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Preferable existence of polyunsaturated lysophosphatidic acids in human follicular fluid from patients programmed with *in vitro* fertilization

Junpei Yamamoto[a], Midori Omura[a], Koichiro Tuchiya[b], Mayumi Hidaka[c], Akira Kuwahara[d], Minoru Irahara[d], Tamotsu Tanaka[a], Akira Tokumura[a,c,*]

[a] *Department of Pharmaceutical Health Chemistry, Institute of Biomedical Sciences, Tokushima University Graduate School, Shomachi, Tokushima 770-8505, Japan*
[b] *Department of Medical Pharmacology, Institute of Biomedical Sciences, Tokushima University Graduate School, Shomachi, Tokushima 770-8505, Japan*
[c] *Faculty of Pharmacy, Yasuda Women's University, Yasuhigashi, Asaminami-ku, Hiroshima 731-0153, Japan*
[d] *Department of Obstetrics and Gynecology, Institute of Biomedical Sciences, Tokushima University Graduate School, Kuramotocho, Tokushima 770-8503, Japan*

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ABSTRACT

Lysophosphatidic acid (LPA) exerts diverse physiological effects on various types of animal cells, including reproductive cells, through its binding to six LPA receptors. We previously found that LPA promoted maturation of the nucleus and cytoplasm of mouse and hamster oocytes surrounded by cumulus cells *in vitro*. Using gas-liquid chromatography, we previously reported detection of several species of LPA by analyzing the fatty acid methyl esters derived from thin layer chromatography-purified LPA in lipid extract from incubated follicular fluids programmed with *in vitro* fertilization. In this study using liquid chromatography- tandem mass spectrometry, we directly detected high levels of linoleoyl, arachidonoyl, and docosahexaenoyl LPAs in human follicular fluid. This unique molecular species composition of LPA was suggested to be due to a balance between the low LPA-degrading activity and high LPA-producing activity of autotaxin in human follicular fluid. Our results suggest that polyunsaturated LPAs produced by autotaxin in human follicular fluid exert unknown physiological effects on cumulus cells.

1. Introduction

In the mammalian ovary, preantral follicles develop to mature follicles with a cumulus-oocyte complex (COC) bathed with follicular fluid (FF) under the control of gonadotropins. The cumulus cells communicate with each other and the oocyte both by formation of a network of gap junctions and secretion of paracrine factors into FF. Thus, FF in the antral follicles provides a special environment for the oocyte [1]. There are many precedents showing that human FF has beneficial effects during human *in vitro* fertilization and oocyte maturation [2] and cumulus expansion [3]. Some of the components in mammalian FF are derived from the corresponding blood plasma and co-exist with other components secreted by follicular cells including granulosa and theca cells. Human FF contains epidermal growth factor-like proteins such as amphiregulin with epidermal growth factor-like activity [4], which affects the growth of follicles and maturation of oocytes [5]. Several protein-bound lipids with a single hydrophobic long chain were found to be physiologically active: free fatty acids [6], platelet-activating factor [7], lysophospholipids [8,9] and sphingosine-1-phosphate [10]. Among the glycerol-backboned lysophospholipid mediators, lysophosphatidic acid (LPA) is the best characterized lipid on the physiological role in the reproductive system [11,12]. We previously found that LPA induces contractions of rat uterine smooth muscle *in vivo* and *in vitro* [13], stimulates preimplantation mouse embryos *in vitro* [14], accelerates ovum transport through the mouse oviduct *in vitro* [15], and activates maturation of golden hamster and mouse oocytes *in vitro* [16,17].

Using gas-liquid chromatography (GLC) of fatty acids derived from thin-layer chromatography (TLC)-purified LPA in the lipid extract of human FF, we found significant elevations of LPA upon incubation of FF at 37 °C, suggesting LPA production by a lysophospholipase D (lysoPLD)-like activity. This suggestion

* Corresponding author at: Faculty of Pharmacy, Yasuda Women's University, 6-13-1 Yasuhigashi, Asaminami-ku, Hiroshima 731-0153, Japan.
E-mail address: tokumura@yasuda-u.ac.jp (A. Tokumura).

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was confirmed by conducting the experiments with radioactive lysophosphatidylcholine (LPC) [8]. We purified human plasma lysoPLD and identified as autotaxin (ATX), the second member of ecto-nucleotide pyrophosphatase/phosphodiesterase family, by tandem mass spectrometry [18]. ATX was originally characterized as a tumor cell-stimulating protein released by the human melanoma cell line A2058 [19], and its original activity was suggested to be mediated by LPA-production [20]. Recently, molecular species analysis of LPA by liquid chromatography-tandem mass spectrometry (LC–MS/MS) with a soft ionization such as electrospray ionization has rapidly advanced due to its high potency as a biomarker of several chronic diseases [21]. This direct quantification of LPA is superior to our previous indirect quantification of LPAs by TLC/GLC, especially for molecular species having a polyunsaturated fatty acyl group. In this study, we re-examined the molecular species composition of LPA in fresh human FF by LC–MS/MS and LPA- producing enzyme activity from LPC by quantification of its degradation products, choline and LPA, to better understand the physiological role of LPA production by lysoPLD in the follicles. Furthermore, we investigated whether the lysoPLD activity in human FF was attributable to ATX.

2. Materials and methods

2.1. Phospholipids

The LPCs caproyl (6:0), capryryl (8:0), lauroyl (12:0), myristoyl (14:0), palmitoyl (16:0), oleoyl (18:1), linoleoyl (18:2) and α-linolenoyl (18:3), 1-*O*-hexadecyl (alkyl-LPC subclass), and bovine heart plasmalogen (alkenyl-LPC subclass) were purchased from Funakoshi Co. (Tokyo, Japan), and butyryl (4:0), capronyl (10:0), heptadecanoyl (17:0), stearoyl (18:0) LPCs were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). Arachidonoyl (20:4) LPC was prepared by hydrolysis of diarachidonoyl phosphatidylcholine (PC) with bee venom phospholipase A_2, as described [22]. PC standards purchased from Sigma-Aldrich were dibutyl (4:0/4:0), dicapryl (6:0/6:0), dicapryryl (8:0/8:0), dicapronyl (10:0/10:0), dilauroyl (12:0/12:0), and dimyristoyl (14:0/14:0) PCs. Hepatadecanoyl-*sn*-glycerol-3-phosphate (17:0 LPA) and palmitoyl-*sn*-glycerol-3-phosphate (16:0 LPA) were prepared by hydrolysis of 17:0 LPC and 16:0 LPC with *Streptomyces chromofuscus* phospholipase D, respectively, as described [23].

2.2. Sample preparation

Human FF was collected from patients in *in vitro* fertilization programs, as previously described [8]. The use of human FF as clinical samples was approved by the Ethics Institutional Review Board for Clinical Research of Tokushima University and performed in accordance with the Declaration of Helsinki. The patients provided written informed consent according to the guidance of the ethical committee of University of Tokushima. They were administered follicle-stimulating hormone or human menopausal gonadotropin. Oocyte retrieval was performed 1 min later by means of ultrasonically guided follicular puncture. Fresh FF were classified into three groups (small, medium and large) on the basis of the volume, collected <2 mL, 2–8 mL and >8 mL, respectively, centrifuged at 10,000*g* for 1 min, and stored −80 °C until use. Human blood was drawn from an antecubital vein of seven healthy female volunteers (age 23 ± 0.3 years old) into two plastic tubes with or without heparin. Centrifugation of the heparinized blood at 1000 *g* for 10 min (4 °C) provided plasma samples. Serum samples were prepared from the blood without heparin treatment by centrifugation at 1000*g* for 10 min (4 °C) after incubation at room temperature for 3 h.

2.3. Molecular species analysis of LPA and LPC in fresh FF

The concentrations of individual molecular species of LPA and LPC were determined by electrospray ionization-LC–MS/MS, as previously described [24]. In brief, lipids were extracted together with known amounts of 17:0 LPC and 17:0 LPA as internal standards, from 0.1-mL aliquots of fresh and incubated human FF (diluted or undiluted) by a two step-extraction of modified from the Bligh and Dyer method. The transitions from $[M+H]^+$ to the phosphocholine cation at m/z 184 was used to identify LPC molecular species in the first organic layer, and the transitions from $[M-H]^-$ ions to the cyclic glycerophosphate anion at m/z 153 were used to identify LPA molecular species recovered in the second organic layer by LC–MS/MS. The molecular species of LPA and LPC were designated in terms of the number of carbon atoms: the number of double bonds in the fatty acyl moiety.

Conversions of 15:0 and 18:3 LPCs added to 3.3-fold diluted FF into corresponding LPAs were monitored immediately after adding the exogenous LPC and after 12 and 24 h incubations (0.15 mM, final). Lipids were extracted from the incubated FF from large human follicles by the modified method of Bligh and Dyer, and LPC and LPA in the lipid extracts were analyzed by LC–MS/MS, as described [24].

2.4. Measurement of lysoPLD activity in FF

In our standard assay without lipid extraction, lysoPLD activity was determined by measuring choline released together with LPA from 0.15 mM exogenous LPC (palmitoyl or linoleoyl) incubated at 37 °C for 24 h in undiluted and diluted FF, essentially as described previously for lysoPLD activity in human sera [25]. In brief, 0.1 mL of FF diluted 3.3-fold with saline was mixed with 0.05 mL of 0.45 mM 16:0 LPC or 18:2 LPC dispersed in saline with 0.25% fatty acid-free bovine serum albumin (BSA) (Sigma-Aldrich). LysoPLD activity against exogenous LPC was calculated by subtracting values measured in the absence of exogenous LPC from those obtained in the presence of LPC. The choline-producing activity against endogenous substrates was also measured for undiluted FF.

2.5. Degradation of LPA by cumulus cells isolated from COC

Cumulus cells were combined after denudation of five COCs collected from large human follicles, and incubated in 1.5 mL of modified human tubal fluid culture medium with 3 μM of fatty acid-free BSA and 3 μM LPA (15:0 or 18:3) for 1.5, 3 and 4.5 h. After the two-step extraction by the modified method of Bligh and Dyer, the remaining exogenous LPA in the incubation mixtures was quantified by LC–MS/MS, as described above.

2.6. Immune-precipitation of ATX

LysoPLD activity of ATX in 3.3-fold diluted human FF (0.3 mL) with saline was tested to determine whether it is immunologically precipitated by various volumes of suspension of Sepharose 4B beads coated with anti-mouse monoclonal ATX antibody (2 μg/mL), essentially as previously described for those in human sera [25]. Human FF (0.3 mL) were incubated with 0.01, 0.02, or 0.04 mL of a suspension of Sepharose 4B beads coating anti-ATX antibody at 4 °C for 2 h under shaking every 10 min. The suspension was centrifuged at 10,000*g* for 1 min (4 °C), and aliquots of the supernatant were used for measurement of remaining lysoPLD activity. The precipitated beads were washed with saline and incubated with 0.15 mL of 100 mM glycine buffer (pH 2.5) for 1 h. After adjusting the pH of the supernatant to 6–7, aliquots were used

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