosphorylation at Ser504/532 may also affect the ability of PDK1 to activate Akt by inhibiting its membrane translocation, which has been shown to be important for PDK1 activity toward Akt in intact cells (4). Further studies will be needed to test these possibilities.

Recent studies suggest that hyperlipidemia and overproduction of proinflammatory cytokines contribute significantly to obesity-induced insulin resistance (34, 35). In the present study, we show that palmitate-induced serine phosphorylation of PDK1 inhibits insulin signaling. This new finding provides a novel mechanism by which obesity leads to insulin resistance. Characterization of the mechanism of FFA-induced PDK1 phosphorylation and its physiological consequences should
provide important information on our understanding of the pathophysiology of insulin resistance and type 2 diabetes.

Acknowledgments—We thank Dr. Wataru Ogawa (Kobe University, Kobe, Japan) for the PKC\(~\varepsilon\)/MEFs and Dr. Jianping Ye (Pennington Biomedical Research Center) for the PKC\(\beta\)/\(\varepsilon\) mice.

REFERENCES

1. Frayn, K. (1993) 
2. Steiner, G., Morita, S., and Vranic, M. (1980) 
3. Reaven, G. M., Hollenbeck, C., Jeng, C. Y., Wu, M. S., and Chen, Y. D. (1988) 
4. Anderson, K. E., Coadwell, J., Stephens, L. R., and Hawkins, P. T. (1998) 
5. Casamayor, A., Morrice, N. A., and Alessi, D. R. (1999) 
6. Currie, R. A., Walker, K. S., Gray, A., Downes, C. P., Cohen, P., Alessi, D. R., and Lucocq, J. (1999) 
7. Wick, M. J., Wick, K. R., Chen, H., He, H., Dong, L. Q., Quon, M. J., and Liu, F. (2002) 
8. Kondo, T., and Kahn, C. R. (2004) 
9. Kenchappa, P., Yadav, A., Singh, G., Nandana, S., and Banerjee, K. (2004) 
10. Aguirre, V., Werner, E. D., Giraud, J., Lee, Y. H., Shoelson, S. E., and White, M. F. (2002) 
11. Lee, Y. H., Giraud, J., Davis, R. J., and White, M. F. (2003) 
12. Gao, Z., Hwang, D., Bataille, F., Lefevre, M., York, D., Quon, M. J., and Ye, J. (2002) 
13. Gao, Z., Wang, Z., Zhang, X., Butler, A. A., Zuberi, A., Gawronska-Kozak, B., Lefevre, M., York, D., Ravussin, E., Berthoud, H. R., McGuinness, O., Cefalu, W. T., and Ye, J. (2007) 
14. Yu, C., Chen, Y., Cline, G. W., Zhang, D., Zong, H., Wang, Y., Bergeron, R., Kim, J. K., Cushman, S. W., Cooney, G. J., Atcheson, B., White, M. F., Kraegen, E. W., and Shulman, G. I. (2002) 
15. Schmitz-Peiffer, C., Craig, D. L., and Biden, T. J. (1999) 
16. Gao, Z., Wang, Z., Zhang, X., Butler, A. A., Zuberi, A., Gawronska-Kozak, B., Lefevre, M., York, D., Ravussin, E., Berthoud, H. R., McGuinness, O., Cefalu, W. T., and Ye, J. (2007) 
17. Dong, L. Q., Zhang, L.-B., Langlais, P., He, H., Clark, M., Zhu, L., and Liu, F. (1999) 
18. Wick, M. J., Dong, L. Q., Riojas, R. A., Ramos, F., and Liu, F. (2000) 
19. Wick, M. J., Ramos, F. J., Chen, H., Quon, M. J., Dong, L. Q., and Liu, F. (2003) 
20. Mao, X., Kikani, C. K., Riojas, R. A., Langlais, P., Wang, L., Ramos, F. J., Fang, Q., Christ-Roberts, C. Y., Hong, J. Y., Kim, D. W., Liu, Z. X., Soos, T. J., Cline, G. W., O’Brien, W. R., Littman, D. R., and Shulman, G. I. (1999) 
21. Koeller, E., Vlahos, L., Bosco, L. A., Comuzzie, A. G., Schwartz, M. B., Altholz, B., Vlassara, H., and DeFronzo, R. A. (1993) 
22. Chalfant, C. E., Ciaraldi, T. P., Watson, J. E., Nikoulina, S., Henry, R. R., and White, M. F. (2002) 
23. Haasch, D., Berg, C., Claepht, J., Pederson, T., Frost, L., Kroeger, P., and Rondinone, C. M. (2006)
24. Hoehn, K. L., Hohnen-Behrens, C., Cederberg, A., Wu, L. E., Turner, N., Yuasa, T., Ebina, Y., and James, D. E. (2008) 
25. Nakamura, K., Sakaue, H., Nishizawa, A., Matsuki, Y., Gomi, H., Watanabe, E., Hiramatsu, R., Tamamori-Adachi, M., Kitajima, S., Noda, T., Ogawa, W., and Kasuga, M. (2008) 
26. Schmitz-Peiffer, C., Browne, C. L., Oakes, N. D., Watson, C., Cheng, H., Shulman, G. I., and Kraegen, E. W. (2002) 
27. Schmitz-Peiffer, C., Browne, C. L., Oakes, N. D., Watson, C., Cheng, H., Shulman, G. I., and Kraegen, E. W. (2002) 
28. Kim, J. K., Fillmore, J. J., Sunshine, M. J., Albrecht, B., Higashimori, T., Cline, G. W., Slezak, L. A., Andersen, D. K., Hundal, R. S., Rothman, D. L., Petersen, K. F., and Shulman, G. I. (1999) 
29. Berti, L., Mosthaf, L., Kroder, G., Kellerer, M., Tippmer, S., Muskack, J., Seifer, E., Seedorf, K., and Haring, H. (1994) 
30. Donnelly, R., and Qu, X. (1998) 
31. Xu, Q., Seale, J. P., and Donnelly, R. (1999) 
32. Guilhaume, A., Virbasius, J. V., Puri, V., and Czech, M. P. (2008) 
33. Ye, J. (2007) 
34. Guilhaume, A., Virbasius, J. V., Puri, V., and Czech, M. P. (2008) 
35. Ye, J. (2007) 
36. Schmitz-Peiffer, C., Craig, D. L., and Biden, T. J. (1999) 
37. Kraegen, E. W., Shulman, G. I., and Biden, T. J. (1997) 
38. Tallquist, M., and Kazlauskas, A. (2004) 
39. Chaffant, C. E., Ciaraldi, T. P., Watson, J. E., Nikoulina, S., Henry, R. R., and White, M. F. (2000) 
40. Haasch, D., Berg, C., Claepht, J., Pederson, T., Frost, L., Kroeger, P., and Rondinone, C. M. (2006) 
41. Hoehn, K. L., Hohnen-Behrens, C., Cederberg, A., Wu, L. E., Turner, N., Yuasa, T., Ebina, Y., and James, D. E. (2008) 
42. Nakamura, K., Sakaue, H., Nishizawa, A., Matsuki, Y., Gomi, H., Watanabe, E., Hiramatsu, R., Tamamori-Adachi, M., Kitajima, S., Noda, T., Ogawa, W., and Kasuga, M. (2008) 
43. Schmitz-Peiffer, C., Browne, C. L., Oakes, N. D., Watson, C., Cheng, H., Shulman, G. I., and Kraegen, E. W. (2002) 
44. Kim, J. K., Fillmore, J. J., Sunshine, M. J., Albrecht, B., Higashimori, T., Cline, G. W., Slezak, L. A., Andersen, D. K., Hundal, R. S., Rothman, D. L., Petersen, K. F., and Shulman, G. I. (1999) 
45. Berti, L., Mosthaf, L., Kroder, G., Kellerer, M., Tippmer, S., Muskack, J., Seifer, E., Seedorf, K., and Haring, H. (1994) 
46. Donnelly, R., and Qu, X. (1998) 
47. Xu, Q., Seale, J. P., and Donnelly, R. (1999) 
48. Guilhaume, A., Virbasius, J. V., Puri, V., and Czech, M. P. (2008) 
49. Ye, J. (2007)