Linoleic Acid Attenuates Endothelium-Derived Relaxing Factor Production by Suppressing cAMP-Hydrolyzing Phosphodiesterase Activity

Jiazhang Wei, MD; Kazuhiko Takeuchi, MD, PhD; Hiroshi Watanabe, MD, PhD

Background: Linoleic acid (LA) promotes monocyte chemotaxis and cell adhesion molecules such as MCP-1 and VCAM-1, which contribute to atherosclerosis. These molecules are restrained by endothelium-derived relaxing factors (EDRFs), such as nitric oxide (NO) and prostaglandin I₂ (PGI₂). Hence, the expressions of MCP-1 and VCAM-1 upregulated by LA may be partly attributable to decreased EDRF production. However, effect of LA on EDRF production remains controversial.

Methods and Results: The present study aimed to examine the effects of LA and other free fatty acids on EDRF production and the endothelial Ca²⁺ responses that mediate EDRF production, using primary cultured porcine aortic endothelial cells (PAECs). LA at 0.1–5 μmol/L attenuated bradykinin (BK)-induced NO and PGI₂ production while suppressing the BK-induced Ca²⁺ response dose-dependently. The inhibitory effect of LA on the Ca²⁺ response was eliminated by adenylate cyclase inhibitor SQ22536, boosted by cAMP-hydrolyzing phosphodiesterase (PDE) inhibitor, rolipram, and mimicked by plasma membrane permeable 8-bromo-cAMP. Moreover, LA was confirmed to dose-dependently increase intracellular cAMP levels and selectively inhibit cAMP-hydrolyzing PDE activity in vitro. In contrast, none of palmitic, stearic, or oleic acid affected BK-induced EDRF production or Ca²⁺ responses, or induced intracellular cAMP accumulation.

Conclusions: LA induced intracellular cAMP accumulation by inhibiting cAMP-hydrolyzing PDE activity, thus resulting in attenuation of Ca²⁺ responses and EDRF production in PAECs. (*Circ J* 2013; **77**: 2823–2830)

Key Words: Cyclic AMP; Endothelial cells; Endothelium-derived relaxing factor; Linoleic acid; Phosphodiesterase

Elevated plasma concentration of free fatty acids (FFAs) has been found to be associated with an increased risk of developing symptomatic ischemic heart disease (IHD).¹ Moreover, in a subanalysis of the relationship between individual FFAs and the risk of major coronary events in the prospective randomized clinical trial, JELIS (the Japan EPA Lipid Intervention Study), only a high concentration of linoleic acid (LA) was found to be an independent risk factor of major coronary events.² Recent, updated data from the Sydney Diet Heart Study revealed that substituting dietary LA for saturated fats increased the rates of death from all causes, coronary artery disease, and cardiovascular disease.³ It was demonstrated that LA promoted monocyte chemotaxis and adhesion to endothelial cells (ECs) and enhanced the expression of cell adhesion molecules, such as monocyte chemoattractant protein 1 (MCP-1) and vascular cell adhesion molecule 1 (VCAM-1), that contribute to atherosclerosis.^{4,5} The expression of MCP-1 and VCAM-1 is restrained by endothelium-derived relaxing factors (EDRFs), such as nitric oxide (NO) and prostaglandin I₂

(PGI₂).^{6–8} Hence, enhanced expression of both MCP-1 and VCAM-1 by LA may be partly attributable to a decreased EDRF production. However, the effect of LA on EDRF production remains controversial.^{9,10} Furthermore, the mechanism by which LA affects EDRF production still needs to be clarified.

Editorial p 2702

The present study used primary cultured porcine aortic endothelial cells (PAECs) to examine the effects of LA and other individual FFA on EDRF production and the endothelial Ca²⁺ responses that mediate it. Oleic, palmitic, linoleic, and stearic acids were used for this study because they account for 32%, 27%, 26%, and 8%, respectively, of human plasma FFAs.¹¹

Methods

Cell Culture

Porcine aortas were collected from a local slaughterhouse and

Received February 19, 2013; revised manuscript received May 14, 2013; accepted June 17, 2013; released online July 25, 2013 Time for primary review: 5 days

Department of Clinical Pharmacology and Therapeutics, Hamamatsu University School of Medicine, Hamamatsu, Japan

Mailing address: Kazuhiko Takeuchi, MD, PhD, Department of Clinical Pharmacology and Therapeutics, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashi-ku, Hamamatsu 431-3192, Japan. E-mail: takeutik@hama-med.ac.jp

ISSN-1346-9843 doi:10.1253/circj.CJ-13-0248

All rights are reserved to the Japanese Circulation Society. For permissions, please e-mail: cj@j-circ.or.jp

the ECs isolated, as previously described,¹² by gently scraping the intima of the descending portion of each aorta. Human umbilical vein endothelial cells (HUVECs) were provided by Lonza Japan Ltd (Tokyo, Japan). The M199 solution (Boehringer Mannheim, Penzberg, DE) used for cell culture contained 150 mmol/L NaCl, 2.7 mmol/L KCl, 1.2 mmol/L MgSO₄, 1.2 mmol/L KH₂PO₄, 10 mmol/L HEPES and 1 mmol/L CaCl₂. After centrifugation at 250 g for 10 min in M199 solution, the sedimented cell fraction was collected and resuspended in M199 solution supplemented with 100 IU/ml penicillin G, 100 µg/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA), and 20% newborn calf serum (NCS, Invitrogen, Carlsbad, CA, USA), seeded onto dishes, and cultured in an incubator at 37°C under 5% CO₂, with the medium renewed daily. The study conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication, 8th edition, 2010). All experiments were performed in accordance with the regulations of the Animal Research Committee of Hamamatsu University School of Medicine.

Reagents and Solution

Linoleic, oleic, palmitic, and stearic acids, as well as bradykinin (BK), thapsigargin, 8-bromoadenosine-3', 5'-cyclic monophosphate (8-bromo-cAMP), adenylyl cyclase inhibitor, SQ22536, and selective phosphodiesterase type IV (PDE4) inhibitor, rolipram, were purchased from Sigma-Aldrich. Oleic, palmitic, linoleic, and stearic acids were dissolved in DMSO at 10 mmol/L as stock solutions, which were further dissolved in a modified Tyrode's solution containing 150 mmol/L NaCl, 2.7 mmol/L KCl, 1.2 mmol/L MgSO₄, 1.2 mmol/L KH₂PO₄, 1 mmol/L CaCl₂ and 10.0 mmol/L *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, with pH 7.4 at 25°C.

Measurement of Endothelial NO Production

NO production in the PAECs was measured using a cell permeable, photo-stable, fluorescent dye for NO, 4-amino-5-methylamino-2', 7'-difluorofluorescein diacetate (DAF-FM/DA, Daiichi Pure Chemicals, Tokyo, Japan), which emits increased fluorescence after reaction with an active intermediate of NO formed during spontaneous NO oxidation to NO₂⁻.¹³ After 2 days of culture, PAECs adhering to glass cover slips were incubated in a modified Tyrode's solution containing 5 µmol/L of DAF-FM/DA for 20 min. The cells were subsequently washed 3 times with the modified Tyrode's solution to remove the DAF-FM/DA from the extracellular fluid and then incubated at room temperature for 75 min in the modified Tyrode's solution containing various concentrations of linoleic, palmitic, stearic, or oleic acid before BK (1 µmol/L) administration to induce NO production in the PAECs. DAF-FM was excited with 490-nm light and its fluorescence emission intensities measured simultaneously at 510 nm. Fluorescent images were acquired and quantified every 6 s from individual cells with a fluorescence imaging and analysis system (Aqua Cosmos 2.5, Hamamatsu Photonics) using an ultrahigh-sensitivity television camera (CCD). Changes in DAF-FM fluorescence intensities (F) in each experiment were normalized to the fluorescence intensity recorded at the experiment beginning (F₀).

Measurement of Endothelial PGI₂ Production

PGI₂ released from PAECs was measured as the concentration of its stable metabolite, 6-keto-PGF₁α, using an enzyme immunoassay, as described previously.¹⁴ PAECs were plated in 12-well culture dishes and grown to confluence in M199 medium at 37°C under 5% CO₂ and saturated humidity. PAECs

were then preincubated in various FFAs as described earlier, and the cell medium was then measured 14 min after BK administration. The 6-keto-PGF₁α concentrations in the cell medium were determined according to the manufacturer's protocol (6-keto-PGF₁α EIA kit, Assay Designs, Ann Arbor, MI, USA). The pH of the samples was adjusted to 7.4 before 6-keto-PGF₁α measurement to eliminate any effects on the immunoassay.

Measurement of Intracellular Ca²⁺ Concentration in ECs

Using the acetoxymethyl ester of fura-2 (fura-2/AM, Dojindo Molecular Technologies, Kumamoto, Japan), intracellular Ca²⁺ concentrations ([Ca²⁺]_i) in individual PAECs were measured as described previously.¹² After 2 days of culture, PAECs adhering to glass cover slips were incubated for 40 min in a modified Tyrode's solution containing 10% NCS and 2 µmol/L fura-2/AM, a fluorescent Ca²⁺ indicator. The cells were subsequently washed 3 times with the modified Tyrode's solution to remove the fura-2/AM and the serum from the extracellular fluid, then left to equilibrate in the cell buffer for 20 min before measurements were started. All experiments were performed at 25°C. The absorption shift of fura-2 that occurs upon binding can be determined by scanning the excitation spectrum between 340 and 380 nm while monitoring the emission at 510 nm. Fluorescent images were acquired and quantified every 30 s from individual cells with a fluorescence imaging and analysis system (Aqua Cosmos 2.5, Hamamatsu Photonics) using an ultrahigh-sensitivity television camera (CCD). After background subtraction, the fluorescence ratio (F₃₄₀/F₃₈₀) was obtained by dividing, pixel by pixel, the 340-nm image by the 380-nm image. Changes in this ratio were used to express changes in the intracellular Ca²⁺ concentration to eliminate potential artifacts caused by variations in cell thickness, intracellular dye distribution or photobleaching. The minimum and maximum F₃₄₀/F₃₈₀ ratios, determined using a Calcium Calibration Buffer Kit (Invitrogen), were 0.46 with no Ca²⁺ and 27.5 at 39 µmol/L Ca²⁺.

Measurement of Endothelial cAMP Production

PAECs were plated in 96-well culture dishes and incubated to confluence with M199 medium at 37°C under 5% CO₂ and saturated humidity. For the experiment, the medium was removed and the cells were washed with a modified Tyrode's solution and then incubated for 75 min in the modified Tyrode's solution containing various concentrations of FFAs in the presence and absence of 200 µmol/L of SQ22536. Intracellular cAMP concentrations in the PAECs were determined using an enzyme immunoassay according to the manufacturer's protocol (Cyclic AMP EIA Kit, Assay Designs).

Measurement of PDE Activity

cAMP/cGMP-hydrolyzing PDE activity was determined using a cyclic nucleotide PDE assay kit according to the manufacturer's protocol (Assay Designs). cAMP and cGMP-hydrolyzing PDE activities were evaluated as the cleavage of cAMP and cGMP through cAMP/cGMP-hydrolyzing PDE in 30 min, respectively. The 5'-nucleotide released was further cleaved into the nucleoside and phosphate by the enzyme 5'-nucleotidase. The phosphate released by enzymatic cleavage was quantified using BIOMOL GREENTM reagent. A nonspecific cyclic nucleotide PDE inhibitor, 3-isobutyl-1-methylxanthine (IBMX) was included as a test control.

Statistical Analysis

Statistical analysis was performed by 1-way ANOVA followed

Figure 1. Effects of free fatty acids (FFAs) on bradykinin (BK)-induced nitric oxide (NO) production in porcine aortic endothelial cells (PAECs). **(A)** Representative traces showing the changes in BK-induced NO production indicated by the F490/F0490 DAF-FM ratio in PAECs pretreated without (control) and with the NO synthase inhibitor, L-NAME (100 μmol/L). **(B)** Summary of the effects of FFAs on BK-induced NO production. PAECs were pretreated with linoleic (LA), palmitic (PA), stearic (SA), or oleic acid (OA) for 75 min at the indicated concentrations. Intracellular NO production stimulated by BK is indicated by the DAF-FM F490/F0490 ratio. The data are the mean±SD of 4 independent observations in separate cell culture wells. *P<0.05 vs. control.

Figure 2. Effects of free fatty acids (FFAs) on bradykinin (BK)-induced prostaglandin I₂ (PGI₂) production in porcine aortic endothelial cells (PAECs) pretreated with FFAs for 75 min at the indicated concentrations. BK-induced PGI₂ production in PAECs was estimated as the concentration of 6-keto-PGF_{1α} by enzyme immunoassay and indicated by the percent of untreated PAECs (control). The data are the mean±SD of 4 independent observations in separate cell culture wells. *P<0.05 vs. control.

by multiple comparisons using Bonferroni's protected least-significant difference test. Statistical analysis was performed using Graph Pad Prism 5 (GraphPad Software, La Jolla, CA, USA). Data are presented as mean±SD and P<0.05 considered statistically significant.

Results

Effects of FFAs on BK-Induced NO Production in PAECs

The effects of individual FFAs on endothelial function were elucidated by determining the effect of each FFA on NO pro-

duction by fluorimetry with DAF-FM/DA. BK at 1 μmol/L increased the F490/F0490 DAF-FM ratio to 1.059±0.019. The BK-induced increase in the F490/F0490 DAF-FM ratio was confirmed to be totally inhibited by pretreatment with 100 μmol/L of L-nitro-arginine methyl ester (L-NAME), an endothelial NO synthase inhibitor (Figure 1A), which was consistent with our previous study's results.¹⁵ LA reduced NO production by 62% and 89% at 0.1 and 1 μmol/L, respectively; however, oleic, palmitic, or stearic acid at 10 μmol/L had no effect on BK-induced NO production in PAECs (Figure 1B).

Figure 3. Effects of free fatty acids (FFAs) on endothelial Ca^{2+} responses in porcine aortic endothelial cells (PAECs). **(A)** Influence of FFAs on bradykinin (BK)-induced changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). PAECs were pretreated with 5 μ mol/L of linoleic (LA), palmitic (PA), stearic (SA), or oleic acid (OA) for 75 min. **(B)** Effect of LA at various concentrations on BK-induced changes in $[Ca^{2+}]_i$. **(C)** Reversible inhibitory effects of LA on BK-induced endothelial Ca^{2+} responses. After the inhibition of BK-induced Ca^{2+} responses caused by 2 μ mol/L of LA was identified, BK and LA were washed out using a modified Tyrode's solution. BK was re-applied 40 min after the washout. **(D)** Effects of LA on thapsigargin (TG)-induced Ca^{2+} responses in PAECs. The data are the mean $\pm$ SD of 4 independent observations in separate cell culture wells.

Effects of FFAs on BK-Induced PGI_2 Production in PAECs

The effect of FFAs on endothelial PGI_2 production was determined by enzyme immunoassay of a stable PGI_2 metabolite, 6-keto- $PGF_{1\alpha}$. BK at 10 nmol/L greatly increased the 6-keto- $PGF_{1\alpha}$ concentration from 116.1 ± 14.9 to 1519.9 ± 117.8 fmol/ 10^6 cells after 14 min. Pretreatment with LA inhibited 6-keto- $PGF_{1\alpha}$ production by 30%, 51%, and 71% at 1, 5, and 10 μ mol/L, respectively (Figure 2). Palmitic, oleic, or stearic acid had no effect on BK-induced PGI_2 production in PAECs.

Effects of FFAs on Intracellular Ca^{2+} Responses in PAECs

To verify whether the inhibitory effects of LA on EDRF production were caused by suppressing the intracellular Ca^{2+} responses that mediate EDRF production,¹⁵ the effects of FFAs on BK-induced Ca^{2+} responses in PAECs were tested. It was found that, in the presence of extracellular Ca^{2+} , 10 nmol/L of BK caused a rapid increase in the F340/F380 ratio of fura-2 from 0.77 ± 0.07 (basal) to 4.81 ± 0.26 (maximal), which then slowly attenuated (Figure 3A). Pretreatment with 5 μ mol/L of

LA inhibited the BK-induced Ca^{2+} responses, but none of the other FFAs exhibited an effect (Figure 3A). Moreover, the intensity and duration of BK-induced Ca^{2+} responses were reduced by LA in a dose-dependent manner (Figure 3B). The inhibition by LA of the BK-induced Ca^{2+} responses was also observed in HUVECs (Figure S1A). To clarify whether this inhibitory effect of LA was reversible or not, the BK-induced Ca^{2+} responses after washout of LA by modified Tyrode's solution were examined. The removal of LA rejuvenated the BK-induced Ca^{2+} responses (Figure 3C).

To evaluate which section of the endothelial Ca^{2+} signaling pathway was being affected by LA, the influence of LA on thapsigargin (TG)-induced Ca^{2+} responses was tested. TG, an irreversible inhibitor of endoplasmic reticulum (ER) Ca^{2+} -ATPase, was used to investigate store-operated Ca^{2+} entry (SOCE). TG increases intracellular Ca^{2+} level through the plasma membrane store-operated Ca^{2+} channel (SOCC) activated by depleting ER Ca^{2+} stores. TG evoked a slightly delayed but long-lasting increase in F340/F380 from 0.80 ± 0.11 (basal) to 4.19 ± 0.55 (maximal) in the presence of extracellular

Figure 4. Involvement of intracellular cAMP formation in the inhibitory effect of linoleic acid (LA) on endothelial Ca^{2+} responses in porcine aortic endothelial cells (PAECs). **(A)** Influences of 8-bromo-cAMP, adenylate cyclase inhibitor and cAMP-hydrolyzing phosphodiesterase (PDE) inhibitor on the inhibitory effect of LA on endothelial Ca^{2+} responses induced by bradykinin (BK). PAECs were pretreated with 8-bromo-cAMP, LA, LA + SQ22563 (adenylate cyclase inhibitor), or LA + rolipram (cAMP-hydrolyzing PDE inhibitor) for 75 min. The figure shows the percent of control (untreated ECs) in the area under the curve (AUC) of the endothelial Ca^{2+} responses from 90 s, at BK administration, to 420 s. Data are the mean $\pm$ SD of 4 independent observations in separate cell culture wells. * $P < 0.05$. **(B)** Intracellular cAMP levels in PAECs treated with various free fatty acids (FFAs: palmitic (PA), stearic (SA), oleic acid (OA)) and/or SQ22563. PAECs were incubated with the various FFAs and/or SQ22563 at the indicated concentrations for 75 min. The intracellular cAMP level (open bar) in the PAECs incubated with a modified Tyrode's solution is indicated as the control. The data are the mean $\pm$ SD of 3 independent observations in separate cell culture wells. * $P < 0.05$ vs. control.

Figure 5. Effects of linoleic acid (LA) on cAMP/cGMP-hydrolyzing phosphodiesterase (PDE) activity in vitro. **(A)** cAMP-hydrolyzing PDE activity without (control) or with various concentrations of LA was evaluated as the cleavage of cAMP in 30 min. IBMX, a nonspecific PDE inhibitor, was used as a test control. The summary indicates the percent of control in the amount of cAMP hydrolysis by cAMP-hydrolyzing PDE in vitro. **(B)** cGMP-hydrolyzing PDE activity in the presence or absence of LA. The data are the mean $\pm$ SD of 6 independent observations in separate wells. * $P < 0.05$ vs. control.

Ca^{2+} . Pretreatment with 1 $\mu\text{mol/L}$ of LA obviously inhibited the TG-induced Ca^{2+} responses, and 5 $\mu\text{mol/L}$ of LA almost abolished them (Figure 3D). The inhibition by LA of TG-induced Ca^{2+} responses was also observed in HUVECs (Figure S1B). These findings indicate that LA suppresses endothelial Ca^{2+} responses by inhibiting the SOCE signaling pathway between

Ca^{2+} depletion of ER Ca^{2+} stores and the SOCC.

Involvement of Intracellular cAMP Formation in the Inhibitory Effect of LA on Endothelial Ca^{2+} Responses in PAECs

The involvement of cAMP-related signaling pathways in the inhibition of endothelial Ca^{2+} responses by LA was explored

because previous studies indicated that intracellular cAMP formation could be involved in the regulation of Ca^{2+} responses in ECs,¹⁶ and that intracellular cAMP levels was increased by LA in neuroblastoma cells.¹⁷ In the present study, the inhibitory effect of LA on BK-induced endothelial Ca^{2+} responses was completely eliminated by an adenylate cyclase inhibitor, SQ22536, at 200 $\mu\text{mol/L}$, and boosted by 50 $\mu\text{mol/L}$ of rolipram, a selective cAMP-hydrolyzing PDE inhibitor (Figure 4A). A plasma membrane permeable cAMP, 8-bromo-cAMP, at 100 $\mu\text{mol/L}$ suppressed the BK-induced Ca^{2+} responses (Figure 4A) and TG (data not shown). Furthermore, LA increased intracellular cAMP levels in a dose-dependent manner, and the intracellular cAMP formation induced by LA was abolished by SQ22536 (Figure 4B). Taken together, these findings strongly suggest that intracellular cAMP formation is involved in the inhibitory effects of LA on endothelial Ca^{2+} responses in PAECs.

Involvement of cAMP-Hydrolyzing PDE Activity in Intracellular cAMP Formation Caused by LA

To explore the mechanisms through which LA increases intracellular cAMP levels, we tested the effects of LA on cAMP-hydrolyzing PDE activity in vitro. In a period of 30 min, LA decreased the cleavage of cAMP through PDE by 27%, 59% and 68% at 1, 5 and 10 $\mu\text{mol/L}$, respectively, as measured by a PDE activity assay (Figure 5A). Other individual FFA at 10 $\mu\text{mol/L}$ did not affect PDE activity (data not shown). A nonspecific PDE inhibitor, IBMX, was used as a test control and pharmacological inhibition of PDE by IBMX was confirmed (Figure 5A). These results suggest that LA directly suppresses cAMP-hydrolyzing PDE activity, which may be one of the mechanisms by which LA increases intracellular cAMP levels in PAECs. The effect of LA on cGMP-hydrolyzing PDE activity in vitro was also examined. We confirmed that LA did not show any inhibitory effects on cGMP-hydrolyzing PDE activity (Figure 5B).

Discussion

The present study aimed to investigate the effects of individual FFAs in human plasma on EDRF production, as well as the mechanisms by which the individual FFAs affected EDRF production. The results demonstrated that (1) among the various FFAs, only LA attenuated NO and PGI_2 production in PAECs, (2) LA suppressed endothelial Ca^{2+} responses by intracellular cAMP accumulation via cAMP-hydrolyzing PDE inhibition, and (3) other FFAs, including palmitic, stearic and oleic acids, did not show any effects on EDRF production or endothelial Ca^{2+} responses.

An elevated plasma FFA level is associated with endothelial dysfunction and cardiovascular diseases.^{18–23} The Quebec Cardiovascular Study indicated that elevated plasma FFA concentrations are associated with an increased risk of developing symptomatic IHD (odds ratio 2.1, $P=0.05$) independent of age, smoking habit, systolic blood pressure, low-density lipoprotein cholesterol level, and family history of IHD.¹ Furthermore, in a subanalysis of the prospective randomized clinical trial JELIS (15,534 subjects), only a high concentration of LA was significantly associated with a higher risk of major coronary events (hazard ratio (HR) 1.33, 95% confidence interval (CI) 1.02–1.74) in the control group of 7,722 subjects, and other FFAs, such as palmitic, stearic, oleic, arachidonic, docosahexaenoic, and eicosapentaenoic acids, did not exhibit any significant correlation with a risk of major coronary events.² Most recently, evaluation of recovered data from the Sydney

Diet Heart Study, a single, blinded, parallel group, randomized controlled trial revealed that replacement of dietary saturated fats with LA resulted in increased rates of death from all causes, coronary artery disease, and cardiovascular disease.³ In the present study, of the major FFAs in human plasma, only LA was confirmed to attenuate EDRF production in PAECs; none of the other FFAs tested showed any inhibitory effects.

In the present study, LA inhibited BK-induced NO and PGI_2 production by suppressing endothelial Ca^{2+} responses. Kim et al demonstrated that LA inhibited insulin-mediated NO production via Akt and eNOS phosphorylation,⁹ which was independent of intracellular Ca^{2+} responses.²⁴ Thus, it is possible that LA suppresses NO production through 2 parallel pathways: the Ca^{2+} response-mediated NO production and that via Akt and eNOS phosphorylation. On the other hand, Saraswathi et al reported that 3 h incubation of LA at 90 $\mu\text{mol/L}$ increased the intracellular Ca^{2+} concentration and Ca^{2+} -dependent NO production in porcine pulmonary arterial ECs.¹⁰ However, we found that LA at 0.1–10 $\mu\text{mol/L}$ did not affect the baseline intracellular Ca^{2+} concentration (data not shown), but did attenuate the BK-induced Ca^{2+} responses and NO production in PAECs (Figures 1B, 3A, 3B). Our present results, together with those from Saraswathi et al study, suggest that the effect of LA on Ca^{2+} -dependent NO production in ECs may have 2 aspects: LA at low doses (approximately 0.1–10 $\mu\text{mol/L}$) attenuates the Ca^{2+} response-mediated NO production stimulated by an agonist such as BK, and LA at higher doses is able to raise intracellular Ca^{2+} levels and therefore increases Ca^{2+} -dependent NO production, which transforms into peroxynitrite reacting with superoxide.¹⁰ LA at both of dosages could disturb vascular homeostasis.

It was recently reported that a 3-day incubation of human aortic ECs with LA increased monocyte chemotaxis and adhesion to ECs, which may exert proinflammatory and proatherogenic effects.⁵ EDRFs such as NO and PGI_2 are known to prevent the abnormal constriction of coronary arteries, inhibit the aggregation of platelets, and suppress the chemotaxis, adhesion and penetration of monocyte to vascular endothelium,^{25,26} thus sustained attenuation of EDRF production, such as results from the exposure of ECs to an high level of LA, may not only be responsible for the promotion of monocyte chemotaxis and adhesion, but also contribute to the multistep process of atherosclerosis.

EDRFs are defined as vasodilators that are synthesized and released by endothelium, and include not only PGI_2 and NO, but also endothelium-derived hyperpolarizing factors (EDHF) such as H_2O_2 and epoxyeicosatrienoic acids. EDHF-mediated vasodilatation is essential for maintaining of cardiovascular homeostasis, especially in microvessels.^{27,28} EDHF-mediated vasodilatation is relatively resistant to atherosclerosis and therefore capable of serving as a back-up system for suppressed NO-mediated vasodilatation. The mechanisms of enhanced EDHF-mediated vasodilatation have been well illustrated.²⁹ The regulation of endothelium function varies according to the site and the type of blood vessel.³⁰ The contribution of each individual EDRF to endothelium-dependent relaxation differs between the aorta and the small vessels; for example, NO plays a prominent role in aorta, but EDHF is prominent in the mesenteric artery.³¹ Our previous study showed that the EDHF-mediated arterial hyperpolarization and relaxation also depends on endothelial Ca^{2+} responses.³² Because of the inhibitory effects on endothelial Ca^{2+} responses exhibited by LA in the present study, LA may also interfere in EDHF-mediated vascular responses in the small blood vessels.

Regarding the mechanism by which LA attenuates EDRF

production, we verified that LA inhibits the endothelial Ca^{2+} responses that regulate EDRF production. LA was also confirmed to inhibit TG-induced Ca^{2+} responses, which suggests that it inhibits the signaling pathways of SOCE between Ca^{2+} depletion of the ER Ca^{2+} stores and the plasma membrane Ca^{2+} -permeable cation channels activated by Ca^{2+} depletion of ER Ca^{2+} stores.^{33,34}

Luckhoff et al reported that a PGI_2 analog (iloprost) and β -adrenergic stimulant (isoproterenol) inhibited endothelial Ca^{2+} responses by intracellular cAMP formation and deduced that enhanced turnover of cAMP in ECs may protect the organism from sudden, overwhelming release of powerful EDRFs.¹⁶ Thus, in the present study, the possible involvement of cAMP-related signaling pathways in the inhibitory effect of LA on endothelial Ca^{2+} responses was examined. Our results showed that (1) LA increased intracellular cAMP levels, (2) the inhibitory effect of LA on endothelial Ca^{2+} responses was eliminated by an adenylate cyclase inhibitor, SQ22563, boosted by a PDE4 inhibitor, rolipram, and mimicked by 8-bromo-cAMP, and (3) LA selectively inhibited cAMP-hydrolyzing PDE activity in vitro. These findings in PAECs suggest that LA suppresses endothelial Ca^{2+} responses by intracellular cAMP formation via cAMP-hydrolyzing PDE inhibition.

The intracellular cAMP concentration results from the balance between its production by adenylate cyclase and its metabolism by cAMP-hydrolyzing PDE. The present results show that LA suppresses cAMP-hydrolyzing PDE activity, which at least in part contributed to the accumulation of intracellular cAMP in PAECs. As shown in Figure 4B, SQ22563 significantly reduced the baseline intracellular cAMP level, which indicates that in the steady state without any stimulants, cAMP is constantly produced via adenylate cyclase. Therefore, suppression of cAMP-hydrolyzing PDE activity by LA could result in an increase in the intracellular cAMP level, even in the steady state, in ECs. Interestingly, LA exhibited a selective inhibitory effect only on cAMP-hydrolyzing PDE, but not on cGMP-hydrolyzing PDE. This selectivity of inhibition by LA on PDEs was demonstrated in vitro for the first time in the present study. The other FFAs used in our study did not show any inhibitory effects on cAMP-hydrolyzing PDE activity.

Study Limitations

There are some limitations of the present study worth mentioning. First, EDRF-mediated modulation of vascular tone varies on the type of blood vessel. The data in our study were only obtained from aortic ECs, so whether LA suppresses EDRF production in ECs from smaller arteries, such as the coronary and mesenteric arteries, remains to be verified. Second, we only showed that LA suppressed NO and PGI_2 production; however, the effects of LA on other EDRFs, such as EDHF, should be further elucidated. Third, our findings should be confirmed in an EDRF-mediated relaxation model, such as the endothelium-dependent relaxation of aorta.

Conclusions

The present study demonstrated that LA induced intracellular cAMP accumulation by selectively inhibiting cAMP-hydrolyzing PDE activity, and thereby attenuating endothelial Ca^{2+} responses and EDRF production in PAECs.

Acknowledgments

This study was supported in part by Grants-in-Aid (22590499) for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan to K. Takeuchi. We thank Ms. Michiko Kageyama

for her help in preparing the porcine endothelial cells.

Disclosures

The authors declare no conflicts of interest.

References

- Pirro M, Mauriege P, Tchernof A, Cantin B, Dagenais GR, Despres JP, et al. Plasma free fatty acid levels and the risk of ischemic heart disease in men: Prospective results from the Quebec Cardiovascular Study. *Atherosclerosis* 2002; **160**: 377–384.
- Itakura H, Yokoyama M, Matsuzaki M, Saito Y, Origasa H, Ishikawa Y, et al. Relationships between plasma fatty acid composition and coronary artery disease. *J Atheroscler Thromb* 2011; **18**: 99–107.
- Ramsden CE, Zamora D, Leelarthaepin B, Majchrzak-Hong SF, Faurot KR, Suchindran CM, et al. Use of dietary linoleic acid for secondary prevention of coronary heart disease and death: Evaluation of recovered data from the Sydney Diet Heart Study and updated meta-analysis. *BMJ* 2013; **346**: e8707.
- Fei J, Cook C, Gillespie M, Yu B, Fullen K, Santanam N. Atherogenic omega-6 lipids modulate PPAR-EGR-1 crosstalk in vascular cells. *PPAR Res* 2011 October 26, doi:10.1155/2011/753917 [E-pub ahead of print].
- Matesanz N, Jewhurst V, Trimble ER, McGinty A, Owens D, Tomkin GH, et al. Linoleic acid increases monocyte chemotaxis and adhesion to human aortic endothelial cells through protein kinase C- and cyclooxygenase-2-dependent mechanisms. *J Nutr Biochem* 2012; **23**: 685–690.
- Takahashi M, Ikeda U, Masuyama J, Funayama H, Kano S, Shimada K. Nitric oxide attenuates adhesion molecule expression in human endothelial cells. *Cytokine* 1996; **8**: 817–821.
- Braun M, Pietsch P, Zepp A, Schror K, Baumann G, Felix SB. Regulation of tumor necrosis factor alpha- and interleukin-1-beta-induced induced adhesion molecule expression in human vascular smooth muscle cells by cAMP. *Arterioscler Thromb Vasc Biol* 1997; **17**: 2568–2575.
- Tsao PS, Wang B, Buitrago R, Shyy JY, Cooke JP. Nitric oxide regulates monocyte chemotactic protein-1. *Circulation* 1997; **96**: 934–940.
- Kim F, Tysseling KA, Rice J, Pham M, Haji L, Gallis BM, et al. Free fatty acid impairment of nitric oxide production in endothelial cells is mediated by IKKbeta. *Arterioscler Thromb Vasc Biol* 2005; **25**: 989–994.
- Saraswathi V, Wu G, Toborek M, Hennig B. Linoleic acid-induced endothelial activation: Role of calcium and peroxynitrite signaling. *J Lipid Res* 2004; **45**: 794–804.
- Miwa H, Yamamoto M, Nishida T, Nunoi K, Kikuchi M. High-performance liquid chromatographic analysis of serum long-chain fatty acids by direct derivatization method. *J Chromatogr* 1987; **416**: 237–245.
- Watanabe H, Takahashi R, Zhang XX, Kakizawa H, Hayashi H, Ohno R. Inhibition of agonist-induced Ca^{2+} entry in endothelial cells by myosin light-chain kinase inhibitor. *Biochem Biophys Res Commun* 1996; **225**: 777–784.
- Kojima H, Nakatsubo N, Kikuchi K, Kawahara S, Kirino Y, Nagoshi H, et al. Detection and imaging of nitric oxide with novel fluorescent indicators: Diaminofluoresceins. *Anal Chem* 1998; **70**: 2446–2453.
- Kaw S, Hecker M. Endothelium-derived hyperpolarizing factor, but not nitric oxide or prostacyclin release, is resistant to menadione-induced oxidative stress in the bovine coronary artery. *Naunyn-Schmiedeberg Arch Pharmacol* 1999; **359**: 133–139.
- Asai M, Takeuchi K, Saotome M, Urushida T, Katoh H, Satoh H, et al. Extracellular acidosis suppresses endothelial function by inhibiting store-operated Ca^{2+} entry via non-selective cation channels. *Cardiovasc Res* 2009; **83**: 97–105.
- Luckhoff A, Mulsch A, Busse R. cAMP attenuates autacoid release from endothelial cells: Relation to internal calcium. *Am J Physiol* 1990; **258**: H960–H966.
- Murphy MG, Byczko Z. Further studies of the mechanism(s) of polyunsaturated-fatty-acid-mediated increases in intracellular cAMP formation in N1E-115 neuroblastoma cells. *Neurochem Res* 1992; **17**: 1069–1077.
- Reaven GM, Hollenbeck C, Jeng CY, Wu MS, Chen YD. Measurement of plasma glucose, free fatty acid, lactate, and insulin for 24 h in patients with NIDDM. *Diabetes* 1988; **37**: 1020–1024.
- Muller JE, Tofler GH, Stone PH. Circadian variation and triggers of onset of acute cardiovascular disease. *Circulation* 1989; **79**: 733–743.
- Steinberg HO, Chaker H, Leaming R, Johnson A, Brechtel G, Baron

- AD. Obesity/insulin resistance is associated with endothelial dysfunction: Implications for the syndrome of insulin resistance. *J Clin Invest* 1996; **97**: 2601–2610.
21. Carlsson M, Wessman Y, Almgren P, Groop L. High levels of non-esterified fatty acids are associated with increased familial risk of cardiovascular disease. *Arterioscler Thromb Vasc Biol* 2000; **20**: 1588–1594.
 22. Kocer A, Ilhan A, Ince N, Bilge C. The related causes in very early morning onset of stroke. *Prog Neuropsychopharmacol Biol Psychiatry* 2005; **29**: 983–988.
 23. Pilz S, Scharnagl H, Tiran B, Seelhorst U, Wellnitz B, Boehm BO, et al. Free fatty acids are independently associated with all-cause and cardiovascular mortality in subjects with coronary artery disease. *J Clin Endocrinol Metab* 2006; **91**: 2542–2547.
 24. Takahashi S, Mendelsohn ME. Synergistic activation of endothelial nitric-oxide synthase (eNOS) by HSP90 and Akt: Calcium-independent eNOS activation involves formation of an HSP90-Akt-CaM-bound eNOS complex. *J Biol Chem* 2003; **278**: 30821–30827.
 25. Vanhoutte PM. Endothelial dysfunction: The first step toward coronary arteriosclerosis. *Circ J* 2009; **73**: 595–601.
 26. Kawabe J, Ushikubi F, Hasebe N. Prostacyclin in vascular diseases: Recent insights and future perspectives. *Circ J* 2010; **74**: 836–843.
 27. Urakami-Harasawa L, Shimokawa H, Nakashima M, Egashira K, Takeshita A. Importance of endothelium-derived hyperpolarizing factor in human arteries. *J Clin Invest* 1997; **100**: 2793–2799.
 28. Shimokawa H. Hydrogen peroxide as an endothelium-derived hyperpolarizing factor. *Pflugers Arch* 2010; **459**: 915–922.
 29. Ohashi J, Sawada A, Nakajima S, Noda K, Takaki A, Shimokawa H. Mechanisms for enhanced endothelium-derived hyperpolarizing factor-mediated responses in microvessels in mice. *Circ J* 2012; **76**: 1768–1779.
 30. Heo KS, Fujiwara K, Abe J. Disturbed-flow-mediated vascular reactive oxygen species induce endothelial dysfunction. *Circ J* 2011; **75**: 2722–2730.
 31. Shimokawa H, Yasutake H, Fujii K, Owada MK, Nakaïke R, Fukumoto Y, et al. The importance of the hyperpolarizing mechanism increases as the vessel size decreases in endothelium-dependent relaxations in rat mesenteric circulation. *J Cardiovasc Pharmacol* 1996; **28**: 703–711.
 32. Fukao M, Watanabe H, Takeuchi K, Tomioka H, Hattori Y. Effects of SK&F 96365 and mefenamic acid on Ca^{2+} influx in stimulated endothelial cells and on endothelium-derived hyperpolarizing factor-mediated arterial hyperpolarization and relaxation. *J Cardiovasc Pharmacol* 2001; **38**: 130–140.
 33. Takeuchi K, Watanabe H, Tran QK, Ozeki M, Uehara A, Katoh H, et al. Effects of cytochrome P450 inhibitors on agonist-induced Ca^{2+} responses and production of NO and PGI_2 in vascular endothelial cells. *Mol Cell Biochem* 2003; **248**: 129–134.
 34. Tran QK, Ohashi K, Watanabe H. Calcium signalling in endothelial cells. *Cardiovasc Res* 2000; **48**: 13–22.

Supplementary Files

Supplementary File 1

Figure S1. Effects of linoleic acid (LA) on endothelial Ca^{2+} responses in human umbilical vein endothelial cells (HUVECs).

Please find supplementary file(s);
<http://dx.doi.org/10.1253/circj.CJ-13-0248>