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HMG-CoA Reductase Inhibitors Suppress Intracellular Calcium Mobilization and Membrane Current Induced by Lysophosphatidylcholine in Endothelial Cells

Keiko Yokoyama, MD; Toshiyuki Ishibashi, MD; Hiroshi Ohkawara, MD; Junko Kimura, MD; Isao Matsuoka, PhD; Takayuki Sakamoto, MD; Kenji Nagata, MD; Koichi Sugimoto, MD; Sotaro Sakurada, MD; Yukio Maruyama, MD

Background—Lysophosphatidylcholine (LPC) is known to increase intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in endothelial cells. This study was conducted to investigate the effects of HMG-CoA reductase inhibitors (statins) on the increase in $[\text{Ca}^{2+}]_i$ and membrane current induced by LPC.

Methods and Results— $[\text{Ca}^{2+}]_i$ was determined in cultured human aortic endothelial cells by fura-2 assay, and membrane current was measured by whole-cell patch clamp. The $[\text{Ca}^{2+}]_i$ increase induced by LPC was abolished by inhibitors of phospholipase C (PLC). Statins markedly decreased the $[\text{Ca}^{2+}]_i$ increase caused by LPC. This suppressive effect was quickly reversed by geranylgeranylpyrophosphate (GGPP) and was mimicked by inhibitors of Rho and Rho kinase. LPC induced the translocation of the GTP-bound active form of RhoA into membranes within 1 minute as determined by a pull-down assay and reduced the levels of RhoA in the cytoplasm, indicating that LPC quickly increases the GTP/GDP ratio of RhoA and induces membrane translocation. Statins prevented the GTP/GDP exchange of RhoA and its membrane translocation from the cytoplasm caused by LPC, and these effects of statins were reversed by GGPP. The responses of RhoA activation to statins and GGPP concurred with their effects on Ca^{2+} mobilization. LPC also induced a nonselective cation current after a lag. Statins prolonged the lag and decreased the current amplitude, and GGPP abolished the inhibitory effect on the current.

Conclusions—LPC induced Ca^{2+} mobilization and membrane current via a Rho activation-dependent PLC pathway in endothelial cells, and statins blocked these effects by preventing the GGPP-dependent lipid modification of Rho. The present study implicates Rho in LPC stimulation of Ca^{2+} movement. (*Circulation*. 2002;105:962-967.)

Key Words: endothelium ■ signal transduction ■ statins ■ calcium ■ ion channels

The dysfunction of endothelial cells is one of the initial steps in atherosclerosis.¹ Hyperlipidemia impairs endothelium-dependent relaxation of coronary arteries in humans.^{2,3} Oxidized low-density lipoprotein (LDL) plays an important role in endothelial dysfunction, and lysophosphatidylcholine (LPC) is a phospholipid component of oxidized LDL. When LDL is oxidized, approximately 60% of the phosphatidylcholine seems to be converted to LPC, which induces atherogenic activities, including the induction of adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1) in endothelial cells.^{4,5}

LPC also increases intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in endothelial cells.⁶ $[\text{Ca}^{2+}]_i$ increase consists of the release of Ca^{2+} from intracellular Ca^{2+} stores and influx of extracellular Ca^{2+} across the plasma membrane. However, the mechanisms by which LPC induces intracellular Ca^{2+} mobilization and the influx of extracellular Ca^{2+} are not clear.

HMG-CoA reductase inhibitors (statins) prevent the incidence of coronary events and improve endothelium-dependent relaxation in hyperlipidemic patients with coronary artery disease.^{2,3,7} In addition, statins have been shown in vitro and in vivo to improve endothelial nitric oxide synthesis by direct actions independent of cholesterol lowering.^{8,9} However, it is not known whether statins alter the effects induced by LPC on $[\text{Ca}^{2+}]_i$ and membrane current in endothelial cells.

In the present study, we examined the effects of LPC on $[\text{Ca}^{2+}]_i$ and membrane current and their possible mechanisms and the effects of statin on the $[\text{Ca}^{2+}]_i$ and membrane current induced by LPC in human aortic endothelial cells.

Methods

Materials

L- α -LPC (palmitoyl, Sigma Chemical Co) was dissolved in chloroform and methanol (3:1) evaporated to dryness and dissolved in

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Tyrode solution. DL-mevalonic acid lactone, geranylgeranylpyrophosphate (GGPP), and farnesylpyrophosphate (FPP) were obtained from Sigma. Fura-2/acetoxymethyl ester, neomycin sulfate, and U-73122 were from Wako Pure Chemicals. Fluvastatin was kindly provided by Novartis Pharm Inc (Basel, Switzerland), cerivastatin was from Bayer, pravastatin from Sankyo Pharmaceutical, and Y-27632, a Rho-kinase inhibitor, from Welfide. C3 exoenzyme, a Rho inhibitor, was purchased from Upstate Biotechnology.

Preparation of Endothelial Cells

Passage 4 endothelial cells from human aortic artery were purchased from Clonetics and cultured according to the supplier's instructions and were used for experiments after 5 to 10 passages.

Measurement of [Ca²⁺]_i

[Ca²⁺]_i was measured as described previously.¹⁰ Briefly, endothelial cells were grown to confluence in 35-mm glass-bottom dishes (MatTek). The cells were incubated at 37°C for 10 minutes with 1 μmol/L fura-2/acetoxymethyl ester and then washed with the Tyrode solution. The dish was mounted on an inverted fluorescence microscope (IX-FLA, Olympus) and then placed in a flow-through chamber (0.2 mL volume). Fura-2-loaded cells were perfused continuously (4 mL/min) with prewarmed (37°C) Ca²⁺-containing or Ca²⁺-free Tyrode solution in the absence or presence of agents. The cells were exposed to alternating excitation wavelengths of 340 and 380 nm, and the fluorescence image was monitored by a cooled CCD camera (Ultra Pix, Olympus). Data were recorded and processed using a MERLIN ratio image system (Olympus), and the 340/380-nm fluorescence emission was calculated.

Western Blotting of RhoA

Western blotting was performed as previously described.¹¹ Briefly, cells were lysed with a hypotonic buffer, and a part of lysate was sonicated and centrifuged at 15 000g for 10 minutes to prepare membrane and cytoplasmic fractions. Aliquots containing 20 μg of proteins were subjected to SDS/polyacrylamide gel electrophoresis (10% running gel, 5% stacking gel, PAGEL, ATTO Co) and were then transferred onto polyvinylidene difluoride membranes (MILLIPORE). After incubating with blocking solution at room temperature for 30 minutes, the membranes were incubated for 60 minutes at room temperature with a mouse monoclonal antibody to RhoA diluted 1:500 (Santa Cruz Biotechnology Inc, Santa Cruz, Calif) and then for 45 minutes with a horseradish peroxidase-conjugated goat anti-mouse IgG diluted 1:10000 (Santa Cruz). The signals from immunoreactive bands were visualized by an Amersham ECL System (Amersham Pharmacia Biotech UK Lit).

Determination of GTP-Rho

RhoA activity was determined by measuring GTP-Rho using the Rho-binding domain of rhotekin, as previously described.¹² Briefly, samples of the membrane fractions of the cells were extracted with a lysis buffer. The extracts were incubated for 45 minutes at 4°C with glutathione-S-transferase (GST)-rhotekin fusion protein bound to glutathione-Sepharose 4B beads. The beads were washed 3 times with a washing buffer and boiled for 5 minutes in a loading buffer. Bound GTP-Rho proteins were quantitatively determined by Western blotting.

Membrane Current Recording

Whole-cell patch clamp was performed using the external and pipette solutions, as previously described.¹³ The external solution was prewarmed to ≈35 to 37°C by a water jacket. The resistance of the pipette filled with the pipette solution was 2 to 4 megaΩ. The patch-clamp amplifier was TM-1000 (Act ME). Ramp pulses were given every 10 seconds from the holding potential of -60 mV, initially depolarizing to +60 mV and then hyperpolarizing to -110 mV and depolarizing back to -60 mV, with a constant speed of 0.72 V/sec. The data were collected online and stored in a computer (PC-9801 RX, NEC).

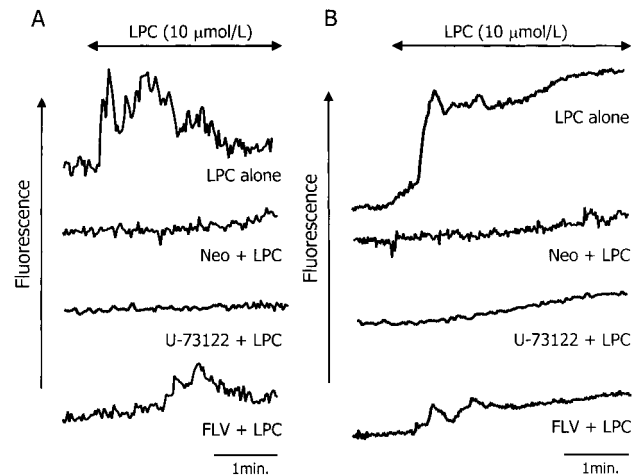


Figure 1. Time course of [Ca²⁺]_i in human aortic endothelial cells treated with 10 μmol/L LPC, 1 μmol/L neomycin (Neo) or U-73122 plus 10 μmol/L LPC, and 5 μmol/L FLV plus 10 μmol/L LPC in the absence (A) or presence (B) of external Ca²⁺. Cells were treated with each agent 3 minutes before LPC perfusion. Each line represents mean fluorescence values of [Ca²⁺]_i in 100 cells.

Statistical Analysis

Statistical analyses were performed using ANOVA with Scheffé's post hoc test, as appropriate. A level of *P* < 0.05 was considered significant. Data are expressed as mean ± SEM.

Results

Effects of LPC on [Ca²⁺]_i

Figure 1 demonstrates the mean fluorescent values indicating [Ca²⁺]_i of 100 endothelial cells each in the presence or absence of external Ca²⁺. LPC (10 μmol/L) induced a rapid and transient increase in [Ca²⁺]_i in the absence of external Ca²⁺ (Figure 1A, top), whereas in the presence of external Ca²⁺, a sustained increase was observed (Figure 1B, top). When 1 μmol/L neomycin or U-73122, PLC inhibitors, perfused the cells starting 3 minutes before LPC treatment, each agent markedly suppressed the increase in [Ca²⁺]_i induced by LPC in the presence or absence of external Ca²⁺ (Figures 1A and 1B, middle). These observations suggest that the increase in [Ca²⁺]_i induced by LPC is mediated by a PLC-signaling pathway.

Effects of Statins and Rho/Rho-Kinase Inhibitors on [Ca²⁺]_i Increase Induced by LPC

Fluvastatin markedly suppressed the [Ca²⁺]_i increase induced by LPC in the presence or absence of external Ca²⁺ (Figure 1, bottom and Figures 2A and 2B). However, fluvastatin (FLV) did not reduce the elevated [Ca²⁺]_i induced by LPC when FLV was perfused after LPC. Moreover, FLV had no effect on [Ca²⁺]_i induced by ATP (100 μmol/L), a G-protein-coupled receptor agonist (data not shown). To examine whether the effect is mediated by a mevalonate pathway, 100 μmol/L mevalonate, 2.5 μmol/L GGPP, or 2.5 μmol/L FPP was added to the cells 3 minutes before FLV and LPC. The suppressive effect of FLV was abolished by mevalonate or GGPP but not by FPP (Figures 2A and 2B), suggesting an involvement of GGPP-dependent isoprenylation of signaling molecules, such as Rho, in LPC-induced effects. Therefore,

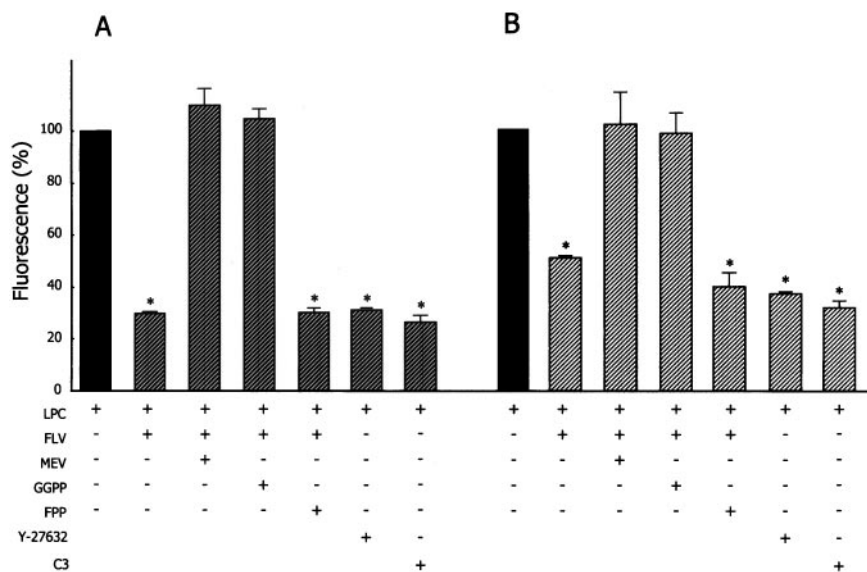


Figure 2. Peak $[Ca^{2+}]_i$ values in the absence (A) or presence (B) of external Ca^{2+} within 3 minutes of LPC stimulation. FLV (5 $\mu\text{mol/L}$) or Y-27632 (10^{-7} mol/L) was perfused 3 minutes before 10 $\mu\text{mol/L}$ LPC. Cells were also treated with 100 $\mu\text{mol/L}$ mevalonate, 2.5 $\mu\text{mol/L}$ GGPP, or 2.5 $\mu\text{mol/L}$ FPP from 3 minutes before being treated with FLV+LPC. Cells were cultured with 0.25 $\mu\text{g/mL}$ C3 exoenzyme overnight and perfused with LPC. MEV indicates mevalonate; GGPP, geranylgeranylpyrophosphate; and FPP, farnesylpyrophosphate. Each bar represents mean \pm SEM of percent fluorescent values of 100 cells compared with those treated with LPC alone. * $P < 0.0001$ vs LPC alone.

we examined the effects of C3 exoenzyme and Y-27632 on the $[Ca^{2+}]_i$ increase evoked by LPC. Treatment with C3 exoenzyme overnight (0.25 $\mu\text{g/mL}$) markedly inhibited the $[Ca^{2+}]_i$ increase induced by LPC (Figures 2A and 2B). Perfusion with Y-27632 (10^{-7} mol/L) induced a suppressive effect similar to statins on $[Ca^{2+}]_i$ increase induced by LPC (Figures 2A and 2B).

Cells were treated with 3 kinds of statins starting 3 minutes before LPC perfusion. Figure 3 shows that there was no significant difference among 3 agents (5 $\mu\text{mol/L}$ FLV, 0.1 $\mu\text{mol/L}$ cerivastatin, and 20 $\mu\text{mol/L}$ pravastatin) in their suppressive effect on $[Ca^{2+}]_i$ increase evoked by 10 $\mu\text{mol/L}$ LPC.

Translocation of RhoA Into Membranes and GTP/GDP Exchange

To examine the correlation between Rho activation and Ca^{2+} mobilization, we determined the translocation of Rho into membranes and the activity of Rho. Western blotting revealed that LPC increased the levels of RhoA in membrane fractions

within 1 minute and decreased its levels in the cytoplasm. A 3-minute treatment with FLV before the addition of LPC blocked the membrane translocation and the reduction of cytoplasmic RhoA induced by LPC (Figure 4A). Figure 4B shows that the translocated RhoA is in a GTP-bound active form, indicating that LPC markedly increases the GTP/GDP ratio of RhoA, which is followed by membrane translocation within 1 minute. A 3-minute incubation with FLV prevented the GTP/GDP exchange and membrane translocation of RhoA. GGPP fully restored the LPC-induced membrane translocation and reduction of cytoplasmic RhoA, which were inhibited by FLV, whereas FPP had no effect (Figure 4C). A 3-minute incubation with FLV alone did not significantly alter the distribution of RhoA in membranes and cytoplasm or the GTP/GDP ratio in the membranes (data not shown).

Membrane Current Induced by LPC

Because the sustained increase in $[Ca^{2+}]_i$ in the presence of external Ca^{2+} is probably mediated by Ca^{2+} influx across the

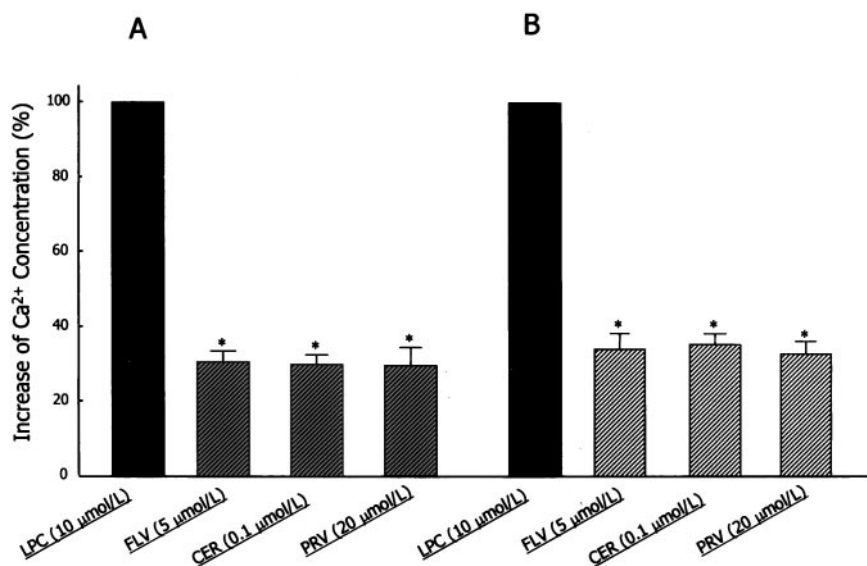


Figure 3. Peak $[Ca^{2+}]_i$ values of cells pretreated with 5 $\mu\text{mol/L}$ FLV, 0.1 $\mu\text{mol/L}$ cerivastatin (CER), or 20 $\mu\text{mol/L}$ pravastatin (PRV) in the absence (A) or presence (B) of external Ca^{2+} within 3 minutes of 10 $\mu\text{mol/L}$ LPC stimulation. Data are expressed as mean \pm SEM of percent fluorescent values compared with those from LPC treatment alone (each group, $n=100$). * $P < 0.0001$ vs LPC alone.

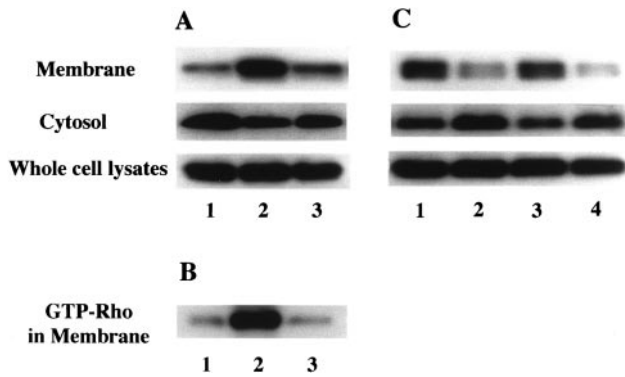


Figure 4. A, Levels of RhoA in membrane (top) and cytoplasmic (middle) fractions and whole-cell lysates (bottom). Cells were incubated for 1 minute in the presence of 10 $\mu\text{mol/L}$ LPC with or without a 3-minute pretreatment with 5 $\mu\text{mol/L}$ FLV. Lane 1, control; lane 2, LPC; and lane 3, FLV+LPC. B, Levels of the GTP-bound form of RhoA in membrane fractions in the same treatment as panel A. Lane 1, control; lane 2, LPC; and lane 3, FLV+LPC. C, Restoring effects of GGPP on the levels of RhoA in the membrane and cytoplasm induced by FLV+LPC. GGPP (lane 3) or FPP (lane 4) was added to cells 3 minutes before FLV and LPC. Cells were then treated with 5 $\mu\text{mol/L}$ FLV for 3 minutes, followed by 1-minute LPC perfusion. Lane 1, LPC; lane 2, FLV+LPC; lane 3, GGPP+FLV+LPC; and lane 4, FPP+FLV+LPC.

plasma membrane, we measured membrane currents. After a lag, LPC increased the membrane current. Figure 5A shows a representative time course of the membrane current induced by 10 $\mu\text{mol/L}$ LPC (top), the current-voltage (*I-V*) curves of the control (a) and during the application of LPC (b) (middle), and the difference in the *I-V* curve before and at 130 seconds after addition of 10 $\mu\text{mol/L}$ LPC (bottom). The reversal potential of the current induced by LPC was 0 mV, suggesting that LPC induces a nonselective cation current (Figure 5A, bottom).

A significant difference in the duration of the lag was observed between the cells treated with 0.2 $\mu\text{mol/L}$ and those treated with >2 $\mu\text{mol/L}$ (Figure 6A, $P < 0.001$). LPC induced

a significant membrane current at concentrations >2 $\mu\text{mol/L}$, and the current magnitude was concentration dependent (Figure 6B). The EC₅₀ of LPC in the nonselective cation current was 2 $\mu\text{mol/L}$.

Effects of Statins on the Current Induced by LPC
Pretreatment with FLV (5 $\mu\text{mol/L}$) for 3 minutes prolonged the lag (Figures 5B and 6C) and markedly suppressed the current density induced by 10 $\mu\text{mol/L}$ LPC (Figures 5B and 6D). Other statins, such as cerivastatin and pravastatin, had similar effects on the current induced by LPC (data not shown). Similar to the effect on [Ca²⁺]_i, FLV could not inhibit the current induced by LPC in the presence of 100 $\mu\text{mol/L}$ mevalonate (Figures 5C, 6C, and 6D).

We added 2.5 $\mu\text{mol/L}$ GGPP to the pipette solution and found that it completely abolished the inhibitory effect of FLV (Figure 7). This suggests that the effect of FLV is mediated through inhibition of geranylgeranylation. Furthermore, Y-27632 partially inhibited the current induced by LPC (data not shown).

Discussion

The present study showed that in human aortic endothelial cells, LPC increased [Ca²⁺]_i via PLC and that this was accompanied by translocation of the GTP-bound active form of RhoA into membranes, providing evidence for a novel signaling pathway in the RhoA-dependent PLC activation of Ca²⁺ regulation induced by LPC. Statins quickly prevented the [Ca²⁺]_i increase and the activation of RhoA caused by LPC, and these effects of statins were reversed by GGPP.

In this study, PLC inhibitors markedly inhibited the stimulatory effect of LPC on [Ca²⁺]_i in the absence or presence of external Ca²⁺. We showed that in the presence of external Ca²⁺, the LPC-induced sustained increase in [Ca²⁺]_i is mediated by Ca²⁺ influx through the nonselective cation channel. Therefore, our findings suggest that in endothelial cells the intracellular Ca²⁺ mobilization and the membrane current induced by LPC are mediated by the PLC-signaling pathway.

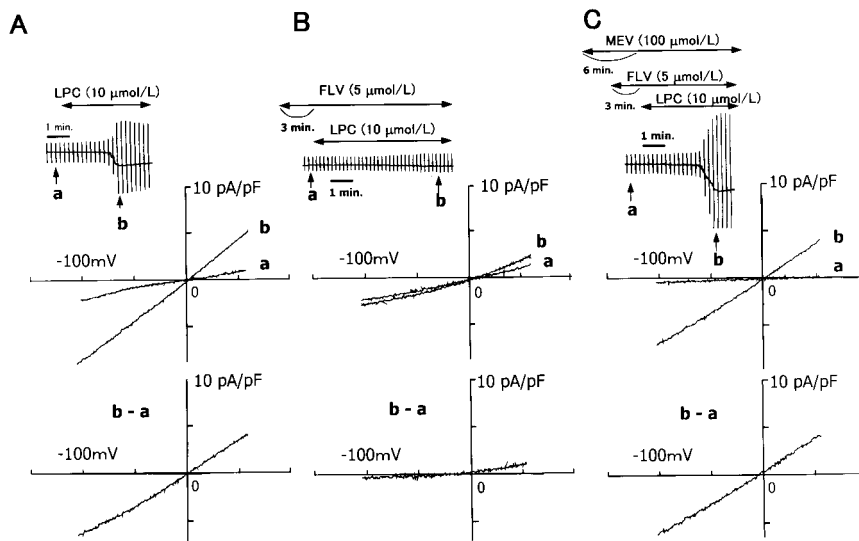


Figure 5. Effect of 10 $\mu\text{mol/L}$ LPC (A), 5 $\mu\text{mol/L}$ FLV plus 10 $\mu\text{mol/L}$ LPC (B), or 100 $\mu\text{mol/L}$ MEV plus 5 $\mu\text{mol/L}$ FLV plus 10 $\mu\text{mol/L}$ LPC (C) on the membrane current under the whole-cell patch clamp of cultured endothelial cells. A, Chart recording of the current (top), *I-V* curves of the control (a) and LPC-induced current density (b) (middle), and the difference in density between b and a of the middle panel (b-a, bottom) in an LPC-treated cell representative of 28 cells. B, Cells were treated with 5 $\mu\text{mol/L}$ FLV 3 minutes before LPC perfusion. Chart recording of the current (top), *I-V* curves during FLV (a) and FLV+LPC application (b) (middle), and the net current density change (b-a, bottom) in a representative cell of 28 cells. C, MEV (100 $\mu\text{mol/L}$) was applied 3 minutes before FLV and 6 minutes before LPC. Chart recording of the current (top), *I-V*

curves during MEV (a) and MEV, FLV+LPC application (b) (middle), and the net current density change (b-a, bottom) in a representative cell of 7 cells. Time points of a and b represent before and after stimulation.

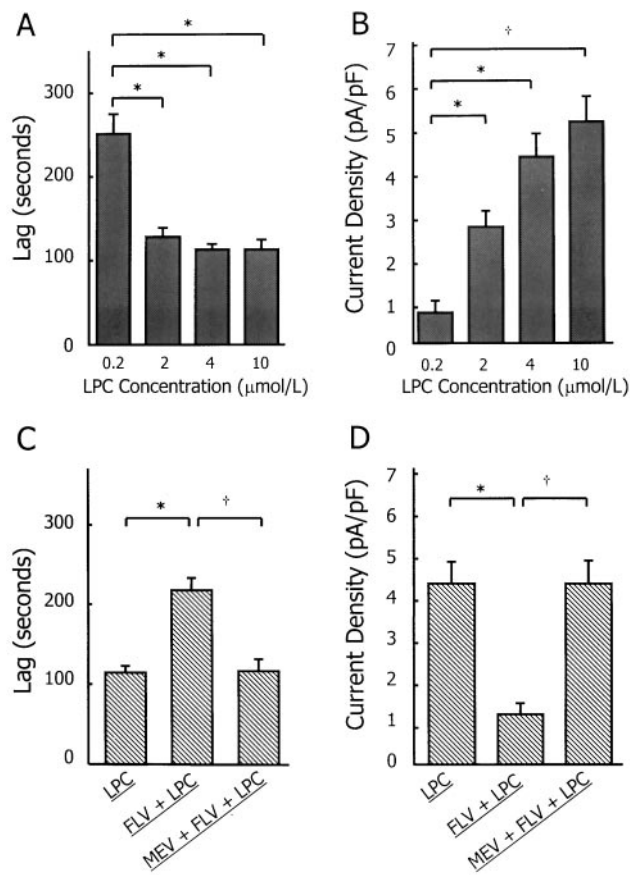


Figure 6. Lag before the appearance of membrane current after the start of LPC perfusion (A and C). Current density at +50 mV induced by LPC (B and D). Cells were treated with 5 $\mu\text{mol/L}$ FLV 3 minutes before 10 $\mu\text{mol/L}$ LPC. In addition, 100 $\mu\text{mol/L}$ MEV was perfused 3 minutes before FLV+LPC. Data are expressed as mean \pm SEM. * P <0.001; † P <0.05. A, Lag in seconds with various concentrations of LPC (0.2 $\mu\text{mol/L}$, 251 \pm 23 seconds, n =17; 2 $\mu\text{mol/L}$, 129 \pm 9 seconds, n =18; 4 $\mu\text{mol/L}$, 114 \pm 6 seconds, n =28; and 10 $\mu\text{mol/L}$, 114 \pm 10 seconds, n =28). B, Current density at +50 mV induced by various concentrations of LPC (0.2 $\mu\text{mol/L}$, 0.71 \pm 0.29 pA/pF, n =17; 2 $\mu\text{mol/L}$, 2.73 \pm 0.34 pA/pF, n =18; 4 $\mu\text{mol/L}$, 4.33 \pm 0.55 pA/pF, n =28; and 10 $\mu\text{mol/L}$, 5.16 \pm 0.61 pA/pF, n =28). C, Effect of 5 $\mu\text{mol/L}$ FLV or 100 $\mu\text{mol/L}$ MEV+FLV on the lag for current activation by LPC (10 $\mu\text{mol/L}$ LPC alone, 114 \pm 6 seconds, n =28; FLV+LPC, 218 \pm 14 seconds, n =28; MEV+FLV+LPC, 117 \pm 14 seconds, n =7). D, Effect of pretreatment with 5 $\mu\text{mol/L}$ FLV or 100 $\mu\text{mol/L}$ MEV plus 5 $\mu\text{mol/L}$ FLV on the current density at +50 mV induced by LPC (10 $\mu\text{mol/L}$ LPC alone, 4.33 \pm 0.55 pA/pF at +50 mV, n =28; FLV+LPC, 1.20 \pm 0.28 pA/pF, n =28; MEV+FLV+LPC, 4.30 \pm 0.73 pA/pF, n =5).

We found that within 1 minute, LPC increased the translocation of RhoA into membranes and that the translocated RhoA was in a GTP-bound active form. This was accompanied by a decrease in the levels of cytoplasmic RhoA. These findings suggest that LPC rapidly activates Rho metabolism, including the GTP/GDP exchange reaction and membrane translocation.

We also found that a 3-minute incubation with FLV prevented the GTP loading, membrane translocation, and reduction of cytoplasmic RhoA induced by LPC and that these were abolished by GGPP. This suggests that there is a pool of ungeranylgeranylated Rho in the cytoplasm and that

the GTP/GDP exchange reaction and the levels of the pool of ungeranylgeranylated Rho are quickly altered by a GGPP-dependent process in LPC stimulation. To prove this, it will be necessary to measure directly the pool of ungeranylgeranylated Rho.

Taken together, our findings suggest that the geranylgeranylation of unprocessed GDP-Rho might limit the rate of GTP/GDP exchange and membrane translocation when stimulated with LPC, although it is generally accepted that the GTP/GDP exchange reaction is a rate-limiting step in Rho activation.¹⁴ In the present study, we describe a novel mechanism of Rho activation. It would be of interest to see if geranylgeranylation is also involved in the activation of Rho by receptor agonists such as sphingosine 1-phosphate and lysophosphatidic acid, which seem to exert biological activities through Rho activation.¹⁵

Statins have been shown to decrease the membrane translocation of Rho slowly, taking several hours.¹⁶ Although a similar phenomenon was observed in cells not stimulated with LPC (data not shown), we show that a GGPP-dependent lipid modification takes place quickly and is associated with Rho activation and Ca^{2+} movement when stimulated with LPC and that statins quickly modulate Rho metabolism and Ca^{2+} movement via inhibiting geranylgeranylation. Moreover, the $[\text{Ca}^{2+}]_i$ increase evoked by ATP was not affected by statins, suggesting that LPC does not directly activate the heterotrimeric Gq family. Thus, the rapid responses of Rho metabolism and Ca^{2+} movement to statins and GGPP seem to be specific for LPC stimulation.

Rho inactivation by C3 exoenzyme inhibited the $[\text{Ca}^{2+}]_i$ increase caused by LPC, suggesting that Rho activation is upstream of Ca^{2+} movement. It is of interest to determine which effector is associated with Rho activation-induced $[\text{Ca}^{2+}]_i$ increase. A Rho-kinase inhibitor induced a similar inhibitory effect as the Rho inhibitor, indicating that Rho/Rho-kinase activation induces the Ca^{2+} mobilization caused by LPC stimulation. Moreover, the responses of Rho activation to statins and GGPP concurred with those of Ca^{2+} mobilization. Therefore, the present study strongly implicates Rho in the LPC stimulation of Ca^{2+} movement.

With respect to LPC-induced Ca^{2+} influx, we found that LPC activated a membrane current in endothelial cells. The current was similar to that reported in ventricular myocytes, in which the reversal potential was 0 mV and a lag was required for the induction of current.¹³ Thus, the current induced by LPC in endothelial cells was characterized as a nonselective cation current. Similar to the $[\text{Ca}^{2+}]_i$ response, the LPC-induced current was inhibited by statins and the inhibitory effect was abolished by GGPP, suggesting that a GGPP-dependent process is also involved in the nonselective cation current induced by LPC.

Intracellular PLC/IP₃ signaling for Ca^{2+} release was immediate in cells stimulated with LPC, whereas there was a lag in the current activation. Although $[\text{Ca}^{2+}]_i$ was markedly suppressed by Y-27632, it only partially inhibited the LPC-induced current. These findings suggest that the Rho/Rho-kinase pathway may play some role in the current induced by LPC and that intracellular Ca^{2+} signaling and membrane

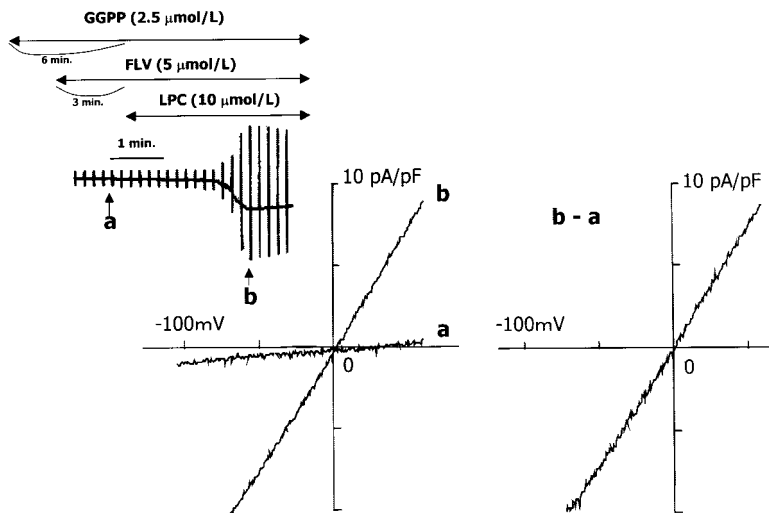


Figure 7. Effect of 2.5 $\mu\text{mol/L}$ GGPP on the current induced by 5 $\mu\text{mol/L}$ FLV plus 10 $\mu\text{mol/L}$ LPC. GGPP was included in the pipette solution, and FLV and LPC were perfused via the external solution. Chart recording of the current (top left insert), I - V curve of the control (a) and during FLV plus LPC application (b) (left), and the net current density change ($b-a$, right) in a representative cell of 5 cells tested.

current may be regulated independently. The detailed signaling pathway in current activation needs to be clarified.

In LPC stimulation, an association of adhesion molecule induction with Ca²⁺ signaling is unknown. A recent study showed that [Ca²⁺]_i increase is required for activation and binding of nuclear factor- κ B for induction of the ICAM-1 and VCAM-1 genes.¹⁷ Together with our findings, it seems that Rho-mediated [Ca²⁺]_i increase may induce adhesion molecule expression in LPC-treated endothelial cells and statins may prevent endothelial dysfunction by suppressing Ca²⁺ signaling through Rho-dependent PLC signaling.

In conclusion, the present study shows that the inhibitory effects of statins on intracellular Ca²⁺ mobilization and nonselective cation current evoked by LPC prevent GGPP-dependent Rho activation in endothelial cells.

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References

- Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature*. 1993;362:801–809.
- Egashira K, Hirooka Y, Kai H, et al. Reduction in serum cholesterol with pravastatin improves endothelium-dependent coronary vasomotion in patients with hypercholesterolemia. *Circulation*. 1994;89:2519–2524.
- Dupuis J, Tardif JC, Cernacek P, et al. Cholesterol reduction rapidly improves endothelial function after acute coronary syndromes: the RECIFE (reduction of cholesterol in ischemia and function of the endothelium) trial. *Circulation*. 1999;99:3227–3233.
- Kugiyama K, Kerns SA, Morrisett JD, et al. Impairment of endothelium-dependent arterial relaxation by lysolecithin in modified low-density lipoproteins. *Nature*. 1990;344:160–162.
- Kume N, Cybulsky MI, Gimbrone MA Jr. Lysophosphatidylcholine, a component of atherogenic lipoproteins, induces mononuclear leukocyte

adhesion molecules in cultured human and rabbit arterial endothelial cells. *J Clin Invest*. 1992;90:1138–1144.

- Inoue N, Hirata K, Yamada M, et al. Lysophosphatidylcholine inhibits bradykinin-induced phosphoinositide hydrolysis and calcium transients in cultured bovine aortic endothelial cells. *Circ Res*. 1992;71:1410–1421.
- Shepherd J, Cobbe SM, Ford I, et al. Prevention of coronary heart disease with pravastatin in men with hypercholesterolemia. *N Engl J Med*. 1995;333:1301–1307.
- Laufs U, La Fata V, Plutzky J, et al. Upregulation of endothelial nitric oxide synthase by HMG CoA reductase inhibitors. *Circulation*. 1998;97:1129–1135.
- Kano H, Hayashi T, Sumi D, et al. A HMG-CoA reductase inhibitor improved regression of atherosclerosis in the rabbit aorta without affecting serum lipid levels: possible relevance of up-regulation of endothelial NO synthase mRNA. *Biochem Biophys Res Commun*. 1999;259:414–419.
- Matsuoka I, Zhou Q, Ishimoto H, et al. Extracellular ATP stimulates adenylyl cyclase and phospholipase C through distinct purinoceptors in NG108-15 cells. *Mol Pharmacol*. 1995;47:855–862.
- Nagata K, Ishibashi T, Sakamoto T, et al. Effects of blockade of the renin-angiotensin system on tissue factor and plasminogen activator inhibitor-1 synthesis in human cultured monocytes. *J Hypertens*. 2001;19:775–783.
- Okamoto H, Takuwa N, Yokomizo N, et al. Inhibitory regulation of Rac activation, membrane ruffling, and cell migration by the G protein-coupled sphingosine-1-phosphate receptor EDG5 but not EDG1 or EDG3. *Mol Cell Biol*. 2000;20:9247–9261.
- Magishi K, Kimura J, Kubo Y, et al. Exogenous lysophosphatidylcholine increases non-selective cation current in guinea-pig ventricular myocytes. *Pflügers Arch*. 1996;432:345–350.
- Kaibuchi K, Kuroda S, Amano M. Regulation of the cytoskeleton and cell adhesion by the Rho family GTPases in mammalian cells. *Annu Rev Biochem*. 1999;68:459–486.
- An S. Molecular identification and characterization of G protein-coupled receptors for lysophosphatidic acid and sphingosine 1-phosphate. *Ann NY Acad Sci*. 2000;905:25–33.
- Noguchi Y, Nakamura S, Yasuda T, et al. Newly synthesized Rho A, not Ras, is isoprenylated and translocated to membranes coincident with progression of the G₁ to S phase of growth-stimulated rat FRTL-5 cells. *J Biol Chem*. 1998;273:3649–3653.
- Quinlan KL, Naik SM, Cannon G, et al. Substance P activates coincident NF-AT- and NF- κ B-dependent adhesion molecule gene expression in microvascular endothelial cells through intracellular calcium mobilization. *J Immunol*. 1999;163:5656–5665.