Original Article

Eicosapentaenoic Acid Prevents Saturated Fatty Acid-Induced Vascular Endothelial Dysfunction: Involvement of Long-Chain Acyl-CoA Synthetase

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***Aim*: Vascular endothelial dysfunction is considered an early predictor of atherosclerosis. It has been proven that elevated blood levels of free fatty acids pose a substantial risk for the development of cardiovascular disease. In this study, we examined the effects of palmitic acid (PA), a saturated fatty acid, on endothelial function by using the expression of adhesion molecule, cytokines, and inflammatory protein as indicators, as well as investigated the effects of eicosapentaenoic acid, an n-3 polyunsaturated fatty acid.**

***Methods*: Human umbilical vein endothelial cells (HUVEC) were exposed to PA and EPA.**

***Results*: When HUVEC were exposed to PA, there was an increase in the expression of adhesion molecule, cytokines, and inflammatory protein (ICAM-1, MCP-1, interleukin-6, PTX3). PA augmented the expression of long-chain acyl-CoA synthetase (ACSL) and the cyclin-dependent kinase inhibitor p21, and enhanced the phosphorylation of p65, a component of NF-κB. ACSL inhibition and siRNA-mediated ACSL3 knockdown suppressed the PA-induced increase in the expression of adhesion molecule, cytokines, and inflammatory protein, and ACSL inhibition suppressed the enhancement of p65 phosphorylation. In addition, p21 knockdown suppressed the PA-induced increase in the expression of MCP-1 and ICAM-1. EPA suppressed the PA-induced increase in the expression of ACSL and p21, the enhancement of p65 phosphorylation, as well as the associated increase in the expression of ICAM-1, MCP-1, interleukin-6, and PTX3.**

***Conclusions*: These results suggest that the ACSL, p21, and NF-κB-dependent pathway may possibly be involved in PA-induced vascular endothelial dysfunction, and that EPA ameliorates this at least in part through the regulation of ACSL3 expression.**

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Key words: Eicosapentaenoic acid, Long-chain acyl-CoA Synthetase, Saturated fatty acid, Vascular endothelial cell

Introduction

It is known that patients with obesity[1] and type II diabetes[2] have elevated blood levels of free fatty acids. It has been reported that higher the level of free fatty acids, the greater is the risk of coronary events[3]. Serum free fatty acids also contribute to the development and progression of atherosclerosis[4-6]. On the other hand, eicosapentaenoic acid (EPA), which is an n-3 polyunsaturated fatty acid, has been shown to significantly suppress the occurrence of coronary events in the Japan EPA Lipid Intervention Study (JELIS), a large-scale clinical trial[7]. In addition, while metabolic syndrome-like patients with high serum triglyceride levels and low high-density lipoprotein cholesterol levels are at high risk for coronary artery disease, EPA reduced the event occurrence by 53% in such a higher risk group of patients[8].

Vascular endothelial cells, macrophages, and vas-

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cular smooth muscle cells play an especially important role in the development and progression of atherosclerosis. In metabolic syndrome-like patients with obesity or diabetes, blood levels of free fatty acids are increased and various inflammatory mediators such as tumor necrosis factor-alpha (TNF-α) and interleukin-1β are secreted from adipocytes and macrophages in which free fatty acids accumulate. It is known that the inflammatory response caused by these mediators induces vascular endothelial dysfunction and macrophage activation[9], leading to the development and progression of atherosclerosis. In particular, an excess of saturated fatty acids is considered a risk factor for cardiovascular disease and has been reported to trigger inflammatory signals in various tissues and promote lipotoxicity[10]. In order to elucidate the mechanism of the anti-atherogenic effect of EPA, we studied lipotoxicity in vascular cells and showed that palmitic acid (PA), a major saturated fatty acid in plasma, stimulates inflammatory cytokine production in macrophages as well as osteoblastic differentiation of vascular smooth muscle cells, and that this action could be mediated by long-chain acyl-CoA synthetase (ACSL), an enzyme that converts free fatty acids into acyl-CoA derivatives[11, 12]. Furthermore, we also reported that EPA may ameliorate lipotoxicity in macrophages and vascular smooth muscle cells through the common mechanism of ACSL expression regulation.

In our present study, we focused on vascular endothelial dysfunction, which is considered an early predictor of atherosclerosis. When vascular endothelial cells are damaged, monocyte migration, the release of adhesion molecules, inflammatory cytokines, coagulation factors, etc., and macrophage infiltration into tissues are initiated, which leads to the promotion of atherosclerosis development and progression[13]. It is known that saturated fatty acids initiate inflammatory signaling and oxidative stress in vascular endothelial cells. While this action is said to be mediated by the Toll-like receptor-4 (TLR-4) or ceramide pathways[14, 15], their involvement is controversial. The TLR-4-mediated pathway is involved in the induction of the inflammatory response associated with the activation of the transcription factor NF-κB, and p21, which is involved in cell cycle regulation, contributes to the increased expression of monocyte chemoattractant protein-1 (MCP-1) and vascular cell adhesion molecule-1 (VCAM-1) induced in vascular endothelial cells by TNF-α[16]; thereby, possibly contributing to the infiltration and adhesion of monocytes in the vascular endothelium. However, the effects of PA on p21 in vascular endothelial cells and its regulatory mechanisms are unclear. In addition, although a number of studies have reported the effects of EPA on vascular endothelial cells, its effect on vascular endothelial dysfunction induced by saturated fatty acids has not been reported.

In this study, we investigated the mechanism of PA-induced vascular endothelial dysfunction and the effect of EPA on this by using the expression of adhesion molecules, cytokines, and inflammatory protein in human umbilical vein endothelial cells (HUVEC) as indicators. The results of our investigation show that PA-induced vascular endothelial dysfunction is partially mediated by the NF-κB-dependent pathway and the p21 pathway through an increase in ACSL expression. In addition, we show that EPA attenuates PA-induced vascular endothelial dysfunction possibly through the regulation of ACSL expression.

Materials and Methods

Materials

Eicosapentaenoic acid sodium salt (EPA-Na) was purchased from Nu-Chek Prep, Inc. (Elysian, MN), sodium palmitate, and lipopolysaccharide (LPS; Escherichia coli 0111:B4) from Sigma (St. Louis, MO), triacsin C from StressMarq Bioscience Inc. (Victoria, BC), hypoestoxide, and myriocin from Merck KGaA (Darmstadt, Germany). For the preparation of medium containing EPA or palmitic acid (PA), sodium eicosapentaenoate and sodium palmitate solutions were prepared in ethanol or methanol, respectively, and added to the cell culture medium before use.

Cell Culture

Human umbilical vein endothelial cells (HUVEC) were obtained from Kurabo Industries Ltd. and maintained in HuMedia-EG2 medium (HuMedia-EB2 supplemented with 2% FBS, 10 ng/mL hEGF, 1.34 μg/mL hydrocortisone, 5 ng/mL hFGF-B, 10 μg/mL heparin, Kurabo) at 37℃ in humidified atmosphere with 5% CO_2. HUVEC were seeded in plates and allowed to attach for 1 day. Next, HUVEC were exposed to PA, LPS, EPA, triacsin C (ACSL inhibitor), hypoestoxide (IκB kinase inhibitor), or myriocin [serine palmitoyltransferase long chain base subunit 1 (SPTLC1) inhibitor] in HuMedia-EG2 medium for 1 day. After treatment, HUVEC were harvested for quantitative real-time polymerase chain reaction (PCR) and western blotting, and conditioned medium was harvested for enzyme-linked immunosorbent assay (ELISA).

ELISA

The concentrations of MCP-1 and IL-6 in cell

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Table 1. List of TaqMan® gene expression assay kits

Gene name	Assay ID
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Hs02758991_g1
Chemokine (C-C motif) ligand 2 (MCP-1)	Hs00234140_m1
Intercellular adhesion molecule 1 (ICAM-1)	Hs00164932_m1
Interleukin 6 (IL-6)	Hs00985639_m1
Pentraxin3, long (PTX3)	Hs00173615_m1
Toll-like receptor 4 (TLR-4)	Hs00152939_m1
Acyl-CoA synthetase long-chain family member 1 (ACSL1)	Hs00960561_m1
Acyl-CoA synthetase long-chain family member 3 (ACSL3)	Hs00244853_m1
Acyl-CoA synthetase long-chain family member 4 (ACSL4)	Hs00244871_m1
Acyl-CoA synthetase long-chain family member 5 (ACSL5)	Hs00212106_m1
Cyclin-dependent kinase inhibitor 1A (p21)	Hs00355782_m1
Cyclin-dependent kinase 2 (CDK2)	HS01548894_m1
Selectin E (E-selectin)	Hs00950401_m1
Nitric oxide synthase 3 (eNOS)	Hs01574659_m1
Serpin peptidase inhibitor (PAI-1)	Hs01126606_m1
Serpine palmitolytransferase, long chain base subunit 1 (SPTLC1)	Hs00272311_m1

culture supernatants were quantified by commercial ELISA kits (Human MCP-1 ELISA Kit, ThermoScientific, Rockford IL, and Human IL-6 Immunoassay, R&D systems, Minneapolis, MN) according to the manufacturer's instructions, and normalized to the total protein content of HUVEC.

Gene Expression Analysis

Total RNA was extracted using TRIzol® reagent (Life Technologies, Carlsbad, CA), and purified by Purelink Micro-to-Midi Total RNA Purification System (Life Technologies) according to the manufacturer's instructions. cDNA was generated using a SuperScript™ Ⅲ First-Strand Synthesis System (Life Technologies). Quantitative real-time PCR was performed using an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Gene expression levels were measured using a TaqMan® Gene Expression Assay (Applied Biosystems) and the specific primers and TaqMan probes used are shown in **Table 1**. The mRNA levels were normalized to GAPDH.

Western Blotting

HUVEC were suspended in RIPA buffer (Thermo Fisher Scientific, Waltham, MA) with protein inhibitors (Thermo Fisher Scientific). The samples were separated by SDS-PAGE. Western blotting was performed using anti-phospho-NF-κB p65 (Ser536) (Cell Signaling Technology, Danvers, MA), anti-p21 (Cell Signaling Technology), and anti-ACSL3 (Santa Cruz Biotechnology, Dallas, TX) antibodies. Anti-β actin antibody (Cell Signaling Technology) was used as the internal control. Blots were visualized with Pierce Western Blotting Substrate Plus (Thermo Fisher Scientific).

Transfection of siRNA

Small interfering RNAs (siRNA) targeted to human ACSL1 (s4995, Ambion, Austin, TX), human ACSL3 (s4997, Ambion), human ACSL4 (s5000, Ambion), CDKN1A (s417, Ambion), SPTLC1 (s20710, Ambion), TLR4 (s14195, Ambion), and control siRNA (Silencer® Select Negative Control #1 siRNA, Ambion) were used in the study. HUVEC were transfected with siRNA using Lipofectamine™ RNAiMAX Reagent (Thermo Fisher Scientific). Several hours after transfection, the medium was replaced by HuMedia-EG2 supplemented with 2% FBS.

Cellular Fatty Acid Composition of HUVEC

Fatty acid composition was analyzed by gas chromatography (GC-2010PIAF Shimadzu Corp.) and normalized by dividing it by the amount of total cellular protein content. Protein concentrations were measured by BCA protein assay kit (Thermo Fisher Scientific).

Statistical Analysis

Results are presented as the means ± S.E.M. Statistical analysis was performed with SAS/STAT® 9.3 Software (SAS institute, Cary, NC). Comparison of the results between two groups was performed using the t-test. The results between multiple groups were compared by the Dunnett's test. Findings with a $p<$

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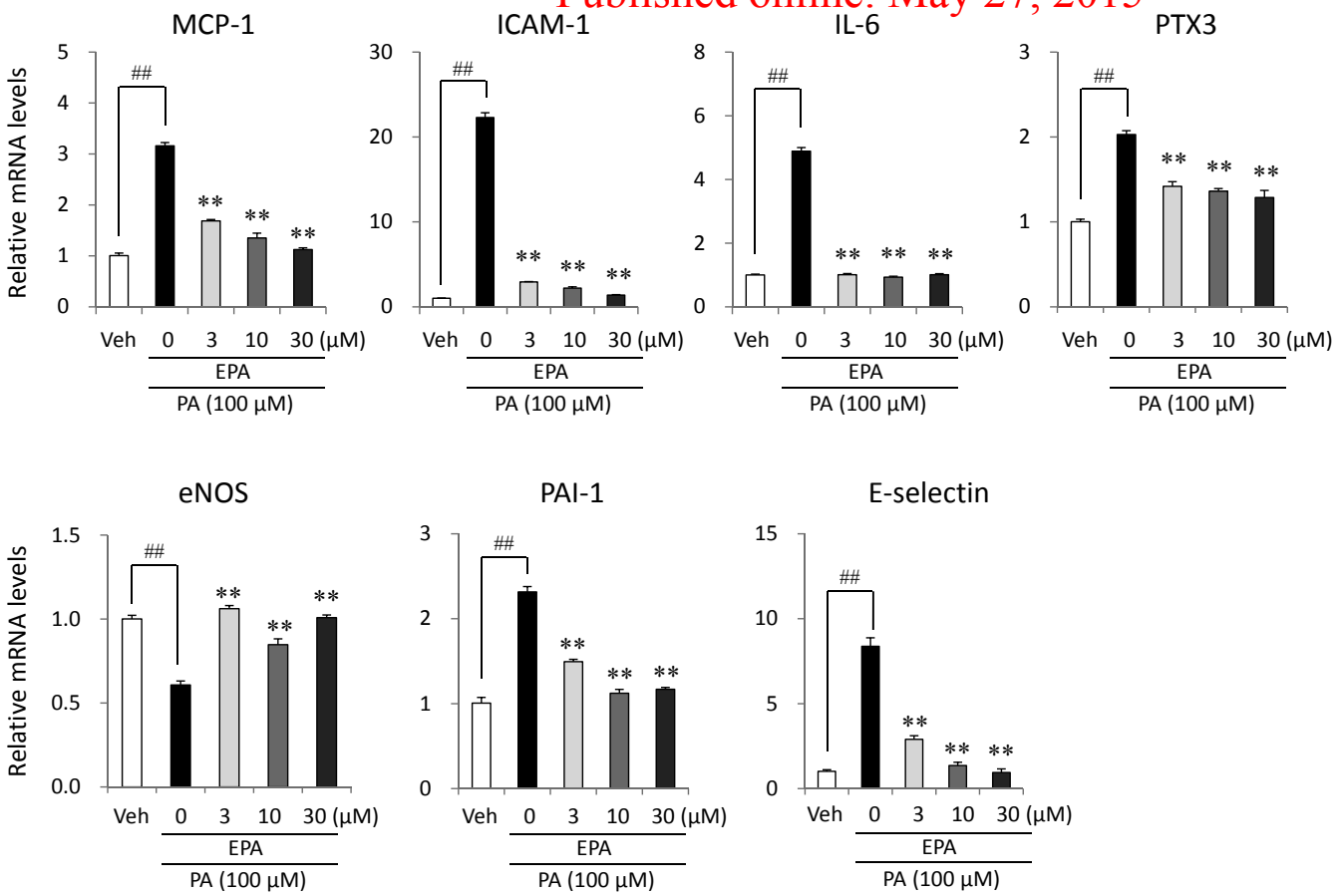

Fig. 1. Effects of EPA on the expressions of vascular endothelial cell function-related genes in PA-stimulated HUVEC.

HUVEC were treated with EPA (3, 10 or 30 μM) in addition to PA (100 μM) for 1 day. The mRNA levels of the indicated factors were analyzed by real-time PCR and normalized to GAPDH. $^{\#\#}p<0.01$ vs vehicle group, $^{**}p<0.01$ vs PA group. All values are expressed as arbitrary units and are presented as the mean ± S.E.M. ($n=4$).

Table 2. Effect of EPA on the secretions of MCP-1 and IL-6 in PA-stimulated HUVEC

	Vehicle	PA (100 μM)	PA (100 μM) + EPA (10 μM)
MCP-1 (pg/mg protein)	5.8 ± 1.1	46.5 ± 9.8$^{\#}$	11.3 ± 4.4*
IL-6 (pg/mg protein)	0.37 ± 0.06	0.90 ± 0.12$^{\#\#}$	0.49 ± 0.04*

HUVEC were treated with EPA (10 μM) in addition to PA (100 μM) for 1 day.
MCP-1 and IL-6 levels in cell culture supernatants were measured by ELISA.
$^{\#}p<0.05$, $^{\#\#}p<0.01$ vs vehicle group, $^{*}p<0.05$ vs PA group.
Values are presented as the mean ± S.E.M. ($n=4$).

0.05 were considered significant ($^{\#}$ or *: $p<0.05$; $^{\#\#}$ or **: $p<0.01$).

Results

EPA Attenuates PA-Induced Vascular Endothelial Dysfunction

To investigate the effects of PA and EPA on vascular endothelial function, HUVEC were stimulated with PA or PA plus EPA. PA increased the gene expressions of MCP-1, intercellular adhesion molecule-1 (ICAM-1), interleukin-6 (IL-6), pentraxin 3 (PTX3), plasminogen activator inhibitor-1 (PAI-1), endothelial-selectin (E-selectin), while the expression of endothelial nitric oxide synthase (eNOS) was decreased. Secretions of MCP-1 and IL-6 increased

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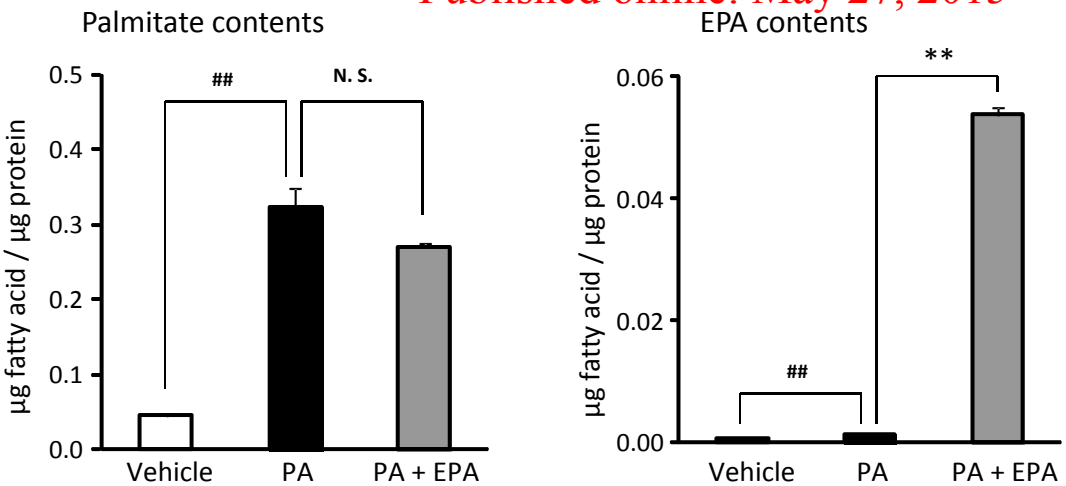

Fig. 2. Cellular fatty acid contents of HUVEC treated with PA and EPA.

HUVEC were treated with EPA (10 μM) and/or PA (100 μM) for 1 day, all cells were then harvested and each fatty acid levels and total cell protein levels were estimated. Fatty acid levels were normalized to total cell protein content. $^{\#\#}p<0.01$ vs vehicle group, $^{**}p<0.01$ vs PA group. N.S., not significant. All values are expressed as the mean ± S.E.M. ($n=3$).

after the addition of PA. In contrast, EPA strongly inhibited these PA-induced changes in gene expression and cytokine secretion (**Fig. 1** and **Table 2**).

Intracellular PA and EPA Contents in Treated HUVEC

The contents of intracellular PA and EPA markedly increased after each was separately added to HUVEC (**Fig. 2**). Similar intracellular contents of PA were attained when PA and EPA were added simultaneously, and the presence of EPA had no effect on the increase in PA content (**Fig. 2**). Therefore, the improved effect of EPA on the PA-induced phenomena does not appear to be due to changes in the uptake of PA.

ACSL is Involved in PA-Induced Vascular Endothelial Dysfunction

Fig. 3A shows changes in the gene expressions of ACSL isoforms in HUVEC stimulated with PA or PA plus EPA. The up-regulation of ACSL1, 3, 4, 5 gene expressions induced by PA was suppressed by EPA. The change in ACSL3 expression was larger than that of the ACSL1, 4, 5 expressions (**Fig. 3A** and **3B**). During the time course of the effect of PA and EPA on ACSL3 and MCP-1, one of the proinflammatory cytokines, the gene expressions were investigated as shown in **Fig. 3D**. This fluctuation of both gene expressions was similar during this time course. EPA suppressed the increases in the gene expression of ACSL3 and MCP-1 by PA in all points. Triacsin C, an inhibitor of ACSL, and siRNA-mediated knockdown of ACSL3 both significantly inhibited PA-induced changes in the expression of vascular endothelial function-related genes (**Fig. 3C**, **4A** and **4B**). We also examined whether ACSL1 and 4 were involved in the vascular endothelial dysfunction caused by PA, and found that siRNA-mediated knockdown of ACSL1, but not ACSL4, suppressed the PA-induced changes in MCP-1 and ICAM-1 mRNA levels (**Fig. 4C** and **4D**).

TLR-4 is Involved in LPS-Induced Vascular Endothelial Dysfunction, but it is not Involved in PA-Induced Vascular Endothelial Dysfunction

We examined whether TLR-4 was involved in PA-induced vascular endothelial dysfunction. The siRNA-mediated knockdown of TLR-4 expression did not significantly suppress the PA-induced changes in the expression of vascular endothelial function-related genes (**Fig. 5A**). LPS is well known as a TLR-4 agonist, and we confirmed that LPS also increased MCP-1, ICAM-1, and IL-6 mRNA levels and that these changes were significantly suppressed by the knockdown of TLR-4 expression (**Fig. 5B**).

Ceramide Synthesis is not Involved in PA-Induced Vascular Endothelial Dysfunction

We next examined whether ceramide, a metabolite of PA, was involved in the vascular endothelial dysfunction caused by PA. HUVEC were treated with PA or PA plus myriocin, an inhibitor of serine palmitoyltransferase. Myriocin did not suppress the PA-induced changes in the expression of vascular endo-

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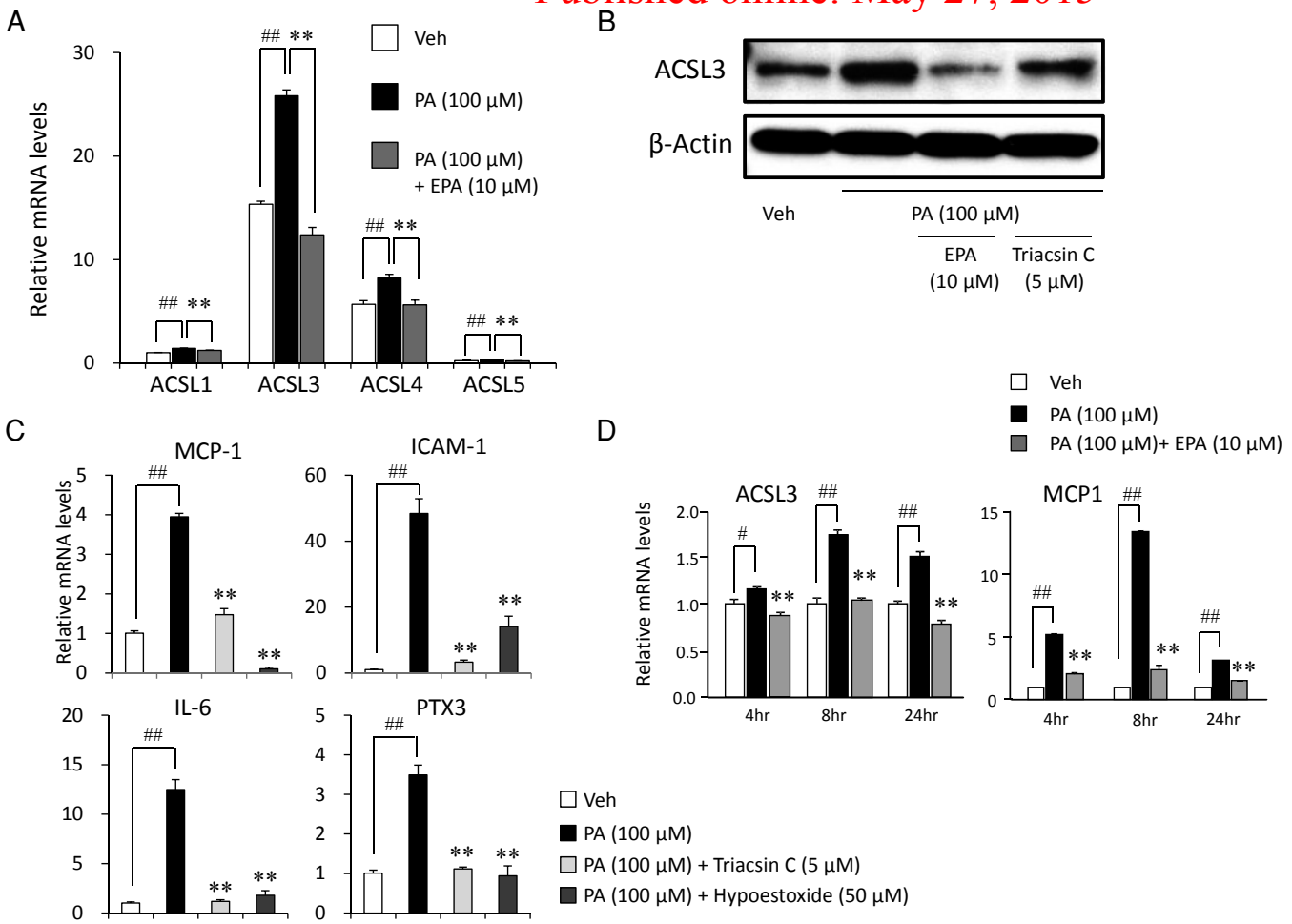

Fig. 3. The effect of EPA on the expressions of ACSLs and the effect of ACSL inhibitor or IκB kinase inhibitor on the expressions of MCP-1, ICAM-1, IL-6, and PTX3 in PA-stimulated HUVEC.

(A) HUVEC were treated with EPA (10 μM) in addition to PA (100 μM) for 1 day. (B, C) HUVEC were treated with the indicated reagents in addition to PA (100 μM) for 1 day. (D) HUVEC were treated with EPA (10 μM) in addition to PA (100 μM) for the indicated period. (B) The expression of ACSL3 protein was determined by western blotting. (A, C, D) The mRNA levels of the indicated factors were analyzed by real-time PCR and normalized to GAPDH. $^{\#\#}p<0.01$ vs vehicle group, $^{**}p<0.01$ vs PA group. All values (A, C, and D) are expressed as arbitrary units and are presented as the mean ± S.E.M. ($n=4$).

thelial function-related genes (**Fig. 6A**), while siRNA-mediated knockdown of SPTLC1 expression also did not suppress the PA-induced changes (**Fig. 6B**).

NF-κB and p21 are Involved in PA-Induced Vascular Endothelial Dysfunction

Fig. 7A and **7B** show the results of western blotting of NF-κB (phospho-p65) and p21. The enhanced NF-κB phosphorylation caused by PA was suppressed by the addition of EPA. Meanwhile, PA increased p21 protein and gene expression, thereby leading to a decrease in the expression of cyclin-dependent kinase 2 (CDK2) (**Fig. 7B** and **7C**). Consistent with these findings, the addition of hypoestoxide, an IκB-kinase inhibitor, inhibited the PA-induced changes in the expression of vascular endothelial function-related genes (**Fig. 3C**). In addition, the siRNA-mediated knockdown of p21 expression suppressed the PA-induced changes in MCP-1 and ICAM-1 mRNA levels (**Fig. 7D** and **7E**). Triacsin C also clearly inhibited the PA-induced NF-κB (p65) phosphorylation (**Fig. 7A**). Furthermore, PA-induced changes in p21 and CDK2 mRNA levels were suppressed by triacsin C but not by hypoestoxide (**Fig. 7F**).

Discussion

The present study demonstrated that EPA sup-

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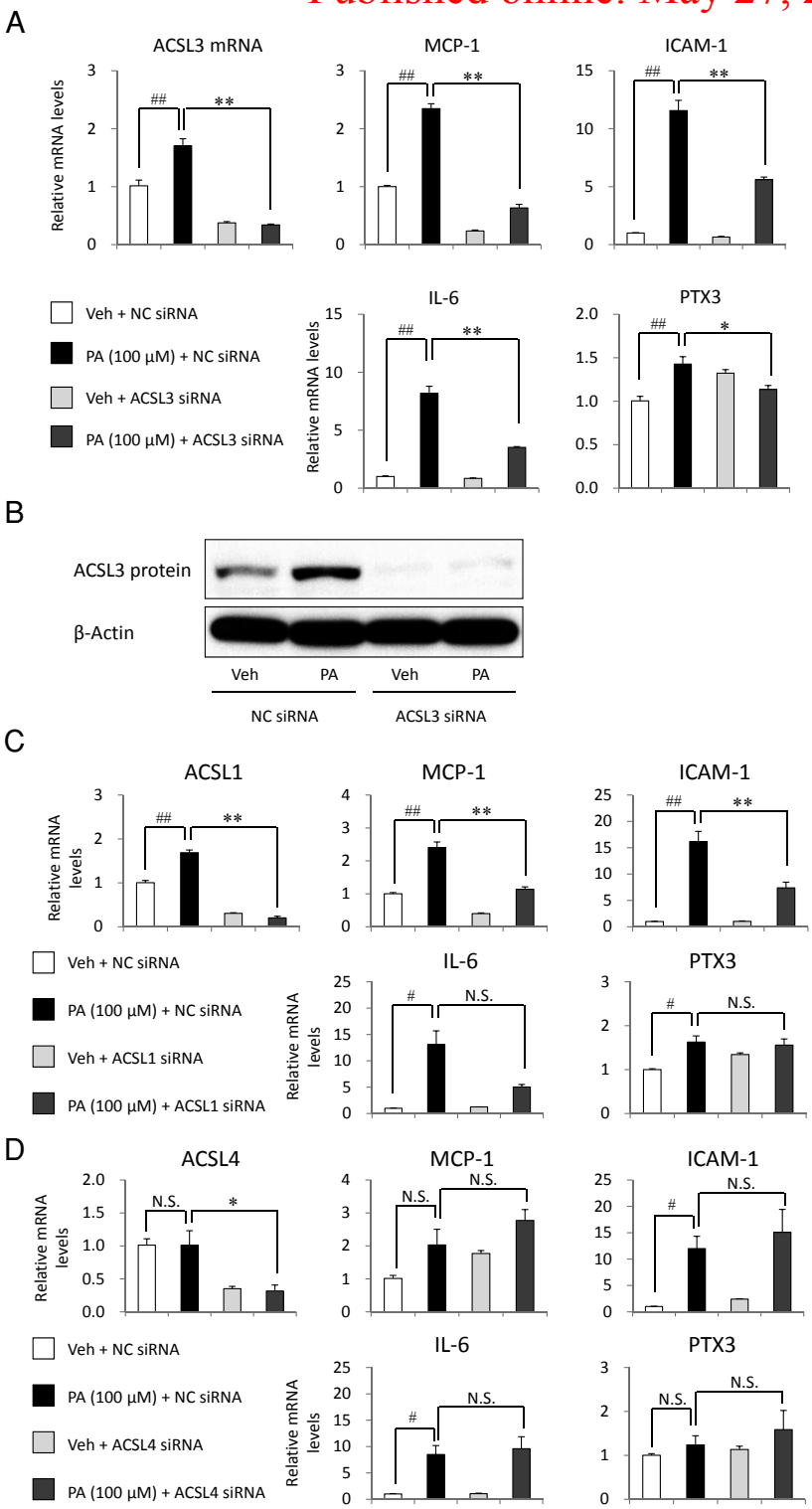

Fig. 4. Effects of ACSLs-knockdown on the expressions of MCP-1, ICAM-1, IL-6, and PTX3 in PA-stimulated HUVEC.

(A, B, C, D) HUVEC were transfected with siRNA targeting ACSL3, ACSL1, ACSL4, or negative control siRNA (NC siRNA), and then treated with PA (100 μM) for 1 day. (B) The levels of ACSL3 protein were determined by western blotting. (A, C, D) The mRNA levels of the indicated factors were analyzed by real-time PCR and normalized to GAPDH. $^{\#}p<0.05$, $^{\#\#}p<0.01$ (vehicle + NC siRNA group vs PA + NC siRNA group), $^{*}p<0.05$, $^{**}p<0.01$ (PA + NC siRNA group vs PA + ACSLs siRNA). N.S., not significant. All values are expressed as arbitrary units and are presented as the mean ± S.E.M. ($n=4$).

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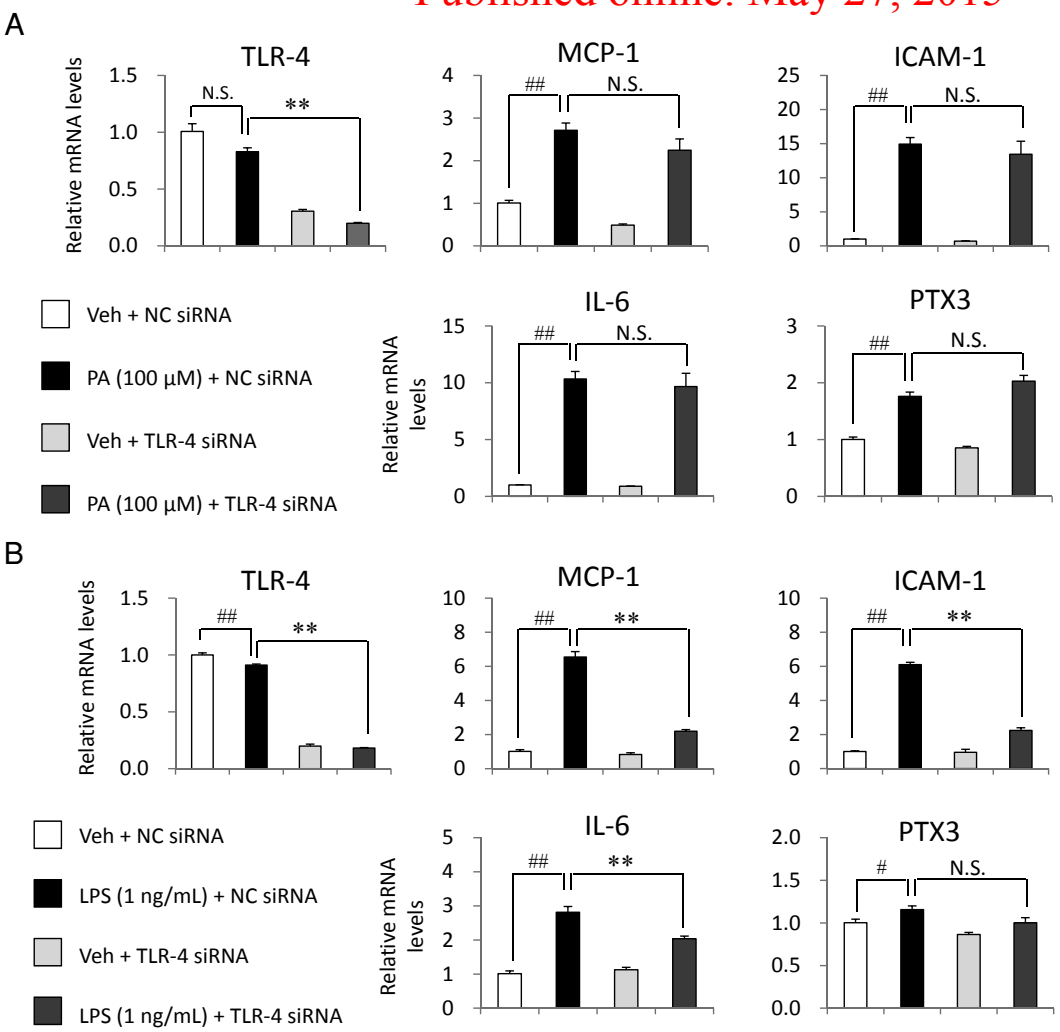

Fig. 5. Effects of TLR-4-knock down on the expressions of MCP-1, ICAM-1, IL-6, and PTX3 in PA- or LPS-stimulated HUVEC.

(A, B) HUVEC were transfected with siRNA targeting TLR-4 or negative control siRNA (NC siRNA), and then treated with PA (100 μM) or LPS (1 ng/mL) for 1 day. The mRNA levels of the indicated factors were analyzed by real-time PCR and normalized to GAPDH. $^{\#}p<0.05$, $^{\#\#}p<0.01$ (vehicle + NC siRNA group vs PA or LPS + NC siRNA group), $^{**}p<0.01$ (PA or LPS + NC siRNA group vs PA or LPS + TLR-4 siRNA). N.S., not significant. All values are expressed as arbitrary units and are presented as the mean ± S.E.M. ($n=4$).

presses the PA-induced up-regulation of the expressions of adhesion molecules, cytokines, and inflammatory protein in vascular endothelial cells. While saturated fatty acid-induced macrophage activation[17], dendritic cell maturation[18], and increased expression of monocyte chemoattractant protein in vascular endothelial cells[19] were previously considered to be mediated by TLR-4, recent reports suggest that PA-induced stimulation of the macrophage inflammatory response may be mediated by other pathways in addition to the TLR-4 pathway[20]. Indeed, because TLR-4 knockdown did not suppress the PA-induced increase in the expression of adhesion molecules, cytokines, and inflammatory protein in the present study, it is possible that saturated fatty acid-induced lipotoxicity may be mediated by a mechanism other than TLR-4. The results of our study indicate that PA-induced vascular endothelial dysfunction is mediated in part by the increased expression of ACSL and the associated activation of NF-κB and p21-dependent pathways, and EPA may attenuate this vascular endothelial dysfunction possibly through the regulation of the expression of ACSL3. We have previously reported that EPA inhibits the saturated fatty acid-induced inflammatory

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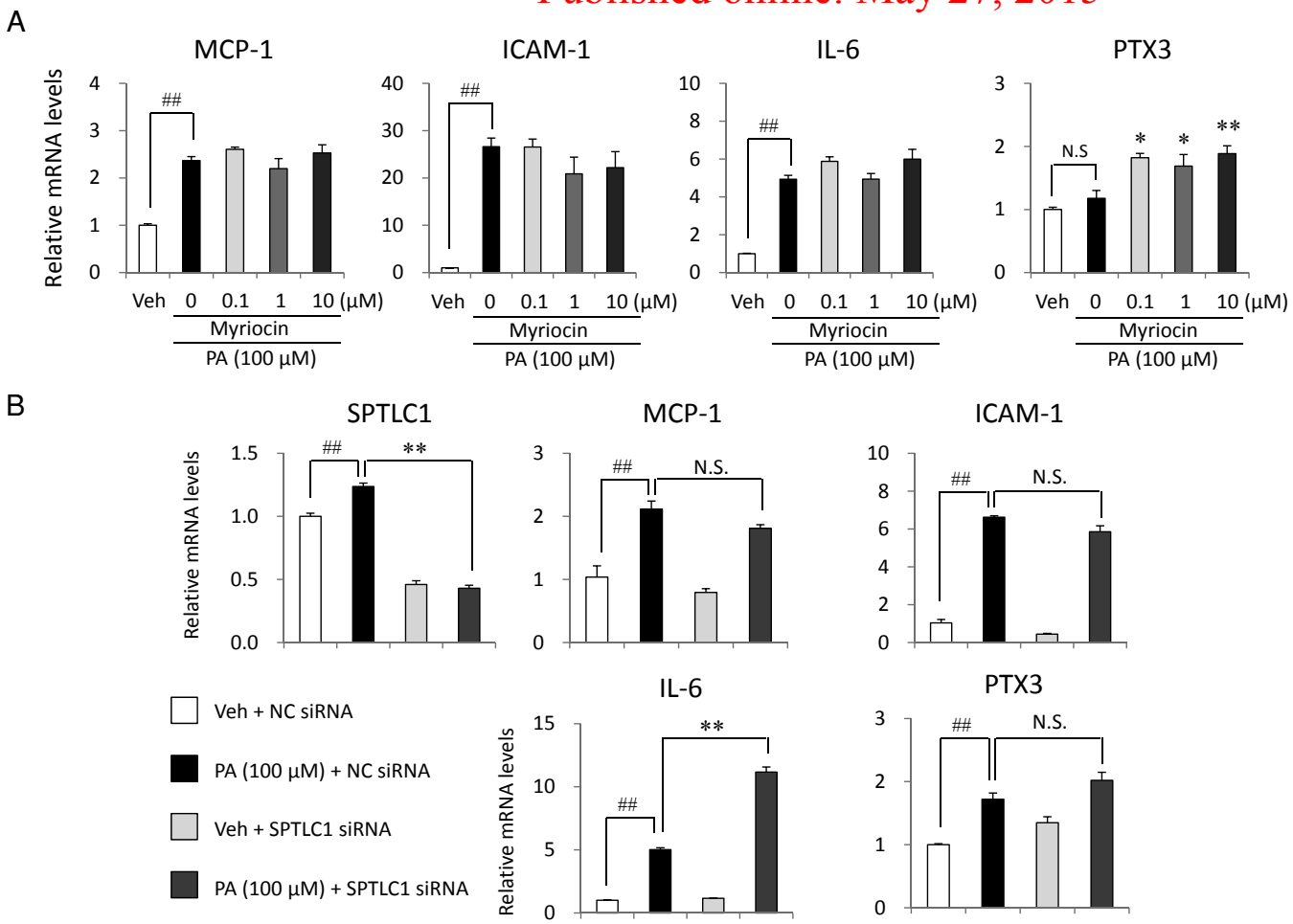

Fig. 6. Effects of ceramide synthesis inhibitor or SPTLC1-knock down on the expressions of MCP-1, ICAM-1, IL-6, and PTX3 in PA-stimulated HUVEC.

(A) HUVEC were treated with myoricin (0.1, 1, or 10 μM) in addition to PA (100 μM) for 1 day. The mRNA levels of the indicated factors were analyzed by real-time PCR and normalized to GAPDH. $^{\#\#}p<0.01$ vs vehicle group, $^{*}p<0.05$, $^{**}p<0.01$ vs PA group. (B) HUVEC were transfected with siRNA targeting SPTLC1 or negative control siRNA, and then treated with PA (100 μM) for 1 day. The mRNA levels of the indicated factors were analyzed by real-time PCR and normalized to GAPDH. $^{\#\#}p<0.01$ (vehicle + NC siRNA group vs PA + NC siRNA group), $^{**}p<0.01$ (PA + NC siRNA group vs PA + SPTLC1 siRNA). N.S., not significant. All values are expressed as arbitrary units and are presented as the mean ± S.E.M. ($n=4$).

cytokine production in macrophages and osteoblastic differentiation of vascular smooth muscle cells through ACSL. The results of this study suggest that saturated fatty acids initiate lipotoxicity in the cells involved in atherosclerosis, including vascular endothelial cells, through ACSL as the common mechanism, and this is inhibited by EPA.

In this study, we first found that in HUVEC, PA induces the expression of MCP-1, ICAM-1, IL-6, PTX3, PAI-1, and E-selectin, down-regulates eNOS expression, and promotes the secretion of MCP-1 and IL-6, while EPA inhibits these changes (**Fig. 1** and **Table 2**). As mentioned previously, it is known that the serum levels of free fatty acids are elevated in metabolic syndrome-like patients such as those with obesity or diabetes, and it has been reported from clinical research that saturated fatty acids impair vascular endothelial function[21]. Because vascular endothelial function is impaired in metabolic syndrome-like patients[22], saturated fatty acid-induced vascular endothelial dysfunction could be occurring in these diseases. On the other hand, EPA showed a marked efficacy in suppressing coronary event occurrence in metabolic syndrome-like patients who had high serum triglyceride levels and low high-density lipoprotein cholesterol levels[8]. Furthermore, it has been reported that when

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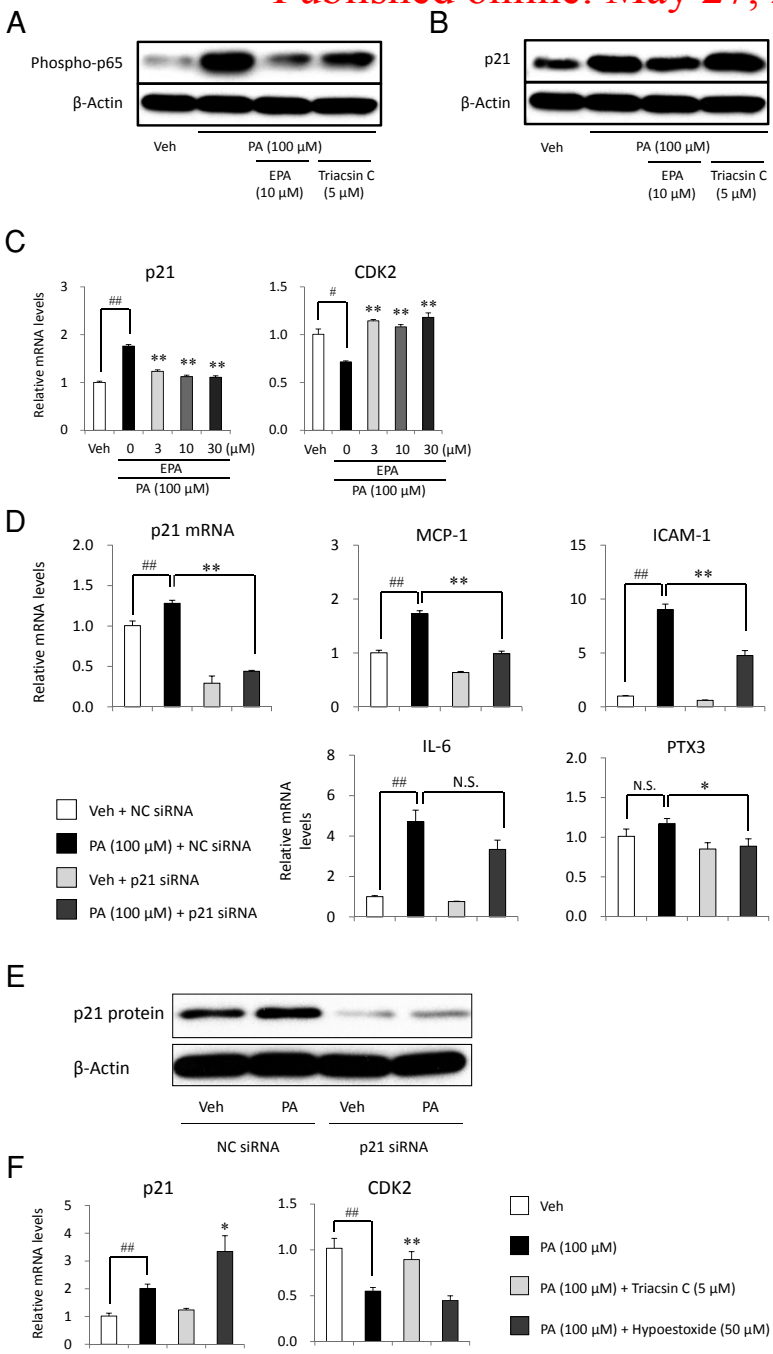

Fig. 7. NF-κB and p21 expressions as well as the effect of p21-knock down on the expressions of MCP-1, ICAM-1, IL-6, and PTX3 in PA-stimulated HUVEC.

(A, B) HUVEC were treated with EPA (10 μM) and triacsin C (5 μM) in addition to PA (100 μM) for 1 day. The expressions of phospho-p65 and p21 protein were determined by western blotting. (C) HUVEC were treated with EPA (3, 10 or 30 μM) in addition to PA (100 μM) for 1 day. The mRNA levels of the indicated factors were analyzed by real-time PCR and normalized to GAPDH. $^{\#}p<0.05$, $^{\#\#}p<0.01$ vs vehicle group, $^{**}p<0.01$ vs PA group. (D, E) HUVEC were transfected with siRNA targeting p21 or negative control siRNA, and then treated with PA (100 μM) for 1 day. (D) The mRNA levels of the indicated factors were analyzed by real-time PCR and normalized to GAPDH. $^{\#\#}p<0.01$ (vehicle + NC siRNA group vs PA + NC siRNA group), $^{*}p<0.05$, $^{**}p<0.01$ (PA + NC siRNA group vs PA + p21 siRNA). (E) The levels of p21 protein were determined by western blotting. (F) HUVEC were treated with triacsin C (5 μM) or hypoestoxide (50 μM) in addition to PA (100 μM) for 1 day. The mRNA levels of the indicated factors were analyzed by real-time PCR and normalized to GAPDH. $^{\#\#}p<0.01$ vs vehicle group, $^{*}p<0.05$, $^{**}p<0.01$ vs PA group. N.S., not significant. All values (C, D, E) are expressed as arbitrary units and are presented as the mean ± S.E.M. ($n=4$).

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EPA is administered to metabolic syndrome patients, the plasma levels of soluble ICAM-1 (sICAM-1) and soluble VCAM-1 (sVCAM-1) decrease[23]. Some clinical studies have reported that PTX3, a vascular-specific inflammatory marker associated with atherosclerosis and ischemic heart disease, shows a negative correlation with blood EPA levels[24]. The results of our basic research may be observed to support these clinical reports, and the suppressive action on coronary events and anti-atherogenic action that EPA demonstrated in JELIS may possibly be attributed to its effect in ameliorating vascular endothelial function.

Saturated fatty acid-induced lipotoxicity has been previously considered to be mediated by the TLR-4 or ceramide pathways[14, 15], although their contributions are controversial. In this study, we focused on ACSL as one of the previously proposed mechanisms of action of PA on macrophages and vascular smooth muscle cells. In mammals, ACSL has five isoforms, each of which has a different tissue distribution and substrate specificity[25]. Triacsin C is known to inhibit ACSL1, 3, and 4 activation[26]. In macrophages and vascular smooth muscle cells, the expression of at least ACSL1, 3, and 4 has been confirmed, and it is known that PA induces ACSL expression in these cells[11, 12]. In this study, we demonstrated that PA augmented the expression of ACSL isoforms in vascular endothelial cells as well, in particular ACSL3, while EPA inhibited the increase in their expressions (**Fig. 3A** and **3B**). We then investigated the effect of the ACSL inhibitor triacsin C and the effect of knockdown of each ACSL isoform on PA-induced lipotoxicity. The results showed that the knockdown of ACSL1 and 3 led to suppression of the PA-induced increase in the expression of adhesion molecule and some cytokines, while the knockdown of ACSL4 only had a minor effect (**Fig. 4A**, **4B**, **4C** and **4D**). Therefore, ACSL1 and ACSL3 may be partially involved in the action of PA, and EPA may act by inhibiting these PA-induced increases in ACSL expression. It was reported that ACSL1 had relatively high substrate specificity for PA compared with ACSL4[26]. This difference may partially contribute to the result that the knockdown of ACSL4 did not affect endothelial dysfunction caused by PA. There are reports that atheroclerotic lesions are significantly suppressed when triacsin C is administered to low density lipoprotein receptor-knockout (LDLR-KO) mice, a mouse model of atherosclerosis[27], and that ACSL1 is involved in the development of atherosclerosis augmented by diabetes in LDLR-KO mice[28]. On the other hand, there are arguments that ACSL1 is not necessarily required for saturated fatty acid-induced inflammation or apoptosis given a report that there is no difference in the degree of saturated fatty acid-induced MCP-1 secretion and degree of apoptosis between microvascular endothelial cells derived from ACSL1-KO or wild-type mice, as well as reports that saturated fatty acid-induced vascular endothelial cell apoptosis and sVCAM-1 or sICAM-1 shedding are not influenced by siRNA-mediated ACSL1 knockdown[29]. In our study, the fatty acid-induced change in ACSL3 expression in human vascular endothelial cells was the greatest, and amongst all the ACSL isoforms, ACSL3 knockdown was the one that clearly inhibited the saturated fatty acid-induced increase in the expression of adhesion molecules, cytokines, and inflammatory protein, thereby suggesting that the involvement of ACSL3 in saturated fatty acid-induced vascular endothelial dysfunction is relatively large. However, further research is needed because the mechanism of ACSL regulation by PA or EPA in cells involved in atherosclerosis has not been elucidated.

We also investigated the role of TLR-4 in PA-induced up-regulation of the expressions of adhesion molecules, cytokines, and inflammatory protein in vascular endothelial cells. The increase in expression of these factors triggered by LPS, the action of which is known to be mediated through the TLR-4-dependent pathway, was significantly inhibited by TLR-4 knockdown (**Fig. 5B**), whereas the siRNA-mediated TLR-4 knockdown did not affect the PA-induced up-regulated expression of these factors (**Fig. 5A**). On the other hand, vascular endothelial dysfunction may be regulated by intracellular palmitoyl-CoA and its metabolites after PA is taken up into cells. Ceramide, one of the palmitoyl-CoA metabolites, has been reported to be involved in the inhibition of the saturated fatty acid-induced Akt/eNOS pathway in vascular endothelial cells[30]. However, PA-induced vascular endothelial dysfunction was not ameliorated by myriocin, which is an inhibitor of SPTLC1, a key enzyme in ceramide synthesis, or by SPTLC1 knockdown in our study (**Fig. 6A** and **6B**). Therefore, we concluded that under the conditions of the present study, TLR-4 and ceramide play a minor role in the PA-induced increase in the expression of adhesion molecules and inflammatory cytokines.

NF-κB is an important transcription factor in the regulation of inflammatory signaling. Hypoestoxide, an IκB kinase (IKK) inhibitor, suppressed the PA-induced increase in the expression of adhesion molecules, cytokines, and inflammatory protein in vascular endothelial cells (**Fig. 3C**), while PA-induced NF-κB activation (p65 phosphorylation) was suppressed by EPA and ACSL inhibitor (**Fig. 7A**). These findings

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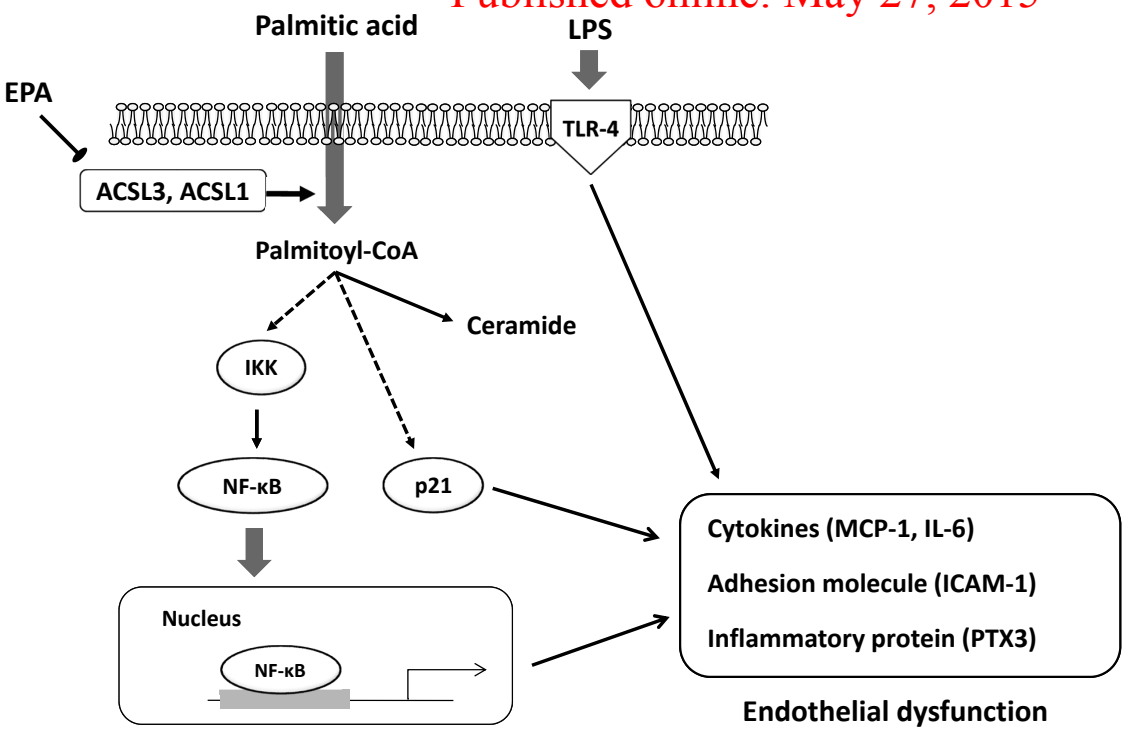

Fig. 8. Pathways for PA-induced vascular endothelial dysfunction.

PA increased the expressions of cytokines, adhesion molecules, and inflammatory protein by activating NF-κB and p21 pathways in HUVEC, where ACSL3 may play an important role in this mechanism. Meanwhile, EPA suppresses the up-regulation of these cytokines, adhesion molecules, and inflammatory protein induced by PA.

suggest that the effect of PA and EPA on vascular endothelial cells is mediated by changes in the IKK-NF-κB pathway that is associated with the regulation of ACSL expression. In addition, we found that PA enhances the expression of p21, which is involved in cell cycle regulation, and inhibits the expression of CDK2 downstream of p21. EPA inhibited these PA-induced changes in expression (**Fig. 7B** and **7C**). It has been reported that p21 is involved in the enhancement of MCP-1 and VCAM-1 expression, which is induced in vascular endothelial cells by TNF-α [16], and the results of experiments that crossed apoE-KO mice with p21-KO mice also suggest that p21 is a proatherogenic molecule [31]. Our study also demonstrated that p21 knockdown significantly suppressed the increase in MCP-1 and ICAM-1 expressions induced by PA (**Fig. 7D** and **7E**). Although EPA showed an inhibitory effect on the PA-induced increase in p21 expression, given the mild inhibitory effect of ACSL inhibitor, there is also the possibility that p21 is mediated by a pathway other than ACSL (**Fig. 7C** and **7F**). The involvement of mitogen-activated protein kinase (MAPK), the transcription factor activator protein 1 (AP-1), and p53 in the regulation of p21 has been suggested [32, 33]. Because p21 expression was not attenuated by IKK inhibitor in our investigation, the PA-induced increase in p21 expression may be a factor independent of the IKK-NF-kB pathway. It has been demonstrated that in THP-1 macrophages, PA stimulates the phosphorylation of p38 MAPK while EPA inhibits it [11], and EPA has also been shown to inhibit LPS-induced AP-1 activation [34]. Therefore, the changes in p21 expression in vascular endothelial cells induced by PA or EPA are consistent with previous reports. Although further research is needed to investigate this phenomenon, the up-regulation of p21 expression in vascular endothelial cells by PA and the inhibitory effect of EPA on this change are new findings. The results described above suggest that the activation of the IKK-NF-kB and p21 pathways accompanying the increase in ACSL expression plays an important role in the induction of vascular endothelial dysfunction by PA, in the form of enhanced expression of adhesion molecules, cytokines, and inflammatory protein (**Fig. 8**).

The above results suggest that the inhibitory effect of EPA against PA-induced vascular endothelial dysfunction is at least partly mediated by the inhibi-

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tion of ACSL expression, and that this involves associated changes in the NF-κB and p21 pathways. In JELIS, EPA was reported to decrease cardiovascular events independent of LDL cholesterol, and as previously mentioned, vascular endothelial dysfunction is involved in the development and progression of atherosclerosis, which leads to cardiovascular events. Therefore, consistent with our previous reports that EPA attenuates saturated fatty acid-induced macrophage cytokine production or the osteoblastic differentiation of vascular smooth muscle cells, it also attenuates vascular endothelial dysfunction with a mechanism of action possibly mediated by ACSL as the common factor. This is important as a new finding to explain the anti-atherogenic action of EPA in the clinical setting.

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Conflicts of Interest

All authors are employees of the Mochida Pharmaceutical Co., Ltd., selling EPA as a drug.

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