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Short communication

CYCLIC PHOSPHATIDIC ACID INDUCES G_0/G_1 ARREST, INHIBITS AKT PHOSPHORYLATION, AND DOWNREGULATES CYCLIN D1 EXPRESSION IN COLORECTAL CANCER CELLS

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Abstract: Lysophosphatidic acid (LPA) and its analogs are well-known mitogens for various cell types. Many reports have confirmed that several types of cancer cell produce LPA to promote survival, growth and tumorigenesis. This indicates that the interface between the LPA signaling pathway and the cell cycle signaling system is critical to the control of cancer cell proliferation. However, our previous study indicated that cyclic phosphatidic acid (cPA), which is structurally similar to LPA, inhibits the proliferation and migration of colon cancer cells. It has been reported that cPA shows several biological activities not shown by LPA. However, understanding of the detailed molecular and cellular mechanism underlying the regulation of the cell cycle by cPA is still in its infancy. In this study, we investigated the effect of cPA treatment on human DLD-1 colon cancer cells by analyzing cell cycle dynamics, gene expression, and AKT phosphorylation. Our findings indicate that cPA inhibits cell cycle progression in DLD-1 colon cancer cells via the downregulation of cyclin D1 and the inhibition of AKT phosphorylation.

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Abbreviations used: AGP – alkyl-glycerophosphate; cPA – cyclic phosphatidic acid; DMEM – Dulbecco's modified Eagle's medium; FBS – fetal bovine serum; LPA – lysophosphatidic acid; PI3K – phosphoinositide 3 kinase; SDS – sodium dodecyl sulfate; siRNA – small-interfering RNA

Keywords: Cyclic phosphatidic acid, Lysophosphatidic acid, Alkyl-glycerophosphate, Colon cancer cells, Cell cycle analysis, Cell proliferation, Cyclin D1, Akt phosphorylation, siRNA, Cancer treatment

INTRODUCTION

The cell cycle is the mechanism by which cells divide. It is driven by a family of proteins called cyclin-dependent kinases (CDKs) [1, 2], which are positively regulated by cyclin A, B, D, and E [3]. Overexpression of cyclin D1 is most frequently associated with human malignancy [4]. The gene was originally cloned by three independent groups (as *CCND1* or *PRAD1*) [5–7] and the *PRAD1* gene product was recognized to have a domain of structural similarity to known cyclins [8]. Cyclin D1 is a critical cell cycle regulating protein that plays a role in cell proliferation and cell cycle progression [9]. Cyclin D1 protein overexpression is observed in human colorectal cancer and adenomatous polyps of the colon [10, 11].

Lysophosphatidic acid (LPA) is a well-known mitogen for various cell types, and multiple reports have confirmed that several types of cancer cell produce LPA to promote survival, growth and tumorigenesis. Interestingly, although cyclic phosphatidic acid (cPA) is structurally close to LPA, it has different biological activities [12–14]. It has been shown to directly inhibit Cdc25 phosphatase, thereby suppressing cell cycle progression at G_1 [15]. Similarly, we previously showed that cPA inhibits proliferation in DLD-1 and HT-29 colon cancer cells [16]. However, the mechanisms underlying the inhibition of cell proliferation and cell cycle progression by cPA have yet to be elucidated.

LPA increased the phosphorylation and activation of the mitogen-activated tyrosine kinase phosphoinositide 3 kinase (PI3K) [17, 18]. PI3K/AKT plays a major role in LPA-induced colon cancer cell migration [19]. Inhibitors of the PI3K/AKT pathway are attractive candidates for cancer drug development, but the clinical efficacy of PI3K inhibitors against various cancers has been moderate.

In this study, we investigated the effect of cPA treatment on cyclin D1 expression and AKT phosphorylation in human DLD-1 colon cancer cells. Our results indicate that cPA inhibited DLD-1 cell cycle arrest via the downregulation of cyclin D1 expression and the inhibition of AKT phosphorylation.

MATERIALS AND METHODS

Materials

A mouse monoclonal anti-β-actin antibody (sc-47778) was purchased from Santa Cruz Biotechnology Inc. A cyclin D1 antibody (#2922) was purchased from Cell Signaling Technology. Cyclic phosphatidic acid (cPA; 18:1) and 2-carba-cPA (2ccPA; 18:1) were donated by Dr. Kimiko Murakami-Murofushi (Ochanomizu University in Tokyo). LPA (18:1) and alky-glycerophosphate (AGP; 18:1) were purchased from Avanti Polar Lipids. Fluorouracil (5-FU) was purchased from Sigma-Aldrich.

Cells and cultures

DLD-1 human colon adenocarcinoma cells were obtained from the Health Science Research Resources Bank in Osaka. Cells were grown in Dulbecco's modified Eagle's medium (Nacalai Tesuque) containing 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin, and 10 μg/ml streptomycin at 37ºC in a humidified incubator with 5% CO_2.

Cell synchronization

DLD-1 cells were synchronized in the G_0/G_1 phase of the cell cycle via serum starvation. Briefly, exponentially grown cells were cultured in serum-free medium for 24 h, followed by the addition of 10% charcoal-stripped FBS (Life Technologies) to stimulate cell proliferation.

Cell cycle analysis

Cell cycle analysis was performed with a MUSE Cell Analyzer from Millipore according to the manufacturer's instructions. Briefly, after a 12-h treatment, DLD-1 cells were harvested, centrifuged at 300 × g for 5 min, washed once with PBS, and then fixed in 70% ethanol overnight at −20ºC. Next, the cells were centrifuged at 300 × g for 5 min and washed once with PBS. Then, 200 μl of MUSE Cell Cycle reagent (MCH100106) was added to each tube. These were then incubated in the dark for 30 min at room temperature before analysis.

Cellular assay for PI3K/AKT pathway

PI3K/AKT phosphorylation analysis was performed using the MUSE PI3K Activation Dual Detection Kit (Millipore) according to the manufacturer's instructions. Briefly, treated or untreated DLD-1 cells were harvested, centrifuged at 300 × g for 5 min, and then washed once with PBS. After the cells were fixed in a fixation buffer for 5 min on ice, they were centrifuged at 300 × g for 5 min, washed once with phosphate-buffered saline, and incubated in a permeabilization buffer for 5 min. Then, 10 μl of an antibody cocktail (anti-phospho-AKT [Ser473] and anti-AKT/PKB, PECy5) and 90 μl of a 1× assay buffer were added to each tube. These were then incubated in the dark for 30 min at room temperature prior to analysis.

Western blotting

The NE-PER Cell Fractionation Kit (Pierce Biotechnology) was used to isolate the cytosolic and nuclear fraction from DLD-1 cells (1×10^5 cells/well), according to the manufacturer's instructions. After the cytoplasmic fraction was separated, the nuclear fraction was subjected to brief centrifugation and the interface was removed to reduce cytoplasmic contamination. The resulting cytosolic and nuclear extracts were assayed for protein content with the Bradford method using the Bio-Rad Protein Assay kit (Bio-Rad Laboratories). The extracts were then separated on 5–20% SDS-PAGE (e-PAGEL; ATTO) and electrotransferred to Immobilon-P membranes (Millipore). The membranes were blocked in 4% (w/v) Block Ace (DS Parma Biomedical Co. Ltd.) for 1 h and

then incubated with a primary antibody in Tris-buffered saline-Tween 20 (TBS-T) for 12 h at 4ºC. After washing, the Immobilon-P membrane was incubated with a horseradish peroxidase-linked species-specific whole secondary antibody (anti-rabbit or anti-mouse IgG; GE Healthcare) for 1 h at room temperature, and then visualized with EzWestLumi plus (ATTO).

Quantitative real-time PCR

Total RNA was prepared from DLD-1 cells using NucleoSpin RNA II (Takara). Then, 0.5 μg of total RNA was used for the subsequent synthesis of cDNA using the ReverTra Ace qPCR RT Kit (Toyobo) as recommended by the manufacturer. mRNA levels were quantified by using an ECO Real-Time PCR system (Illumina, Inc.) and the SYBR Green Real-time PCR Master Mix Plus (Toyobo) with the following primers: cyclin D1, 5'-GCTGCGAAGTGGAAACCATC-3' (F) and 5'-CCTCCTTCTGCACACATTTGAA-3' (R), 18S rRNA, 5'-CAGCCACCCGAGATTGAGCA- 3' (F) and 5'-TAGTAGCGACGGGC GGTGTG- 3' (R). All of the PCR was performed in 10-μl volumes using 48-well PCR plates (Illumina). The cycling conditions were 95ºC for 1 min (polymerase activation) followed by 40 cycles of 95ºC for 15 sec, 55ºC for 15 sec, and 72ºC for 30 sec. The relative mRNA quantification was calculated using the arithmetic formula $2^{-\Delta\Delta Cq}$, where ΔCq is the difference between the threshold cycle of a given target cDNA and an endogenous reference cDNA.

RNA interference

We suppressed AKT expression in DLD-1 cells by transfecting the cells with small interfering RNAs (siRNAs) targeting AKT (Santa Cruz Biotechnology). Lipofectamine RNAiMAX (Invitrogen) was used for transfections. Cells were plated in 6-well plates (Iwaki) at a density of 5×10^4 cells/well in DMEM containing 10% FBS and then transfected with 100 pmol/ml of mRNA-specific siRNAs or scrambled (control) siRNAs. Reduction of the AKT levels was confirmed by western blotting.

Statistical analysis

Student's *t*-test was applied for statistical comparisons. Differences were considered significant when p was less than 0.05.

RESULTS AND DISCUSSION

LPA is a potent regulator of normal and cancer cell proliferation and a major signal transducer for LPA-induced cell migration [17]. Although cPA, which can be generated by phospholipase D2 [20], is similar in structure to LPA, it has different biological activities. It has been reported that cPA attenuates cancer cell invasion. A metabolically stabilized derivative of cPA was shown to suppress cancer cell metastasis [15, 21]. Moreover, our previous study showed that cPA inhibited cell growth in colon cancer cells [16]. A previous study of LPA-treated cells showed a dose-dependent increase in the S phase population,

accompanied by an increase in the G_0/G_1 population [22]. Therefore, to examine the effects of cPA on the cell cycle more closely, DLD-1 cells were exposed to cPA (20 μM) for 24 h in a medium with 10% charcoal-stripped FBS and then analyzed for cell cycle distribution using flow cytometry.

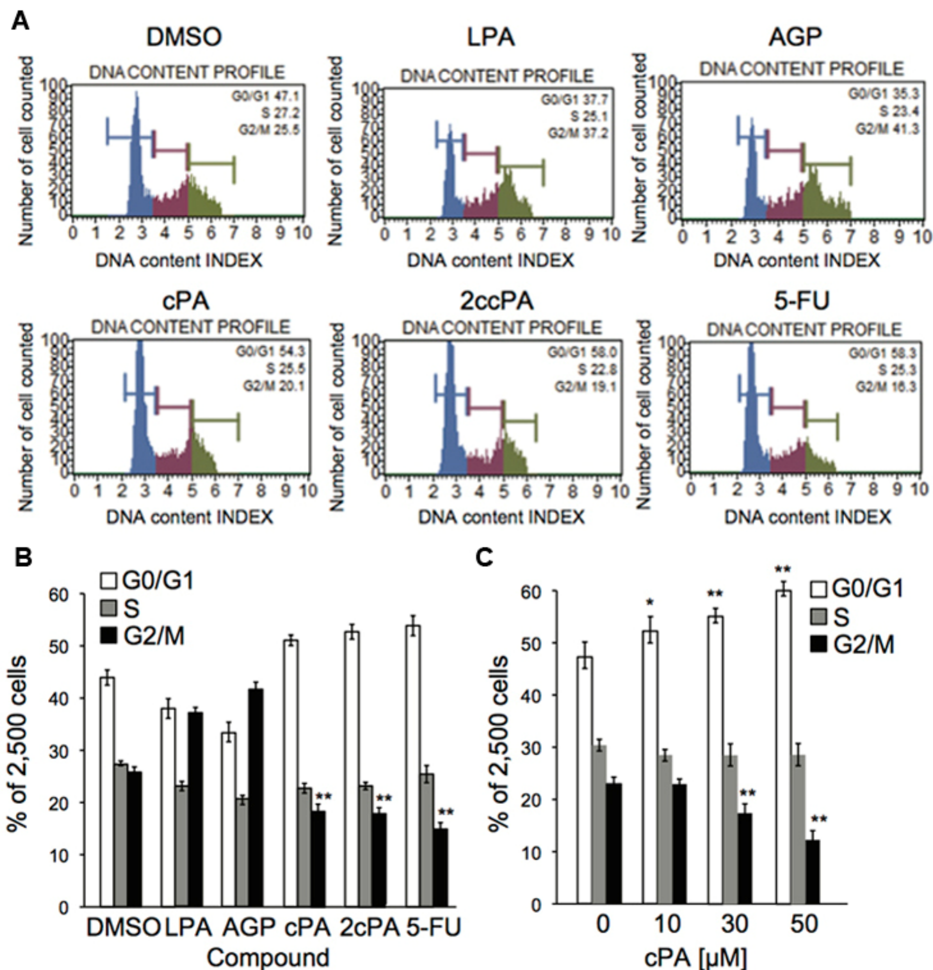

Fig. 1. Effects of LPA, AGP, cPA, 2ccPA and 5-FU on cell cycle distribution in DLD-1 cells. A – Cell cycle analysis of control (DMSO) and DLD-1 cells treated with 20 μM LPA, AGP, cPA, 2ccPA or 5-FU for 48 h was performed via flow cytometry based on propidium iodide intercalation into the cellular chromatin (for details see the Materials and Methods section). The percentages of cells in (1) G_0/G_1, (2) S phase, and (3) G_2/M phase are shown. B – Cell cycle distribution after treatment with different compounds (20 μM each) for 48 h. Data are presented as means ± SEM (n = 3), $^{**}p < 0.01$. C – Cell cycle arrest at the G_0/G_1 phase in DLD-1 cells was induced by cPA for 48 h in a dose-dependent manner (0, 10, 30, and 50 μM). Data are presented as means ± SEM (n = 3), $^{*}p < 0.05$, $^{**}p < 0.01$.

The cell cycle represents one of the most critical and fundamental processes in mammalian cells. Recently, cell cycle analysis has become increasingly important in understanding the action of anti-cancer drugs.
As shown in Fig. 1A, the number of cells in G_0/G_1 significantly arrested after exposure to cPA, 2-carba-cPA (2ccPA), or 5-FU (used as a positive control). By contrast, compared to the control cells, approximately 35% of DLD-1 cells treated with LPA or alkyl-LPA (AGP) were in G_0/G_1 phase (47% at DMSO control) and the number of cells in S phase was not altered significantly. In addition, the proportion of cells in G_2/M phase significantly increased after exposure to LPA or AGP (Fig. 1B). We also found that cPA mediated cell cycle arrest at the G_2/M phase in a dose-dependent manner (Fig. 1C). These results demonstrate that cPA arrests DLD-1 cells in the G_0/G_1 phases of the cell cycle.

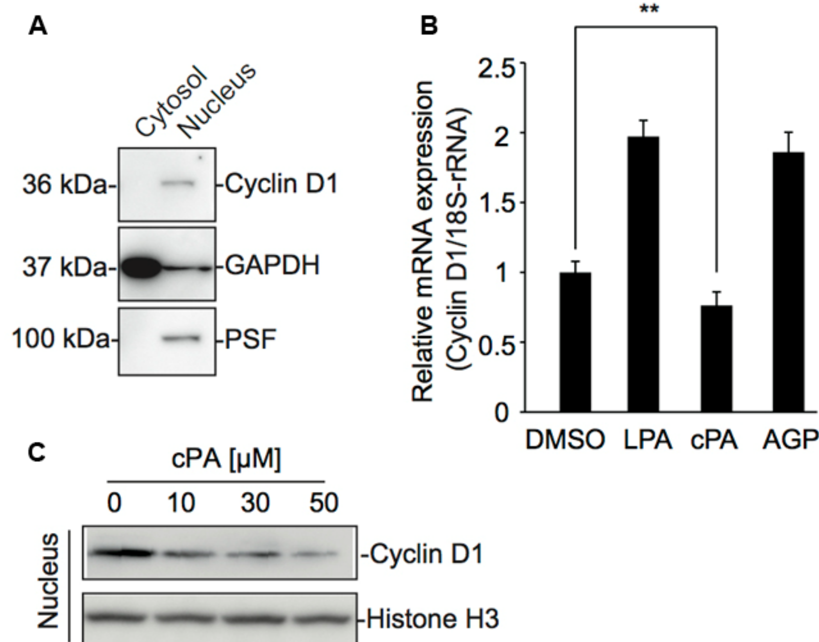

Fig. 2. cPA decreased cyclin D1 expression. A – Verification of the localization of cyclin D1 in nuclear and cytosolic extracts from DLD-1 cells. Cytosolic extracts and nuclear extracts were prepared from cells and analyzed by immunoblotting using an antibody against human cyclin D1. GAPDH was used as a loading control for the cytoplasmic fraction and PTB-associated splicing factor (PSF) was used as a loading control for the nuclear fraction. B – cPA downregulates cyclin D1 mRNA expression. Real-time PCR quantification of the expression of cyclin D1 mRNA and 18S rRNA (internal control) after treatment of DLD-1 cells with 20 μM LPA, AGP or cPA for 24 h. The mRNA levels were normalized to those of 18S rRNA (means ± SEM; n = 3; $^{**}p < 0.01$). C – cPA decreases nuclear cyclin D1 protein expression in a dose-dependent manner. Protein levels were analyzed using SDS-PAGE and western blot and visualized with enhanced chemiluminescence reagent. Each lane was loaded with 20 μg cytosolic extracts or nuclear extracts. Histone H3 was used as a loading control for the nuclear fraction.

Cyclin D1 is a key cell cycle regulatory protein, and its expression and subcellular localization is often altered in colon cancer cells [23–25]. Therefore, we further investigated cyclin D1 protein expression in DLD-1 cells. As shown in Fig. 2A, in DLD-1 cells, cyclin D1 localized predominantly within the nuclear pellet. We then examined the effect of cPA on cyclin D1 expression and found that cPA downregulates cyclin D1 mRNA and protein expression (Fig. 2B and C). Our findings also indicate that LPA or AGP induced cyclin D1 mRNA expression. Hu et al. reported that LPA treatment increased cyclin D1 mRNA and protein in a dose- and time-dependent manner in ovarian carcinoma cells [26]. They also demonstrated that LPA specifically stimulates cyclin D1 promoter activity. cPA is a structural analog of LPA or AGP. Nonetheless, many of its effects on cells are the opposite to those of its non-cyclic cousin, including inhibition of cancer cell growth and invasion [27]. Thus, our results suggest that cPA may inhibit cell cycle progression in DLD-1 cells via the downregulation of cyclin D1.

It has been reported that the PI3K/AKT pathway is required for the G_1-to-S phase cell cycle progression stimulated by LPA, and it is implicated in the activation of several downstream effectors [19]. Thus, we investigated whether the PI3K/AKT pathway would be affected by cPA. DLD-1 cells were cultured for 24 h with either LPA or cPA. As shown in Fig. 3A and B, the phosphorylation was significantly high in the non-treated DLD-1 cells. Interestingly, LPA treatment significantly increased AKT phosphorylation in DLD-1 cells (data not shown), whereas cPA treatment reduced AKT phosphorylation. As positive controls, we also used wortmannin (1 μM) or LY294002 (50 μM), two potent and structurally different inhibitors of PI3K. Both significantly inhibited AKT phosphorylation. As shown in Fig. 3C and D, reduction of AKT expression by transfection of cells with AKT siRNA had no significant effect on DLD-1 cell proliferation, suggesting that the remaining AKT activity is high enough to maintain proliferation of the cells. However, 24-h treatment with cPA significantly inhibited DLD-1 cell proliferation in AKT siRNA-transfected cells compared to the effect on control siRNA-transfected cells. This may be due to cPA treatment having decreased the level of AKT activity below the threshold for maintaining normal cell proliferation under these conditions.

Our results suggest that cPA may block cell cycle progression in DLD-1 cells by inhibiting AKT activity. In summary, this study provides evidence that cPA-mediated DLD-1 cell cycle arrest may occur via the downregulation of cyclin D1 expression and the inhibition of PI3K activation. Inhibitors of the PI3K/AKT pathway are attractive candidates for cancer drug development [28]. In addition, knowledge of the effect of cPA on cyclin D1 may lead to the use of cPA in colon cancer therapy. Further studies focusing on the molecular target(s) and the mechanisms underlying the inhibition of cell cycle regulation and proliferation in colon cancer cells by cPA will increase the feasibility of using cPA for the treatment of cancer.

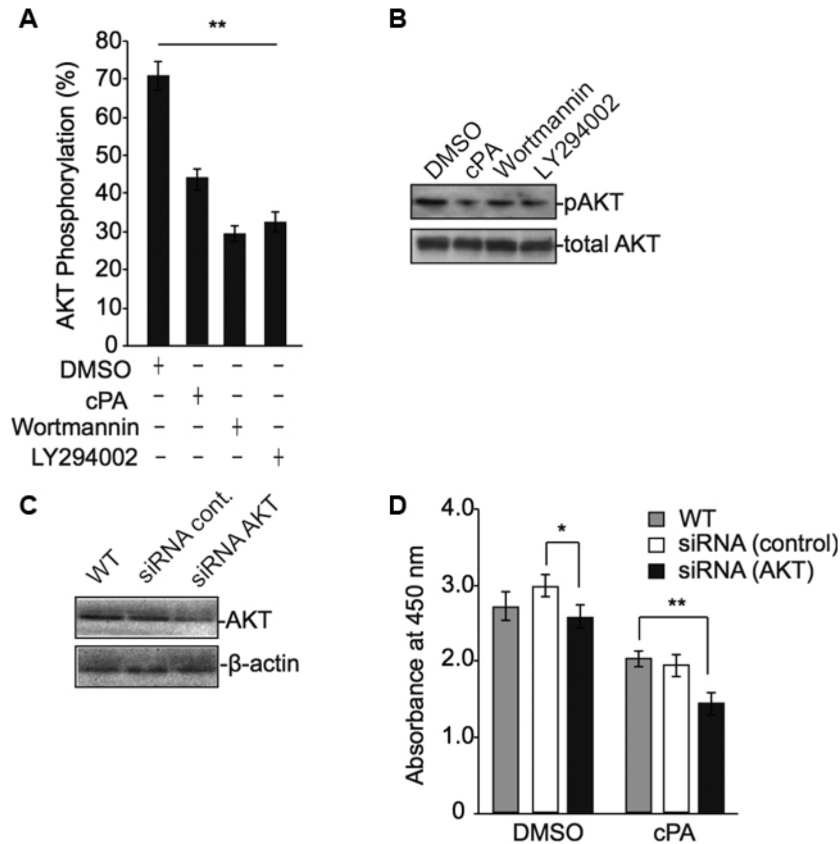

Figure 3. cPA inhibits AKT phosphorylation. A – AKT phosphorylation analysis was performed using a MUSE PI3K Activation Dual Detection Kit according to the manufacturer's instructions. Wortmannin (1 μM) and LY294002 (50 μM) were used as positive controls. Data are presented as means ± SEM (n = 3), **p < 0.01. B – Phosphorylated AKT (pAKT) and total AKT were analyzed via western blotting with an anti-AKT and anti-pAKT antibody. C – Genetic suppression of AKT by AKT siRNA transfection. D – Inhibition of cell growth by cPA in control and AKT siRNA-transfected DLD-1 cells. Cell growth inhibition was measured using a Cell Counting Kit-8 at 24 h after siRNA transfection. Data are presented as means ± SEM (n = 3), *p < 0.05, **p < 0.01.

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