Effects of C18 Fatty Acids on Intracellular Ca\textsuperscript{2+} Mobilization and Histamine Release in RBL-2H3 Cells

Myung Chul Kim, Min Gyu Kim, Young Soo Jo, Ho Sun Song, Tae In Eom, and Sang Soo Sim

College of Pharmacy, Chung-Ang University, Seoul 156-756, Korea

To investigate the underlying mechanisms of C18 fatty acids (stearic acid, oleic acid, linoleic acid and \(\alpha\)-linolenic acid) on mast cells, we measured the effect of C18 fatty acids on intracellular Ca\textsuperscript{2+} mobilization and histamine release in RBL-2H3 mast cells. Stearic acid rapidly increased initial peak of intracellular Ca\textsuperscript{2+} mobilization, whereas linoleic acid and \(\alpha\)-linolenic acid gradually increased this mobilization. In the absence of extracellular Ca\textsuperscript{2+}, stearic acid (100 \(\mu\)M) did not cause any increase of intracellular Ca\textsuperscript{2+} mobilization. Both linoleic acid and \(\alpha\)-linolenic acid increased intracellular Ca\textsuperscript{2+} mobilization, but the increase was smaller than that in the presence of extracellular Ca\textsuperscript{2+}. These results suggest that C18 fatty acid-induced intracellular Ca\textsuperscript{2+} mobilization is mainly dependent on extracellular Ca\textsuperscript{2+} influx. Verapamil dose-dependently inhibited stearic acid-induced intracellular Ca\textsuperscript{2+} mobilization, but did not affect both linoleic acid- and \(\alpha\)-linolenic acid-induced intracellular Ca\textsuperscript{2+} mobilization. These data suggest that the underlying mechanism of stearic acid, linoleic acid and \(\alpha\)-linolenic acid on intracellular Ca\textsuperscript{2+} mobilization may differ. Linoleic acid and \(\alpha\)-linolenic acid significantly increased histamine release. Linoleic acid (C18:2: \(\omega\)-6)-induced intracellular Ca\textsuperscript{2+} mobilization and histamine release were more prominent than \(\alpha\)-linolenic acid (C18:3: \(\omega\)-3). These data support the view that the intake of more \(\alpha\)-linolenic acid than linoleic acid is useful in preventing inflammation.

Key Words: Ca\textsuperscript{2+} mobilization, C18 fatty acids, Histamine release, PLC assay

INTRODUCTION

Mast cells play a central role in the innate immune response. They are particularly important in helping to recruit other cells, such as basophils, neutrophils and lymphocytes. Mast cells play an important role in a number of allergic diseases including atopic dermatitis and asthma, and contribute to chronic inflammatory conditions like atherosclerosis, vasculitis and rheumatoid arthritis. Mast cells have a number of secretory granules including histamine and 5-hydroxytryptamine, and rapidly synthesize lipid-derived mediators, such as prostaglandins and leukotrienes from arachidonic acid (C20:4: \(\omega\)-6) released by the activation of phospholipase A\(_2\) [1]. The addition of arachidonic acid to RBL-2H3 cells distinctly altered the fatty acid composition of the cell membrane and enhance Fe\(\varepsilon\)RI-mediated degranulation and tumor necrosis factor-alpha (TNF-\(\alpha\)) release with augmented tyrosine phosphorylation of some proteins and Ca\textsuperscript{2+} influx [2]. We previously reported that exogenous arachidonic acid significantly increases histamine release from RBL-2H3 cells [3].

On the other hand, \(\omega\)-3 polysaturated fatty acids (PUFAs) reportedly decrease the production of arachidonic acid-derived eicosanoids. These fatty acids decrease the production of inflammatory cytokines, such as TNF, interleukin (IL)-1, and IL-6, and the expression of adhesion molecules involved in inflammatory interactions between leukocytes and endothelial cells [4]. Babcock et al. reported that \(\omega\)-3 PUFAs, such as eicosapentaenoic acid and docosahexaenoic acid, significantly inhibit the production of TNF-\(\alpha\) and IL-10 in monocytes [5]. The anti-inflammation effects of \(\omega\)-3 eicosapentaenoic acid and the inflammation reactions of \(\omega\)-6 arachidonic acid have been studied largely, but \(\alpha\)-linolenic acid (a precursor of eicosapentaenoic acid) and linoleic acid (a precursor of arachidonic acid) had been not. In this study, to investigate the underlying mechanisms of C18 fatty acids on anti-inflammatory response, we measured the effect of C18 fatty acids such as stearic acid (C18, saturated fatty acid), oleic acid (C18:1: \(\omega\)-9), linoleic acid (C18:2: \(\omega\)-6) and \(\alpha\)-linolenic acid (C18:3: \(\omega\)-3) on intracellular Ca\textsuperscript{2+} mobilization and histamine release in RBL-2H3 mast cells.

ABBREVIATIONS: IP\(_3\), inositol 1,4,5-triphosphate; PKC, protein kinase C; PLC, phospholipase C; PUFA, polyunsaturated fatty acid; ROCC, receptor operated Ca\textsuperscript{2+} channels; VDCC, voltage-operated Ca\textsuperscript{2+} channels.
METHODS

Materials

C18 fatty acids (stearic acid, oleic acid, linoleic acid and α-linolenic acid), verapamil, 8-(diethylamino)octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8), bisindolylmaleimide (BIM), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and o-phthalaldehydesulfonic acid (OPT) were obtained from Sigma Chemical (St. Louis, MO, USA). Fura-2/AM was purchased from Enzo Life Science (Farmingdale, NY, USA). [3H] Inositol was obtained from PerkinElmer (Waltham, MA, USA). AG 1-X8 resin was purchased from Bio-Rad (Hercules, CA, USA). The materials for cell culture were purchased from Life Technologies (Grand Island, NY, USA). Unless otherwise stated, all reagents were of the highest purity and were purchased from Sigma Chemical.

Cell culture

Rat basophilic leukemia (RBL)-2H3 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured as recommended by ATCC at 37°C in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics (100 U/mL penicillin G, 100 μg/mL streptomycin) in a 5% CO2 incubator. Cells between 5 and 20 passages were used for experiments at a density of 1 ×10^6 cells/mL.

Measurement of cell viability

Cell viability testing was performed using a MTT-based colorimetric assay [6]. Cells were diluted and cultured in 96-well plates (1×10^4 cells/mL), with the select C18 fatty acid added (100 ~ 200 μM). An equal amount of ethanol was added in the control group, which resulted in a final concentration of 0.1% in the culture medium. MTT was added to the cells to dissolve the formazan. The absorbance of each supernatant and pellet were measured with 0.1 mL of 1% OPT (Molecular Devices, Sunnyvale, CA, USA) at wavelength of 520 nm [7].

Measurement of intracellular Ca^{2+} mobilization

The intracellular Ca^{2+} level was measured using Fura-2/AM by monitoring a fluorospectrometer [8]. Briefly, culture medium was replaced and cells were washed three times with phosphate buffered saline (PBS). Cells were detached using 0.5% trypsin/EDTA buffer and suspended in Krebs buffer [120.8 mM NaCl, 4.5 mM KCl, 1.2 mM MgCl2, 1.8 mM CaCl2, 5.6 mM glucose, 1.2 mM NaH2PO4 and 25 mM HEPES (pH 7.4)]. Fura-2/AM was added to a final concentration of 2 μM and incubated at 37°C for 1 h. The cells were washed twice with Krebs buffer and centrifuged at 3,000×g for 10 min. For the experiments performed in the absence of external calcium, Krebs-EGTA buffer [120.8 mM NaCl, 4.5 mM KCl, 1.2 mM MgCl2, 1.0 mM EGTA, 5.6 mM glucose, 1.2 mM NaH2PO4 and 25 mM HEPES (pH 7.4)] was used. Fura-2 fluorescence was monitored by a Quanta Master device (Photon Technology Inter-

RESULTS

Effects of C18 fatty acids on the viability of RBL 2H3 cells

To confirm cytotoxicity of C18 fatty acids, cell viability

Statistical analyses

Results are presented as mean±S.D. and significance was determined by analysis of variance (ANOVA) and differences between groups were determined with a Newman-Keuls’s test. The level of significance was set at less than 5% (p<0.05).
C18-fatty Acids and Intracellular Ca\textsuperscript{2+} Mobilization

Fig. 1. Cytotoxicity of C18 fatty acids in RBL-2H3 cells. Cells were incubated with C18 fatty acids (A: stearic acid, B: oleic acid, C: linoleic acid, D: \(\alpha\)-linolenic acid) for 24 h in a 5% CO\textsubscript{2} incubator at 37°C. The number of living cells was measured using MTT. Results are mean±S.D. from four separate experiments.

was determined by the ability of the cells to metabolically reduce MTT to a formazan dye. It was performed after 6, 12 and 24 h exposure to the C18 fatty acid stearic acid, oleic acid, linoleic acid or \(\alpha\)-linolenic acid. After 24 h exposure to oleic acid, linoleic acid and \(\alpha\)-linolenic acid, which contain a double bond, no cytotoxicity was evident at a concentration of 200 \(\mu\)M. In contrast, stearic acid, which lacks a double bond, reduced the cell viability by 16% at 200 \(\mu\)M (Fig. 1). However, stearic acid showed no cytotoxicity at 6 h.

C18 fatty acid-induced intracellular Ca\textsuperscript{2+} mobilization

To investigate the effects of C18 fatty acids on intracellular Ca\textsuperscript{2+} concentration, we measured Ca\textsuperscript{2+} mobilization in Fura-2/AM-loaded RBL-2H3 cells. As shown in Fig. 2A, stearic acid rapidly increased the initial peak of intracellular Ca\textsuperscript{2+} mobilization, whereas linoleic acid and \(\alpha\)-linolenic acid gradually increased intracellular Ca\textsuperscript{2+} mobilization. Oleic acid (100 \(\mu\)M) slightly increased intracellular Ca\textsuperscript{2+} concentration, which was smaller than the effects of other C18 fatty acids (Fig. 2A). In the next experiment, we excluded oleic acid. Stearic acid, linoleic acid and \(\alpha\)-linolenic acid dose-dependently increased intracellular Ca\textsuperscript{2+} mobilization (Figs. 2B-2D). In the absence of extracellular Ca\textsuperscript{2+}, stearic acid (100 \(\mu\)M) did not cause any increase of intracellular Ca\textsuperscript{2+} mobilization, but both linoleic acid and \(\alpha\)-linolenic acid increased intracellular Ca\textsuperscript{2+} mobilization; the increase was smaller than that in the presence of extracellular Ca\textsuperscript{2+} (Fig. 3). These data suggest that C18 fatty acid-induced intracellular Ca\textsuperscript{2+} mobilization is mainly dependent on extracellular calcium influx.

Effects of calcium antagonists and BIM on C18 fatty acid-induced intracellular Ca\textsuperscript{2+} mobilization

Verapamil (10 \(\mu\)M), a voltage-dependent calcium channel blocker, dose-dependently inhibited stearic acid-induced intracellular Ca\textsuperscript{2+} mobilization (Figs. 4A and 5), but did not affect linoleic acid- and \(\alpha\)-linolenic acid-induced intracellular Ca\textsuperscript{2+} mobilization (Figs. 4B and 4C). At 10 \(\mu\)M TMB-8, an intracellular calcium release blocker, did not influence intracellular Ca\textsuperscript{2+} mobilization induced by stearic acid, linoleic acid and \(\alpha\)-linolenic acid (data not
Fig. 2. C18 fatty acid-induced intracellular Ca\(^{2+}\) mobilization. Intracellular Ca\(^{2+}\) mobilization was measured in Fura-2/AM-loaded RBL-2H3 cells. Intracellular Ca\(^{2+}\) concentration induced by a 100-sec exposure to the C18 fatty acids [A: C18 fatty acids, B: stearic acid (SA), C: linoleic acid (LA), D: α-linolenic acid (LN)] was expressed as the ratio of F340/F380. Results are the representative data of four separate experiments.

Fig. 3. C18 fatty acids-induced intracellular Ca\(^{2+}\) mobilization in the presence or absence of extracellular Ca\(^{2+}\). Intracellular Ca\(^{2+}\) concentration was induced by a 100-sec exposure to the C18 fatty acids [A: stearic acid (SA, 100 μM), B: linoleic acid (LA, 100 μM), C: α-linolenic acid (LN, 100 μM)]. Results are the representative data of four separate experiments.
C18-fatty Acids and Intracellular Ca\(^{2+}\) Mobilization

Fig. 4. Effect of verapamil on intracellular Ca\(^{2+}\) mobilization induced by C18 fatty acids. After a 50-sec treatment with 10 \(\mu M\) verapamil (VP), intracellular Ca\(^{2+}\) concentration was induced by a 100-sec exposure to the C18 fatty acids [A: stearic acid (SA), B: linoleic acid (LA), C: \(\alpha\)-linolenic acid (LN)]. Results are the representative data of four separate experiments.

Fig. 5. (A) Dose-response of verapamil (VP) to intracellular Ca\(^{2+}\) mobilization and (B) peak ratio induced by stearic acid (SA, 100 \(\mu M\)). Results are the representative data of four separate experiments. *Significantly different from control (\(p<0.05\)).

Fig. 6. Effect of bisindolylmaleimide (BIM) on intracellular Ca\(^{2+}\) mobilization induced by a 100-sec exposure to C18 fatty acids [A: stearic acid (SA), B: linoleic acid (LA), C: \(\alpha\)-linolenic acid (LN)]. Results are the representative data of four separate experiments.
shown). At 10 μM, BIM, a protein kinase C (PKC) inhibitor, significantly inhibited both linoleic acid- and α-linolenic acid-induced intracellular Ca²⁺ mobilization, but did not affect stearic acid-induced intracellular Ca²⁺ mobilization (Fig. 6).

**Effects of C18 fatty acids on [³H]inositol phosphates formation in RBL-2H3 cells**

The phospholipase C (PLC) pathway is closely involved in the intracellular Ca²⁺ mobilization. In [³H]inositol-labeled cells, melittin as a positive control significantly increased [³H]inositol phosphate formation by 245%. However, three C18 fatty acids did not increase [³H]inositol phosphate formation (Table 1), indicating that C18 fatty acids-induced intracellular Ca²⁺ mobilization is not associated with intracellular Ca²⁺ release by the PLC pathway.

**Effects of C18 fatty acids on histamine release in RBL-2H3 cells**

At 100 μM, linoleic acid and α-linolenic acid significantly increased histamine release from 15.2±4.8% (control) to 54.3±7.7% and 44.2±8.8%, respectively, which was smaller than the effect of 100 μM arachidonic acid (69.7±2.4%). However, stearic acid, which lacks a double bond, did not induce histamine release at a concentration of 100 μM (Fig. 7A). At 10 μM, the BIM, PKC inhibitor, significantly inhibited linoleic acid- and α-linolenic acid-induced histamine release (Fig. 7B), suggesting the involvement of PKC in linoleic acid- and α-linolenic acid-induced histamine release in RBL-2H3 cells.

**DISCUSSION**

In the measurement of histamine release, it is important to confirm the cytotoxicity of C18 fatty acids. To measure histamine release from RBL-2H3 cells, C18 fatty acids were exposed to the cells for 1 h. This exposure was not toxic, since no cytotoxicity was apparent after 6 h exposure of the tested fatty acids. These data suggest that exocytosis of histamine may be independent on cell membrane rupture. It has been reported that exposure of stearic acid at 150 μM for 24 h induces cell death and that ω-6 PUFAs have a greater protective effect on the deleterious effect caused by stearic acid in ECV-304 endothelial cells than ω-3 PUFAs [13].

Stearic acid rapidly increased the initial peak of intracellular Ca²⁺ mobilization, whereas linoleic acid and α-linolenic acid gradually increased intracellular Ca²⁺ mobilization. In the absence of extracellular Ca²⁺, stearic acid (100 μM) did not cause any increase of intracellular Ca²⁺ mobilization. Both linoleic acid and α-linolenic acid increased intracellular Ca²⁺ mobilization, but the increase was smaller than that in the presence of extracellular Ca²⁺. These results suggest that C18 fatty acid-induced intracellular Ca²⁺ mobilization is mainly dependent on extracellular calcium influx. A prior study reported that 40 μM of three ω-6 polyunsaturated fatty acids (arachidonic acid, γ-linolenic acid and linoleic acid) increased intracellular calcium concentration, while 40 μM ω-3 and ω-9 PUFAs did not [14]. The discrepancy between the present and previous study may be due to the concentration of C18 fatty acids because we used 100 μM of the C18 fatty acids.

At 10 μM, verapamil, a voltage-dependent calcium chan-

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**Table 1. Production of [³H]inositol phosphates by C18 fatty acids**

| Concentration (μM) | [³H]Inositol phosphates (dpm/mg protein) |
|-------------------|------------------------------------------|
| Control           | 0                                        |
| Stearic acid      | 100                                      |
| Linoleic acid     | 100                                      |
| α-Linolenic acid  | 100                                      |
| Melittin          | 10                                       |

Results are mean±S.D. from four separate experiments.

*Significantly different from control (p<0.05).

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**Fig. 7.** C18 Fatty acid-induced histamine release in RBL-2H3 cells. (A) Histamine release induced by arachidonic acid (AA, 100 μM), stearic acid (SA, 100 μM), linoleic acid (LA, 100 μM) and α-linolenic acid (LN, 100 μM). (B) Effect of 10 μM bisindolylmaleimide (BIM) on histamine release induced by linoleic acid (LA, 100 μM) and α-linolenic acid (LN, 100 μM). *Significantly different from Control (p<0.05); †Significantly different from LA (p<0.05).
The PKC inhibitor BIM used at 10 μM significantly inhibited both linoleic acid and α-linolenic acid-induced intracellular Ca\(^{2+}\) mobilization. These data suggest that the underlying mechanism of stearic acid, linoleic acid and α-linolenic acid on intracellular Ca\(^{2+}\) mobilization may be different from each other. Also, C18 fatty acids-induced intracellular Ca\(^{2+}\) mobilization appeared not to be associated with intracellular Ca\(^{2+}\) release by the PLC pathway, since they failed to induce [\(^{3}H\)]inositol phosphates formation in [\(^{3}H\)]inositol-labeled cells. This finding is consistent with a previous report that 40 α arachidonic acid did not result in the formation of inositol-1,4,5-triphosphates [14]. Long chain fatty acids and ω-3 PUFAs (EPA and DHA) have been demonstrated to act as ligands of several G-protein-coupled receptors, such as GRP40 and GRP120, respectively [16,17]. Especially GRP40 activation which is strongly expressed in the pancreas results in an increase of phosphatidylinositol 3,4,5-trisphosphate-sensitive calcium entry system. Biochemistry. 2004;43:11701-11708.

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