Activation of Protein Phosphatase 2A by Palmitate Inhibits AMP-activated Protein Kinase*

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Elevated levels of free fatty acids contribute to cardiovascular diseases, but the mechanisms remain poorly understood. The present study was aimed to determine if free fatty acid inhibits the AMP-activated kinase (AMPK). Exposure of cultured bovine aortic endothelial cells (BAECs) to palmitate (0.4 mM) but not to palmitoleic or oleic acid (0.4 mM) for 40 h significantly reduced the Thr172 phosphorylation of AMPK and acetyl-CoA carboxylase. Further, 2-bromopalmitic or oleic acid (0.4 mM) for 40 h significantly reduced the Thr172 phosphorylation of AMPK-Thr172 and endothelial nitric-oxide synthase of BAECs. This study was aimed to determine if free fatty acid inhibits the activation of protein phosphatase 2A (PP2A) and AMPK. Inhibition of AMPK in parallel with increased phosphorylation of AMPK-Thr172 and endothelial nitric-oxide synthase of BAECs via ceramide-dependent PP2A activation.

This article has been withdrawn by the authors. The Journal raised questions regarding Figs. 1A, 4C, 5A, and 8 (A and C). Twelve years after the publication, the authors were able to locate some, but not all, of the original data and were able to locate some repeated experiments performed at the time of the original work, which the authors state support the conclusions of the paper. The authors state that the results of this paper are confirmed by the results of complementary experiments presented in the manuscript, and the principal observations of this paper were further confirmed in the publications of other laboratories (Bharath, L. P. et al. (2015) Diabetes 64, 3914-3926; Joseph, B. K. et al. (2015) J. Biol. Chem. 290, 10588-10598; and Wang, T. et al. (2010) PLoS One 5, e13096). The authors stand by the conclusions of the paper.
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acid availability independent of the cellular AMP levels. Indeed, a reduced fat oxidative capacity has been reported in type 2 diabetic patients (12). Also, high fat diet feeding significantly decreases phospho-AMPK in the liver and muscles of rodents (13, 14). AMPK activity is reduced in aortic endothelium or skeletal muscle of obese rats compared with lean animals (15, 16). These data raise the possibility that chronic exposure of fatty acids to cells inhibit AMPK activation. However, the mechanisms by which fatty acids inhibit AMPK are poorly understood.

In an attempt to understand the mechanism underlying which chronically increased FFA inhibits AMPK, we examined the effects of the saturated fatty acid palmitate, which makes up 30–40% of plasma FFA, in cultured endothelial cells and in mice. Here we report that palmitate inhibited AMPK via ceramide-dependent PP2A activation in vivo.

EXPERIMENTAL PROCEDURES

Materials—Bovine aortic endothelial cells (BAECs) and cell culture media were obtained from Clonetics Inc. (Walkersville, MD). BAECs were maintained in EBM with 2% serum and growth factors. HeLa-S3 and A549 cells were obtained from ATCC (Manassas, VA). Dulbecco’s modified Eagle’s medium/ Ham’s F-12 medium was purchased from Mediatech, Inc. (Herndon, VA). 5-Aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside (AICAR) was purchased from Toronto Research Chemicals, Inc. (Toronto, Canada). FFA-free bovine albumin (BSA), palmitic acid, oleic acid, palmito-leamic acid, C2-ceramide, fumonisin B1, and EDTA were obtained from Sigma. Peroxynitrite (ONOO\textsuperscript{-}) was from Calbiochem (La Jolla, CA). 2-Bromopalmitic acid (Sigma, St. Louis, MO), phospho-ACC (Ser\textsuperscript{79}), phospho-AMPK (Thr\textsuperscript{172}), AMPK, phospho-LKB1 (Ser\textsuperscript{428}), LKB1, and PP2A antibodies against ACC were obtained from Alpha Diagnostic International, Inc. (San Antonio, TX). Antibodies against PP2A was obtained from Upstate Biotechnology (Lake Placid, NY). Other chemicals and organic solvents, if not indicated, were obtained from Sigma with the highest grade. Other assay kits or antibodies, if not mentioned here, are indicated under “Experimental Procedures.”

Cell Culture and Treatments—BAECs were grown in EBM supplemented with 2% fetal bovine serum. HeLa-S3 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% serum. A549 cells were grown in Ham’s F-12 medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 \mu g/ml). Rat vascular smooth muscle cells (VSMCs) were cultured from rat thoracic aortas as described previously (17). Cultured cells were used between passages 5 and 10. When 70% confluent, the cells were washed with serum-free medium and then maintained in Dulbecco’s modified Eagle’s medium/F-12 with 0.1% fetal calf serum for 24 h. All cells were incubated in a humidified atmosphere of 5% \text{CO}_2/95% air at 37 °C. When the cells were 60–70% confluent, the maintenance medium was removed and cells were treated with fatty acids (palmitic acid, oleic acid, or palmitoleic acid, 0.4–0.8 mM) for 1–40 h.

Fatty acids were added to the culture medium as a fatty acid-BSA complex. Briefly, a stock solution of fatty acids was directly prepared in 95% ethanol and kept at 4 °C. The fatty acids were added to the cell cultures coupled to fatty acid-free bovine serum albumin (BSA) in the ratio of 2 mol of fatty acid to 1 mol of albumin. These complexes were constituted in stopper-covered flasks by adding the appropriate volume of the ethanolic FFAs solution to the albumin previously dissolved in culture medium containing no fetal bovine serum (here termed SF-EBM). These solutions were gently stirred and sterilized by filtration through 0.2-\mu m filters. Final concentrations of ethanol in the culture medium were kept to <0.1%. The concentrations of endotoxins in BSA were very low, as assessed by the supplier (3 enzyme units/mg BSA versus ~30–60 enzyme units/mg of BSA for standard albumin preparations).

Fatty acid-BSA complexes were added to culture dishes at a final concentration of 0.4 mM fatty acid. Controls were incubated with equal concentrations of FFA-free albumin as present in fatty acid-treated cells. In some experiments, BAECs were incubated in the presence of the presence of either 2-bromopalmitate (2-BrP, 0.4 mM), fumonisin B1 (15 \mu M), AICAR (2 mM), okadaic acid (OA, 2 nM), and peroxynitrite (ONOO\textsuperscript{-}, 5 mM), AICAR (2 mM), fumonisin B1 were added to the cell culture medium. The final Me\textsubscript{3}SO concentration in all experiments, BAECs were exposed to solvent alone (0.1% Me\textsubscript{3}SO).

Cell Viability and Apoptosis—To exclude the potential contribution of cell death to the effects of FFA on AMPK phosphorylation, in our experimental conditions, we first verified cell viability after 40 h of culture in the presence of either 0.4 mM palmitic or oleic acid. Cells were rinsed with phosphate-buffered saline, trypsinized, washed with medium, centrifuged, and resuspended in phosphate-buffered saline. Next, cells were mixed with the same volume of 0.25% trypan blue and transferred to a slide for 3 min. A total of 300 cells was microscopically counted using a hemocytometer to determine the dead cell rate. The experiments were performed in triplicate. Compared with the control (cells not exposed to FFA), no significant differences were detected for cell viability after exposing the cells for 40 h to the treatment conditions.

Apoptosis was measured using the Cell Death Detection ELISA (Roche Diagnostics, Mannheim, Germany) to detect cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) according to the manufacturer’s protocol. Data were normalized for comparison by total protein concentration (Bio-Rad).

Animals—Male C57BL/6j mice were purchased from the Jackson Laboratory. The animals were maintained in a temperature-controlled room (22 °C) on a 12-h light-dark cycle. The study was approved by the Institutional Animal Care and Use Committee at University of Oklahoma Health Sciences Center. One week after arrival, mice were divided into three groups and were fed with normal chow, diet rich in palmitic acid (palmitate-HFD, high palmitate, 16:0 = 50% of total fatty acids) or diet rich in oleic acid (oleate-HFD, high oleic, 18:1 = 80% of total fatty acids, Research Diets, New Brunswick, NJ) for 3 months.
Food intake and body weight were measured once a week. The intraperitoneal glucose tolerance test was performed after 12 weeks in 6 mice from each dietary group. Briefly, mice fasted for 12 h and blood was drawn from the tail vein at 0, 5, 15, 30, 60, and 120 min after intraperitoneal injection of glucose (2 g/kg body weight). The blood glucose was assayed using a blood glucose meter (LifeScan, Inc.). The trapezoid rule was used to determine the area under the curve (AUC) for glucose concentrations in each animal.

Finally, after 12 weeks, blood samples were taken from the intraorbital, retrobulbar plexus from non-fasted, anesthetized mice to measure basal plasma levels of glucose and insulin. Insulin was determined using a Mouse Insulin Elisa Kit (Linco Research, St. Charles, MO). The mice were sacrificed, and thoracic aortas were immediately isolated and snap frozen in liquid nitrogen. The manipulation times were reduced to the minimum between aorta isolation and storage in liquid nitrogen. To avoid additional phosphorylation/dephosphorylation, Krebs-Ringer bicarbonate solution, which was used to rinse isolated aortas, was insufflated with 95% O₂ and 5% CO₂, and the mixtures were placed on ice to prevent tissue hypoxia. Mouse aortas were subsequently homogenated for determination of AMPK, ACC, and eNOS. In some experiments, the adventitia was removed from the isolated aortas. Aortas were then cut open along the ordinate axis, and the endothelium was removed by gentle rubbing of the intima with curved forceps.

**Immunohistochemistry**—Ceramide formation was determined using immunohistochemistry. After incubation with media containing palmitic, palmitoleic, or oleic acid, the cells were fixed with 100% methanol for 3 min at −20 °C and then blocked with 1% normal goat serum in phosphate-buffered saline. Ceramide was detected using a specific anti-ceramide antibody (Alexis, Carlsbad, CA; clone MID 15B4, 1:200 dilutions in blocking buffer) and assayed using an Alexa 488-labeled anti-mouse antibody (Molecular Probes, 1:200 dilution in blocking buffer). Cells were examined under a fluorescence microscope (Olympus IX71) and pictures were taken for analysis.

**siRNA Construction and Infection**—Double-stranded RNA with sequence 5′-CCAUUCUUCCAGGAAAAACAtt-3′ was designed to target the open reading frame of the bovine PP2A catalytic subunit Cα (GenBank™ accession number M16968) and purchased from Ambion (Austin, TX). A scrambled sequence served as control. Transient infection of siRNA was carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Cells were plated in a 6-well plate at 2.0 × 10⁵ cells/well in 1.5 ml of EBM supplemented with 10% serum without antibiotics. BAECs were transfected with either
scrambled or PP2A-specific siRNA oligonucleotides at a final concentration of 80 nM. No cell toxicity was observed at the concentrations of siRNA used. The PP2A expression was verified in Western blots. We found the maximal inhibition of PP2A was achieved after 2 days infection, and cells were treated on day 3 post infection.

For mice, Double-stranded RNA, with sequence 5'H11032/GC-CUCUUGUCAUCAACAGCtt-3'H11032, was designed to target the open reading frame of the mouse PP2A catalytic subunit C (GenBankTM accession number NM019411) and was purchased from Ambion. 50 g of siRNAs was mixed with in vivojetPEITM (Qbiogene, Carlsbad, CA) at an N/P ratio of 5 at room temperature for 15 min. For intravenous administration, 200 l of the mixture containing the indicated amounts of siRNA was injected retro-orbitally. After 48 h, the mice were anesthetized and sacrificed. Aortas were isolated for biochemical assays.

Assay of Phosphatase Activities—PP2A activity was measured by using threonine phosphopeptide (KRpTIRR) as the substrate with the PP2A Immunoprecipitation Phosphatase Assay Kit (Upstate Biotechnology). The cells were lysed in lysis buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EGTA, 10% glycerol, 1.5 mM magnesium chloride, 1% Triton X-100, 1 µg of leupeptin/ml, 50 units of aprotinin/ml, 1 mM phenylmethylsulfonyl fluoride). Clarified supernatants were incubated with anti-PP2A antibody and protein A-agarose for 2 h at 4 °C. A sf or mice, PP2A immunoprecipitates in total aorta were prepared as described previously (18). The washed immunoprecipitates were resuspended in p-nitrophenyl phosphate Ser/Thr assay buffer, provided by the kit, and incubated for 2 h at 4 °C. After washing the beads three times, the diluted phosphopeptide (750 µM) and Ser/Thr assay buffer were added, and the mixture was incubated at 30 °C followed by addition of the Malachite Green Phosphate Detection Solution. PP2A activity was assayed spectrophotometrically at 405 nm.

Western Blot Analysis—BAEC, HeLa-S3, A549, and VSMC cells were washed twice with cold phosphate-buffered saline and lysed in cold radioimmune precipitation assay buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and a mixture of protease inhibitors (Roche Applied Science). The protein concentrations were determined with a bicinchoninic acid protein assay system (Pierce). Proteins were subjected to Western blots. The antibody bindings were detected by using ECL-Plus, as described previously (20).

Expression of Data and Statistics—Data are expressed as mean ± S.E. Intergroup comparisons were performed by Student’s paired t test. p < 0.05 was considered significant.

RESULTS

Effects of Palmitate on Phosphorylation of AMPK and Its Downstream Enzyme, ACC-Ser79—Previous studies had demonstrated that palmitate activates AMPK in rats heart (10) and
AMPK-Thr172 and ACC-Ser79 phosphorylation. The levels of both AMPK-Thr172 and ACC-Ser79 (Fig. 1, A and B) caused a transient increase in both AMPK and ACC phosphorylation. As depicted in Fig. 1 (E and F), 0.4 mM palmitate also reduced both basal and AICAR-stimulated AMPK and ACC phosphorylation at 40 h. Because 0.4 mM palmitate caused the greatest extent of inhibition on the phosphorylation of AMPK and ACC at 40 h, we exposed BAECs to 0.4 mM palmitate in the rest of the study.

**Inhibition of AMPK by Palmitate Is LKB1-independent**—Two recent studies (6, 24) had identified that LKB1 acts as one of the AMPK upstream kinases, AMPK kinase. In the current study, we determined whether or not palmitate inhibited AMPK activation by measuring the phosphorylation of LKB1 at Ser428, which is essential for ONOO- induced AMPK activation (25). As shown in Fig. 2, A and B, ONOO- significantly increased the levels of LKB1-Ser428 phosphorylation compared with its control. In contrast, palmitate did not alter the levels of LKB1-Ser428 phosphorylation.

We next determined if palmitate inhibited AMPK in either HeLa-S3 or A549, two LKB1-deficient cells. Similar to that seen in BAECs, palmitate (0.4 mM) inhibited the Thr172 phosphorylation of AMPK in both HeLa-S3 and A549 by 60 and 70%, respectively (Fig. 2, C and E). Because palmitate inhibited AMPK Thr172 phosphorylation in both BAEC and LKB1-depleted cells, these results suggest that palmitate-induced reduction of AMPK-Thr172 might be independent of its upstream kinase, LKB1.

**Inhibition of AMPK by Palmitate Requires Palmitate Activation but Is β-Oxidation-independent**—The inhibitory effects of saturated FFA on AMPK phosphorylation could either be due to a direct action or due to products generated by its metabolism. To distinguish between direct and indirect effects of FFA we determined the effects of a non-metabolizable analog of palmitate, 2-BrP, on AMPK and ACC phosphorylation. As shown in Fig. 3, 2-BrP, unlike palmitate, did not suppress the phosphorylation of both AMPK and ACC, suggesting that the effect of palmitate was dependent on its metabolism in endothelial cells.

AMPK, as defined by its name, is regulated by the ratios of AMP to ATP. Increased oxidation of palmitate might inhibit AMPK by increasing ATP via enhanced mitochondrial oxidation of palmitate. To this end, BAECs were preincubated with 2-BrP (0.4 mM) 2 h prior to the addition of palmitate. 2-BrP inhibits long-chain fatty acid β-oxidation and consequent ATP synthesis, decreasing the AMP/ATP ratio and thereby inhibiting AMPK activation. Inhibition of synthesis of AMP by the addition of 2-BrP (0.4 mM) for 2 h restored the basal levels of AMPK phosphorylation and ACC phosphorylation in BAECs (Fig. 3, A and B).

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Palmitate Inhibits AMPK

**FIGURE 4.** PP2A-mediated reduction of AMPK phosphorylation in BAECs exposed to palmitate. A, inhibitory effect of OA on AMPK and ACC phosphorylation in BAECs. The blot is a representative of three blots obtained from three independent experiments. B, summary data (n = 4; *p < 0.05 versus control groups; #p > 0.05 versus non-OA-treated groups). C, inhibition of PP2A activity with siRNA abolished the effects of palmitate on AMPK and ACC phosphorylation. The blot is a representative of three blots obtained from three independent experiments. D, inhibition of PP2A activity with siRNA suppressed the effects of palmitate on AMPK and ACC phosphorylation, whereas scrambled siRNA had no effect (n = 3; *p < 0.05 versus control groups; #p < 0.05 versus corresponding non-siRNA-treated groups).

To further examine if palmitate inhibited AMPK by activating PP2A in BAECs, we suppressed the expression of PP2A by applying PP2A siRNA in BAECs. As shown in Fig. 4C, PP2A siRNA suppressed the expression of PP2A by 68%. As expected, suppression of PP2A by PP2A siRNA increased both AMPK and ACC phosphorylation in both unstimulated and AICAR-treated BAECs, confirming that PP2A might negatively regulate AMPK phosphorylation in BAECs in basal conditions. Further, infection of PP2A siRNA but not scrambled siRNA reversed the inhibitory effect of palmitate on AMPK and ACC, implying that palmitate-induced AMPK inhibition might be due to activation of PP2A (Fig. 4D).

**Inhibition of AMPK by Palmitic Acid**

Ceramide-dependent AMPK Inhibition by Palmitate—We next determined if ceramide mimicked the effects of palmitate on AMPK phosphorylation. To this end, BAECs were exposed to exogenous C2-ceramide, a cell-permeable ceramide analog. Compared with control, C2-ceramide (15 μM) significantly suppressed the levels of AMPK-Thr172, whereas neither 0.4 mM palmitoleate nor oleate had effects. Because palmitate but not the monounsaturated palmitoleate (16:0) but not its monounsaturated counterpart palmitoleate (16:1) or oleate (18:1), is correlated with de novo synthesis of ceramide (30, 31), we employed both ceramide analogs to test if ceramide participates in palmitate-induced AMPK inhibition. As shown in Fig. 5A and B, chronic incubation of BAECs with 0.4 mM palmitate attenuated the levels of AMPK-Thr172, whereas neither 0.4 mM palmitoleate nor oleate had effects. Because palmitate but not the monounsaturated palmitoleic and oleic acid increases the levels of ceramide, these results suggest that palmitate might inhibit AMPK by increasing ceramide synthesis.

Ceramide-dependent AMPK Inhibition by Palmitate—We next determined if ceramide mimicked the effects of palmitate on AMPK phosphorylation. To this end, BAECs were exposed to exogenous C2-ceramide, a cell-permeable ceramide analog. Compared with control, C2-ceramide (15 μM) significantly suppressed the levels of AMPK-Thr172 phosphorylation without altering the expression of AMPK α expression (Fig. 5, C and D). In contrast, the metabolically inactive ceramide analog, C2-di-hydroceramide, did not alter palmitate-induced AMPK phosphorylation (data not shown).

We next assayed if selective inhibition of ceramide synthesis attenuated the effects of palmitate on AMPK phosphorylation. Fumonisin B1, a specific inhibitor of ceramide synthase, can inhibit ceramide de novo formation. Concurrently, administration of fumonisin B1 (15 μM) with 0.4 mM palmitate for 40 h significantly reduced the inhibitory effect of palmitate on AMPK phosphorylation (Fig. 5, E and F). In addition, fumonisin B1 also increased AMPK phosphorylation in non-stimulated cells by 40% (p < 0.05, Fig. 5, E and F).
Taken together, these findings implied that palmitate attenuated AMPK via de novo ceramide synthesis.

**Palmitate Induces de Novo Ceramide Synthesis in BAECs**—Prior studies had implicated that palmitoyl-CoA, the activated form of palmitate, is the substrate of de novo formation of ceramide (32). We next determined if exposure of BAECs to palmitate increased de novo synthesis of ceramide. As shown in Fig. 6 (A and B), compared with the controls, palmitate instead of palmitoleate and oleate increased the formation of ceramide in BAECs.

**Activation of PP2A by Ceramide in BAECs**—We next determined if palmitate activated PP2A in BAECs. To directly assess PP2A activity in response to different factors, immunoprecipitation of PP2A was performed with a peptide antibody directed against the C subunit of PP2A. The immunoprecipitated PP2A activity was further assayed by using the specific phosphopeptide as its substrate, as described under “Experimental Procedures." As indicated in Fig. 7 A, pretreatment with palmitate increased PP2A activity by 50% compared with the control, whereas oleate did not change PP2A activity. Direct exposure of cells with C2-ceramide mim-
icked the effect of palmitate and caused a 2-fold elevation in PP2A activity. In contrast, fumonisin B1, which suppresses ceramide formation, caused a 60% reduction in PP2A activity. Furthermore, fumonisin B1 abolished palmitate-induced PP2A activation. Palmitate did not change the abundance of the PP2A catalytic subunit (Fig. 7B), suggesting that palmitate activated AMPK without altering its expression.

**PP2C Is Not Involved in Palmitate-induced Decrease in AMPK Phosphorylation**—There is evidence that another type of phosphatase, protein phosphatase 2C (PP2C) negatively regulates AMPK in vitro and in skeletal muscle (33). To determine whether or not PP2C was involved in AMPK inhibition induced by palmitate, we next determined the effects of palmitate on PP2C expression and activity. As shown in Fig. 7 (C and D), palmitate altered neither PP2A expression nor the PP2C activity. PP2C was known to be OA-insensitive but Mg²⁺-sensitive (34), and no specific inhibitors for PP2C were available. High concentrations (10 mM) of EDTA were reported to inhibit PP2C by chelating Mg²⁺, which is required for PP2C activity. High concentrations of EDTA had no effect on palmitate-induced AMPK inhibition (Fig. 7, E and F). These results suggest that Mg²⁺-sensitive phosphatases, like PP2C, might not be involved in palmitate-induced AMPK inhibition.

**Palmitate-induced Inhibition of AMPK Is Associated with PP2A Activation in Mice**—We next determined the effects of palmitate on AMPK and the roles of PP2A. Palmitate but not oleate significantly inhibited AMPK and ACC phosphorylation in aorta from C57BL/6J mice (Fig. 8, A and B) in parallel with increased aortic PP2A activity (Fig. 8, E and F). The PP2A activity was inversely related to the levels of phosphorylated AMPK suggesting that PP2A played an important role in palmitate-induced AMPK inhibition. Previous studies from us and others (20, 35) suggested that AMPK activation might improve vascular endothelial functions by increasing endothelial nitric-oxide synthase (eNOS) phosphorylation on Ser¹¹⁷⁷. Therefore, effects of palmitate or oleate on eNOS Ser¹¹⁷⁷ phosphorylation on mouse aortas were also assayed in these mice. As indicated in Fig. 8 (A and B), palmitate significantly decreased eNOS Ser¹¹⁷⁷ phosphorylation by 65%, and, to a less extent, oleate decreased it by 25% (p < 0.05) compared with control groups.
In addition, we explored the effects of these fatty acids on another important cell type of aorta, VSMCs. Interestingly, palmitate inhibited AMPK phosphorylation (by 28%, \( p < 0.05 \)) in cultured VSMC but had no effects in endothelium-denuded aortas from palmitate-fed mice (Fig. 8, C and D), suggesting that endothelium-derived AMPK might be a primary target of HFD-induced PP2A activation.

Inhibition of PP2A with PP2A-specific siRNA Reverses Palmitate-induced AMPK Inhibition in Mice—We next determined if inhibition of PP2A reversed the inhibitory effect of palmitate on AMPK as well as eNOS phosphorylation. Normal chow or palmitate-HFD fed mice were injected retro-orbitally with NP-specific PP2A siRNA in complexes with jetPEI™. As controls, mice were injected retro-orbitally with scrambled siRNA in complexes with jetPEI™. Forty-eight hours after infection, PP2A protein content in aortas was measured by Western blot. Administration of PP2A-specific siRNA diminished aortic PP2A protein expression by 50%. As depicted in Fig. 9 (A and B), PP2A siRNA not only increased AMPK phosphorylation in mice fed with normal chow fed mice (\( \sim 2.7\text{-fold}, p < 0.05 \)) but also abolished palmitate-induced inhibition of AMPK phosphorylation in mouse aortic tissues (Fig. 9, A and B). In parallel with the change of AMPK phosphorylation, eNOS Ser\(^{1177} \) phosphorylation was decreased in response to HFD rich in palmitate, whereas inhibition of PP2A with PP2A-specific siRNA but not scrambled siRNA reversed HFD-triggered reduction of eNOS-Ser\(^{1177} \) phosphorylation in mice fed with HFD (Fig. 9, A and B). These data indicated that HFD inhibited eNOS phosphorylation, likely via a PP2A-dependent inhibition on AMPK in vivo.

**Palmitate Causes Insulin Resistance in Vivo**—Plasma glucose, insulin, food intake, body weight, and AUC\(_{\text{glucose}}\) during intraperitoneal glucose tolerance test measured after 3 months of normal chow, diet rich in palmitic acid, or oleic acid are shown in Table 1. No differences in food intake were observed among the three groups. Both palmitate-HFD and oleate-HFD increased body mass relative to normal chow (28 \( \pm \) 2 g and 22.8 \( \pm \) 1.5 g versus 18.9 \( \pm \) 0.8 g, \( p < 0.01 \)). However, body weights were significantly higher in the palmitate-HFD than in the oleate-HFD group after 3 months of the diet (\( p < 0.05 \)). The changes in net weight gain were similar to those in body mass among these groups. These data indicated that the mice were more prone to obesity when fed with palmitate rather than oleate. After 3 months, levels of plasma glucose and insulin
were significantly higher in the palmitate-HFD group than in the control and oleate-HFD group (p < 0.01 and p < 0.05, respectively). At 3 months, AUCglucose was ~1.5-fold higher in the palmitate-HFD group than in the control group (p < 0.01), but there was no difference in AUCglucose between the oleate-HFD and control groups. In palmitic acid HFD-fed mice, a marked rise in both plasma glucose and insulin levels plus impaired glucose tolerance implied that mice fed with palmitic acid developed the phenotypes of insulin resistance. These results are consistent with the hypothesis that a decreased AMPK activity induced by palmitate contributes to excess lipid accumulation and insulin resistance in vivo.

**DISCUSSION**

The data presented in the study have demonstrated that prolonged exposure of endothelial cells to palmitate significantly suppresses the phosphorylation of AMPK and its downstream enzyme, ACC. In addition, we have for the first time provided compelling evidence obtained in vitro and in vivo that palmitate inhibits both AMPK and eNOS phosphorylation by ceramide-dependent PP2A activation. Thus, our results might provide a novel mechanism by which palmitate inhibits AMPK in endothelial cells.

One metabolic fate of the palmitate taken up by cells is that, following its conversion into palmitoyl-CoA, it can be condensed with acetyl-CoA synthetase; this reaction catalyzed by serine palmitoyl transferase to generate 3-ketodihydrosphingosine. This latter molecule can be reduced to sphinganine whose acylation produces dihydroceramide, which can be desaturated to yield ceramide (37). The present study suggests that palmitate, instead of palmitoleate and oleate, increase de novo synthesis of ceramide in BAECs (Fig. 6). Ceramide is a second messenger in the sphingomyelin signaling pathway and has been implicated in the regulation of diverse cellular responses, including cell death, differentiation, and in the pathogenesis of insulin resistance (38) and lipotoxicity (39). Our data suggest that ceramide may be the intermediate molecule mediating palmitate-induced PP2A activation and subsequent AMPK inhibition (Fig. 8). This notion is supported by several lines of evidence. First, incubation of BAECs with palmitate promotes intracellular accumulation of ceramide and inhibits AMPK phosphorylation (Figs. 5 and 6), and palmitoleate or oleate, which cannot produce ceramide, have no effect. Second, a cell-permeable analog of ceramide, C2-ceramide, was capable of mimicking the effect of palmitate on AMPK phosphorylation. Conversely, fumonisin B1, a ceramide synthase inhibitor, prevented the inhibitory effect of palmitate on AMPK phosphorylation (Fig. 5). In addition, we also found palmitate-induced PP2A activation is ceramide-dependent in cultured BAECs (Fig. 7). The latter observation provides further, albeit indirect, support for the concept that PP2A is an important component of dephosphorylating and inactivating AMPK. Third, the inhibition of PP2A, by either a specific inhibitor, OA, or by PP2A siRNA, increased AMPK phosphorylation and normalized palmitate-induced increase in AMPK Thr172 dephosphorylation (Fig. 4), suggesting that PP2A is important for AMPK phosphorylation in vivo. Fourth, high fat diet rich in palmitate...
rather than oleate lead to activation of PP2A and inhibition of AMPK in C57BL/6J mice (Fig. 8). Moreover, introduction of PP2A siRNA to the mice reversed the palmitate-induced decrease in AMPK phosphorylation (Fig. 9). The important finding of the present study is that PP2A may directly modulate AMPK function. In support of this idea, it has been reported that the PP2A complex is involved in regulating the interaction between AMPK α2 and γ1 (40) and inactivating of AMPK in pancreatic β-cells (41), and the active phosphorylated form of AMPK can be inactivated in cell-free assays by PP2A (42). Thus, we conclude that PP2A might be a physiologically relevant AMPK phosphatase. Our data suggest that palmitate inhibits AMPK phosphorylation by activating the serine/threonine AMPK phosphatase, PP2A, instead of inhibiting the AMPK kinase, LKB1 (Fig. 2), or enhancing ATP levels after its metabolism and β-oxidation (Fig. 3).

PP2C is an Mn2+- or Mg2+-dependent protein serine/threonine phosphatase. Its exact physiological role is still unclear. It has been shown that PP2C is responsible for dephosphorylation of AMPK (33). Wang et al. (43) have demonstrated that AMPK phosphorylation was significantly reduced in both ZDF fa/fa rats and ob/ob mouse hearts compared with lean, wild-type controls, and the reduction in active P-AMPKα is associated with an increase in PP2C. In the present study, our data indicate that a decrease in AMPK phosphorylation after chronic exposure of endothelial cells to high levels of palmitate is caused by PP2A inhibition rather than with PP2A activation instead of PP2C. The discrepancy may be due to the differences in species, organs, and nutrition levels. PP2A is responsible for dephosphorylation of AMPK activity, but PP2C is responsible for dephosphorylation of AMPK activity, which is ceramide-dependent, as we demonstrate in the present study.

The physiological relevance of these findings is obvious and important. On the one hand, high circulating plasma fatty acid levels are a hallmark of obesity and poorly controlled Type II diabetes. Fatty acid oversupply is also associated with fatty acid metabolite accumulation in non-adipose tissues, which causes insulin resistance (44). There is growing acceptance of the idea that excessive exposure of non-adipose tissues to lipids in excess of their oxidative or storage capacities causes cell dysfunction or death. These FFA-induced disturbances are referred to as lipotoxicity. The lipotoxicity is the main causation of endothelial dysfunction and development of cardiovascular diseases (45). However, the concrete mechanisms mentioned above are not elucidated clearly. On the other hand, AMPK pathways play an important role in development of insulin resistance and endothelial damage. Activation of AMPK induced by exercise, hypoxia, and physiological or pharmacological inducers can improve insulin sensitivity by increasing glucose transport and oxidation, fatty acid oxidation, and subsequent decrease in lipid accumulation in non-adipose tissues. More importantly, AMPK can inhibit the mTOR-S6 kinase 1 pathway, which phosphorylates insulin receptor substrate proteins on serine residues resulting in decreasing tyrosine phosphorylation and insulin signaling (8). The exact role of AMPK in endothelial cells is not very clear, but it may have some potential benefits. The most important aspect is that activated AMPK increases fatty acid oxidation by phosphorylating and inhibiting ACC leading to a decrease in the concentration of malonyl-CoA (46). In addition, it decreases fatty acid incorporation into glycerolipids, either secondary to its effect on fatty acid oxidation or by virtue of the fact that in some tissues it phosphorylates and inhibits sn-glycerophosphate acyltransferase, the first committed enzyme in diacylglycerol and triglyceride synthesis (47). An additional benefit of endothelial AMPK activity is that it may inhibit glycerol-3-phosphate acyltransferase, required for de novo synthesis of diacylglycerol (47). In other words, AMPK may lessen endothelial diacylglycerol production (and thus protein kinase C activation) both by diminishing availability of the FFA substrate for this synthesis, and by directly inhibiting the enzyme that catalyzes it. Endothelial AMPK has the further advantage of activating nitric-oxide synthase via phosphorylation on Ser1177 (35), thus opposing the adverse impact of protein kinase C on this enzyme. Consistent with these findings, our data indicated that inhibition of AMPK activation by palmitate blocked eNOS phosphorylation, and increasing AMPK phosphorylation by PP2A siRNA administration could increase eNOS phosphorylation (Fig. 9, A and B) suggesting that AMPK phosphorylation is associated with the status of eNOS phosphorylation. There are other possibilities that AMPK phosphorylation directly (48) or through altering protein kinase B/Akt activity (49), an important regulator of eNOS activity. Therefore, it is possible that AMPK reduction by palmitate can cause a direct dephosphorylation of eNOS, which suggests that phosphatidylinositol 3-kinase and protein kinase C isoenzymes may directly contribute to eNOS phosphorylation or endothelium dysfunction deserves further investigation. Taken together, our studies might reveal a novel mechanism by which long term high levels of FFA trigger a PP2A-dependent inhibition of both AMPK and eNOS, which might contribute to vascular endothelial injury and dysfunction (Fig. 9C).

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