Akt/eNOS signaling pathway mediates inhibition of endothelial progenitor cells by palmitate-induced ceramide

Minghuan Fu,1 Zhihong Li,2 Tao Tan,3 Weixin Guo,4 Nanzi Xie,5 Qing Liu,1 Hua Zhu,3* Xiaoyun Xie,5* and Han Lei1*

1The First Affiliated Hospital, Chongqing Medical University, Chongqing, China; 2Division of General Surgery, Chenzhou First People’s Hospital, Chenzhou, Hunan, China; 3Department of Surgery, Davis Heart and Lung Research Institute, The Ohio State University Wexner Medical Center, Columbus, Ohio; *Guangdong Geriatrics Institute, Guangdong General Hospital, Guangdong Academy of Medical sciences, Guangzhou, China; and 5Division of Geriatrics, Tongji Hospital and Tongji University School of Medicine, Shanghai, China

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Fu M, Li Z, Tan T, Guo W, Xie N, Liu Q, Zhu H, Xie X, Lei H. Akt/eNOS signaling pathway mediates inhibition of endothelial progenitor cells by palmitate-induced ceramide. Am J Physiol Heart Circ Physiol 308: H11–H17, 2015. First published November 7, 2014; doi:10.1152/ajpheart.00503.2014.—Palmitate (PA) impairs endothelial progenitor cells (EPCs). However, the molecular mechanism underlying the suppressive function of PA remains largely unknown. Ceramide, a free fatty acid metabolite, mediates multiple cellular signals. We hypothesized that ceramide acts as an intermediate molecule to mediate inhibition of EPCs by PA. We first demonstrated that PA could inhibit the attachment, migration, and tube formation of EPCs through suppression of the Akt/endothelial nitric oxide (NO) synthase (eNOS) signaling pathway. In addition, we observed that PA could induce ceramide accumulation in EPCs. To test whether the accumulation of ceramide causes EPC dysfunction, the ceramide synthesis inhibitors myriocin and fumonisin B1 were used. We found both inhibitors could effectively abolish PA-mediated EPC inhibition. Furthermore, the ceramide deacylation inhibitor N-oleoyl ethanolamine could augment the inhibitory effect of PA on EPCs, indicating that it is ceramide, not its metabolites, that mediates the suppression of EPCs by PA. We have previously shown that Akt/eNOS phosphorylation was reduced after PA treatment, which, in turn, hampered the normal bioavailability of NO, leading to impaired functions of EPCs. To test the role for ceramide in this process, a clinically used NO donor, sodium nitroprusside, was used. We found that sodium nitroprusside could rescue the suppressive effects of ceramide on EPCs, suggesting that ceramide-mediated EPC inhibition might be through reduction of NO production. Taken together, our findings indicated that ceramide-induced reduction of NO might be the molecular mechanism for PA-mediated EPC inhibition; thus, targeting either ceramide or NO production might be an effective means for improvement of EPC functions in diseases.

palmitate; ceramide; endothelial progenitor cells; stem cells; nitric oxide

ENDOTHELIAL PROGENITOR CELLS (EPCs) were initially described and defined as a special type of stem cell by Asahara et al. (2). EPCs have been found in the bone marrow, spleen, umbilical cord blood, and peripheral blood and can contribute to the formation of new blood vessels in postnatal life and incorporate into injured vessels to form mature endothelial cells (ECs) in response to tissue ischemia (9, 28). Increasing evidence indicates that incorporation of EPCs plays an important role in postnatal neovascularization (1, 12).

In some pathological conditions, such as diabetes and coronary artery disease, the number of circulating EPCs is decreased and their functions are impaired (8, 22). As previously highlighted, circulating EPCs in diabetes are subjected to many biochemical alterations attributable to the unfavorable vascular environment. Among these factors, elevated levels of free fatty acids (FFAs) are commonly observed in human patients with diabetes, obesity, and dyslipidemias. A high FFA level is an independent risk factor for atherosclerosis, hypertension, and sudden cardiac death (5). FFAs may create a state of continuous and progressive damage to circulating cells. Therefore, FFAs may be one of the factors that influences the growth and bioactivity of EPCs. Indeed, it has been previously reported that the ability of diabetic EPCs to integrate into vascular networks was reduced after FFA treatment (26). In addition, circulating EPCs and EPC colony formation were found to be reduced in type 2 diabetic patients suffering from high circulating fatty acids (3). A study from our group (13) has also shown that palmitate (PA), one of the most common FFAs, impaired the adherence, migration, and tube formation capacity of EPCs. However, the molecular mechanisms of inhibitory effects of PA on EPCs have not yet been addressed.

Ceramide, one of the key FFA metabolites, is an important bioactive molecule participating in a number of physiological and pathophysiological events (20). Studies have revealed that ceramide as a potentially important molecule mediating cellular dysfunction induced by PA in multiple cell types. For example, PA blocked insulin activation of phosphatidylinositol 3-kinase/PKB by promoting the accumulation of ceramide and diacylglycerol in 3T3-L1 adipocytes and C2C12 myocytes (7). In the present study, we hypothesized that ceramide, as a potential intermediate molecule, is responsible for PA-induced EPC dysfunction through inhibiting the Akt/endothelial nitric oxide (NO) synthase (eNOS) signaling pathway. Our findings might shed lights on development of effective treatments for disorders with impaired EPCs.

MATERIALS AND METHODS

EPC isolation and culture. The study was approved by the Independent Ethics Committee of Tongji Hospital affiliated with Tongji University. Peripheral blood was collected from healthy adult volunteers, and informed consent was signed before the study. The EPC isolation protocol was modified from previous studies (21, 23, 27) and established in our laboratory (13). In brief, peripheral blood mononu-
clear cells from healthy volunteers were isolated by lymphoprep (1.077 g/ml) density barrier centrifugation. The low-density fraction (<1.077 g/ml) was carefully removed from the interface and washed three times with PBS containing 2% FBS. Immediately after isolation, cells were counted, and 8 × 10^5 cells were plated on fibronectin-coated 12-well plates containing 1 ml medium 199 (Invitrogen, Carlsbad, CA) supplemented with 20% FBS, 100 U/ml penicillin-streptomycin (Invitrogen), 0.05 mg/ml bovine pituitary extract (Invitrogen), 10 μg/ml EGF (Invitrogen), 50 μg/ml gentamicine (Invitrogen), 50 μg/ml amphotericin-B (Invitrogen), 1 μg/ml hydrocortisone (Sigma), and 10 ng/ml human VEGF-165 (Sigma). Before use, the medium was passed through a 0.2-μm filter. After 4 days in culture, nonadherent cells were removed and fresh medium was added. The medium was changed on day 6, and cells were kept in culture until day 7. The EPC phenotype was confirmed by the presence of endothelial markers and the uptake of 1,19-dioctadecyl-3,39-tetramethylindocarbocyanine-labeled acetyl low-density lipoprotein (di-Ac-LDL) and binding of Ulex europaeus agglutinin-1 as previously described. To detect the uptake of di-Ac-LDL, cells were incubated with di-Ac-LDL (2.4 mg/ml) at 37°C for 1 h. Cells were then fixed with 2% paraformaldehyde for 10 min, and lectin staining was performed by incubation with FITC-labeled Ulex europaeus agglutinin I (lectin 10 mg/ml, Sigma) for 1 h. After being stained, samples were viewed with an inverted fluorescent microscope. Dual-stained cells positive for both lectin and di-Ac-LDL were judged to be EPCs. Two to three independent investigators evaluated the number of EPCs per well by counting three randomly selected high-power fields. To detect the expression of marker proteins, EPCs were detached with 1 mmol/l EDTA in PBS followed by repeated gentle flushing through a pipette tip. Cells were incubated for 15 min in the dark with monoclonal antibodies against human kinase insert domain receptor, the FITC-labeled monoclonal antibody against human CD34, and the phycoerythrin-conjugated monoclonal antibody against human CD133. Isotype-identical antibodies served as controls (IgG1-phycoerythrin and IgG2a-FITC). Each analysis included 60,000 events.

**Preparation of media containing fatty acid-albumin complexes.** Saturated PA used in this study was purchased from Sigma. Lipid-containing media were prepared by conjugating FFA to BSA. Briefly, PA was dissolved in ethanol at 200 mmol/l and then combined with 10% FFA-free low endotoxin BSA to a concentration range of 1–10 mmol/l. All stock solutions were adjusted to a pH value of 7.5, filter sterilized, and stored at −20°C. Control solution containing ethanol and BSA without PA was similarly prepared. Fresh working solutions were prepared by diluting stock solution (1:10) in medium 199 supplemented with 0.5% or 2% FCS as appropriate. All final PA media contained 1% BSA, whereas the ratio of FFA to BSA varied depending on the concentrations of PA.

**Ceramide assay.** The ceramide isolation protocol was established from our previous study (29). EPCs were incubated at 37°C for 30 min in serum-free medium 199, EPCs were then detached by 0.05% trypsin and 0.53 mM EDTA (GIBCO-BRL) and pelleted by centrifugation. The pellet was washed three times with 10 ml of cold PBS and centrifuged again. Washed cells (5 × 10^6 cells) were resuspended in 220 μl of cold 0.25 M sucrose in PBS, transferred into a microtube, and disrupted by sonication. After the cell lysate was centrifuged at 800 g for 10 min, the supernatant containing the cytosolic fraction with the cytoplasm and organelles was withdrawn. The pellet containing the membrane fraction was then washed three times with 1 ml of cold PBS, centrifuged, and suspended in 220 μl of PBS. Lipid extractions and ceramide

![Fig. 1. Effect of palmitate (PA) on tube formation, adherence, migration capacities, and ceramide concentration of endothelial progenitor cells (EPCs). A: PA inhibited EPC adhesion and migration in a dose-dependent manner. Data are presented as means ± SE; n = 3/group. **P < 0.01; *P < 0.05. B: PA treatment impaired the vasculogenesis abilities of EPCs. Representative figures for tube formation of different groups are shown. Magnification: ×100. Scale bar = 50 μm. C: summary of tube formation for the indicated groups. D: PA inhibited the proliferation of EPCs in a dose-dependent manner. Data are presented as means ± SE; n = 3/group. *P < 0.05.](http://ajpheart.physiology.org/)
determinations from each of above fractions were carried out according to the method of Zhou et al. (30). Briefly, a 200-μl sample of each fraction was mixed with 4 ml chloroform-methanol (2:1) and extracted for its lipid contents for 30 min. After the addition of 1 ml water, the sample was vortexed and centrifuged. The lower phase containing the lipids was collected and evaporated to dryness under a nitrogen stream. Dry lipids were dissolved in 100 μl chloroform and reacted with 100 mM (+)-6-methoxy-α-methyl-2-naphthaleneacetic acid, 100 mM N,N′-dicyclohexylcarbodiimide, and 100 mM 4-dimethylaminopyridine (10 μl each) at −20°C for 3 h. After evaporation of the solvent under a nitrogen stream, the dry residue was suspended in 15 μl chloroform, mixed with 2 ml hexane, and then centrifuged. The supernatant was removed, vigorously mixed with 5 ml methanol-water (4:1), and then centrifuged again. A fraction of 1 ml of the upper phase was collected and filtered through a 0.45-μm membrane filter. The filtrate (20 μl) was injected into an Econosphere CN 5 μ column (4.6 × 250 mm, Alltech) in a HPLC system equipped with a model 600 solvent delivery system (Waters), a model 474 fluorescence detector (Waters), and a model 805 data station (Waters). Derivatized ceramide was separated from the byproducts with 3% 2-propanol in n-hexane as the mobile phase. The flow rate was 2.0 ml/min, and the eluted compounds were monitored by a fluorescence spectrophotometer at an excitation wavelength of 230 nm and emission wavelength of 352 nm.

Tube formation assay. An ECM gel (Sigma) was thawed at 4°C overnight and placed on a 96-well culture plate at 37°C for 1 h to allow solidification. EPCs treated with or without PA were harvested and replated (10,000 cells/well) on the top of the solidified ECM gel in EBM-2 medium supplemented with 0.5% BSA and VEGF (100 ng/ml). Cells were incubated at 37°C for 12 h. Tube formation was defined as a structure exhibiting a length four times its width. Networks of tubes were photographed from six randomly chosen fields with a microscope. The total length of the tube structures in each photograph was measured using Adobe Photoshop software (Adobe, San Jose, CA).

Adhesion assay. After detachment and centrifugation, EPCs were resuspended in adhesion buffer (0.5% BSA in EBM-2), and identical numbers of cells were replated onto fibronectin-coated 24-well culture plates, incubated for 30 min at 37°C, and then washed three times carefully with adhesion buffer to remove nonadherent cells. Adherent cells were counted by independent blinded investigators. Six independent fields were assessed for each well, and the average number of adherent cells was determined.

Migration assay. Migration of EPCs was performed in a Transwell chamber (Corning Costar, NewYork, NY). Briefly, EPCs were gently detached, harvested, and resuspended in chemotaxis buffer (EBM-2 with 0.5% BSA); 100 ml chemotaxis buffer containing 1 × 10⁵ cells was added to the upper compartment, and 600 ml chemotaxis buffer with 100 ng/ml VEGF was added to the lower compartment. After incubation at 37°C for 4 h, the filters were removed, and cells in the lower compartment were counted using flow cytometry with appropriate gating for 20 s at a high flow rate. The migratory rate was expressed as the percentage of input cells migrating into the lower chamber. All groups of experiments were performed in triplicate.

Proliferation assay. EPCs were isolated and incubated with different concentrations of PA for 48 h. A Vybrant MTT Cell Proliferation Assay (Life Technologies) was performed for EPC proliferation activity. Cell numbers were normalized to cells incubated in control medium.

Western blot analysis. EPCs were harvested in Western blot lysis buffer, and lysates were cleared by centrifugation at 12,000 g for 10 min at 4°C. Proteins were separated by 10% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and then probed with one of the following primary antibodies against total Akt, total eNOS, phosphorylated (p-)Akt, p-eNOS, and inducible NO synthase (iNOS). All these antibodies were polyclonal antibodies from Santa Cruz Biotechnology (Santa Cruz, CA). Primary antibodies bound to the target proteins were then detected by horseradish peroxidase-conjugated anti-rabbit IgG (Promega, Madison, WI) and visualized with enhanced chemiluminescent detection (Pierce Biotechnology, Rockford, IL). Intensities of all target bands were normalized with that of the protein loading control GAPDH band calculated by the FluorChem 8900 software system (Alpha Innotech, San Leandro, CA) (16).

Determination of NO generation. NO is unstable, but it produces the stable end products nitrite and nitrate. Hence, the best index of NO generation is the sum of nitrite and nitrate. Culture media were harvested and stored at −80°C until used for the assays. Levels of nitrite and nitrate were measured as previously described (14). Briefly, nitrate was converted to nitrite with nitrate reductase, and total nitrite was reacted with Griess reagent. Absorbance of the color product was determined at 540 nm with a spectrophotometer.

Statistical analysis. Data are generally expressed as means ± SD. SPSS software (version 11.0, SPSS, Chicago, IL) was used for statistical analyses. Statistical significance among mean values was evaluated by one-way ANOVA tests for measurement data and the Statistical analysis presented as means ± SE; n = 3/group. **P < 0.01; *P < 0.05.
least-significant-difference t-test for comparison between each other. Differences were considered significant when P values were <0.05.

RESULTS

**PA induces EPC dysfunction.** To determine the effects of PA on EPCs, we tested the adherence, migration, and tube formation abilities of EPCs with or without PA treatments. Adhesion of EPCs to the extracellular matrix is important for EPC-mediated blood vessel formation. When treated with PA, EPCs exhibited impaired adhesion to fibronectin. PA inhibited adhesion at a concentration as low as 0.2 mM (Fig. 1A). Migration is another critical function for EPCs. To assess the effect of PA on EPC migration, we tested the migratory activity of EPCs in response to VEGF. The effect of PA on EPC migration was evaluated using a modified Boyden chamber assay with VEGF as a chemotact factor. As shown in Fig. 1A, VEGF-induced augmentation of EPC migration was significantly inhibited in the PA-treated group compared with the control group. Finally, in vitro tube formation assay was also performed. As shown in Fig. 1, B and C, the capacity for tube formation of EPCs on ECM gel was significantly reduced with PA treatment. The effect of PA on proliferation of EPCs was also determined by MTT assay. As shown in Fig. 1D, PA inhibited the proliferation of EPCs in a dose-dependent manner. Taken together, our data suggest that PA treatment impaired normal functions for EPCs. Since there was no obvious difference in the inhibitory effect of PA on EPC functions between concentrations of 0.4 and 0.6 mM, we used 0.4 mM PA for the following experiments.

**PA inhibits the Akt/eNOS pathway and decreases NO production in EPCs.** The Akt/eNOS pathway plays a pivotal role in the regulation of EPC functions. We therefore investigated the effects of PA on the Akt/eNOS signaling pathway in EPCs. Immunoblot analysis showed that PA treatment inhibited Akt activation, as evidenced by the reduction of specific p-Akt (activated Akt) at Ser473 and Thr308 phosphorylation sites in a time-dependent manner (Fig. 2A).

It has been shown that FFAs can inhibit eNOS activity by reducing posttranslational phosphorylation at its Ser1177 site in ECs (29). To determine whether PA had any effect on eNOS activity in EPCs, we examined eNOS activation by measuring its phosphorylation at Ser1177. As shown in Fig. 2B, a significant reduction of eNOS phosphorylation was observed with PA treatment in a time-dependent manner. PA inhibited eNOS phosphorylation as soon as 15 min at a concentration of 0.4 mM. Since the Akt/eNOS signaling pathway was inhibited by PA, we further assessed the effect of PA on NO production. Indeed, we found that the reduction of NO was associated with...
the reduction of p-eNOS (Fig. 2, B and D). Since both eNOS and iNOS can regulate NO production, we also checked the effects of PA on iNOS expression (Fig. 2C). We found that PA treatment failed to alter iNOS expression, suggesting the selective inhibition of eNOS activity by PA (Fig. 2, B and C). We have found that PA induces the accumulation of ceramide, which mediates detrimental effects of PA in other cell types, such as ECs (29). Thus, we hypothesized that PA would induce ceramide accumulation in EPCs. Indeed, as shown in Fig. 3A, PA induced ceramide accumulation by fourfold over basal levels in EPCs.

Based on this finding, we further hypothesized that ceramide was the principal factor responsible to the inhibitory effects of PA on EPCs. To manipulate intracellular ceramide levels in EPCs, we relied on the use of a number of enzyme inhibitors to block ceramide synthesis. Ceramide biosynthesis requires the coordinate action of two enzymes, serine palmitoyltransferase and ceramide synthase. Serine palmitoyltransferase catalyzes the initial step, which involves the condensation of serine and palmitoyl-CoA to form 3-keto-sphinganine. Keto-sphinganine is a sphingolipid that is subsequently reduced to form the sphingoid base sphinganine. Ceramide synthase catalyzes sphinganine acylation, producing dihydroceramide, which is then converted to ceramide by the introduction of a trans-4,5 double bond in the sphinganine moiety. Pretreatment of EPCs with myriocin, a fungal toxin that inhibits serine palmitoyltransferase, completely prevented the PA-induced increase in ceramide levels. Fumonisin B1, a fungal toxin that inhibits ceramide synthase, also abolished the PA effect on ceramide (Fig. 3D). These results suggest that PA induced ceramide accumulation and that this was mediated by the actions of the two enzymes responsible for ceramide biosynthesis. To test the effects of ceramide on EPCs, cells were cotreated with PA and inhibitors. We found that ceramide synthesis inhibitors abolished the inhibition of the Akt/eNOS signaling pathway by PA (Fig. 3, B and C), indicating that ceramide might mediate PA-induced dysfunction of EPCs.

Ceramide metabolites do not contribute to EPC dysfunction. It has been demonstrated that ceramide can be rapidly deacylated and glucosylated to give rise to a broad array of sphingolipid-derived molecules. Thus, one question remained: is ceramide itself or its metabolite the principal mediator of the inhibitory effects of PA? To answer this question, we treated cells with an inhibitor of ceramide deacylation, N-oleoylethanolamine (NOE), that has been previously shown to increase endogenous ceramide levels by blocking its metabolism. Treatment of EPCs with NOE increased cellular ceramide to a level comparable with PA treatment alone (Fig. 3D), and NOE markedly improved the inhibition of p-Akt by PA (Fig. 3, B and C) but did not affect the total protein expression of Akt and eNOS. Thus, we demonstrated that PA induces the accumulation of ceramide, which appears to mediate PA inhibitory effects on the Akt/eNOS pathway. Furthermore, eNOS function was tested by NO generation. Consistent with the effects on eNOS phosphorylation, we found that blockade of ceramide synthesis could attenuate the inhibitory effects of PA on the generation of NO, whereas blockade of ceramide metabolism could further inhibit NO production (Fig. 3D).

**Fig. 4.** Effect of ceramide biosynthesis or ceramidase inhibitors on EPC function after PA treatment. A: PA inhibited EPC adhesion and migration. Ceramide biosynthesis inhibitor (myriocin or FB1) rescued adherence and migration abilities of EPCs after PA injury. Treatment of EPCs with NOE increased the inhibitory effect of PA. Pretreatment of EPCs with sodium nitroprusside (SNP) restored the impaired EPC function after PA and NOE treatment. Data are presented as means ± SE; n = 3/group. **P < 0.01; *P < 0.05. B: tube formation ability was obviously decreased in the PA-treated group and recovered by myriocin or FB1 treatment. The ceramidase inhibitor NOE significantly improved the inhibition of tube formation by PA. Pretreatment of EPCs with SNP restored the impaired EPC function induced by PA and NOE. Scale bar = 50 μm. C: summary of EPC tube formation in the indicated groups.
**NO production rescues ceramide-mediated EPC dysfunction.**

Ceramide mediates the inhibition effect of PA on the Akt/eNOS pathway and downstream NO production. Next, we asked whether ceramide inhibited EPCs through the Akt/eNOS pathway or whether it could work directly on EPCs. To test this hypothesis, a NO donor, sodium nitroprusside, was used. As shown in Fig. 4, B and C, pretreatment with sodium nitroprusside significantly restored the ability of EPCs for adherence, migration (Fig. 4A), and tube formation, suggesting that the inhibitory effect of ceramide on EPCs is through the Akt/eNOS pathway.

**DISCUSSION**

In the present study, we showed that Akt/eNOS phosphorylation was reduced after PA treatment, which hampered the normal bioavailability of NO and led to impaired adherence, migration, and tube formation capacity of EPCs. In addition, our results demonstrated that PA induced accumulation of ceramide in EPCs. The detrimental effects of PA on EPCs were mediated by ceramide, not its metabolites, via inhibition of the Akt/eNOS pathway.

The effect of PA on ceramide accumulation in different cell types was not extensively studied. We have previously shown that PA caused ceramide accumulation in ECs (29). It has been previously demonstrated that when grown in medium with EC growth supplement, EPCs express endothelium-specific markers and show endothelial biological characters (15). Thus, we hypothesized that PA may induced ceramide accumulation in EPCs. In the present study, we demonstrated that PA treatment significantly increased ceramide concentration in EPCs. To minimize the possibility that our observations were the result of nonspecific pharmacological effects, we used inhibitors capable of blocking separate enzymes in the various pathways. First, the fungal toxins myriocin and fumonisin B1 inhibit separate enzymes that are required for de novo ceramide synthesis (19, 25), and both protected EPCs from the accumulation of ceramide induced by PA. Second, NOE, an inhibitor of ceramidase (4), was able to aggravated the effect of PA-induced ceramide accumulation. Thus, by using various inhibitors, our data indicated that PA can induce the accumulation of ceramide in EPCs.

Akt, a serine/threonine kinase, regulates several biological processes, including cellular growth, proliferation, and survival in multiple organs (18). Akt regulates EPC functions and survival through the phosphorylation/activation of eNOS. Established evidence indicates that the Akt/eNOS pathway plays a pivotal role in the regulation of EPCs (10). In turn, eNOS modulates EPC mobilization, migration, and survival via the generation of NO (10). In an ex vivo study (17), the migration and tube formation ability of early and late EPCs were related to NO. In line with these findings, our results showed that the Akt/eNOS pathway plays an important role in the PA-induced EPC dysfunction. Adherence, migration, and tube formation capacities, eNOS and Akt phosphorylation, as well as NO synthesized by EPCs were decreased by PA treatment. Furthermore, decreased NO production seems to play a pivotal role in adherence, migration, and tube formation deficit, because preincubation of EPCs with sodium nitroprusside, a NO donor, restored proper function of EPCs. Nonetheless, the clear mechanism of PA regulating Akt/eNOS and NO release has not been addressed. Previous studies (14, 24) have indicated ceramide accumulation with simultaneous inhibition of Akt in cultured myotubes, L6 skeletal muscle cells, 3T3-L1 adipocytes, and cardiac myocytes exposed to PA. Based on our results that PA induced inactivation of the Akt/eNOS pathway accompanied by accumulation of ceramide, we hypothesized that ceramide might be one of the mechanisms that mediates cross-talk between PA and the Akt/eNOS pathway.

In humans, ceramides are collectively involved in physiological processes, such as growth regulation and apoptosis, and in pathological conditions, such as diabetes and cancers (11). A previous study (6) has also shown that ceramides mediate a multitude of distinct cellular signals. The present study revealed the relationship of ceramide accumulation and Akt/eNOS dephosphorylation in EPCs. However, we cannot study every aspect of activity of PA-ceramide signaling in EPCs, for example, PA-induced accumulation of ceramide might also cause apoptosis in EPCs and, therefore, suppress EPC functions; ceramide has also been shown to induce oxidative stresses in ECs, which might happen in EPCs as well. All of these possibilities will require further studies.

In conclusion, the present study revealed a novel mechanism that PA induced accumulation of ceramide, which appears to mediate PA inhibitory effects on the Akt/eNOS pathway, leading to an overall dysfunction of EPCs. A better understanding of the regulation of EPC function will enable the design of new pharmacological therapies or regeneration strategies related to EPCs.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**


**REFERENCES**


