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). 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. 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. It was demonstrated that LA promotes monocyte chemotaxis and adhesion molecules such as monocyte chemotactic protein 1 (MCP-1) and vascular cell adhesion molecule 1 (VCAM-1), which contribute to atherosclerogenesis. These molecules are restrained by endothelium-derived relaxing factors (EDRFs), such as nitric oxide (NO) and prostaglandin I\(_2\) (PGI\(_2\)). 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 Ca\(^{2+}\) responses that mediate EDRF production, using primary cultured porcine aortic endothelial cells (PAECs). LA at 0.1–5 \(\mu\)mol/L attenuated bradykinin (BK)-induced NO and PGI\(_2\) production while suppressing the BK-induced Ca\(^{2+}\) response dose-dependently. The inhibitory effect of LA on the Ca\(^{2+}\) response was eliminated by adenylyl 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 Ca\(^{2+}\) responses, or induced intracellular cAMP accumulation.

Conclusions: LA induced intracellular cAMP accumulation by inhibiting cAMP-hydrolyzing PDE activity, thus resulting in attenuation of Ca\(^{2+}\) 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
the ECs isolated, as previously described, 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 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-aminophenyl-5-methylamino-2′, 7′-difluorofluorescein diacetate (DAF-FM/DA, Daichi Pure Chemicals, Tokyo, Japan), which emits increased fluorescence after reaction with an active intermediate of NO formed during spontaneous NO oxidation to NO₂−. After 2 days of culture, PAECs adhering to glass cover slips were incubated for 40 min in a modified Tyrode’s solution containing 100 μmol/L. 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 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 GREEN™ 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
Effect of LA on EDRF

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).

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.

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 production 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. 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).
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 endothelial PGI\(_2\) production was examined. LA inhibited the BK-induced PGI\(_2\) production, 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).

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 the 6-keto-PGF\(_{1\alpha}\) production by 30%, 51%, and 71% at 1.5, and 10 μmol/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, 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 μmol/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
Ca\textsuperscript{2+}. Pretreatment with 1 \(\mu\text{mol/L}\) of LA obviously inhibited the TG-induced Ca\textsuperscript{2+} responses, and 5 \(\mu\text{mol/L}\) of LA almost abolished them (Figure 3D). The inhibition by LA of TG-induced Ca\textsuperscript{2+} responses was also observed in HUVECs (Figure S1B). These findings indicate that LA suppresses endothelial Ca\textsuperscript{2+} responses by inhibiting the SOCE signaling pathway between Ca\textsuperscript{2+} depletion of ER Ca\textsuperscript{2+} stores and the SOCC.

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

The involvement of cAMP-related signaling pathways in the inhibition of endothelial Ca\textsuperscript{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, and that intracellular cAMP levels was increased by LA in neuroblastoma cells. 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 μmol/L, and 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 μmol/L, respectively, as measured by a PDE activity assay (Figure 5A). Other individual FFA at 10 μmol/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 cAMP-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. 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. 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.

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. 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, which was independent of intracellular Ca^{2+} responses. 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 μmol/L increased the intracellular Ca^{2+} concentration and Ca^{2+}-dependent NO production in porcine pulmonary arterial ECs. However, we found that LA at 0.1–10 μmol/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 μmol/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. 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. 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. Since EDRF-mediated arterial hyperpolarization and relaxation also depends on EDHF-mediated arterial hyperpolarization and relaxation also depends on endothelial Ca^{2+} responses. 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...
Thus, in the present study, the possible involvement of cAMP-depletion of the ER Ca²⁺ stores and the plasma membrane confirmed to inhibit TG-induced Ca²⁺ responses, which suggests responses that regulate EDRF production. LA was also confirmed to inhibit TG-induced Ca²⁺ responses, which suggests responses and EDRF production in PAECs.

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 expression between its production by adenylate cyclase and its metabolism by 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.

Conclusions

The present study demonstrated that LA induced intracellular cAMP accumulation by selectively inhibiting cAMP-hydrolyzing PDE activity, and thereby attenuating endothelial Ca²⁺ responses and EDRF production in PAECs.

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Disclosures

The authors declare no conflicts of interest.

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Supplementary Files

Supplementary File 1

Figure S1. Effects of linoleic acid (LA) on endothelial Ca2+ responses in human umbilical vein endothelial cells (HUVECs).

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