Diacylglycerol kinase-dependent formation of phosphatidic acid molecular species during interleukin-2 activation in CTLL-2 T-lymphocytes

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Abstract

Although effective liquid chromatography (LC)/mass spectrometry (MS) methods enabling the separation of phospholipid molecular species have been developed, there are still problems with an intracellular signaling molecule, phosphatidic acid (PA). In this study, we optimized LC/MS conditions to improve the quantitative detection of PA molecular species from a cellular lipid mixture. Using the newly developed LC/MS method, we showed that stimulation of CTLL-2 murine T-lymphocytes by interleukin-2 (IL-2) induced a significant increase of 36:1-, 36:2-, 40:5- and 40:6-diacyl-PA. A diacylglycerol kinase (DGK) inhibitor, R59949, attenuated the increase of 36:1-, 40:5-, 40:6-diacyl-PA, suggesting that DGK IL-2-dependently and selectively generated these diacyl-PA species.

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1. Introduction

Phosphatidic acid (PA) is an important intermediate in the synthesis of all phospholipids and an intracellular signaling molecule in mammalian cells. PA as a lipid second messenger performs specific tasks in a wide range of biological processes [1–3]. For example, PA regulates phosphatidylinositol-4-phosphate-5-kinase, Ras and phosphatidylinositol-3-kinase, and cytoskeleton reorganization, glucose incorporation and carcinogenesis [5–10]. For example, the α-isozyme of DGK regulates the proliferation of melanoma [11] and hepatocellular carcinoma [12]. Moreover, PA produced by DGKα, which is abundantly expressed in T-lymphocytes and the thymus [13,14], is necessary for the interleukin-2 (IL-2)-induced G1-to-S transition of T-lymphocytes [15,16]. Furthermore, Jones et al. revealed that IL-2 causes an increase of PA in CTLL-2 murine T-lymphocytes that lack the IL-2-stimulating PLD activity [17,18]. These studies indicate that the DGK-dependent accumulation of PA is important for IL-2-induced T-cell proliferation. However, it remains unclear what PA molecular species are involved during IL-2-dependent T-cell proliferation.

To detect molecular species of phospholipids in cells, liquid chromatography/electrospray ionization mass spectrometry (LC/ESI-MS) is a powerful tool [19,20]. However, there are still problems with detecting PA. Because PA is a minor component of phospholipids, and because PA contains a variety of fatty acids, extensively broad PA peaks inevitably overlap with other major phospholipid peaks, causing inferior detection, quantification and reproducibility. Because of these methodology limitations, the behavior of different PA molecular species during cell stimulation is poorly understood.

In this study, we optimized LC conditions to quantitatively and reproducibly detect PA molecular species using LC/ESI-MS. To test the developed LC/ESI-MS method, we examined phospholipid mixtures from various mammalian cells including CTLL-2 T-lymphocytes, and confirmed that PA species from m/z 591.41 (28:0-PA) to m/z 759.59 (40:0-PA) were quantitatively and reproducibly detected. As a result, the PA diacyl and alkyl-acyl species profiles were found to vary between different mammalian cell lines. Moreover, we found that IL-2 stimulation caused a DGK-dependent increase of limited molecular species of PA in CTLL-2 cells.
2. Materials and methods

2.1. Cell culture

CO2-7 (a simian virus 40-transformed simian kidney cell line), C2C12 (a mouse myoblast cell line) and HeLa (a human epithelial carcinoma cell line) cells as well as mouse embryonic fibroblasts (MEFs), obtained from fetal C57/BL6 mice, were maintained on 100-mm dishes in Dulbecco’s modified Eagle’s medium (DMEM, Wako Pure Chemicals, Tokyo, Japan) containing 10% fetal bovine serum (FBS) at 37 °C in an atmosphere with 5% CO2. C2L-2 (an IL-2-dependent mouse cytotoxic T-cell line) and Jurkat (a human T cell lymphoblast-like cell line) cells were maintained in 75-cm² flasks in RPMI-1640 medium (Wako Pure Chemicals) containing 10% FBS, 2 mM sodium pyruvate and 50 μM 2-mercaptoethanol. For C2L-2 cell culture, 100 U/ml IL-2 (human recombinant, Wako Pure Chemicals) was added to the medium.

2.2. IL-2 stimulation

C2L-2 cells (grown to 80% confluence) were washed twice with RPMI-1640. To starve the cells, the washed cell were incubated in serum- and IL-2-free RPMI-1640 for 90 min. The starved cells were pre-incubated with or without 25 μM DGK inhibitor R59949 (Merk Biosciences-Calbiochem, Tokyo, Japan) for 30 min and stimulated with 200 U/ml IL-2 (Wako Pure Chemicals) for 15 min.

2.3. Lipid extraction and measurement of the phospholipid amount

Cells (grown to 80% confluence) were harvested in phosphate-buffered saline. Total lipids were extracted from the cells according to the method of Bligh and Dyer [21]. An aliquot of the extracted lipids was used for measurement of the amount of inorganic phosphate in the phospholipid preparation as previously described [22].

2.4. Liquid chromatography

The extracted cellular lipids (5 μl) containing 40 pmol of the 28:0-PA internal standard (Sigma–Aldrich, Tokyo, Japan), were separated on the LC system (Accela LC Systems, Thermo Fisher Scientific, Tokyo, Japan) using a UK-Silica column (3 μm, 150 × 2.0 mm i.d., Intakt, Kyoto, Japan). Mobile phase A consisted of chloroform/methanol/ammonia (89:10:1), and mobile phase B consisted of chloroform/methanol/ammonia/water (55:39:1:5). The gradient elution program was as follows: 30% B for 5 min, 30–60% B over 25 min, 60–70% over 5 min, followed by 70% B for 10 min. The flow rate was 0.3 ml/min, and the chromatography was performed at 25 °C.

2.5. Mass spectrometry

The LC system described above was coupled online to an Exactive Orbitrap MS (Thermo Fisher Scientific) equipped with an ESI source. The ion spray voltage was set to –5 and 5 kV in the negative and positive ion modes using an Orbitrap Fourier Transform MS with a resolution of 50,000. The MS peaks were identified based on their mass/charge (m/z) ratio and were presented in the form of X:Y, where X is the total number of carbon atoms and Y is the total number of double bonds in both acyl chains of the phospholipid.

Fig. 1. Separation of PA and identification of PA molecular species by LC/ESI-MS. (A) PAs in MEFs were separated from other phospholipids by the Accela LC System using a UK-Silica column with mobile phases containing 0.28% ammonia. (B) Negative-ion ESI-MS spectra with PA species from m/z 591.41 (28:0-PA) to m/z 759.59 (40:0-PA) were identified. Abbreviations: t, internal standard, PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidyglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin.

2.6. Statistics

All LC/ESI-MS data were normalized based on the inorganic phosphate content and the intensity of the internal standard and were represented as the mean ± SD. Statistical analysis was performed by the two-tailed t-test.

3. Results

3.1. Improvement of PA species detection using LC/ESI-MS

Pettitt et al. and Shui et al. reported that PA molecular species can be separated from total lipid extracts of eukaryotic cells using silica column LC/MS [23,24]. We attempted to separate PA species from total lipids extracted from MEFs according to the previously reported LC conditions. However, because PA elutes relatively close to phosphatidylcholine and sphingomyelin using these conditions, PA often overlapped with the major phospholipids (Suppl. Fig. 1), causing inferior reproducibility. Thus, we optimized the LC conditions to establish an improved PA separation. We found that the PA peaks were completely separated from other phospholipid species when mobile phases A and B were changed to chloroform/methanol/ammonia (89:10:1) and chloroform/methanol/ammonia/water (55:39:1:5), respectively (Fig. 1A).

The detected diacyl- and alkyl–acyl-PA species were listed in Tables 1 and 2 and all had mass accuracies less than ±6 ppm. In addition, the representative MS spectra pattern of the PA species from m/z 591.41 (28:0-PA, internal standard) to m/z 759.59 (40:0-PA) was shown in Fig. 1B. Moreover, we tested the reproducibility of quantitative detection of cellular diacyl-PA species in MEFs. The repeated
experiments between periods on a given study day (within-day reproducibility) and between the three study days (between-day reproducibility) exhibited excellent reproducibility (Suppl. Fig. 2). These results indicate that our LC/ESI-MS method provided reproducible and quantitative detection of cellular PA species profiles.

To determine recovery rate, a mixture of standard 28:0-, 36:2-, 38:4-PA (80 pmol of each) were added to MEFs before and after the extraction process. The calculated recovery rates were 74.4 ± 10.1% for a mixture of 28:0-, 36:2 and 38:4-PA. Moreover, we established standard curves using different amounts (0.59 – 146 pmol) of standard 28:0-PA and 38:4-PA. Both 28:0- and 38:4-PA displayed linear curves in the high and low amount range tested and the slopes of the linear curves of 28:0-PA and 38:4-PA were very similar (Fig. 2).

In addition, standard curves using various amount of both 28:0- and 38:4-PA showed good linearity at the high (9.50–146 pmol, r > 0.995) and low ranges (0.59–9.50, r > 0.985), suggesting that the length of fatty acyl chains and double bond did not affect ionization efficiency significantly.

### 3.2. MS profiles of PA species in various mammalian cells

To test the new LC/ESI-MS method, we detected and quantitated PA molecular species in various mammalian cells. The primary common components in adherent cells (COS7, C2C12 and HeLa cells (Suppl. Fig. 3) and MEFs (Fig. 3A)) were diacyl-PA species with saturated and mono- and/or di-unsaturated fatty acids (32:1-, 34:1-, 34:2-, 36:1- and 36:2-PA). In addition, several PA species varied in abundance in the different cell lines: 30:0- and 32:0-PA, which consisted of 34:0-, 34:1-, 34:2-, 36:1-, 36:2- and 36:3-PA, comprising common components in adherent cells (COS7, C2C12 and HeLa cells). Moreover, we established standard curves using various amount of both 28:0- and 38:4-PA and the results indicated that the PA species differed moderately in the different adherent cell lines and the differences between the MS profiles of PA species in various mammalian cells.

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#### Table 1

Identification of alkyl–acyl-PA species in MEFs.

| Species | Theoretical m/z | Measured m/z | Mass error a (ppm) |
|---------|----------------|--------------|--------------------|
| 28:0 (I.S.) | 591.4022 | 591.4052 | 5.0 |
| 30:0 | 615.4022 | 615.4049 | 4.3 |
| 30:1 | 617.4179 | 617.4209 | 4.9 |
| 30:2 | 619.4335 | 619.4364 | 4.6 |
| 32:3 | 641.4179 | 641.4195 | 2.5 |
| 32:2 | 643.4335 | 643.4371 | 5.6 |
| 32:1 | 645.4492 | 645.4524 | 5.0 |
| 30:0 | 674.4492 | 674.4516 | 3.6 |
| 31:0 | 676.4648 | 676.4683 | 5.2 |
| 31:1 | 678.4804 | 678.4831 | 3.9 |
| 34:0 | 697.4961 | 697.4985 | 0.6 |
| 36:0 | 695.4968 | 695.4973 | 3.7 |
| 36:4 | 695.4968 | 695.4973 | 3.7 |
| 36:3 | 697.4968 | 697.4985 | 3.6 |
| 36:2 | 699.4968 | 699.4985 | 4.5 |
| 36:1 | 701.5117 | 701.5138 | 3.0 |
| 36:0 | 703.5274 | 703.5285 | –1.2 |
| 38:7 | 717.4492 | 717.4523 | 4.4 |
| 38:6 | 719.4648 | 719.4682 | 4.7 |
| 38:5 | 721.4804 | 721.4836 | 4.4 |
| 38:4 | 723.4961 | 723.4992 | 4.3 |
| 38:3 | 725.5117 | 725.5139 | 3.0 |
| 38:2 | 727.5274 | 727.5301 | 3.8 |
| 38:1 | 729.5430 | 729.5461 | 4.2 |
| 38:0 | 731.5586 | 731.5615 | 3.9 |
| 36:0 | 733.5743 | 733.5773 | 4.0 |
| 36:1 | 735.5902 | 735.5927 | 3.7 |

### Table 2

Identification of alkyl–acyl-PA species in MEFs.

| Species | Theoretical m/z | Measured m/z | Mass error a (ppm) |
|---------|----------------|--------------|--------------------|
| 30:1 | 603.4386 | 603.4417 | 5.1 |
| 30:0 | 605.4543 | 605.4575 | 5.4 |
| 32:3 | 627.4386 | 627.4399 | 2.1 |
| 32:1 | 631.4699 | 631.4734 | 5.6 |
| 30:0 | 633.4855 | 633.4890 | 5.5 |
| 34:0 | 653.4543 | 653.4588 | 2.4 |
| 34:3 | 655.4699 | 655.4721 | 3.4 |
| 34:2 | 657.4855 | 657.4893 | 5.3 |
| 34:1 | 659.5012 | 659.5046 | 5.2 |
| 34:0 | 661.5168 | 661.5198 | 4.3 |
| 36:6 | 677.4543 | 677.4571 | 4.2 |
| 36:5 | 679.4699 | 679.4730 | 4.6 |
| 36:4 | 681.4855 | 681.4884 | 4.4 |
| 36:3 | 683.5012 | 683.5050 | 5.6 |
| 36:2 | 685.5168 | 685.5203 | 5.1 |
| 36:1 | 687.5325 | 687.5360 | 5.2 |
| 36:0 | 689.5481 | 689.5508 | 3.9 |
| 38:7 | 703.4699 | 703.4731 | 4.6 |
| 38:6 | 705.4855 | 705.4892 | 5.2 |
| 38:5 | 707.5012 | 707.5050 | 5.4 |
| 38:4 | 715.5437 | 715.5463 | 0.8 |
| 40:2 | 741.5794 | 741.5824 | 4.1 |
| 40:1 | 743.5950 | 743.5985 | 4.7 |
| 40:0 | 745.6107 | 745.6142 | 4.8 |

a Difference between theoretical m/z and measured m/z.
Fig. 3. Profiles of PA species in mammalian cells (MEFs, and CTLL-2 cells and Jurkat cells). (A, C and E) The molecular species composition of diacyl-PAs in MEFs (A), CTLL-2 cells (C) and Jurkat cells (E). (B and D) The molecular species composition of alkyl–acyl-PAs in MEFs (B) and CTLL-2 cells (D). The values are presented as the mean ± SD (n = 3). N.D., not detected.

Fig. 3). Intriguingly, 38:7-, 36:6-, 38:2-, 40:4- and 40:3-alkyl–acyl-PA species were enriched in COS7 cells (Suppl. Fig. 3). In summary, we concluded that the diacyl and alkyl–acyl-PA profiles are quite different among different mammalian cell lines, and that our new LC/ESI-MS method reproducibly and quantitatively detected cellular PA species profiles.

3.3. Alteration of PA species levels in CTLL-2 cells by IL-2 stimulation

IL-2 causes an increase in PA [18]. Hence, we analyzed the induction of PA species by IL-2 stimulation in CTLL-2 cells using our new LC/ESI-MS method. As shown in Fig. 4A, IL-2 stimulation for 15 min broadly increased diacyl-PA species, in particular, 36:2-, 36:1-, 40:5- and 40:6-diacyl-PA with statistical significance. Interestingly, IL-2 did not augment the amount of 38:4-PA (1-stearoyl-2-arachidonoyl-PA), which is generated from phosphatidylinositol turnover-derived 38:4-diacylglycerol. In addition, the stimulation also failed to alter the amounts of 34:0-, 32:1-, 36:5-, 36:4- and 36:3-diacyl-PA as well as 30:0-, 32:1- and 32:2-alkyl–acyl-PA were inhibited by R59949, whereas those of 34:2, 36:3, 38:6 and 38:5 were not inhibited (data not shown). Therefore, these R59949-inhibited PA species are likely generated by the R59949-sensitive DGK.

4. Discussion

Because PA eluted relatively close to phosphatidylcholine and/or sphingomyelin using previously established methods [23,24], PA often overlapped with these major phospholipids, causing inferior reproducibility. For this study, we thus optimized the LC conditions, including the mobile phase composition and the solvent program to establish a new LC/ESI-MS system suitable for analysis of PA molecular species (Fig. 1). The LC/ESI-MS system stably and reproducibly detected PA species from various mammalian cell lines (Figs. 2 and 3 and Suppl. Fig. 3). Using the developed method, we obtained the following information.

PAs in various mammalian cell lines (MEFs and COS7, C2C12, HeLa,
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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fosbio.2012.08.006.

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