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Increase of Intracellular Ca^{2+} Concentration Induced by Lysophosphatidylcholine in Murine Aortic Endothelial Cells

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Effects of oxidized low-density lipoprotein (ox-LDL), l-α-stearoyl-lysophosphatidylcholine (LPC), on intracellular Ca^{2+} concentration were examined in mouse endothelial cells by measuring intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) with fura 2-AM and reverse transcription-polymerase chain reaction (RT-PCR). LPC increased $[Ca^{2+}]_i$ under the condition of 1.5 mM $[Ca^{2+}]_o$ but did not show any effect under the nominally Ca^{2+}-free condition. Even after the store depletion with 30 μM 2,5-di-tert-butylhydroquinone (BHQ) or 30 μM ATP, LPC could still increase the $[Ca^{2+}]_i$ under the condition of 1.5 mM $[Ca^{2+}]_o$. The time required to increase $[Ca^{2+}]_i$ (about 1 minute) was longer than that for ATP-induced $[Ca^{2+}]_i$ increase (10~30 seconds). LPC-induced $[Ca^{2+}]_i$ increase was completely blocked by 1 μM La^{3+}. Transient receptor potential channel(trpc) 4 mRNA was detected with RT-PCR. From these results, we suggest that LPC increased $[Ca^{2+}]_i$ via the increase of Ca^{2+} influx through the Ca^{2+} routes which exist in the plasma membrane.

Key Words: Oxidized low-density lipoprotein (ox-LDL), Lysophosphatidylcholine (LPC), 2.5-di-tert-butylhydroquinone (BHQ), Endothelial cell (EC)

INTRODUCTION

In hypercholesterolemia and atherosclerosis, low-density lipoproteins (LDL) are converted to oxidized LDL (ox-LDL), including lysophosphatidylcholine (LPC). In atherosclerotic arteries, several fold increase of LPC content were demonstrated in nutritionally induced atherosclerosis (Portman & Alexander, 1969). According to these observations, LPC might be an important factor for the impairment of endothelial cells function in atherosclerotic arteries.

Lysophosphatidylcholine (LPC) is a naturally occurring soluble polar phospholipid component in mammalian cell membrane. LPC has previously been shown to activate a verapamil-sensitive Ca^{2+} influx pathway in vascular smooth muscle cells (Stoll et al, 1993). Several effects of LPC on membrane currents in cardiac cells have been reported, including activation of a non-selective cation current (I_{NSC}) (Magishi et al, 1996). It has been reported that alteration of endothelial Ca^{2+} regulation may play a role in LPC-induced impairment of endothelial cell. There are quite different results on the role of $[Ca^{2+}]_i$ in LPC-induced impairment of endothelial cell. LPC (2~20 μM) inhibited both acetylcholine (ACh)-induced endothelium-dependent relaxation (EDR) and an increase in endothelial $[Ca^{2+}]_i$ in a dose-dependent manner (Murohara et al, 1994; Miwa et al, 1997; Froese et al, 1999). The mechanism by which LPC causes an impairment of EDR is due to the inhibition of production/release of the EDRF and EDHF, which is dependent upon the cytosolic Ca^{2+} concentration and Ca/calmodulin. There are also several lines of evidences to suggest that LPC is involved in the inhibition of inositol 1,4,5-trisphosphate ($InsP_3$) generation in bovine aortic endothelial cells (EC) (Inoue et al, 1992), inhibition of protein kinase C in human umbilical vein EC (Kugiyama et al, 1995), and receptor-G protein uncoupling in porcine aortic EC (Flavahan et al, 1993; Freemen et al, 1996). On the other hand, like in smooth muscle and ventricular myocytes, LPC induced a biphasic increase in $[Ca^{2+}]_i$, which consisted of a rapid increase in $[Ca^{2+}]_i$ followed by a sustained increase (Inoue et al, 1992). Furthermore, LPC inhibited dose-dependently both phosphoinositide hydrolysis and subsequent increases in $[Ca^{2+}]_i$ by bradykinin in endothelial cells. Thus, how LPC interacts with the endothelial plasma membrane to bring along LPC-induced impairment of endothelial cell and the role of $[Ca^{2+}]_i$ in LPC-induced impairment of endothelial cell is not fully understood.

LPC increased $[Ca^{2+}]_i$ in freshly isolated murine aortic endothelial cells. In the present study, effects of LPC on the Ca^{2+} signaling were studied in freshly isolated mouse aortic endothelial cells. The results of the study show that LPC increased $[Ca^{2+}]_i$ via an increase of Ca^{2+} influx through the Ca^{2+} routes which exist in the plasma membrane.

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ABBREVIATIONS: ox-LDL, oxidized low-density lipoprotein; LPC, Lysophosphatidylcholine; BHQ, 2.5-di-tert-butylhydroquinone; EC, Endothelial cell; RT-PCR, reverse transcription-polymerase chain reaction; EDRF, endothelium-dependent relaxation factor; EDHF, endothelium-derived hyperpolarization factor.

METHODS

Isolation of cells

We used the primary explanted technique to study on freshly isolated endothelial cells obtained from the mouse aorta. This technique has been described elsewhere (Suh et al, 1999). The mouse was anesthetized with 0.4 ml Entobar (5,000 U/ml heparin 0.1 ml added to the injection), and vessel was isolated from the aorta. After cleaning, the vessel was opened longitudinally and cut into two or three small pieces. These pieces were placed with intima side down on Matrigel-coated 24 well plate, containing a very small volume of growth medium [mix for 100 ml: medium including 80 ml Dulbecco's minimum essential medium (DMEM, GIBCO-BRL 41965), plus 10 ml fetal calf serum (FCS, GIBCO-BRL 10270), 7.5 mg endothelial cell growth supplement (ECGS, Sigma E-2759), 200 μl heparin (10 U/ml final), 2 ml penicillin/streptomycin (100 U/ml final, GICO-BRL 15070), 1 ml L-glutamine (100X, GICO-BRL 25030-024), 1 ml minimal essential amino acid (100X, GIBCO-BRL 11140-035)], so that the aortic pieces were adhered to substratum. We used Matrigel-coated plates containing endothelial growth factors because under these circumstances the endothelial cells grow efficiently out of the vessel intima (Fig. 1) (Marigel, Becton Dickinson, Two Oak Park, Bedford, Mass., USA). Matrigel, solubilised from EHS mouse sarcoma cells, contains transforming growth factor β, fibroblast growth factor, tissue plasmogen activator and other growth factors that occur naturally in extracellular matrix-rich tumors. When the pieces were well- attached, more medium was added. The cells were incubated at 37°C for 4~7 days, and the aortic pieces were removed from Matrigel coated well. When the endothelial cells grew to confluence, we used 20 ml/10 cm^2 (100 U) dispase (Becton Dickinson) to recover the cultured cells from Matrigel, and then these cells were seeded on the 12 wells (5,000 cells/well). In this study, we only used cultured endothelial cells of passage 1~2.

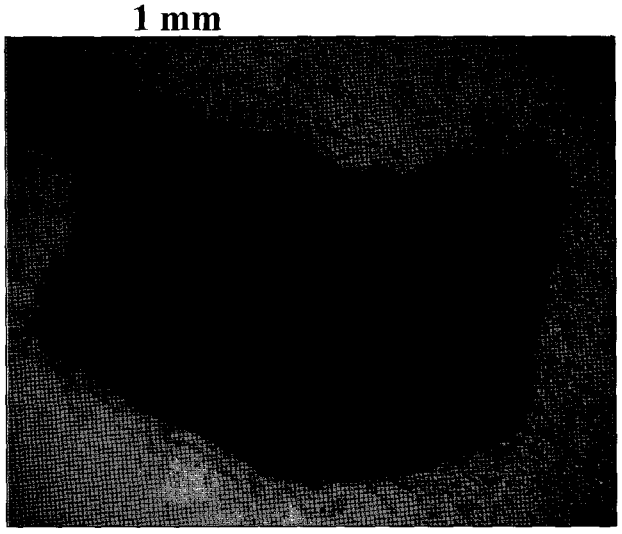

Fig. 1. Piece of mouse aorta explanted on Matrigel support. Endothelial cells migrating out of the aortic intima can be seen at the edge (after 2 or 3 days).

Cell-staining

To identify the nature of the cells, immunohistochemical techniques were used to detect von Willebrand's factor (vWF) and acetylated low-density lipoprotein (Dil-Ac-LDL). Murine aortic endothelial cells at passage 1~2 were used for cell immunostaining. Murine aorta endothelial cells were washed with tris (hydoxymethyl) aminomethane (TRIS)-bufferd saline (TBS, 50 mM TRIS, 150 mM NaCl, pH 7.5). After fixation with mixture of methanol/acetone (7 : 3) for 20 min, at −20°C, cells were permeabilised with 0.25% Triton X100/TBS for 30 min, then blocked by 5 % bovine serum albumin (BSA, in TBS) for 1 hour. Rabbit

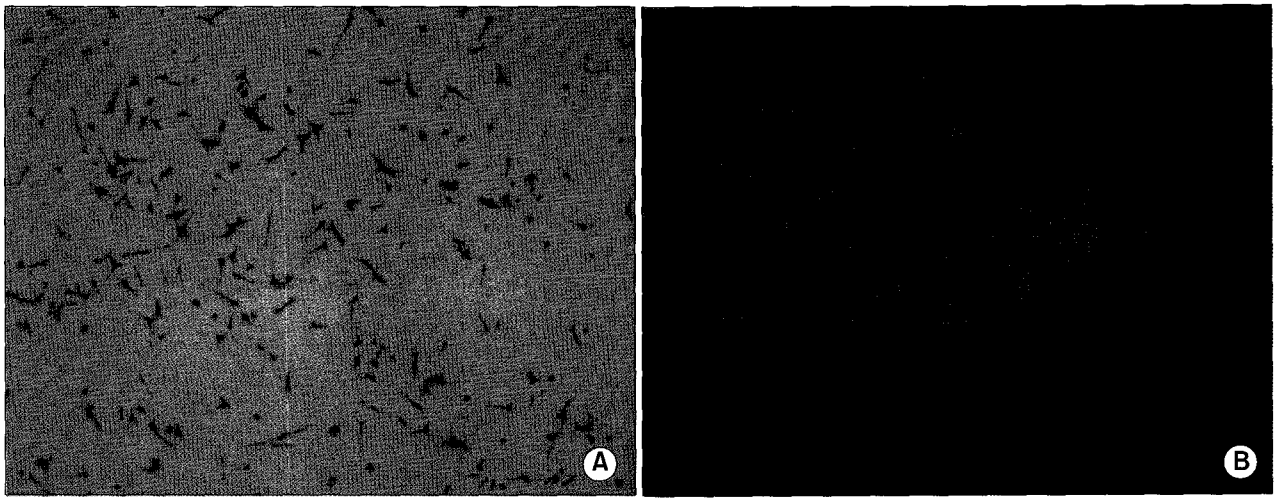

Fig. 2. Characterization of endothelial phenotype of the cells isolated from the inner layer of mouse aorta. A and B are the representative staining of the isolated cells with anti-von Willebrand factor antibody (vWF) and Dil-Ac-LDL using immunohistochemical technique.

anti-human vWF Ab (DAKO) (1 : 300 in TBS/1% BSA) was added to the mixture and incubated for overnight at 4°C. Subsequently, cells were washed with 0.1% Tween (in TBS) and incubated with either an alkaline phosphatase-conjugated monoclonal anti-rabbit IgG (1 : 200 in TBS/1% BSA) or goat anti-rat Ig-specific polyclonal Ab (PharMingen, 1 : 20 in TBS/1% BSA) at room temperature for 1hour. The washed cells (with TBS 3X) were stained with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyphosphate-p-toluidin (NBT) in alkaline phosphatase buffer (100 mM TRIS-HCl, pH 9.5, 10 mM NaCl, 5 mM $MgCl_2$). vWF was also directly detected in cells 1 day after removing the aortic pieces from the Matrigel surface. Except endothelial cells, fibroblast and smooth muscle are stained by vWF antibody, but cannot take up LDL into their cytoplasm for LDL staining.

Mouse aorta endothelial cells were incubated with Dil-Ac-LDL (10 μg/ml) in growth medium for 4~6 hours, washed with PBS, and incubated with 3% Hoechst (Sigma, in PBS) for 5 min. DAKO fluorescent mounting medium was added to the cells, and the cells were detected by fluorescence microscope (Fig. 2)

Electrophysiology

Membrane currents were recorded using the whole-cell configuration of the patch-clamp technique at room temperature (18~25°C). Currents and voltages were monitored with an EPC-9 (HEKA Elektronik, Lambrecht, Germany, sampling rate 1ms, 8-pole Bessel filter 2.9 kHz). We used patch pipette with a resistance of 4~5 MΩ when filled with the pipette solution. The holding potential of whole-cell experiments was 0 mV, and ramp pulse from −100 to 100 mV was applied for 650 ms. Data were digitized with pClamp software 6.0 and Digidata 1200 (Axon Instruments, USA) at a sampling rate of 1~2 kHz, and filtered at 5 kHz.

Solutions

Normal Tyrode solution contained (mM) 150 NaCl, 6 KCl, 1 $MgCl_2$, 1.5 $CaCl_2$, 10 glucose, 10 HEPES, and was adjusted to pH 7.4 with NaOH. The osmolality of this solution was 320±5 mOsm. Ca^{2+}-free solution was identical to the normal Tyrode solution except that $CaCl_2$ was omitted. We omitted KCl and $MgCl_2$ from normal Tyrode solution, and added 100 mM mannitol to eliminate the major endogenous currents and Ca^{2+}-activated Cl^- current of ECs. The large conductance Ca^{2+} activated K^+ channels were abolished by removing K^+ from both sides of the membrane and by including Cs^+ (Suh et al, 1998). The high Cs^+ internal solution contained (mM) 145 Cs-glutamic acid, 8 NaCl, 2 $MgCl_2$, 4 Na_2ATP, 10 HEPES, 12 BAPTA and was adjusted to pH 7.2 with CsOH.

Ca^{2+} measurement

For $[Ca^{2+}]_i$ measurement, the cells were loaded with fura-2/AM (the acetoxymethyl ester form). Fura-2/AM (2 μM) was added to the bath solution, and the cells were loaded for 20 min at 37°C incubator. After loading, the cells were illuminated at wavelengths of 340 and 380 nm through a rotating filter wheel. The fluorescence was measured at 510 nm and autofluorescence was subtracted from the signals. The free $[Ca^{2+}]_i$ was calculated from the ratio of fluorescence signals emitted by the excitation at each wave length. Results are shown as the mean±standard error. Significant differences were detected using Student's t-test. All experiments were performed at room temperature (20~22°C).

RNA Preparation and Reverse Transcription-Polymerase Chain Reaction Analysis (RT-PCR)

Total RNAs were extracted from murine endothelial cells and brain, using a SNAP Total RNA Isolation Kits (Invitrogen, Carlsbad, CA, USA) following the procedures of the manufacturer as described previously (Sim et al, 2002). First-strand cDNA was synthesized from the RNA preparations with a Superscript II RNase Transcriptase kit (Gibco BRL, Gaithersburg, MD); RNA (1 pg) was reverse transcribed by using random hexamers (50 μg/ μl). To perform nested PCR, the following sets of primers were used: mtrp1 forward, nucleotide (1583-1600, 1601-1608), and reverse, (2283-2300, 2301-2318, gene accession number NM_011643); mtrp2 forward, nucleotide (2783-2800, 2801-2818), and reverse, (3483-3500, 3501-3518, AF111107); mtrp3 forward, nucleotides (1030-1047, 1048-1065), and reverse, (1749-1966, 1749-1966, AF190645); mtrp4 forward, nucleotides (1483-1500, 1501-1518), and reverse, (2183-2200, 2201-2218, AF190646); mtrp5 forward, nucleotides (1749-1766, 1767-1784), and reverse, (2449-2466, 2467-2484, AF060107); mtrp6 forward, nucleotides (603-620, 621-638), and reverse, (1303-1320, 1321-1338, AF057748) and mtrp8 forward, nucleotides (2065-2082, 2083-2100), and reverse, (2765-2782, 2783-2800, NM_012035). Complementary DNA (20% of the first-strand reaction) was combined with 1st sense and antisense primers (20 μM), 1 mM deoxynucleotide triphosphates, 60 mM Tris-HCl (pH 8.5), 15 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 2.5 units of Taq (Bioneer), and RNase-free water to a final volume of 50 μl. The reaction was carried out in a Perkin-Elmer Thermal Cycler under the following conditions: an initial denaturation at 94°C for 4 min, followed by 40 cycles at 94°C for 30 s, 42°C for 30 s, 72°C for 1 min, with a final extension step at 72°C for 7 min. Five microliters of the first-round PCR product were then added to a new reaction mixture containing all of the components listed above except for 2nd sense and antisense primers (20 μM), and 40 additional cycles of PCR were then performed. PCR products were separated by 2% agarose gel electrophoresis. The sets of primers for mtrp1, mtrp2, mtrp3, mtrp4, mtrp5, mtrp6 and mtrp8 were predicted to yield 700 bp, 700 bp, 700 bp, 718 bp, 700 bp, 700 bp and 700 bp products, respectively. Two sets of negative control experiments were performed by including primers without cDNA or with RNA that had not been reversely transcribed (no reverse transcriptase added). To confirm murine trp channels, PCR products of mtrp1~8 were digested with restriction enzymes. PCR product of mtrp1 was digested into 395 bp and 305 bp by EcoRI, PCR product of mtrp2 into 378 bp and 322 bp, PCR product of mtrp3 into 391 bp and 319bp, PCR product of mtrp4 into 400bp and 318bp, PCR product of mtrp5 into 400 bp and 300 bp, PCR product of mtrp6 into 400 bp and 300 bp, and PCR product of mtrp8 into 400 bp and 300 bp as expected from the nucleotide sequences of murine trp channels. Primers were designed by the designer program 'Primer3' at http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi using the corresponding human mRNA sequences. The specificity of the primers for the target gene was checked against the databases using 'Fasta3' at http://www2.ebi.ac.uk/fasta3/ and primers were checked for hairpin loops and palindromes using the

Cybergene Utility at http://www.cybergene.se/primer.html. The oligonucleotides were synthesized by Bionics, Seoul, Korea.

RESULTS

Intracellular Ca^{2+} response to ATP

Endothelial cells (ECs) in primary explant were studied after the first or the second passage with dispase on a gelatin support. ATP-induced Ca^{2+} transients in ECs were shown in Fig. 3. ATP (30 μM) induced a fast peak followed by a sustained plateau, a typical response observed in endothelial cells (Revest et al, 1992). It is well known that Ca^{2+} influx during the sustained plateau is a necessary condition for the essential endothelial functions, such as secretion of nitric oxide and prostacyclin (PGI_2). There was no change in $[Ca^{2+}]_i$ when 10 μM verapamil was applied to the bath solution during the plateau phase, reflecting that the ECs do not have voltage-operated Ca^{2+} channel, but other channels contributing to Ca influx during the sustained plateau.

Effect of LPC on the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$)

Three types of lysophosphatidylcholines (LPC, each 2 μM) were applied to non-clamped endothelial cells (ECs). While palmitoyl (C16 : 0)-LPC and stearoyl (C18 : 0)-LPC increased $[Ca^{2+}]_i$ (Fig. 4A), lauroyl (C12 : 0)-, myristoyl (C1 4 : 0)- and oleoyl (C18 : 1, [cis]-9)-LPC did not (Fig. 4B). Effect of LPC on $[Ca^{2+}]_i$ increase was dependent upon the fatty acid side chain of LPC. Therefore, stearoyl-LPC was used to investigate for the mechanism of stearoyl-LPC-induced $[Ca^{2+}]_i$ increase. Extracellular application of 1-α-stearoyl lysophosphatidylcholine (LPC, 2 μM) increased $[Ca^{2+}]_i$. in non-clamped ECs (Fig. 5). Although LPC-induced $[Ca^{2+}]_i$ increase was delayed, the increase was remarkable compared to the ATP-induced $[Ca^{2+}]_i$ increase. It was irreversible and gradually decreased even in the presence of LPC. Resting $[Ca^{2+}]_i$ (R of F340/380) was 0.52 $\pm$ 0.03 (mean $\pm$ S.E.M., n=5). The ratio increments after the application of 30 μM ATP and 2 μM LPC were 1.16 $\pm$ 0.26 and 2.2 $\pm$ 0.18 (mean $\pm$ S.E.M., n=5) under physiological condition (1.5 mM Ca^{2+}), respectively (Fig. 5A). LPC-induced increase in $[Ca^{2+}]_i$ was dependent on the extracellular Ca^{2+} which is shown in Fig. 5B. LPC-induced increase in $[Ca^{2+}]_i$ was not developed in the absence of extracellular Ca^{2+}. When the extracellular Ca^{2+} was raised from 0 to 1.5 mM, LPC-induced $[Ca^{2+}]_i$ increase was slower compared with that in Fig. 5A. The R 340/380 in the presence of extracellular Ca^{2+} after the application of 2 μM LPC was 1.87 $\pm$ 0.35 (n=3).

Ca^{2+} sources of LPC-induced increase in $[Ca^{2+}]_i$

Application of LPC also increased the Ca^{2+} influx even after store depletion (Fig. 6A). After intracellular Ca^{2+} store was depleted by 30 μM 2.5-di-tert-butylhydroquinone (BHQ) in the absence of extracellular Ca^{2+}, addition of 1.5 mM extracellular Ca^{2+} induced Ca^{2+} influx (0.19 $\pm$ 0.06, n=6). During the Ca^{2+} influx evoked under the condition of BHQ and 1.5 mM extracellular Ca^{2+}, application of 2 μM LPC induced an additional elevation of $[Ca^{2+}]_i$ (1.03 $\pm$

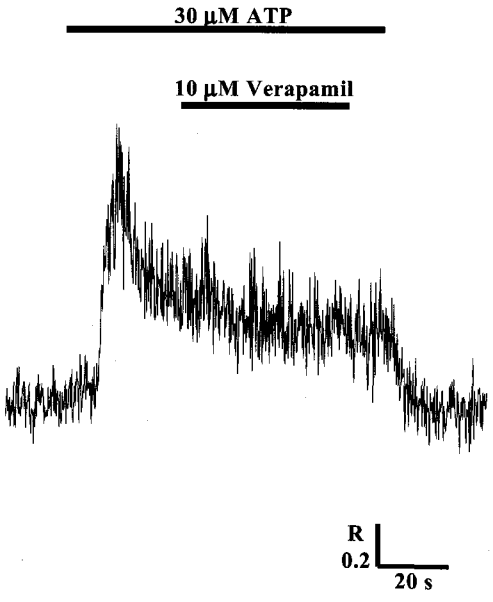

Fig. 3. Effect of verapamil on ATP-induced $[Ca^{2+}]_i$ increase in endothelial cells (ECs). Verapamil (10 μM) has no effect on Ca^{2+} signaling. R means the ratio of emission (510 nm) for 340 nm/380 nm excitation ($F_{340/380}$).

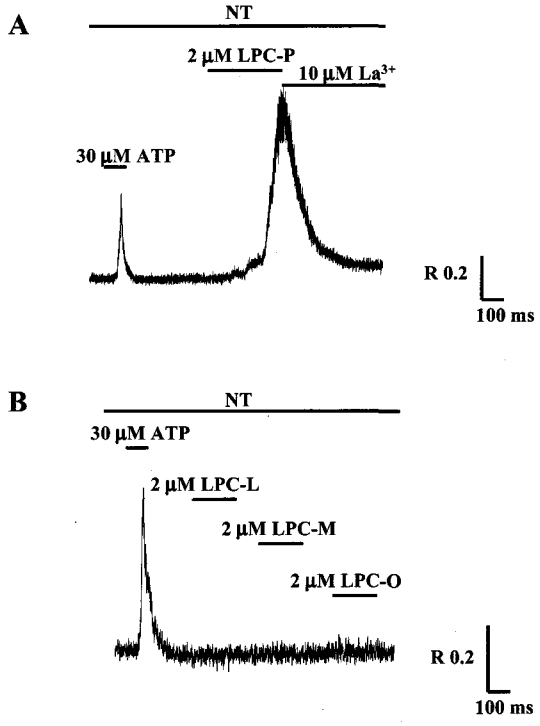

Fig. 4. Effect of different L-α-lysophosphatidylcholines (LPC) on cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$). A; palmitoyl-LPC (LPC-P). B; lauroyl-LPC (LPC-L), myristoyl-LPC (LPC-M) and oleoyl-LPC (LPC-O). R means the ratio of emission (510 nm) for 340 nm/380 nm excitation ($F_{340/380}$).

0.13, n=6) (Fig. 6A). This result suggested that LPC can activate some Ca^{2+} influx pathways additionally even after store depletion.

Fig. 6B showed that La^{3+} blocks the LPC-induced increase in $[Ca^{2+}]_i$. La^{3+} at the micromolar concentration is well known to be a blocker of Ca^{2+}-release activated Ca^{2+} channel (CRAC) and nonselective cation channel (NSC). LPC-induced increase in $[Ca^{2+}]_i$ was completely and reversibly blocked by 1 μ M La^{3+} in the normal Tyrode solution. These results suggested that extracellular La^{3+} -sensitive Ca permeable channels are responsible for the LPC-induced increase in $[Ca^{2+}]_i$.

RT-PCR and expression of transient receptor potential channels

We used RT-PCR to examine whether trp4 mRNA existed in murine endothelial cells, since it was suggested to be a molecular identity for the La^{3+} sensitive Ca^{2+} entry pathway (Freichel et al, 2001). To test whether each primer can detect each trp mRNA, murine brain was used. All 7 types of trp mRNA were detected in brain (data not shown).

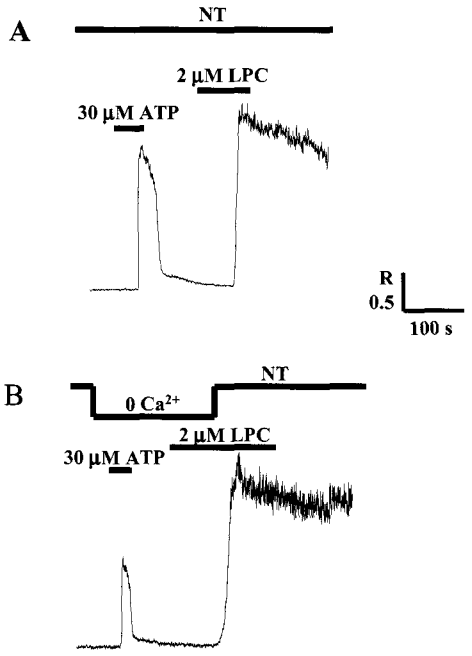

Fig. 5. Effect of 1-α-stearoyl-lysophosphatidylcholine (LPC) on cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$). A; After the Ca^{2+} store depletion with 30 μM ATP, 2 μM LPC can increase $[Ca^{2+}]_i$ in the normal Tyrode solution (1.5 mM Ca^{2+}). B; Application of 2 μM LPC does not increase $[Ca^{2+}]_i$ in Ca^{2+}-free condition, but when Ca^{2+} concentration increased from 0 to 1.5 mM in normal Tyrode solution, 2 μM LPC induced $[Ca^{2+}]_i$ increase. R means the ratio of emission (510 nm) for 340 nm/380nm excitation ($F_{340/380}$).

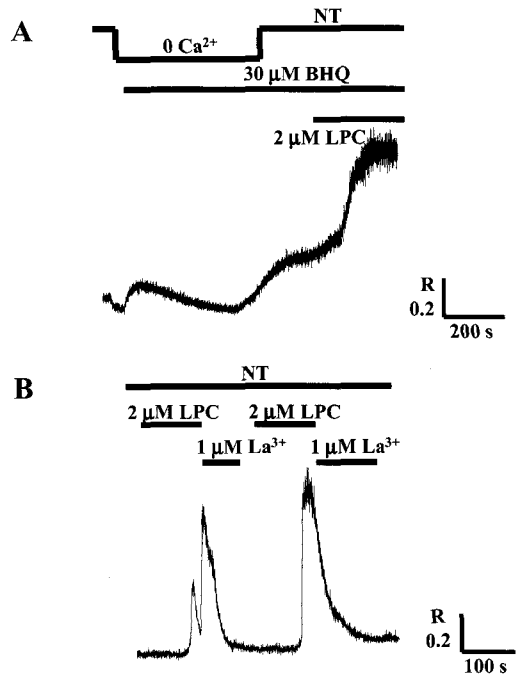

Fig. 6. Effect of LPC on cytosolic Ca^{2+} concentration after Ca^{2+} store depletion and that of La^{3+} on LPC-induced $[Ca^{2+}]_i$ increase. A; After the Ca^{2+} store depletion with 30 μM 2,5-di-tert-butylhydroquinone (BHQ), 2 μM LPC also induced $[Ca^{2+}]_i$ increase. B; 2 μM LPC-elevated $[Ca^{2+}]_i$ was completely blocked by 1 μM La^{3+}. R means the ratio of emission (510 nm) for 340 nm/380 nm excitation ($F_{340/380}$).

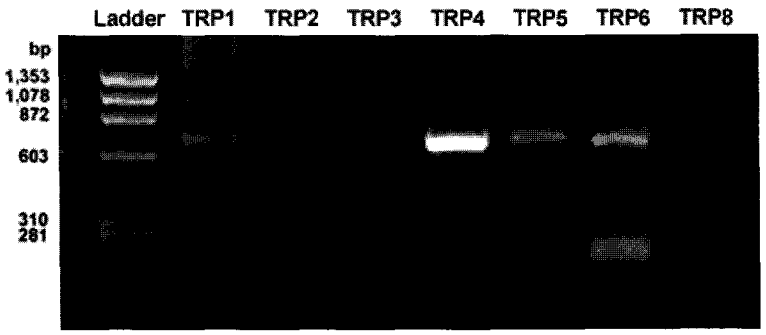

Fig. 7. Detection of trp mRNA with RT-PCR in murine endothelial cells. Trp4 mRNA was detected with RT-PCR. Trp1, 5, and 6 mRNA were also detected in murine endothelial cells.

These primers were used to detect trp mRNA in murine endothelial cells. In endothelial cells, trp4 mRNAs was detected (Fig. 7), although trp 1, 5 and 6 mRNAs were also detected. To confirm the nucleotide sequences of the PCR products, the PCR products were digested with restriction enzyme. We found the PCR product of each trp subtype was digested into two fragments of the expected size from the nucleotide sequences (data not shown).

DISCUSSION

Several disorders, such as hypertension, atherosclerosis, cardiovascular diseases, diabetes, and hypercholesterolemia, are associated with a dysfunctional endothelium (Born et al, 1997). The role of endothelium in these disorders depends on a balance between the release of various factors, such as NO and endothelin-1 (Suh et al, 2000). It has been known that synthesis and/or release of vasoactive compounds can be modulated by the changes in free intracellular Ca^{2+} concentration. Sources for an increased $[Ca^{2+}]_i$ are primarily release of Ca^{2+} from intracellular stores via an Ins $(1,4,5)P_3$-dependent mechanism and an influx of extracellular Ca^{2+} (Inagami et al, 1995; Nilius et al 1997).

LPC is a major component of oxidized low density lipoprotein found in artheroslerotic arterial walls (Portman et al, 1969). LPC increases cytoplasmic free Ca^{2+} concentration in several types of cells including endothelial cells. The atherogenic and inflammatory LPC exerted both stimulatory and inhibitory actions on the phospholipase C/Ca^{2+} system depending on the species of fatty acid residue of the lipid; the stimulatory effect was possibly mediated through G-protein-coupled receptors; the inhibitory effect might be caused by dysfunction of the component involved in the enzyme system owing to the amphiphilic nature of the lipid (Okajima et al, 1998). Ox-LDL (including LPC) elevated $[Ca^{2+}]_i$ in smooth muscle and inhibited endothelium-dependent relaxation, the prominent mechanism of which included activation of protein kinase C, inhibition of nitric oxide (NO) production and/or release, and altered production of superoxide anion (Jabr et al, 2000). The effects of LPC were also dependent upon the concentration. Low (less than 20 μM) and high (more than 30 μM) concentration activated and inhibited PKC (Prokazova et al, 1997), respectively.

In the present study, effects of lysophosphatidylcholine (LPC) on signaling in ECs were characterized. LPC was shown to increase $[Ca^{2+}]_i$ by the activation of Ca^{2+}influx via Ca^{2+} -permeable membrane pathway (Figs. 5 and 6). In vascular smooth muscle cells, LPC caused abnormal sarcoplasmic reticulum Ca^{2+} regulation, leading to appearance of spontaneous transient outward currents (STOCs) and spontaneous transient inward currents (STICs), and following activation of large voltage-independent current with a reversal potential close to 0 mV (I_{NSC}) (Jabr et al, 2000). LPC activation of I_{NSC} was based upon several criteria: (1) the current was close to 0 mV, (2) the channels mediating the LPC-activated current exhibited permeability to Na^+, K^+, Cs^+ and Ca^{2+}, but were impermeant to anions such as Cl^-, and (3) the LPC-activated current was completely blocked by Ga^{3+} (Carl et al, 1996). In endothelial cells, Ca^{2+} entry pathways comprise non-selective cation channels (NSC), Ca^{2+} release-activated Ca^{2+}-channels (CRAC), and agonist-activated non-selective Ca^{2+} permeable cation channels. CRAC is a highly Ca^{2+} permeable influx pathway and is controlled by the refilling degree of intracellular Ca^{2+} stores (Dolor et al, 1992). Thus, CRAC were activated by application of store-depleting inhibitor of sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA), such as tBHQ (tert-butyl-benzohydroquinone) or thapsigargin. Agonist-activated nonselective cation channels are activated by vasoactive agonists, are dependent on IP_3 production and are permeable for Ca^{2+} (Nilius et al, 1990; Nilius et al, 1991). In our experiments, application of 2 μM LPC after store depletion by tBHQ or ATP evoked an additional elevation of $[Ca^{2+}]_i$ (Fig. 6A). This result suggests that LPC can activate Ca^{2+} influx pathways other than CRAC or agonist-activated non-selective Ca^{2+} permeable cation channels. We have also shown that LPC-elevated $[Ca^{2+}]_i$ was completely blocked by 1 μM La^{3+} in normal Tyrode solution or by Ca^{2+}-free solution. Trpc4 mRNA, a molecular identity for La^{3+}-sensitive Ca^{2+} entry pathway (Freichel et al, 2001), was also detected in murine endothelial cells (Fig. 7). In conclusion, these results suggested that LPC increased $[Ca^{2+}]_i$ via La^{3+}-sensitive calcium permeable routes which exist in the plasma membrane in endothelial cells.

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REFERENCES

Born GVR, Schwartz CJ. Vascular endothelium: Physiology, Pathology and Therapeutics, Stuttgart: Schatthauern Verlag, 1997

Born G, Rabelink T, Smith T. Endothelium and cardiovascular disease. London: Science Press, 1998

Carl A, Lee HK, Sanders KM. Regulation of ion channel in smooth muscle by calcium. *Am J Physiol* 271: C9−C34, 1996

Cox DA, Cohen ML. Effects of oxidized low-density lipoproteins on vascular contraction and relaxation: clinical and pharmacological implication in atherosclerosis. *Pharmacol Rev* 48: 3−19, 1996

Dolor RJ, Hurwitz LM, Nirza Z, Strauss HC, Whorton AR. Regulation of extracellular calcium entry in endothelial cells-Role of intracellular calcium pool. *Am J Physiol* 262: C171−C181, 1992

Flavahan NA. Lysophosphatidylcholine modifies G protein-dependent signaling in porcine endothelial cells. *Am J Physiol* 264 (3 Pt 2): H722−H727, 1993

Freeman JE, Kuo WY, Drenger B, Barnett TN, Levine MA, Flavahan NA. Analysis of lysophophatidylcholine-induced endothelial dysfunction. *J Cardiovasc Pharmacol* 28 (3): 345−352, 1996

Freichel M, Suh SH, Pfeifer A, Schweig U, Trost C, Weissgerber P, Biel M, Philipp S, Freise D, Droogmans G, Hofmann F, Flockerzi V, Nilius B. Lack of an endothelial store-operated Ca^{2+} current impairs agonist-dependent vasorelaxation in TRP4$^{-/-}$ mice. *Nature Cell Biol* 3: 121−127, 2001

Froese DE, McMaster J, Man RY, Choy PC, Kroeger EA. Inhibition of endothelium-dependent vascular relaxation by lysophosphatidyl-choline: impact of lysophosphatidylcholine on mechanisms involving endothelium-derived nitric oxide and endothelium derived hyperpolarizing factor. *Mol Cell Biochem* 197 (1−2): 1−6, 1999

Fukao M, Hattori Y, Knno M, Sakuma I, Kitabatake A. Evidence

for selective inhibition by lysophosphatidylcholine of acetylcholine-induced endothelium-dependent hyperpolarization and relaxation in rat mesenteric artery. *Br J Pharmacol* 116: 1541–1544, 1995

Inagami T, Naruse M, Hoover R. Endothelium: As an endocrine organ. *Annu Rev Physiol* 157: 171–189, 1995

Inoue N, Hirata K, Yamada M, Hamamori Y, Matsuda Y, Akita H, Yokoyama M. Lysophosphatidylcholine inhibits bradykinin-induced phosphoinositide hydrolysis and calcium transients in cultured bovine aortic endothelial cells. *Circ Res* 71(6): 1410–1421, 1992

Jabr RI, Yamazaki J, Hume JR. Lysophosphatidylcholine triggers intracellular calcium release and activation of non-selective cation channels in renal arterial smooth muscle cells. *Pflgers Arch* 439(4): 495–500, 2000

Kita T, Kume N, Yokode M, Ishii K, Arai H, Horiuchi H, Moriwaki H, Minami M, Kataoka H, Watatsuki Y. Oxidized-LDL and atherosclerosis. Role of LOX-1. *Ann N Y Acad Sci* 902: 95–100, 2000

Kugiyama K, Murohara T, Yasue H, Kimura T, Sakaino N, Ohgushi M, Sugiyama S, Okumura K. Increased constrictor response to acetylcholine of the isolated coronary arteries from patients with variant angina. *Int J Cardiol* 52(3): 223–233, 1995

Magishi K, Kimura J, Kubo Y, Abiko Y. Exogenous lysophosphatidylcholine increases non-selective cation current in guinea-pig ventricular myocytes. *Pflgers Arch* 432: 345–350, 1996

Miwa Y, Hirata K, Kawashima S, Akita H, Yokoyama M. Lysophosphatidylcholine inhibits receptor-mediated Ca^{2+} mobilization in intact endothelial cells of rabbit aorta. *Arterioscler Thromb Vasc Biol* 17(8): 1561–1567, 1994

Murohara T, Kugiyama K, Ohgushi M, Sugiyama S, Ohta Y, Yasue H. LPC in oxidized LDL elicits vasocontraction and inhibits endothelium-dependent relaxation. *Am J Physiol* 267(6 Pt 2): H2441–H2449, 1994

Nilius B. Permeation properties of a non-selective cation channel in human vascular endothelial cells. *Pflgers Arch* 416: 609–611, 1990

Nilius B. Regulation of transmembrane calcium fluxes in endothelium. *News Physiol Sci* 6: 110–114, 1991

Nilius B, Viana F, Droogmanns G. Ion channels in vascular endothelium. *Annu Rev Physiol* 59: 145–170, 1997

Okajima F, Sato K, Tomura H, Kuwabara A, Nochi H, Tamoto K, Kondo Y, Tokumitsu Y, Ui M. Stimulatory and inhibitory actions of lysophosphatidylcholine, depending on its fatty acid residue, on the phospholipase C/Ca^{2+} system in HL-60 leukaemia cells. *J Biochem* 336: 491–500, 1998

Prokazova NV, Zvezdina ND, Korotaeva AA. Effect of lysophosphatidylcholine on transmembrane signal transduction. *Biochemistry (Moscow)* 63(1): 31–38, 1997

Portman OW, Alexander M. Lysophosphatidylcholine concentrations and metabolism in aortic intima plus inner media: effect of nutritionally induced atherosclerosis. *J Lipid Res* 10(2): 158–65, 1996

Revest PA, Abbott NJ. Membrane ion channels of endothelial cells. *Trends Pharmacol Sci* 13: 404–407, 1992

Sim JH, Yang DK, Kim YC, Park SJ, Kang TM, So I, Kim KW. ATP-sensitive K channels composed of Kir6.1 and SUR2B subunits in guinea pig gastric myocytes. *Am J Physiol Gastrointest Liver Physiol* 282: G137–G144, 2002

Stoll LL, Spector AA. Lysophosphatidylcholine causes cGMP-dependent verapamil-sensitive Ca^{2+} influx in vascular smooth muscle cells. *Am J Physiol* 264: C885–893, 1993

Suh SH, Droogmans G, Nilius B. Effect of cyanide and deoxyglucose on the Ca^{2+} signaling in macrovascular endothelial cells. *Endothelium* 7(3): 155–168, 2000

Suh SH, Vennekens R, Manolopoulos VG, Freichel M, Schweig U, Prenen J, Flockerzi V, Droogmans G, Nilius B. Characterization of explanted endothelial cells from mouse aorta: electrophysiology and Ca^{2+} signaling. *Pflgers Arch-Eur J Physiol* 438: 612-620, 1999