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Unsaturated Free Fatty Acids Inhibit Ca^{2+} Mobilization and NO Release in Endothelial Cells

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Circulating free fatty acids (FFAs) are elevated in various disorders like states of insulin resistance, and an increase of FFAs has been reported to be associated with endothelial dysfunction. To investigate the effect of FFAs on vascular endothelial cells, we measured nitric oxide (NO) release and intracellular free calcium concentration ($[Ca^{2+}]i$) in cultured bovine aortic endothelial cells (BAECs). Monounsaturated FFAs such as oleic acid (OA) and polyunsaturated FFAs such as linoleic acid (LA) dose-dependently (10-100 μmol/L) inhibited NO release from BAECs stimulated by adenosine 5'-(3-*O*-thio)triphosphate (ATPγS) whereas saturated FFAs such as palmitic acid had no effect on NO release. ATPγS induced a biphasic increase in $[Ca^{2+}]i$, which consisted of a rapid increase followed by a sustained increase. OA and LA inhibited ATPγS-induced Ca^{2+} release from intracellular Ca^{2+} stores and increase in Ca^{2+} influx from extracellular space. In addition, OA and LA rapidly decreased sustained increase in $[Ca^{2+}]i$ induced by ATPγS when OA or LA was added after stimulation with ATPγS. Impaired Ca^{2+} mobilization by unsaturated FFAs (UFFAs) was completely reversed by treatment with bovine serum albumin (1 mg/ml) indicating that inhibitory effect of FFAs was not caused by cytotoxic effect. In spite of this inhibitory effect of UFFAs, neither OA nor LA had any effect on phosphoinositide hydrolysis evoked by ATPγS. From these results, elevated UFFA may contribute to impaired NO production through inhibition of receptor-mediated Ca^{2+} mobilization and Ca^{2+} influx in pathological conditions such as multiple risk factor syndrome and insulin resistance.

Circulating free fatty acids (FFAs) are elevated in many obese and hypertensive individuals and higher FFAs may cause endothelial dysfunction such as impaired endothelium-dependent relaxation and contribute to cardiovascular diseases (3,15).

Nitric oxide (NO) accounts for the biological activity of endothelium-derived relaxing factor (EDRF), and regulates the vascular tone (12). In addition, NO has also been shown to inhibit platelet aggregation, smooth muscle proliferation, and adhesion of leukocyte to endothelial cells (5). In endothelial cells, NO is synthesized by endothelial constitutive type of NO synthase (eNOS) and intracellular calcium concentration plays a pivotal role in the activation of eNOS (14). Agonists that release NO share in common an ability to activate phospholipase C. This activation produces two distinct second messengers: inositol 1,4,5-trisphosphate (IP3), which elevates intracellular free calcium concentration ($[Ca^{2+}]i$) (1) and diacylglycerol which activates protein kinase C (13).

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The aim of our present study is to clarify the effect of FFAs on endothelial function, especially NO release and intracellular signal transduction. We demonstrated that unsaturated FFAs (UFFAs) inhibit NO release in cultured endothelial cells, and the mechanism of inhibitory action by UFFAs was thought to be the inhibition of agonist-induced calcium mobilization in vascular endothelial cells.

MATERIALS AND METHODS

Materials

Sodium salt of oleic acid (OA), linoleic acid (LA) and palmitic acid (PA), adenosine 5'-(3-*O*-thio) triphosphate (ATPγS), and ionomycin were purchased from Sigma Chemical Co.(St. Louis, MO). Fura 2 acetoxymethyl ester was obtained from Dojindo Laboratories (Kumamoto, Japan). Myo-[2-^{3}H]inositol (20 Ci/mmol) was obtained from Amersham (Arlington Heights, IL). Ionomycin was dissolved in ethanol. Maximal ethanol concentration in each experiment was 0.01 %, which did not induce any changes in [Ca^{2+}]i or NO production. Other materials and chemicals were obtained from commercial sources. Cultured bovine aortic endothelial cells (BAECs) used in the present study were 5 th to 11 th passage.

Measurement of NO Release by Chemiluminescence

The release of NO from the cultured BAECs was measured by examining production of nitrite, the stable degradation product of NO, with a NOx analyzer as previously described (9). At the time of study, the BAECs (3 x 10^6) in 60-mm dish were rinsed twice with 2 ml of physiological saline solution (PSS) (pH 7.4), and were incubated with 2 ml of PSS at 37 °C with various drugs as follows. To examine the effect of FFAs on NO release, the cells were preincubated with FFAs such as OA, LA and PA for 10 min before addition of 10 μM ATPγS, and then cells were incubated for 60 min. After incubation, an aliquot of 1 ml was used to measure NO release by chemiluminescence. To standardize the protein concentration the BAECs were solubilized with 1 ml of 1 N NaOH for determination of protein. Protein concentrations were determined with BSA as a standard protein as described (2).

Measurement of Intracellular Free Calcium Concentration

The [Ca^{2+}]i level was monitored by measuring the fluorescence of fura 2 (7). BAECs were harvested by enzymatic digestion (0.25 % trypsin and 0.02 % EDTA) and resuspended in PSS containing 2 mmol/L fura 2-AM. After 30 min at 37 °C, BAECs were washed with DMEM with 0.1 % BSA and PSS and resuspended at a final concentration of 1.25 x 10^6 cells/mL. Fura 2 fluorescence was monitored by the ratio-fluorescence spectroscopy technique with a dual-wavelength spectrofluorometer (HITACHI F2000). The fluorescent Ca^{2+} indicator was excited alternately at 340 and 380 nm, and emission was collected at 500 nm. The signal ratio (340:380) was determined after autofluorescence correction, and free intracellular Ca^{2+} concentration was calculated according to Grynkiewiecz et al, a dissociation constant of 224 nmol/L was assumed for the fura 2/Ca^{2+} complex at 37 °C. Calibration factors Rmax and Rmin (maximum and minimum fluorescence ratio, respectively) and Sf2/Sb2 (the ratio of fluorescence values at 380 nm in the absence and presence of saturating Ca^{2+} concentrations) were determined in situ by addition of 1 μmol/L ionomycin in the presence of 2.5 mmol/L Ca^{2+} (to obtain Rmax and Sb2) and by subsequent addition of excess (20 mmol/L) of EGTA (to obtain Rmin and Sf2).

Assay for Phospholipase C Reaction

The cells were plated into 35-mm dishes. After 2 days the cultured cells reached confluence. The final cell density on the day of the assay was 1 X 10^6 cells per dish. Phospholipase C reaction was measured by the methods previously described (8).

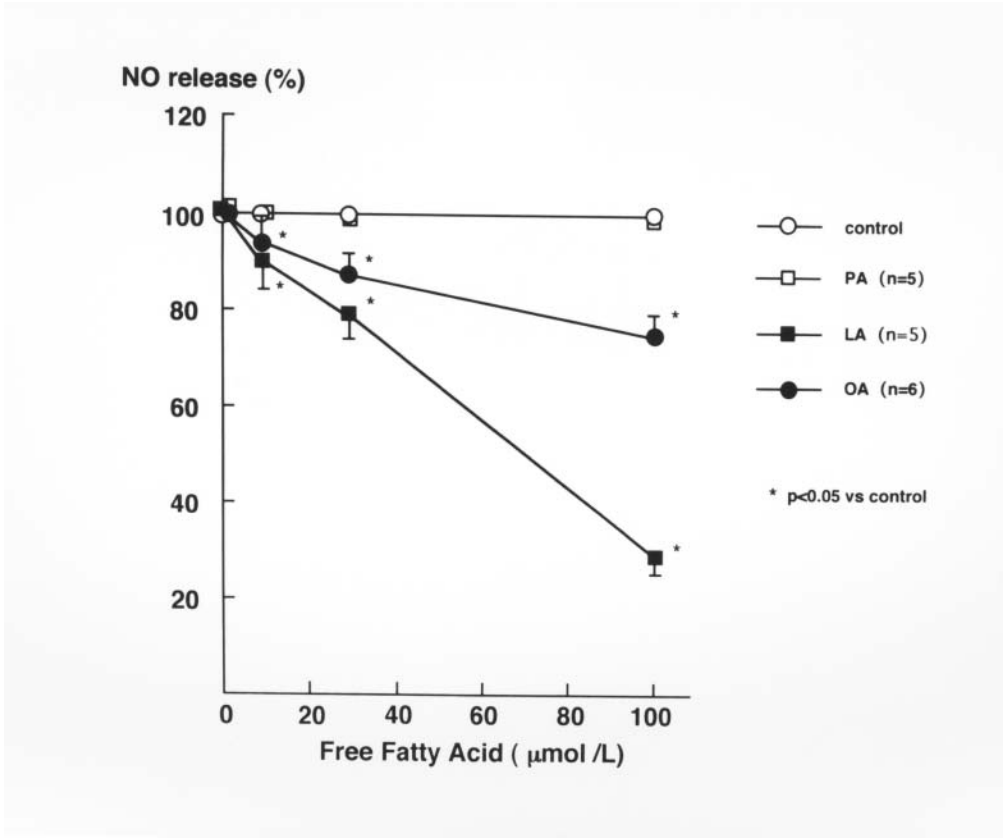

Figure 1. Effect of free fatty acids on nitric oxide (NO) release.

We examined the effect of fatty acids on accumulation of NO release induced by 10 μmol/L ATPγS from bovine aortic endothelial cells (BAECs) (○). The amount of NO release stimulated by 10 μmol/L ATPγS for 60 minutes is 904.8 ± 75.2 pmol/mg protein. BAECs were preincubated for 10 minutes with oleic acid (OA, n=6) (●), linoleic acid (LA, n=5) (■), and palmitic acid (PA, n=5) (□) at the concentrations shown before addition of ATPγS.

* P<0.05 compared with NO release without FFAs.

Determinations

Results were expressed as mean ± SEM. Statistical evaluation of the data was performed by Student's t-test for unpaired observation. When more than two groups were compared, the significance of the difference between group means was analyzed by one-way analysis of variance and the Bonferroni test for samples. Values were considered to be statistically different at $p<0.05$.

RESULTS

Nitrite Release from BAECs

A standard curve for nitrite over 100 pmol was obtained in response to infusion of standard quantities of sodium nitrite. NOx signals linearly increased with concentrations of sodium nitrite (data not shown). The release of nitrite stimulated by ATPγS from BAECs increased lineally with incubation time. We examined the effect of FFAs on accumulation of nitrite release induced by 10 μmol/L ATPγS for 60 minutes. The amount of NO release

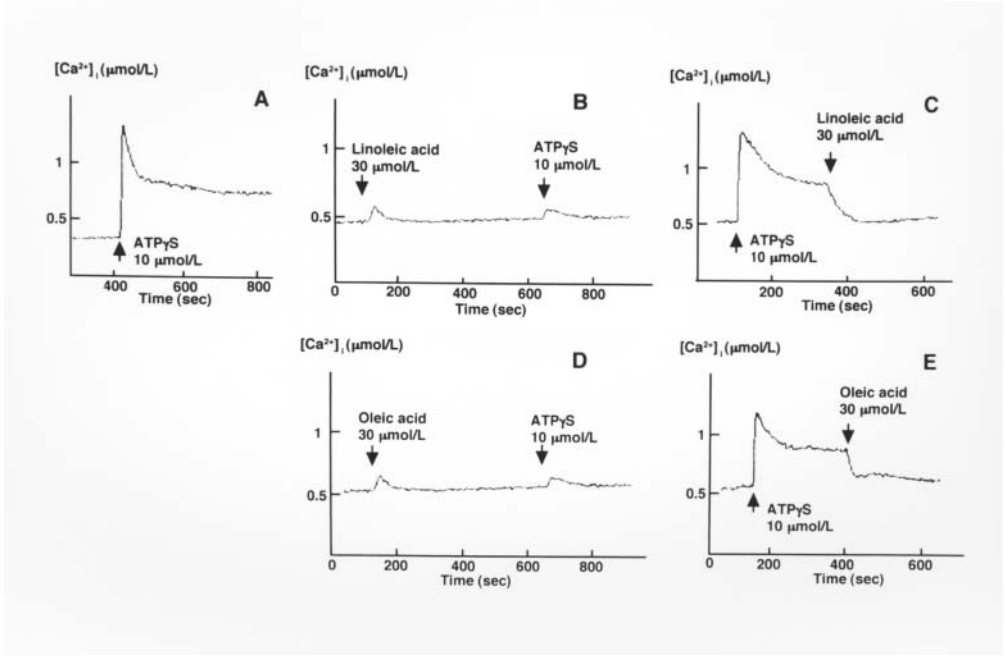

Figure 2. Representative tracings showing the effect of free fatty acids on ATPγS-induced intracellular calcium concentrations ($[Ca^{2+}]i$) in bovine aortic endothelial cells (BAECs).

ATPγS (10 μmol/L) was added to the fura 2-loaded BAECs at the time indicated by the arrow (A). After preincubation with 30 μmol/L linoleic acid (LA) (B) and 30 μmol/L oleic acid (OA) (D) for 5 minutes, 10 μmol/L ATPγS was added. OA (30 μmol/L) (C) and LA (30 μmol/L) (E) were added during the ATPγS-induced sustained phase of $[Ca^{2+}]i$.

Table Ⅰ. Effects of free fatty acids on ATPγS-induced $[Ca^{2+}]i$ rise.

		phasic (nmol/L)	sustained (nmol/L)
Control		1569 ± 191	319 ± 25
Palmitic acid	100 μmol/L	1540 ± 145	267 ± 39
Oleic acid	30 μmol/L	699 ± 83*	112 ± 24*
	100 μmol/L	89 ± 13**	79 ± 11*
linoleic acid	30 μmol/L	647 ± 184*	212 ± 57*
	100 μmol/L	98 ± 13**	81 ± 13*

Suspended bovine aortic endothelial cells (BAECs) were preincubated with free fatty acids for 3 minutes at the concentrations indicated and 10 μmol/L ATPγS was added. Results are expressed as mean ± SEM of five independent experiments.

* $P<0.05$ vs. control

** $P<0.03$ vs. control

stimulated by 10 μmol/L ATPγS for 60 minutes is 904.8 ± 75.2 pmol/mg protein. FFAs (10-100 μM) themselves did not increase nitrite release in this experimental condition. Preincubation with OA (10-100 μM) or LA (10-100 μM) dose-dependently reduced nitrite release stimulated by 10 μmol/L ATPγS, whereas preincubation with PA (10-100 μM) had no effect on the nitrite release by ATPγS (Fig. 1). To investigate the influences of oxidation

of FFAs on the inhibitory effect, we measured the ATPγS-induced NO release from BAECs preincubated with FFAs in the presence of 100 μmol/L butylated hydroxytoluene (BHT), antioxidant. The reduction of the ATPγS-induced NO release by FFAs was not changed by coincubation with BHT (data not shown). We measured lactate dehydrogenase (LDH) concentrations released from BAECs after each experiment to assess the cytotoxic effect. UFFAs or agonists did not release LDH from BAECs during the incubation at the concentration used in this experiment.

Effects of FFAs on the Elevation of $[Ca^{2+}]i$

We measured $[Ca^{2+}]i$ in BAECs to clarify the mechanism of the inhibitory effect of UFFAs on nitrite release. Representative tracings are shown in Figure 2. After stabilization of $[Ca^{2+}]i$ for several minutes, the median baseline value was measured. ATPγS (0.1-10 μmol/L) induced a biphasic rise of $[Ca^{2+}]i$, i.e., a rapid increase followed by a sustained increase. OA (10-100 μM) and LA (10-100 μM) themselves also elicited increases in $[Ca^{2+}]i$ in a dose-dependent manner, whereas PA (10-100 μM) had no significant effect on $[Ca^{2+}]i$. OA (30-100 μM) and LA (30-100 μM) inhibited ATPγS-induced increase in $[Ca^{2+}]i$, both in rapid phase and in sustained phase, in a dose dependent manner (Fig. 2, Table Ⅰ). Moreover,

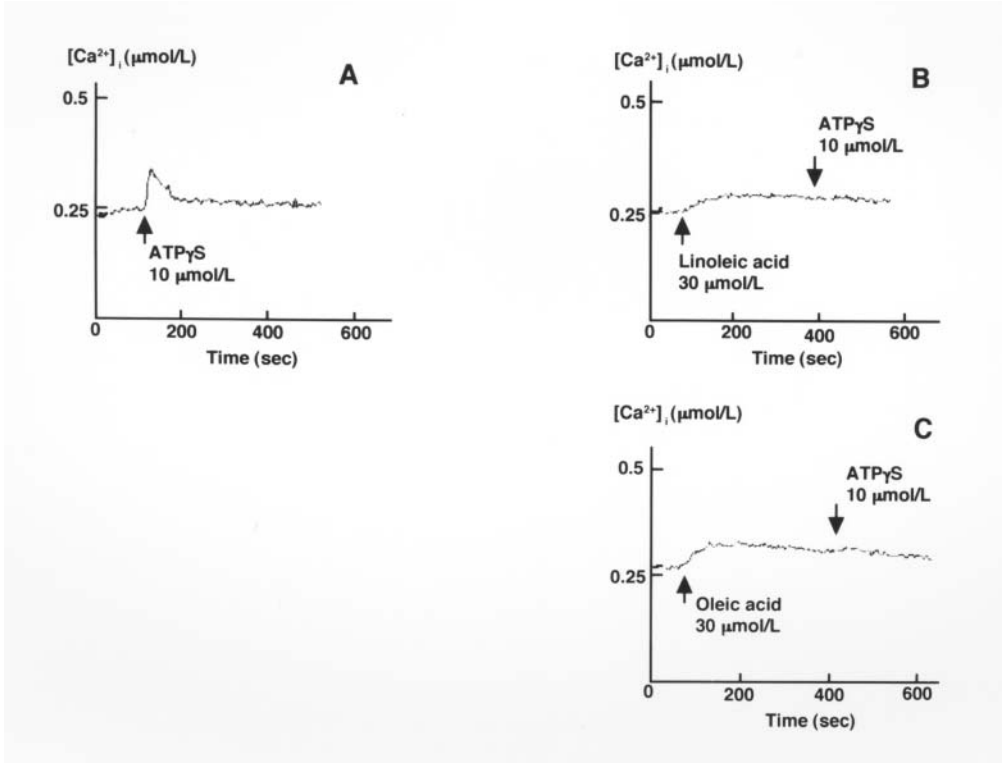

Figure 3. Representative tracings showing the effect of free fatty acids on ATPγS-induced intracellular calcium concentration ($[Ca^{2+}]i$) in bovine aortic endothelial cells (BAECs) in the absence of Ca^{2+}.

ATPγS (10 μmol/L) was added to the fura 2-loaded BAECs at the time indicated by the arrow (A). After preincubation with 30 μmol/L linoleic acid (LA) (B) or 30 μmol/L oleic acid (OA) (C) for 3 minutes, 10 μmol/L ATPγS was added.

UFFAs induced a rapid inhibition of the $[Ca^{2+}]i$ rise when OA and LA were added during the course of ATPγS-induced $[Ca^{2+}]i$ increase (Fig. 2). In contrast, PA had no effect on the increase in $[Ca^{2+}]i$ evoked by ATPγS. The reduced Ca^{2+} mobilization by UFFAs was completely reversed by treatment with bovine serum albumin (1 mg/ml), a well-known fatty acid-binding protein (data not shown).

In Ca^{2+} free conditions, OA (10-100 μM) and LA (10-100 μM) themselves also elicited increases in $[Ca^{2+}]i$ in a dose-dependent manner. An increase of $[Ca^{2+}]i$ by ATPγS was almost abolished after the incubation of BAECs with UFFAs (Fig. 3).

Effect of FFAs on Ionomycin-induced $[Ca^{2+}]i$ Elevation

To clarify whether the inhibitory property of FFAs depends on the receptor mediated phospholipase C-coupled pathway, we investigated the effect of FFAs on ionomycin-induced $[Ca^{2+}]i$ rise. As shown in figure 4, neither OA nor LA had any effect on ionomycin-induced $[Ca^{2+}]i$ rise.

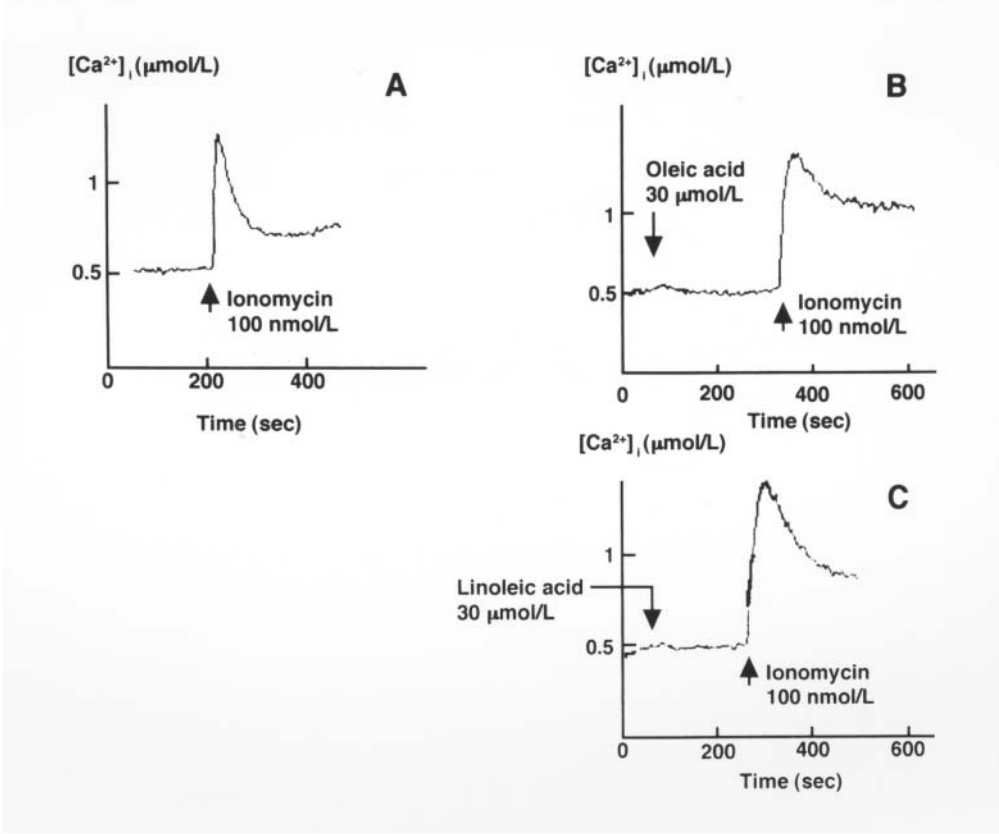

Figure 4. Representative tracing showing the effect of free fatty acids on ionomycin-induced intracellular calcium concentration in bovine aortic endothelial cells.

Ionomycin (100 nmol/L) was added to the fura 2-loaded BAECs at the time indicated by the arrow (A). After preincubation with 30 μmol/L oleic acid (OA) (B) and 30 μmol/L linoleic acid (LA) (C) for 3 minutes, 100 nmol/L ionomycin was added.

Table Ⅱ. Effects of free fatty acids on ATPγS-induced accumulation of inositol phosphates.

		IP1 (dpm)	IP2 (dpm)	IP3 (dpm)
Control		13753 ± 927	4478 ± 909	4550 ± 541
oleic acid	30 μmol/L	13675 ± 636	4422 ± 603	4737 ± 382
linoleic acid	30 μmol/L	13255 ± 823	4185 ± 824	4397 ± 527

Bovine aortic endothelial cells were preincubated with 30 μmol/L oleic acid and 30 μmol/L linoleic acid for 10 minutes and then stimulated with 10 μmol/L ATPγS for 30 seconds.
IP1; inositol 1-monophosphate. IP2; inositol 1,4-bisphosphate. IP3; inositol 1,4,5-trisphosphate.
Results are expressed as mean ± SEM of five independent experiments.

Effect of FFAs on ATPγS-induced Accumulation of Inositol Phosphates

Incubation of cultured BAECs with 10 μmol/L ATPγS induced accumulation of IP1, IP2, and IP3. IP2 and IP3 production was rapid and reached a peak at 30 seconds with an approximately threefold increase and thereafter declined. The decline of accumulated IP2 and IP3 might be due to the conversion of IP2 to IP1 and IP3 to IP2, as demonstrated in other cell types. On the other hand, without ATPγS, no significant increase in any of the inositol phosphates was induced in BAECs even in the presence of LiCl. We examined the effect of FFAs on the phospholipase C-mediated reaction in BAECs. OA and LA themselves had no effects on the phospholipase C-mediated phosphoinositide hydrolysis (Table Ⅱ). Moreover, neither OA nor LA had any effects on the ATPγS-induced inositol phosphates accumulation.

DISCUSSION

There is accumulating evidence that elevation of circulating FFAs causes endothelial cell dysfunction. However, the mechanism of endothelial dysfunction including reduced endothelium-dependent relaxation is unresolved (4). In the present study we demonstrated that UFFAs reduces ATPγS-induced NO release in BAECs. We also showed that UFFAs inhibit Ca^{2+} mobilization from intracellular Ca^{2+} stores and Ca^{2+} influx from extracellular space. UFFAs did not alter IP3 formation evoked by ATPγS.

Because long chain UFFAs including OA contains an amphiphile molecule with detergent like effect and may easily penetrate into and damage the endothelium. In our present study, LDH, a cytosolic enzyme, was not released from BAECs during incubation with UFFAs and the inhibitory effects of UFFAs on Ca^{2+} mobilization was reversed by treatment with BSA. Therefore the effects of UFFAs on NO production and Ca^{2+} mobilization in BAECs were not due to cytotoxic effects, and cell viability was preserved. This effect appeared to be specific to the unsaturation of the FFAs since saturated ones such as PA did not affect the Ca^{2+} mobilization and NO release. This inhibitory effect is not due to the oxidation of UFFAs in our experimental system because co-incubation of BHT, an antioxidant, with UFFAs had no effect on the inhibition of Ca^{2+} mobilization and NO release by UFFAs.

In the present study, we measured $[Ca^{2+}]i$ in the suspensions of cultured BAECs by the fluorescent calcium indicator fura 2. Although the cell suspension system is not physiological condition for vascular endothelial cells, this system has been employed to demonstrate that the some substances inhibit NO production and agonist-induced $[Ca^{2+}]i$ in cultured endothelial cells (6,11). Thus, we speculate that the inhibition of receptor-mediated

Ca^{2+} mobilization and Ca^{2+} influx by UFFAs may contribute to inhibitory effect of FFAs on agonist-induced NO production in endothelial cells.

The results of the present study indicate that UFFAs inhibited the agonist-induced increase in $[Ca^{2+}]i$, and finally NO release from BAECs by mechanisms independent of the receptor-mediated phospholipase C-coupled system, as demonstrated by the unchanged IP3 production. The finding that UFFAs did not inhibit ionomycin-induced Ca^{2+} increase indicates that UFFAs don't affect Ca^{2+} extrusion. UFFAs inhibited both the rapid and the sustained increase in $[Ca^{2+}]i$ evoked by ATPγS. The sustained increase in $[Ca^{2+}]i$ reflects Ca^{2+} influx from extracellular space, which is believed to be triggered by Ca^{2+} release from the intracellular Ca^{2+} stores. We found that, in Ca^{2+} free condition, UFFAs inhibited Ca^{2+} release from the intracellular Ca^{2+} stores. Thus, UFFAs inhibit Ca^{2+} mobilization from intracellular Ca^{2+} stores and Ca^{2+} influx from extracellular space. The inhibitory mechanism of UFFAs on Ca^{2+} mobilization is still unknown. However, UFFAs themselves induced a small but significant Ca^{2+} release from intracellular Ca^{2+} stores. Therefore, the inhibitory effect of UFFAs may be partially due to the depletion of the IP3-sensitive intracellular Ca^{2+} stores（10）.

Plasma FFA concentration is elevated after the meal and in the state of insulin resistance, which underlies various disorders such as hypertension and non-insulin dependent diabetes mellitus. Moreover, plasma FFA concentration was also elevated during the intravenous infusion of heparin through the activation of lipoprotein lipase. From the present study, it is thought that the elevated FFAs, particularly UFFAs, may cause endothelial dysfunction in those pathological conditions through the impairment of Ca^{2+} regulation in vascular endothelial cells.

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