Full Paper

The Action Mode of Lysophosphatidylcholine in Human Monocytes

Mi-Ran Yun¹, Fumikazu Okajima², and Dong-Soon Im^{1,*}

¹Laboratory of Pharmacology, College of Pharmacy, Pusan National University, Busan, 609-735, Republic of Korea

²Laboratory of Signal Transduction, Institute for Molecular and Cellular Regulation, Gunma University, Maebashi 371-8512, Japan

Received July 17, 2003; Accepted November 25, 2003

Abstract. To elucidate the action and signal transduction of lysophosphatidylcholine (LPC), we challenged a set of LPC on U937 human monocytes and found that LPC mobilized Ca^{2+} . The Ca^{2+} response was not blocked by pertussis toxin, an inhibitor of $\text{G}_{i/o}$ proteins, or by U73122, a phospholipase C inhibitor. Furthermore, the response was totally blocked by addition of EGTA to the extracellular media, suggesting that Ca^{2+} influx across the plasma membrane was the only source of LPC-induced Ca^{2+} response in the U937 cells. 16:0 and 18:0 LPC induced similar responses. Recently it has been suggested that two G protein-coupled receptors function as LPC receptors in the plasma membrane. RT-PCR analysis indicated that neither the G2A receptor nor the GPR4 receptor is expressed in the U937 monocytes. Our data suggests that another action mechanism of LPC may be involved in the LPC response in the U937 cells.

Keywords: lysophosphatidylcholine, atherosclerosis, G protein-coupled receptor, calcium influx, signal transduction

Introduction

Atherosclerosis is the main cause of cardiovascular diseases (that is, angina, hypertension, cardiac infarction) and stroke. Oxidized low-density lipoprotein (LDL) has been shown to be an atherogenic lipoprotein (1). Lysophosphatidylcholine (LPC), a phospholipid component of oxidized LDL, has been demonstrated to induce multiple functional alterations of the vasculature that are potentially involved in atherosclerosis. LPC acts on several types of cells involved in atherosclerosis. For example, LPC increases the cytoplasmic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in endothelial cells, leukocytes, and macrophages (2, 3). Recently, research has unveiled some roles of LPC in endothelial cells and smooth muscle cells (2, 4, 5). However, the action of LPC in monocytes has been poorly studied. LPC has been shown to be a chemoattractant for monocytic cells and T-lymphocytes and also a regulator of the proliferation of T-lymphocytes and macrophages (6–8).

Several lysolipid molecules including lysophosphatidic acid, sphingosine 1-phosphate, and LPC have been

recognized as inter-cellular mediators regulating cell functions such as cell growth, apoptosis, and cell movement (9, 10). Two subgroups of G protein-coupled receptors (GPCR) in the plasma membrane bind the lysolipids and signal to cells (10). The Edg receptor family is the group of receptors for lysophosphatidic acid and sphingosine 1-phosphate (9). OGR-1 and GPR4 have been shown to be sphingosylphosphorylcholine receptors (11, 12). G2A and GPR4 bind to LPC specifically and regulate cell growth and immunologic responses (12, 13).

In this study, we tested a set of LPC in U937 human monocytes and found that LPC mobilized Ca^{2+} across the plasma membrane. The present data suggests that the LPC-induced response in monocytes may not be mediated through GPCR and also that another action mechanism of LPC may be involved in the LPC response in U937 cells.

Materials and Methods

Materials

1-Stearoyl ($\text{C}_{18:0}$) LPC, 1-palmitoyl ($\text{C}_{16:0}$) LPC, and 1-myristoyl ($\text{C}_{14:0}$) LPC were purchased from Avanti Polar lipids (Alabaster, AL, USA); and fura 2/acetox-

*Corresponding author. FAX: +82-51-513-6754

E-mail: imds@pusan.ac.kr

methyl ester was acquired from Calbiochem (Darmstadt, Germany). U73122, U73343, lysoPAF ($C_{16:0}$), platelet-activating factor (PAF), and lysoPAF ($C_{18:0}$) were purchased from Biomol (Plymouth Meeting, PA, USA). Primers were synthesized and provided by Bioneer Corp. (Dae-Jun, Korea). All other materials were purchased from Sigma-Aldrich Korea (Seoul, Korea).

Cell culture

U937 cells were maintained in modified Eagles medium containing 10% (v/v) fetal bovine serum, 100 units/ml penicillin, 50 μ g/ml streptomycin, 2 mM of glutamine, and 1 mM of pyruvate sodium at 37°C in a humidified 5% CO₂ incubator. Treatment of the cells with pertussis toxin was performed by adding the toxin (100 ng/ml) to the medium 24 h before the experiments.

Measurement of $[Ca^{2+}]_i$

The cells were sedimented, resuspended with a Hepes-buffered medium consisting of 20 mM of Hepes (pH 7.4), 103 mM NaCl, 4.8 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 0.5 mM CaCl₂, 25 mM NaHCO₃, 15 mM glucose, and 0.1% bovine serum albumin (fatty acid free); and they were then incubated for 40 min with 5 μ M of fura 2/acetoxymethyl ester. Fluorescence emission at 510 nm wavelength from two excitation wavelengths (340 and 380 nm) were measured every 0.1 s (F4500; Hitachi, Tokyo). The ratio of fluorescence intensities from the two wavelengths was monitored as an estimate of $[Ca^{2+}]_i$ (14).

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

The total RNAs and subsequent messenger RNAs were purified from the U937 cells by using the mRNA isolation system (Novagen, Darmstadt, Germany). We followed the manufacture's instructions. Three microliters of mRNAs was used to make cDNA with oligo dT primers. One microliter of the synthesized cDNA was used as a template for the PCR reaction. Specific primers for human OGR1 (sense 5'-TTC CTG CCC TAC CAC GTG TTG C-3', antisense 5'-TGG CGA GTT AGG GGT CTG GAA G-3'); human GPR4 (sense 5'-ACC TCT ATC GGG TGT TCG TG-3', antisense 5'-TTC CTC TTG GAG GTG AGT GG-3'); human G2A (sense 5'-CCG CCT ACA TCT TCT TCT GC-3', antisense 5'-ACA GGC ACA GAA ACA CCA CA-3'); or TDAG8 (sense 5'-TGC AAA GGG AGT GCT TTT CT-3', antisense 5'-CAC CTC CCA GTG CAG AAT TT-3') were used to amplify 341-, 400-, 500-, and 600-bps of fragments of OGR1, GPR4, G2A, and TDAG8, respectively. The reaction mixture contained 1 $\times$ i-Taq buffer, 2.5 U of i-Taq DNA polymerase (iNtRon Bio-

technology, Seoul, Korea), 300 nM of primers, and 250 μ M of dNTPs. The PCR reaction was performed by 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 1 min, and elongation at 72°C for 2 min in a PTC-100 DNA amplification system (MJ Research, Inc., Watertown, MA, USA). Seven microliters of the aliquots were electrophoresed in a 1.2% agarose gel and stained with ethidium bromide.

Data presentation

The representative traces for $[Ca^{2+}]_i$ were chosen out of 3–5 separate experiments and shown in the Figs. 2–5.

Results

LPC mobilizes Ca^{2+} in U937 monocytes

In the present study, we used a set of LPC compounds. They included 14:0 LPC, 16:0 LPC, 18:0 LPC, lysoPAFs (ether-linked LPCs) with 16:0 or 18:0 fatty chain and PAF with 16:0 fatty chain. Their structures are shown in Fig. 1. The structure of LPC is composed of four different parts. Glycerol, a backbone of glycerolipids, is a part of the linkage, which bridges a hydrophobic lipid chain part and a negatively charged phosphate part. These three parts make up the structure of lysophosphatidic acid, a well-known bioactive lysolipid mediator. LPC additionally has a choline group attached to the phosphate. Therefore, LPC has a positive charge in biological fluids.

First of all, we tested whether LPC increases $[Ca^{2+}]_i$. LPCs (16:0 and 18:0) induced Ca^{2+} increase in the U937 monocytes at 10 μ M, but not 14:0 LPC. Representative Ca^{2+} traces with 10 μ M of each compound are shown in Fig. 2. The response was specific to the U937 cell line. In Jurkat T cells, LPC-induced Ca^{2+} increase was not observed, consistent with the previous report of Xu et al. (8) (data not shown). Xu et al. reported that lysolipids, including sphingosylphosphorylcholine, lysophosphatidic acid, and sphingosine 1-phosphate, but not LPC induced Ca^{2+} increase in Jurkat cells (8). LysoPAFs also induced similar responses. The responses to 16:0 LPC and 16:0 lysoPAF were greater than to 18:0 LPC or 18:0 lysoPAF. By less than 10 μ M of 18:0 LPC or lysoPAF, there was no significant Ca^{2+} change, but 5 μ M of 16:0 LPC or 16:0 lysoPAF increased $[Ca^{2+}]_i$. Furthermore the height of Ca^{2+} responses by 10 μ M of 16:0 compounds were greater than those of 18:0 compounds (Fig. 2).

Signaling of LPC-induced Ca^{2+} increase

To elucidate the signaling cascade for LPC-induced Ca^{2+} increase, we next tested the involvement of several signaling molecules, for example, G proteins and

Fig. 1. Structures of LPC, lysoPAF, PAF, and lysophosphatidic acid (LPA).

Fig. 2. Representative Ca^{2+} traces with 10 μM LPC and related compounds.

phospholipase C (PLC). We treated cells with pertussis toxin (100 ng/ml, 24 h), a specific inhibitor of $\text{G}_{i/o}$ -type trimeric G proteins, and monitored their Ca^{2+} responses. In the toxin-treated cells, basal activity of p42/44 MAP was diminished about 50%, and serum-induced activation was further blocked (data not shown). However, there was no significant blockage on LPC-induced Ca^{2+} increase by the toxin treatment (Fig. 3), excluding the involvement of $\text{G}_{i/o}$ -type trimeric G proteins. Using

U73122, a specific inhibitor of PLC, we tested the involvement of PLC in the $\text{G}_{q/11}$ -type G proteins – inositol 1,4,5-triphosphate (IP_3) – Ca^{2+} signaling pathway of the LPC-induced Ca^{2+} increase. The treatment of U73122 changed the shape of the Ca^{2+} trace by PAF (Fig. 4). There were two phases of the Ca^{2+} increase by PAF: a fast, robust increase and a delayed, sustained one. The former may be evoked by Ca^{2+} release from internal Ca^{2+} stores via PLC- IP_3 and the latter by Ca^{2+}

Fig. 3. Effect of pertussis toxin treatment on the LPC-induced Ca^{2+} increase. Representative Ca^{2+} traces with 10 μM LPC and related compounds in the cells treated with pertussis toxin (100 ng/ml, 24 h).

Fig. 4. Effect of U73122 treatment on the LPC-induced Ca^{2+} increase. Representative Ca^{2+} traces with 10 μM LPC and related compounds in the cells after treatment of 5 μM U73122.

influx across the plasma membrane (Fig. 2). In the U73122-treated monocytes, the fast phase of the Ca^{2+} response disappeared, although the delayed Ca^{2+} increase was intact (Fig. 4). In this condition, the LPC-induced Ca^{2+} increases were observed to be the same as those in non-treated cells, implying that PLC might not be a step

in the LPC-induced Ca^{2+} increase in the cells. Finally we monitored the LPC-induced Ca^{2+} increase after chelating the extracellular Ca^{2+} with EGTA (Fig. 5). There was a small increase of Ca^{2+} by PAF. However, not all of the LPC-induced Ca^{2+} increases were observable, implying that the Ca^{2+} influx across the plasma membrane was the

Fig. 5. Effect of EGTA treatment on the LPC-induced Ca^{2+} increase. Representative Ca^{2+} traces with 10 μM LPC and related compounds in the cells after addition of 5 mM EGTA.

Fig. 6. Expression analysis of four GPCR in U937 monocytes. RT-PCR was performed using mRNAs extracted from U937 cells. For positive controls, 10 ng of the expression vectors for each receptor were used as PCR templates. The data is a representative of three independent experiments with similar results.

only source of the LPC-induced Ca^{2+} increase.

Involvement of GPCR

Recently, two GPCR in the plasma membrane have been identified as receptors for LPC (10). G2A and GPR4 bind to LPC specifically and regulate cell growth and immunologic responses (12, 13). GPR4 also recognizes a structurally related lysolipid, sphingosylphosphorylcholine, as a ligand. Both are members of a

subfamily of GPCR (12). OGR1, a third member of this subfamily, has been reported to be a receptor for sphingosylphosphorylcholine (11). The fourth member, TDAG8, is a receptor for psychosine, D-galactosyl β , β' -sphingosine (15). To test the involvement of LPC receptors in the Ca^{2+} increase in monocytes, we conducted RT-PCR in order to amplify the messenger RNAs for G2A and GPR4. Primers for each receptor of the subfamily work very well in control DNA templates (Fig. 6). From the mRNAs of U937 cells, a strong signal was detected for the TDAG8 receptor, but there was no signal for GPR4, OGR1, or G2A.

Discussion

In the present study, we found that 16:0 and 18:0 LPC increase the intracellular Ca^{2+} concentration in U937 human monocytes. The response was not blocked by treatments of pertussis toxin or U73122, specific inhibitors for $\text{G}_{i/o}$ -type G proteins or PLC. However, it was totally blocked by the chelation of the extracellular Ca^{2+} ions with EGTA. Therefore, it might be assumed that the influx of Ca^{2+} across the plasma membrane is the only source of Ca^{2+} mobilized by LPC in those cells.

A subfamily of GPCR, consisting of OGR1, GPR4, G2A, and TDAG8, has been identified as receptors for LPC, sphingosylphosphorylcholine, and psychosine (10). RT-PCR analysis of those receptor expressions in U937

cells implied no expression of GPR4 or G2A. Previous Northern blot analyses of each LPC receptor in various tissues have indicated G2A expression but no expression of GPR4 in immune cells and immune tissues. No expression of the known LPC receptors, G2A and GPR4, excludes the possibility of GPCR involvement in the LPC response in U937 monocytes. Furthermore, involvement of PLC or $G_{i/o}$ proteins was excluded. Analysis of the signaling cascades of G2A and GPR4 in cells expressing each recombinant LPC receptor suggested the involvement of pertussis toxin-sensitive G proteins in both receptors' signaling. Considering the present results with previous signaling cascades for the suggested LPC receptors, LPC might induce a Ca^{2+} increase via a novel target of LPC, which may not be the receptors in the plasma membrane.

The response was also observable with lysoPAFs, which have an ether link between the glycerol and the fatty chain (16:0 or 18:0) instead of an ester bond. PAF showed a similar Ca^{2+} increase in terms of maximum response, that is, the height of the Ca^{2+} increase. However, there are several differences between the PAF response and the LPC-induced Ca^{2+} response. The fast Ca^{2+} increase by PAF was not blocked by EGTA treatment. Furthermore, the fast response was also eliminated by U73122 treatment. These observations suggest that the fast response was triggered through PLC activation, subsequent IP_3 formation, and IP_3 -mediated Ca^{2+} release from the internal Ca^{2+} stores.

Acknowledgment

This work was supported by a Korea Research Foundation Grant (KRF-2002-041-E00048).

References

- 1 Steinberg D, Parthasarathy S, Carew TE, Khoo JC, Witztum JL. Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. *N Engl J Med*. 1989;320:915–924.
- 2 Yokoyama K, Ishibashi T, Ohkawara H, et al. HMG-CoA reductase inhibitors suppress intracellular calcium mobilization and membrane current induced by lysophosphatidylcholine in endothelial cells. *Circulation*. 2002;105:962–967.
- 3 Okajima F, Sato K, Tomura H, et al. Stimulatory and inhibitory actions of lysophosphatidylcholine, depending on its fatty acid residue, on the phospholipase C/ Ca^{2+} system in HL-60 leukaemia cells. *Biochem J*. 1998;336:491–500.
- 4 Hsieh CC, Yen MH, Liu HW, Lau YT. Lysophosphatidylcholine induces apoptotic and non-apoptotic death in vascular smooth muscle cells: in comparison with oxidized LDL. *Atherosclerosis*. 2000;151:481–491.
- 5 Takahashi M, Okazaki H, Ogata Y, Takeuchi K, Ikeda U, Shimada K. Lysophosphatidylcholine induces apoptosis in human endothelial cells through a p38-mitogen-activated protein kinase-dependent mechanism. *Atherosclerosis*. 2002;161:387–394.
- 6 Quinn MT, Parthasarathy S, Steinberg D. Lysophosphatidylcholine: a chemotactic factor for human monocytes and its potential role in atherogenesis. *Proc Natl Acad Sci USA*. 1988;85:2805–2809.
- 7 Sakai M, Miyazaki A, Hakamata H, et al. The scavenger receptor serves as a route for internalization of lysophosphatidylcholine in oxidized low density lipoprotein-induced macrophage proliferation. *J Biol Chem*. 1996;271:27346–27352.
- 8 Xu Y, Casey G, Mills GB. Effect of lysophospholipids on signaling in the human Jurkat T cell line. *J Cell Physiol*. 1995;163:441–450.
- 9 Lynch KR, Im DS. Life on the edge. *Trends Pharmacol Sci*. 1999;20:473–475.
- 10 Hla T, Lee MJ, Ancellin N, Paik JH, Kluk MJ. Lysophospholipids – receptor revelations. *Science*. 2001;294:1875–1878.
- 11 Xu Y, Zhu K, Hong G, et al. Sphingosylphosphorylcholine is a ligand for ovarian cancer G-protein-coupled receptor 1. *Nat Cell Biol*. 2000;2:261–267.
- 12 Zhu K, Baudhuin LM, Hong G, et al. Sphingosylphosphorylcholine and lysophosphatidylcholine are ligands for the G protein-coupled receptor GPR4. *J Biol Chem*. 2001;276:41325–41335.
- 13 Kabarowski JH, Zhu K, Le LQ, Witte ON, Xu Y. Lysophosphatidylcholine as a ligand for the immunoregulatory receptor G2A. *Science*. 2001;293:702–705.
- 14 Im DS, Fujioka T, Katada T, Kondo Y, Ui M, Okajima F. Characterization of sphingosine 1-phosphate-induced actions and its signaling pathways in rat hepatocytes. *Am J Physiol*. 1997;272:G1091–G1099.
- 15 Im DS, Heise CE, Nguyen T, O'Dowd BF, Lynch KR. Identification of a molecular target of psychosine and its role in globoid cell formation. *J Cell Biol*. 2001;153:429–434.