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Extracellular lipid metabolism influences the survival of ovarian cancer cells

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ABSTRACT

Lysophosphatidic acid (LPA) is an extracellular lipid mediator consisting of a fatty acid and a phosphate group linked to the glycerol backbone. Here, we show that 1-oleoyl- and 1-palmitoyl-LPA, but not 1-stearoyl- or alkyl-LPA, enhance HNOA ovarian cancer cell survival. Other lysophospholipids with oleic or lauric acid, but not stearic acid, also induce the survival effects. HNOA cells have the lipase activities that cleave LPA to generate fatty acid. Oleic acid stimulates HNOA cell survival via increased glucose utilization. Our findings suggest that extracellular lysolipid metabolism might play an important role in HNOA cell growth.

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1. Introduction

Lysophosphatidic acid (LPA) is a growth factor-like phospholipid that interact with at least six types of LPA receptors (LPA_1–LPA_6) [1,2]. LPA receptor activation exerts diverse cellular effects in normal and cancer cells, which include increased cell proliferation, enhanced cell migration, actin rearrangements, and peptidergic growth factor production [3,4]. Production of extracellular LPA has been demonstrated via two independent pathways: autotaxin (ATX)-mediated conversion of lysophosphatidylcholine (LPC) and membrane-bound phosphatidic acid-preferring phospholipase A1 (mPA-PLA1)-mediated conversion of PA [5,6]. Produced LPA activates LPA receptors and then is degraded by PA phosphatase 2 to be eliminated [7]. Thus, a cycle of LPA production to degradation might control LPA-dependent cellular function, such as cell proliferation, survival, and migration.

Many ovarian cancer cell lines have been reported to show aberrant expressions of LPA receptor genes, and blood and peritoneal fluid from patients with ovarian cancer have been known to contain higher levels of LPA than those from patients without cancer [4]. We have been investigating roles of LPA receptor-mediated signaling in five distinct, human ovarian cancer cell lines. In the present study, we show that, unexpectedly, LPA metabolism to generate fatty acids is likely to be important for HNOA ovarian cell survival.

2. Materials and methods

2.1. Reagents

1-Oleoyl-LPA (18:1-LPA), 1-stearoyl-LPA (18:0-LPA), 1-oleoyl-LPC (18:1-LPC), 1-lauroyl-LPC (12;0-LPC), 1-palmitoyl-LPC (16:0-LPC), 1-stearoyl-LPC (18:0-LPC), 1-oleoyl-lysophosphatidylserine (18:1-LPS), 1-oleoyl-lysophosphatidylethanolamine (18:1-LPE), 1-palmitoyl-2-oleoyl-PA (16:0,18:1-PA), dioleoyl-PA (18:1,18:1-PA), were purchased from Avanti Polar Lipid (Alabaster, AL). 1-Palmitoyl-LPA (16:0-LPA), 1-hexadecyl-LPA (16:0-O-LPA), Ki16425 and S32826 were from Cayman Chemical Co (Ann Abor, MI). Stearic acid (18:0) was from Tokyo Chemical Industry (Tokyo, Japan). Lauric acid (12:0) and palmitic acid (16:0) were from Nacalai Tesque (Kyoto, Japan). Oleic acid (18:1) was from Sigma (St. Louis, MO). 1-Oleyl-O-LPA (18:1-O-LPA) and bromo-hydroxy-(palmitoyloxy)butylphosphonate (BrP-LPA) was from Echelon (Salt Lake City, UT). Etomoxir was from Merck (Tokyo, Japan). 2-Deoxylglucose was from Wako Pure Chemicals (Osaka, Japan). Cell counting kit-8 (CCK-8) was from Dojin Chemical (Kumamoto, Japan).

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2.2. Cell cultures

Human ovarian cancer cell lines (KF28, HAC2, RMG-1, HNOA, and HMOA) were maintained in Dulbecco's modified Eagle's medium (D-MEM; Wako Pure Chemicals) containing 10% fetal calf serum and penicillin/streptomycin (Nacalai Tesque).

2.3. RT-PCR

Total RNAs (1 μg) were prepared using Tri Reagent (Sigma), treated with RNase-free DNase, and reverse-transcribed with oligo(dT)12–18 and Superscript reverse transcriptase (all from Invitrogen, Tokyo, Japan). The resultant cDNAs, derived from 12.5 ng total RNA, were amplified by PCR using GoTaq DNA polymerase (Promega, Tokyo, Japan). The cycling protocol was 60 s at 94 °C; 35 cycles of 30 s at 94 °C, 30 s at 55 °C, and 90 s at 72 °C; and 7 min at 72 °C at the end of cycling to complete extension. The amplified products were then separated on 1.5% agarose gels containing 0.5 μg/ml ethidium bromide. The primers used (Nippon EGT, Toyama, Japan) and expected sizes are summarized in Table 1.

2.4. Cell growth assay

Cells were plated in 48-well plates at the cell density of 7500 cells/well. Next day, cells were washed once with D-MEM and further incubated in 150 μl of D-MEM for 24 h. Lipids were mixed with 1% fatty acid-free bovine serum albumin (FAFBSA, Nacalai Tesque) in PBS and added at 1/20 of volume. Inhibitors were pretreated 10 min prior to addition of lipids. For counting the number of cells, cells were incubated with 7 μl of CCK-8 for 1 h and the absorbance of culture supernatants was measured at 450 nm. Experiments were always performed in triplicates or quadruplicates and repeated at least three times. All data shown in figures are representative.

2.5. Lipid extraction from culture supernatants and thin layer chromatography (TLC) analysis

Cells were cultured in 0.3 ml of D-MEM for 24 h in a 24 well plate and then incubated with 20 μM 1-oleolyl-LPA for the indicated times. Culture supernatants were collected and centrifuged at 15,000 rpm to remove cell debris. The resultant supernatants were mixed with 10 μl of 3 M acetic acid, 0.75 ml of methanol, and 0.4 ml of chloroform. The mixture was further combined with 0.4 ml chloroform and 0.4 ml of water, and then centrifuged at 3000 rpm. The organic phase was evaporated by blowing nitrogen gas, and separated on TLC using chloroform:methanol:water (65:25:4), followed by detection under iodine exposure. In the quantitative experiments, 0.1 μM [^{3}H]-1-oleoly-LPA (0.1 μCi/well, Perkin Elmer, Tokyo, Japan) was added to HNOA cells or precleared culture supernatants. Following lipid extraction and TLC separation, radioactivities in the oleic acid fractions were counted.

2.6. Statistical analysis

Analysis of variance (ANOVA) followed by a post hoc test was applied to data to determine statistical significance using the statistical software StatView 4.5 (Abacus Concepts).

3. Results

3.1. Gene expression for LPA receptors, LPA-producing enzymes, and LPA-degrading enzymes in ovarian cancer cells

Many cancer cell lines show aberrant expressions of LPA receptor genes, depending on cell types, and activation of one or more LPA receptors leads to cell type-specific responses. We first examined the expressions of LPA receptor genes in human ovarian cancer cell lines, KF28, HAC2, RMG-1, HNOA, and HMOA. RT-PCR analyses revealed that KF28, HAC2, and HMOA cells expressed genes of all LPA receptor subtypes except for *LPAR4*, RMG-1 cells expressed *LPAR1*, *LPAR2*, *LPAR5*, and *LPAR6* genes, and HNOA cells expressed *LPAR2 LPAR5*, and *LPAR6* genes (Fig. 1A).

We next examined the gene expression of *ENPP2* encoding ATX, *LIPH* encoding mPA-PLA1α, and *LIPI* encoding mPA-PLA1β in these ovarian cancer cells. Only HMOA cells expressed *ENPP2* gene (Fig. 1). All tested cells expressed *LIPH* gene as well as a possible short splicing variant. Only HMOA cells expressed *LIPI* gene. Extracellular LPA is degraded by PA phosphatase 2a, b, and c, encoded by *PPAP2a*, *b*, and *c* genes, respectively. All genes were expressed in all tested cell types (Fig. 1A).

3.2. Effects of 1-oleoyl-LPA, 1-oleoyl-LPC, and dioleoyl-PA on HNOA cell growth

We focused on a role of LPA signaling in HNOA cell growth, because this cell type showed relatively limited expression profiles of LPA receptor genes, compared with those in other cell types. Furthermore, HNOA cells showed cell death under serum-free conditions, being contrast to other cell types that showed continued cell proliferation under the same conditions (Fig. 1B). Treatment of HNOA cells with 1-oleoly-LPA significantly inhibited cell death (Fig. 1B and C). 1-Oleoyl-LPC treatment also enhanced HNOA cell survival, while dioleoyl-PA showed no effect on cell survival.

Because no specific antagonists for LPA_2, LPA_5 or LPA_6 were commercially available, we tested BrP-LPA, a pan LPA receptor

Table 1
Primers used in this study.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Expected size (bp)
LPAR1	TCTTCTGGGCCATTTTCAAC	TGCCTGAAGGTGGCGCTCAT	349
LPAR2	CCTACCTCTTCCTCATGTTC	TAAAGGGTGGAGTCCATCAG	785
LPAR3	GGAATTGCCTCTGCAACATCT	GAGTAGATGATGGGGTTCA	382
LPAR4	TGGTGACACCCTCTGTAAGATCTC	GGAGAAGCCTTCAAAGCAAGTGG	262
LPAR5	GGTGAGCGTGTACATGTGCAA	GCTGCCGTACATGTTCATCTGG	157
LPAR6	CAGCATGGTGTTTGTGCTTGGGT	AAACGGCGGGTGCACTTCCT	457
ENPP2	TGGATTACAGCCACCAAGCAAG	GCCCCACAATTTTGTCGATTTCCC	387
LIPH	CTAGGAGCCCACATAGCTG	CTGACACACTTGCCATTCC	296
LIPI	CACGGATGCAAAGTTTGTGG	ACACAGTGGTCCTAAGAGGT	374
LYPLA1	GGCTATGCCTTCATGGTTTG	CTATTAGCACCACCGATAGGAC	285
LYPLA2	CATCAAGAAGGCAGCAGAGA	CCGGGTATGTCTTGAACTGG	347
PLB1	GTGACGGTGCTTGAGATCGT	CCTGCATGCTGTTCTTGTAGG	472
FFAR1	TGGTCTACGCCCTGAACCT	AGCCTCCAACCCAAAGACC	314
GPR84	GTTTTCAGTGCCAAGGGGATAG	CAGGCATGGCCTCATCAGTC	316
GPR119	GCTTCACCTTGAATCTGG	GCATAGATGAGTGGGTTGAG	715
GPR120	CCATTCCTGGAGAGATCTCGT	GCACAGTGTCATGTTGTAGAGG	375, 428

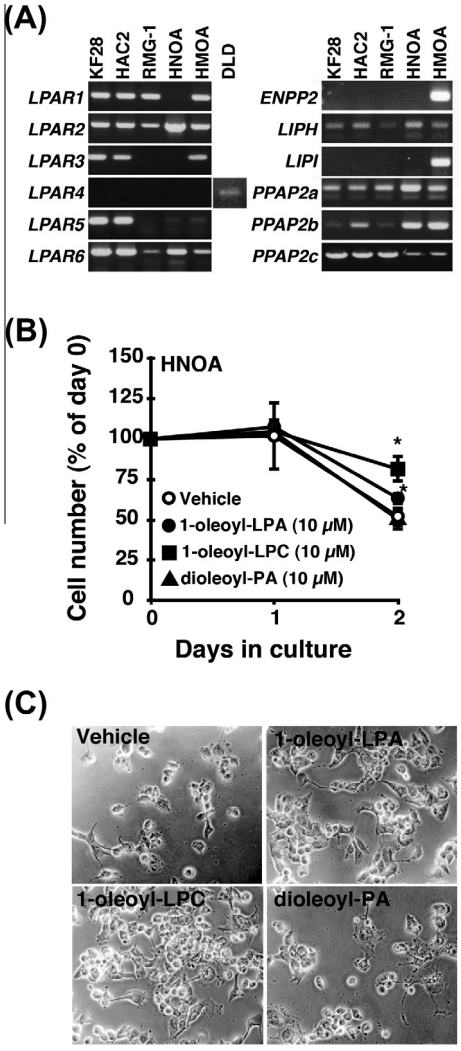

Fig. 1. 1-Oleoyl-LPA and -LPC enhances cell survival of HNOA ovarian cancer cells. (A) RT-PCR analyses for genes of LPA receptors (*LPAR1–LPAR6*), LPA-producing enzymes (*ENPP2*, *LIPH*, *LIPI*), and LPA-degrading enzymes (*PPAP2a*, *b*, *c*) in KF28, HAC2, RMG-1, HNOA, and HMOA cells, and DLD colon cancer cells (as *LPAR4* positive cells). Cells were serum-starved for 1 day and subjected to RNA extraction, followed by RT-PCR analyses. (B) Effects of 1-oleoyl-LPA, 1-oleoy-LPC, or dioleoyl-PA on HNOA cell survival. Cells were serum-starved for 1 day and further cultured without or with each lipid at 10 μM. The number of cells was determined at days 0, 1, and 2. Data were expressed as% of day 0 values and the mean ± S.D. $^{*}p < 0.05$ vs vehicle. (C) Cell morphologies cultured without or with each lipid for 2 days.

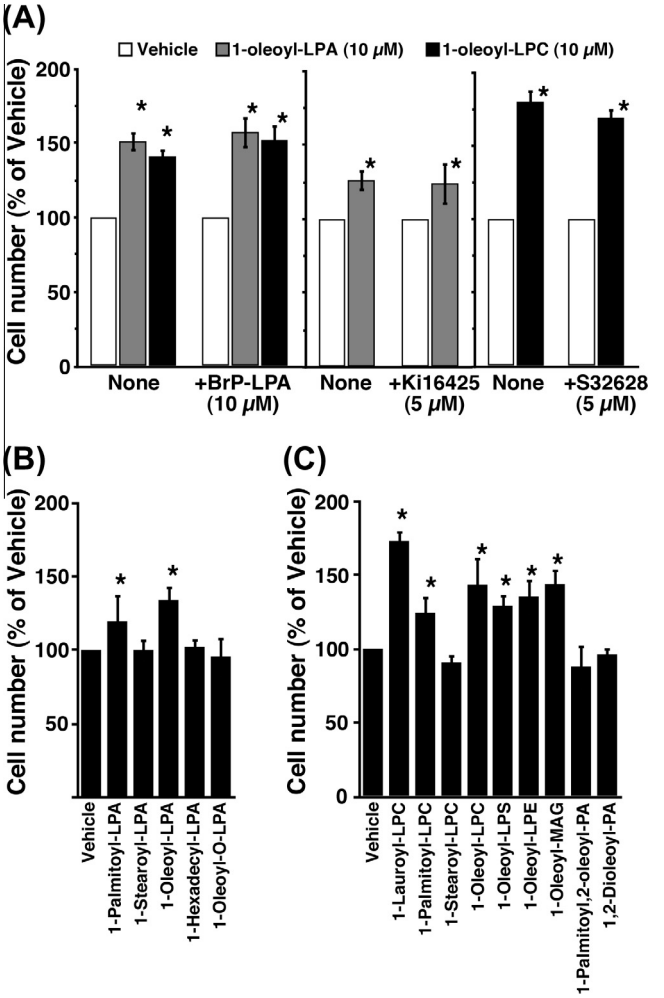

Fig. 2. Effects of 1-oleoyl-LPA or -LPC might not be mediated through LPA receptors. (A) Effects of LPA antagonists or an autotaxin inhibitor on 1-oleolyl-LPA- or -LPC-induced HNOA cell survival. Cells were pretreated with BrP-LPA, Ki16425, or S32628 for 10 min and then treated with 1-oleoyl-LPA or -LPC for 2 days. Data were expressed as% of vehicle and the mean ± S.D. $^{*}p < 0.05$ vs vehicle. (B) Effects of various species of lysophosphatidic acid on HNOA cell survival. Cells were treated with the indicated lipids at 10 μM for 2 days. Data were expressed as% of vehicle and the mean ± S.D. $^{*}p < 0.05$ vs vehicle. (C) Effects of various species of lysophospholipids, monoacylglycerol, or phosphatidic acids on HNOA cell survival. Cells were treated with the indicated lipids at 10 μM for 2 days. Data were expressed as% of vehicle and the mean ± S.D. $^{*}p < 0.05$ vs vehicle.

antagonist. The effects of 1-oleoyl-LPA or -LPC on HNOA cell survival were not inhibited by pretreatment with BrP-LPA (Fig. 2A). Ki16425, an $LPA_{1/3}$ antagonist, expectedly failed to inhibit the effects of both lysolipids (Fig. 2A). S32628, an ATX inhibitor, also failed to inhibit the survival effects of 1-oleoyl-LPC (Fig. 2A). To further explore the involvement of LPA receptors in LPA-induced cell survival, the effects of other LPA species having distinct acyl chain on HNOA cell survival were examined. For this purpose, we employed 1-palmitoyl-LPA, 1-stearoyl-LPA, 1-hexadecyl-LPA, and 1-oleoyl-O-LPA, all of which have been shown to activate LPA receptors, including LPA_{1-6}. However, no significant survival effects were observed for these LPA species except for 1-palmitoyl-LPA (Fig. 2B). In addition, 1-lauroyl-LPC, 1-palmitoyl-LPC, 1-oleoly-LPS, and 1-oleoly-LPE as well as 1-oleoyl-glycerol significantly enhanced HNOA cell survival (Fig. 2C). On the other hand, 1-palmitoly, 2-oleoyl-PA showed no survival effect. These results suggested that there might be non-LPA receptor mechanisms accounting for LPA- and LPC-induced effects on cell survival in HNOA.

3.3. Conversion of LPA to fatty acid in HNOA cell cultures

Oleic acid is known to be an extracellular mediator as well as energy source, and affect cell growth in many cell types, including cancer cells [8,9]. Therefore, we addressed the possibility that 1-oleoyl-LPA was cleaved to oleic acid, leading to inhibition of cell death. When 1-oleoyl-LPA was added to HNOA cell cultures, production of oleic acid was observed (Fig. 3A). Quantitative analyses using [^{3}H]-1-oleoyl-LPA revealed that more than 87% of [^{3}H]-1-oleoyl-LPA was cleaved at 1 h in HNOA cells (Fig. 3B). This conversion to oleic acid was not detected in HONA cell culture supernatant, indicating that the lipase activity was not released from cells. The well-known enzymes having lysophospholipase activities include lysophospholipase A1 (lysoPLA1), lysoPLA2, and

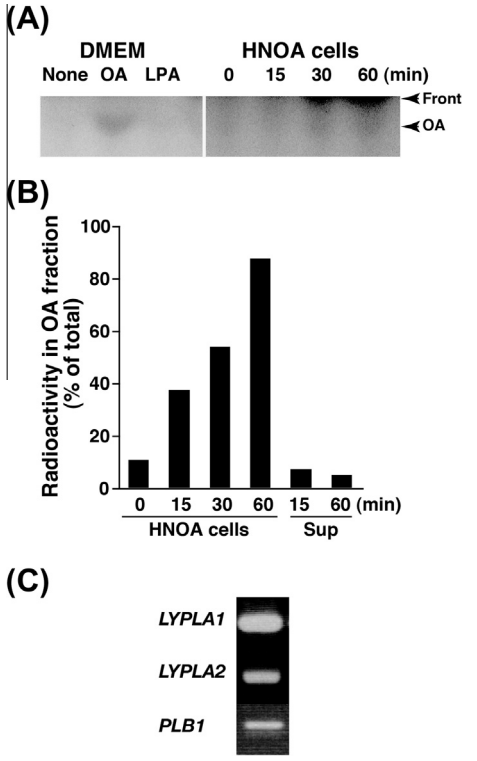

Fig. 3. HNOA cells produce functional lysolipase activities that cleave 1-oleoyl-lysophosphatidic acid to oleic acid. (A and B) Thin layer chromatography analyses for oleic acid produced from 1-oleoyl-LPA in HNOA cells. (A) 1-oleoyl-LPA (20 μM) was added to serum-starved HNOA cell cultures for the indicated times, followed by lipid extraction and TLC analyses. Then, oleic acid (OA) was visualized under iodine vapor. (B) [^{3}H]-1-oleoyl-LPA (0.1 μM) was added to serum-starved HNOA cell cultures or culture supernatants (Sup) for the indicated times, and radioactivities in oleic acid fraction on TLC was measured. (C) RT-PCR analyses for *LYPLA1*, *LYPLA2* or *PLB1* gene in HNOA cells. Cells were serum-starved for 1 day and subjected to RNA extraction, followed by RT-PCR analyses.

phospholipase B1 (PLB1) [10,11]. Therefore, the gene expression for these enzymes were examined in HNOA cells. *LYPLA1* encoding lysoPLA1, *LYPLA2* encoding lysoPLA2, and *PLB1* were expressed in HNOA cells (Fig. 3C).

3.4. Effects of various fatty acids on HNOA cell survival

Because 1-oleoyl-LPA conversion to oleic acid was observed in HNOA cells, we next tested various fatty acids on HNOA cell survival. Oleic acid showed enhanced cell survival as well as 1-oleoyl-LPA did (Fig. 4A and B). Lauric and palmitic acid also significantly stimulated cell survival. By contrast, stearic acid showed no significant effects on HNOA cell survival. Species for active fatty acids were quite consistent with those for acyl chains of active lysophospholipids shown in Fig. 2B and C, indicating that fatty acids cleaved from LPA- or LPC might account for lysolipids-induced survival effects in HNOA cells.

Fatty acids are known to be an energy source by generating ATP via β-oxidation after fatty acid incorporation into a cell. However, this would be not the case, because stearic acid was ineffective. In addition, etomoxir, an inhibitor for carnitine palmitoyltransferase that is required for fatty acid incorporation into mitochondria [12], showed no effect on oleic acid- or lauric acid-induced HNOA cell survival (Fig. 4C). On the other hand, the effects of oleic or lauric acid were blocked by co-treatment with 2-deoxyglucose, a glycolysis inhibitor, indicating that these fatty acids enhanced glucose utilization (Fig. 4C).

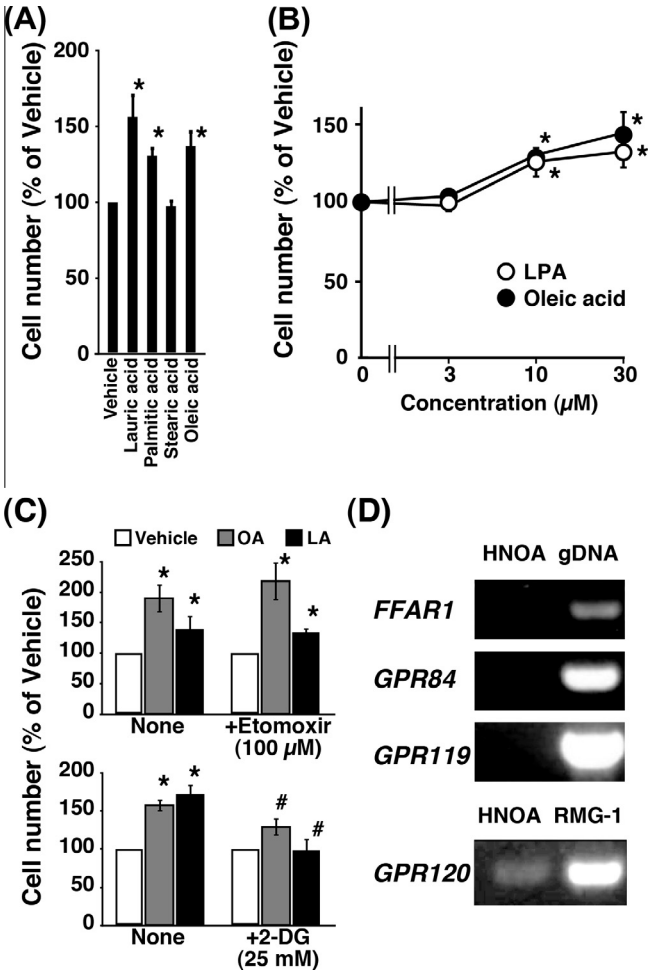

Fig. 4. Lauric, palmitic, and oleic, but not stearic acid were effective for HNOA cell survival. (A) Effects of various fatty acids on HNOA cell survival. Cells were serum-starved for 1 day and further cultured without or with lipids at 10 μM for 2 days. Data were expressed as% of vehicle and the mean ± S.D. $^*p < 0.05$ vs vehicle. (B) Effects of varying concentrations of oleic acid or 1-oleoyl-lysophosphatidic acid on HNOA cell survival. Cells were serum-starved for 1 day and further cultured with lipids at the indicated concentrations for 2 days. Data were expressed as% of vehicle and the mean ± S.D. $^*p < 0.05$ vs vehicle. (C) Effects of etomoxir or 2-deoxylglucose on fatty acid-induced HNOA cell survival. Cells were pretreated with etomoxir or 2-deoxyglucose (2-DG) for 10 min and then treated with oleic (OA) or lauric acid (LA) at 10 μM for 2 days. Etomoxir and 2-DG treatment showed no marked influence on HNOA cell survival (101.4% and 118.8% of vehicle in None treatment, respectively). Data were expressed as% of vehicle and the mean ± S.D. $^*p < 0.05$ vs vehicle. $^\#p < 0.05$ vs OA or LA in None treatment. (D) RT-PCR analyses for fatty acid receptor genes (*FFAR1*, *GPR84*, *GPR119*, *GPR120*) in HNOA cells. Cells were serum-starved for 1 day and subjected to RNA extraction, followed by RT-PCR analyses. Genomic DNA (gDNA) was used as PCR references.

Recent studies have identified G protein-coupled membrane receptors for short to long chain fatty acids. These include FFAR1, FFAR2, FFAR3, GPR84, GPR119, and GPR120 [13]. Because FFAR1, GPR84, GPR119, and GPR120 are known to bind medium and long chain fatty acids, we examined whether HNOA cells expressed the genes for these receptors. RT-PCR analyses revealed that HNOA cells expressed no FFAR1, GPR84, and GPR119, but expressed short form of GPR120 (Fig. 4D).

4. Discussion

In the present study, we have found that five different types of human ovarian cancer cells show diverse expression profiles of LPA signaling-related genes, which include LPA-producing enzymes, LPA receptors, and LPA-degrading enzymes. However, LPA

signaling might not play an important role in cell survival of HNOA cells. Rather, fatty acids cleaved from LPA by lysophospholipase activities seem to be involved in HNOA cell survival. In addition to lysophospholipids, monoacylglycerol also showed the survival effects. Thus, it is also possible that lysophospholipids are first hydrolyzed by PPAP2 expressed in HNOA cells, then the resultant monoacylglycerols are hydrolyzed by lysolipase. In any case, these findings provide a new view that lysophospholipase or lysolipase-mediated cleavage of lysolipids to fatty acids would not be a simple mechanism that digests lysolipids to terminate signaling, but a novel, extracellular lipid signaling machinery that regulates cellular functions, as proposed for ATX-mediated LPA signaling [5]. In future, direct evidence would be needed to show that LPA-derived fatty acids are of biologically and/or pathophysiologically relevant. Furthermore, the possibility that LPA_2 or LPA_6-mediated signaling is involved in HNOA cell survival cannot be excluded. LPA receptor gene knockout or knockdown experiments would also be needed.

The molecular identification of lysophospholipase or lysolipase activity that cleaves LPA to liberate fatty acid remains to be done. LYPLA1 and LYPLA2 are cytosolic enzymes, whereas PLB1 is a membrane-spanning protein and its catalytic domain exists in the extracellular region [10,14]. Thus, one strong candidate accounting for cleavage of LPA would be PLB1. However, PLB1 has been reported to have PLA_2 activities toward dioleoyl-PA using micelle preparation [11]. On the other hand, in our experiment, we used dioleoyl-PA suspended in FAFBSA-containing PBS, and observed no survival effects. Thus, the difference in PA preparation might be the reason why we did not observe no cleavage of dioleoyl-PA. This question should be further explored.

Fatty acids serve as substrates for β-oxidation generating ATP, substrates for cell membrane lipids, and extracellular mediators to activate their cognate G protein-coupled receptors. Although we have not yet fully addressed the cellular mechanisms for fatty acid-induced cell survival, our results indicate that fatty acids stimulates glucose utilization. Thus, fatty acids are likely to enhance glucose uptake or glycolysis. Such signaling might be mediated through GPR120 expressed in HNOA cells, because this receptor has been shown to enhance glucose uptake in 3T3-L1 adipocytes [15]. Furthermore, GPR120 is also demonstrated to mediate long chain fatty acid-dependent cell survival [16]. Our findings indicate that fatty acids are effective at the concentrations of 10–30 μM, which are considered to be physiological concentrations sufficient for activating fatty acid receptors. We are currently investigating the involvement of GPR120 in fatty acid-mediated HNOA cell survival as well as the intracellular signaling pathway.

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