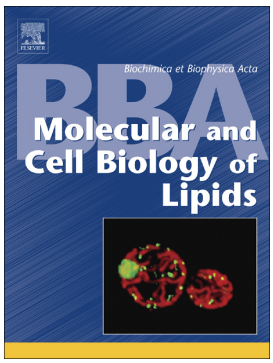

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Title

Palmitoyl lactic acid induces adipogenesis and a brown fat-like phenotype in 3T3-L1 preadipocytes

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Running title: Palmitoyl lactic acid induces **adipogenesis**

Keywords: krill oil; beige; adipogenesis; PPARγ agonist; PRDM16; palmitoyl lactic acid;

Abstract

Brown adipose tissue is specialized to generate heat by dissipating chemical energy and may provide novel strategies for the defense against human obesity. Recent advances have been made in the understanding of pharmacological and dietary agents that contribute to the browning of white adipose tissue to alleviate obesity by promoting energy expenditure. Krill oil is widely used as a human health supplement. In this study, we screened components from krill oil that promote adipogenesis of 3T3-L1 cells identified palmitoyl lactic acid (PLA). The PLA-induced adipocytes were filled with a large number of small lipid droplets. Moreover, as with the peroxisome proliferator-activated receptor (PPAR)γ agonists pioglitazone and rosiglitazone, PLA significantly enhanced adipogenesis in the presence of dexamethasone, compared with treatment with PLA alone. Treatment of PLA causes a brown fat-like phenotype in 3T3-L1 cells via enhanced expression of various brown/beige cells-specific genes, such

as PR domain containing 16 (*Prdm16*) and peroxisome proliferative activated receptor, gamma, coactivator 1 alpha (*Pgc1a*), as well as adiponectin gene. The expression profile of the brown/beige cell-specific genes induced by PLA was similar to that by the PPARγ agonist in 3T3-L1 cells. Our findings suggest that PLA induces the brown fat-like phenotype and thus likely has therapeutic potential for the treatment of obesity.

1. Introduction

Obesity is an important risk factor for cardiovascular and kidney diseases, diabetes, some cancers, and musculoskeletal disorders [1-5] and has emerged as a leading cause of staggering health care expenses along with the expanding population of chronically ill people [1,2]. One of the major causes of obesity is the accumulation of excessive calories as fat mainly due to the loss of balance between caloric intake and expenditure. In addition to lifestyle modification to reduce caloric intake and/or increase caloric expenditure by exercise, it is highly desired to develop new therapies for obesity.

An adipocyte is an endocrine cell that secretes a number of mediators, including metabolites, cytokines, lipids, and coagulation factors [6]. In general, adipocytes in mammals are roughly divided into two types: white and brown [7,8]. Brown adipocytes express constitutively high levels of thermogenic genes and contain a

large amount of uncoupling protein-1 (UCP1), which plays an important role in thermogenesis [2,7]. Therefore, enhancement of thermogenesis in brown adipocytes is considered to be an attractive target for anti-obesity therapy [2]. It was revealed that white adipose tissue harbors brown-like adipocytes [7,8], which are referred to as "beige" or "brite" (from brown-in-white) adipocytes and are characterized by uncoupled respiration [7-9]. The beige/brite adipocytes in mice have similar molecular characteristics to the brown adipocytes of adult humans, such as increased expression of specific genes, which include tumor necrosis factor receptor superfamily member 9 (*Tnfrsf9/CD137*), transmembrane protein 26 (*Tmem26*), and T-box 1 (*TBX1*); however, the origin of beige/brite adipocytes remains unclear [2, 8-10]. One possibility is white-to-brown transdifferentiation, in which "britening" occurs through a direct interconversion of a white adipocyte into a brown adipocyte phenotype [8]. Another possibility is that beige/brite adipocytes are recruited via the de novo differentiation of preadipocytes [11]. An increase in the abundance of beige/brite adipocytes in white adipose tissue (browning) has been observed in tissue in response to low temperatures. Notably, such increases have also been observed in white adipose tissue stimulated by pharmacological and dietary agents [10,11]. For example, capsaicin, berberine, irisin, monoterpene limonene, curcumin, and chrysin have been reported as natural products

that promote browning [9,12-17]. The activities of beige/brite adipocytes were shown to reduce metabolic disease in mice and have been correlated with leanness in humans [2]. These lines of evidence have raised the possibility of the discovery of new compounds that promote browning. Studies to elucidate the molecular mechanism underlying the ability of such compounds, in addition to known agents, to induce browning may lead to new therapies for metabolic diseases, including obesity.

Krill oil, extracted from the Antarctic krill *Euphasia superba*, a shrimp-like zooplankton, represents a valuable source of omega-3 polyunsaturated fatty acids [18], in amounts similar to those present in fish oil. Krill oil is widely used as a human health supplement. Bunea *et al.* reported that krill oil was significantly more effective than fish oil for the reduction of glucose, triglycerides, and low-density lipoprotein levels in humans [19]. Berge *et al.* showed that dietary supplementation with a krill powder persistently ameliorated high triglycerides without weight loss in obese men to an extent similar to that observed following lifestyle-induced weight loss in a previous study of dyslipidemic overweight/obese men [20]. Moreover, Li *et al.* demonstrated that krill oil reduced plasma cholesterol and glucose levels in rats fed a high-cholesterol diet [21]. One possible reason for the beneficial effects unique to krill oil is that omega-3 polyunsaturated fatty acids exist in phospholipid form rather than as a triglyceride in

contrast to fish oil supplements [22,23]. However, the mechanisms of action of krill oil remain poorly understood.

In a previous report, krill oil supplementation was shown to modulate key metabolic pathways, including glucose metabolism, lipid metabolism, and the mitochondrial respiratory chain [23,24]. Surprisingly, an equimolar dose of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) derived from fish oil did not modulate key metabolic pathways regulated by krill oil [24], suggesting that krill oil contains unidentified active compounds other than EPA and DHA that modulate key metabolic pathways as a signaling molecule. In line with this concept, we screened components from krill oil that promote adipogenesis and identified palmitoyl lactic acid (PLA). Moreover, PLA was found to induce a brown fat-like phenotype in 3T3-L1 preadipocytes via enhanced expression of brown/beige cell-specific genes, such as PR domain containing 16 (*Prdm16*), cytochrome c oxidase subunit VIIa 1 (*Cox7a1*), peroxisome proliferative activated receptor, gamma, coactivator 1α (*Pgc1a*), Cbp/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain 1 (*Cited1*), fibroblast growth factor 21 (*Fgf21*), uncoupling protein 1 (*Ucp1*), cell death-inducing DNA fragmentation factor, alpha subunit-like effector A (*Cidea*), and transmembrane protein 26 (*Tmem26*), as well as the proteins PRDM16, PGC-1α, and

UCP1. Our findings suggest that PLA promotes the induction of the brown fat-like phenotype and thus may have potential therapeutic implications for treatment of obesity.

2. Materials and Methods

2.1. Reagents

Mouse 3T3-L1 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Dulbecco's modified Eagle's medium (DMEM) with high glucose (11965-092), 100 mM sodium pyruvate, penicillin-streptomycin (liquid, cat# 15070063), and BODIPY® 493/503 were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Fetal bovine serum (FBS) was obtained from Nichirei Biosciences, Inc. (Tokyo, Japan). 3-Isobutyl-1-methylxanthine (IBMX), insulin, and dexamethasone were obtained from Sigma-Aldrich Corporation (St. Louis, MO, USA). Perilipin (D1D8) XP® rabbit monoclonal antibody (mAb) and PPARγ (C26H12) rabbit mAb were purchased from Cell Signaling Technology (Beverly, MA, USA). UCP1 (4E5) mouse mAb was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against PGC1α (NBP1-04676) and PRDM16/MEL1 (NBP1-77096) were purchased from Novus Biologicals LLC (Littleton, CO, USA). Pioglitazone hydrochloride was obtained from LKT Laboratories, Inc. (St. Paul, MN, USA).

Rosiglitazone was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Krill oil capsules containing processed krill oil extracted from Antarctic krill (*Euphausia superba*) were provided by Sunsho Pharmacetical Co. Ltd. (Shizuoka, Japan). All reagents were of the highest grade available from commercial sources.

2.2. Isolation of PLA from krill oil

Krill oil (10 g) was dissolved in chloroform and subjected to flash chromatography on a silica gel column and eluted with a stepwise gradient of methanol in chloroform. The fractions eluted with 10%–20% methanol were combined and loaded on a Sephadex LH-20 column (ϕ3.2 × 115 cm) and fractionated with 50% methanol in chloroform to obtain nine fractions (L1–L9). The fractions eluted at a Kd of 1–1.5 were combined (L9, 7.5 mg) and the major components in the fraction were identified to be acyl lactic acids by nuclear magnetic resonance (NMR) spectroscopy and liquid chromatography–mass spectrometry (LC–MS) analyses.

2.3. Synthesis of PLA

Benzyl bromide (56.4 mg, 0.33 mmol) and Cs_2CO_3 (107 mg, 0.33 mmol) were added to a solution of L-lactic acid (27 mg, 0.3 mmol) in dimethylformamide (1 ml). The

reaction mixture was stirred for 4 h at room temperature. After the addition of ethyl acetate, the organic layer was washed with saturated aqueous ammonium chloride and saturated aqueous sodium chloride. The organic phase was concentrated *in vacuo*. The resulting residue was fractionated by silica gel column chromatography to yield benzyl lactate (39.3 mg, 73% yield).

To synthesize benzyl palmitoyl lactate, the solution of palmitic acid (25.6 mg) in dichloromethane (5 ml) was treated with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (19 mg) and 4-dimethylaminopyridine (2.4 mg). Benzyl lactate (39 mg) was then added to the resulting solution and the reaction mixture was stirred overnight at room temperature and then quenched with saturated aqueous ammonium chloride. The resulting mixture was extracted with ethyl acetate, and the organic layer was further washed with saturate aqueous sodium chloride and dried over sodium sulfate. The organic phase was fractionated by silica gel column chromatography to yield benzyl palmitoyl lactate (32.8 mg, 55% yield).

To remove the benzyl groups, 10% Pd/C (1 mg) was added to the solution of benzyl palmitoyl lactate (32.8 mg) in chloroform (5 ml). The resulting solution was stirred under H_2 atmosphere for 40 min at room temperature. The Pd catalyst was filtered, and the filtrate was concentrated and fractionated by silica gel chromatography

to yield palmitoyl lactatic acid (7.3 mg, 28% yield).

2.4. Cell culture and treatment

Unless otherwise specified, cell culture and further analyses were conducted at 37°C under an atmosphere of 5% CO_2 and 95% air. 3T3-L1 cells were maintained as subconfluent cultures in DMEM supplemented with 25 mM glucose, 10% FBS, 1 mM sodium pyruvate, and 100 U/ml penicillin-100 μg/ml streptomycin (medium A). The 3T3-L1 cell line is a widely used model system for the analysis of adipocyte differentiation. To differentiate into mature adipocytes, these cells were seeded at a density of 4×10^5 cells/well in 35-mm dishes and then cultured in medium A to confluency. The cells were incubated for at least 2 days and then cultured for 3 days with 10 μg/ml of insulin, 0.25 μg/ml dexamethasone, and 0.5 mM IBMX in medium A (henceforth called MIX). The cells were further incubated for 4 days in medium A, as described previously [25]. The differentiated adipocytes were characterized on day 7 by assessing the intracytoplasmic accumulation of lipid droplets, as indicated by positive staining with Oil Red O [26].

2.5. Immunofluorescence

3T3-L1 cells were plated in eight-chamber plastic slides (LAB-TEK II, #154534) at a concentration of 5×10^3 cells/well/0.3 ml of medium A. The cells were differentiated to adipocytes by the methods described above. At day 7 after stimulation of differentiation, the cells were fixed with 4% paraformaldehyde at 37°C for 10 min and then subjected to permeabilization with 0.5% Triton X-100/Tris-buffered saline (TBS). Afterward, the cells were washed with TBS containing 0.1% Triton X-100 (TBS-T) three times, blocked with 2% goat serum in TBS-T for 1 h, and incubated with polyclonal anti-perilipin, anti-PPARγ, anti-PRDM16, and anti-PGC1-α antibodies (1:100 dilution) overnight at 4°C, followed by three washes with TBS-T. The cells were then incubated with Alexa Fluor® 594 secondary antibody goat anti-rabbit immunoglobulin G (1:600 dilution). The cells were stained with DAPI (nuclei) and BODIPY® 493/503 lipid stain (1 μg/ml of phosphate-buffered saline (PBS); Molecular Probes, Eugene, OR, USA) for 30 min. Finally, the cells were washed with TBS-T and treated with mounting agent. Images were acquired using the Olympus FV10i confocal microscope (Olympus Corporation, Tokyo, Japan), which has an automated scanning confocal system and is equipped with a × 600 water immersion objective 1.345 numerical aperture.

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2.6. AdipoRed™ assays

Lipid content was measured using a commercially available kit (AdipoRed™; Lonza Walkersville, MD, USA) according to the manufacturers' protocol. 3T3-L1 cells were seeded at a density of 3×10^3 cells/well of 96-well plates with black walls and clear bottoms and then cultured in medium A to confluency. The cells were treated with 3 μM pioglitazone, 3 μM rosiglitazone, or various concentration of PLA for 7 days. The medium was changed every 3 days. In contrast, the cells were treated with MIX for 3 days and then cultured in fresh medium. On day 7, the cells were fixed with 4% paraformaldehyde for 30 min and then washed with PBS (pH 7.4), and 200 μl of PBS was added to the wells. Then, 5 μl of AdipoRed reagent was added to each well. After 20 min, the plates were placed in the ARVO plate reader (PerkinElmer Life and Analytical Sciences, Boston, MA, USA), and fluorescence was measured with an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

2.7. BODIPY staining and quantitation

The size and adiposity of the cells were determined using the fluorescent dye BODIPY® 493/503. Cells were cultured in six-well plates and differentiated for 7 days. For the fluorescence-activated cell sorting (FACS) experiment, the cells were incubated with DMEM containing with 0.25 μg/ml BODIPY® 493/503 for 30 min at 37°C in the

dark and then rinsed twice with PBS (pH 7.4) and trypsinized with phenol red-free 0.05% trypsin-ethylenediaminetetraacetic acid (Invitrogen Corporation, Grand Island, NY, USA) for 3 min at 37°C. The cells were then suspended in 1 mL of PBS and immediately put on ice. The samples were analyzed with a FACSCanto II (Becton Dickinson, Franklin Lakes, NJ, USA) using BD FACSDiva software, version 6.0. The cell size distribution was determined with a forward scatter (FSC) detector.

2.8. Real-time quantitative polymerase chain reaction (qRT-PCR) analysis

qRT-PCR was performed to determine gene expression levels of target genes in mouse 3T3-L1 cells using an ABI7300 RT-PCR System (Applied Biosystems, Foster City, CA, USA). The qRT-PCR reactions were performed in 10-μl reaction volumes containing the following components: 1 μl of cDNA solution, 5 μl of SYBR® Green PCR Master Mix (Applied Biosystems), 300 nM of each primer (qRT-PCR primer set for adipose (mouse); Hokkaido System Science Co., Ltd., Sapporo, Japan, or specific primers synthesized by Eurofins Genomics K.K., Tokyo, Japan), and deionized water. The sequences of all primers are listed in Table 1. The cDNA amplification conditions were 95°C for 2 min followed by 40 cycles at 95°C for 15 s and 60°C for 60 s. The expression levels of the target genes were normalized to that of β-actin, as an internal control.

Relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method. qRT-PCR analysis was performed in triplicate with a mean of at least three separate experiments.

Table. 1

Primer Sequences used in real-time PCR

Gene	Forward Primer	Reverse Primer	Rf.
Prdm16	GATGGGAGATGCTGACGGAT	TGATCTGACACATGGCGAGG	12
Cox7a1	AAAGTGCTGCACGTCCTTG	TTCTCTGCCACACGGTTTTC	30
Cited1	CGCTTCGTCCGTACCTCAGCT	CAGCTGGGCCTGTTGGTCTC	30
Fgf21	CGTCTGCCTCAGAAGGACTC	TCTACCATGCTCAGGGGGTC	12,17
Ucp1	CCTGCCTCTCTCGGAAACAA	GTAGCGGGGTTTGATCCCAT	17
Cidea	CGGGAATAGCCAGAGTCACC	TGTGCATCGGATGTCGTAGG	12,17
Tmem26	GAAACCAGTATTGCAGCACCC	CCAGACCGGTTCACATACCA	12,17
Tbx1	CGAATGTTCCCCACGTTCCA	GTCTACTCGGCCAGGTGTAG	12,17
ATGL	CACTTTAGCTCCAAGGATGA	TGGTTCAGTAGGCCATTCCT	37

Real-time PCR primer set for adipose (mouse) are obtained from Hokkaido System Sci. Co., Ltd (*PGC1A*, *ACTB*, *ADIPOQ*, *PLIN*, *CEBPA*, *LIPE*, and *PPARG*)

2.9. Immunoblot analysis

Cells were cultured in six-well plates, differentiated for 7 days, and then trypsinized with phenol red-free 0.05% trypsin-ethylenediaminetetraacetic acid for 3 min at 37°C. According to the manufacturer's instructions of a nuclear extraction kit (Cayman

Chemicals, Ann Arbor, MI, USA), the detached cells were washed twice with ice-cold PBS, homogenized in 500 μl of ice-cold complete hypotonic buffer for 15 min, combined with 100 μl of NP-40, and then centrifuged at 14,000 × g for 30 s at 4°C. The supernatant containing the cytoplasmic protein fraction was used for determination of PPARγ. The remaining nuclear pellet was resuspended in 100 μl of ice-cold complete nuclear extract buffer for 30 min and then centrifuged at 14,000 × g for 15 min at 4°C. The supernatant containing the nuclear fraction was used for determination of PPARγ. For immunoblot analyses of UCP1, cells were lysed in radioimmunoprecipitation assay buffer. The protein concentration of the supernatant was determined with a bicinchoninic acid protein assay kit (Takara Bio Inc., Otsu, Japan). Each protein sample (5 μg) was separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride (PVDF) filter membrane (Millipore, Billerica, MA), which was subjected to immunoblot analyses with each primary antibody (anti-PPARγ antibody or anti-UCP1 antibody) and horseradish peroxide-conjugated secondary antibody. The ECL Prime Western Blotting Detection Reagent kit (GE Healthcare Life Sciences, Marlborough, MA, USA) was used for visualization. The intensity of each immunoreactive band was measured with Image Gauge software and an Amersham Imager 600 system (GE Healthcare Life Sciences).

2.10. Statistical Analysis

The results are expressed as the mean ± standard error of mean (SEM). Prism 6 software (Graph Pad Software, Inc., San Diego, CA, USA) was used to perform all statistical analyses. Comparisons between groups were performed by one-way analysis of variance and the Dunnett's post-hoc test. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Identification of active components that induce adipogenesis

To identify the active constituents responsible for the adipogenesis in 3T3-L1 cells, krill oil was fractionated by silica gel chromatography and Sephadex LH-20 gel filtration, which resulted in nine fractions (L1–L9). Fraction L9, which was eluted in the latter part by Sephadex LH-20 column chromatography, showed the most potent adipogenic activity, as indicated by positive staining with Oil Red O (Fig. 1A). Under these conditions, fraction L1 exhibited cytotoxicity. Based on the ^{1}H NMR spectrum, the active compounds contain a common structural unit, lactic acid, of which hydroxyl group is further acylated with several different fatty acids. The major ion peaks detected by LC–MS analysis were consistent with the molecular weights of acyl lactic acids

having different acyl unit compositions, including palmitic acid, oleic acid, EPA, and DHA. Furthermore, PLA was chemically synthesized from commercially available palmitic acid and L-lactic acid. The LC–MS analysis confirmed that the major component of active fraction was PLA (Fig. 1B).

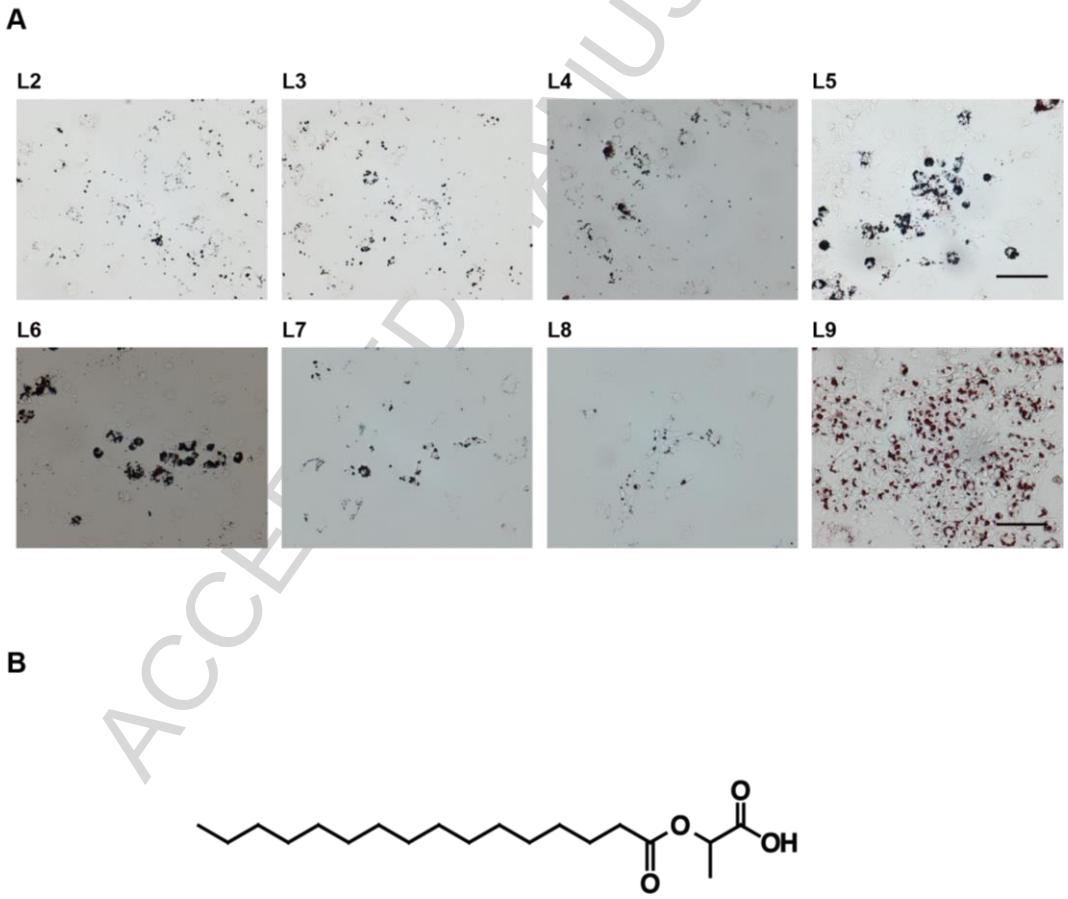

Figure 1. Adipogenic activity of each fraction and palmitoyl lactic acid

3.2. PLA-induced differentiation into adipocytes

To investigate differentiation of adipocytes by PLA, 3T3-L1 cells were treated with 100 μM PLA for 7 days. The PLA-treated cells were accumulated in intracytoplasmic lipid droplets that were positively stained with Oil Red O (Fig. 2A). The lipid accumulation was significantly increased by PLA in a dose-dependent manner (Fig. 2B, E, and F). Conversely, both 100 μM lactose and 100 μM palmitic acid did not affect accumulation in 3T3-L1 cells (Fig. 2A). We then examined the effect of PLA on typical expression patterns of adipocyte markers, including perilipin and fatty acid-binding protein 4 (FABP4; Fig. 2C, D, and 4D). PLA significantly enhanced perilipin expression at both the mRNA and protein levels (Fig. 2C and D) and enhanced FABP4 expression at the mRNA level (Fig. 4D). Under these conditions, PLA used for stimulation had no effect on cytotoxicity, as assessed by the WST-8 cell proliferation assay (Supplementary Fig. 1A). FACS analysis showed that the population of control cells (Fig. 2E) exhibited a wide heterogeneity in size and low BODIPY fluorescence (Q3 and Q4), consistent with the undifferentiated state. Upon differentiation by MIX and PLA treatment, the population of small cells with high BODIPY fluorescence (Q1) increased in a dose-dependent manner (Fig. 2E and F). PLA treatment did not have a noticeable effect

on the appearance of Q2 cells. Thus, PLA promoted fat accumulation without increasing cell size, which is thought to be controlled by a mechanism independent of fat accumulation. Conversely, cell populations in the undifferentiated state (Q3 and Q4) were reduced by PLA and MIX treatment, and a particularly large size cell population (Q4) was significantly reduced. These results indicate that PLA promoted the accumulation of intracellular lipid droplets of preadipocytes and induced the expression of the adipogenesis markers perilipin and FABP4. PLA showed potent 3T3-L1 differentiation activity.

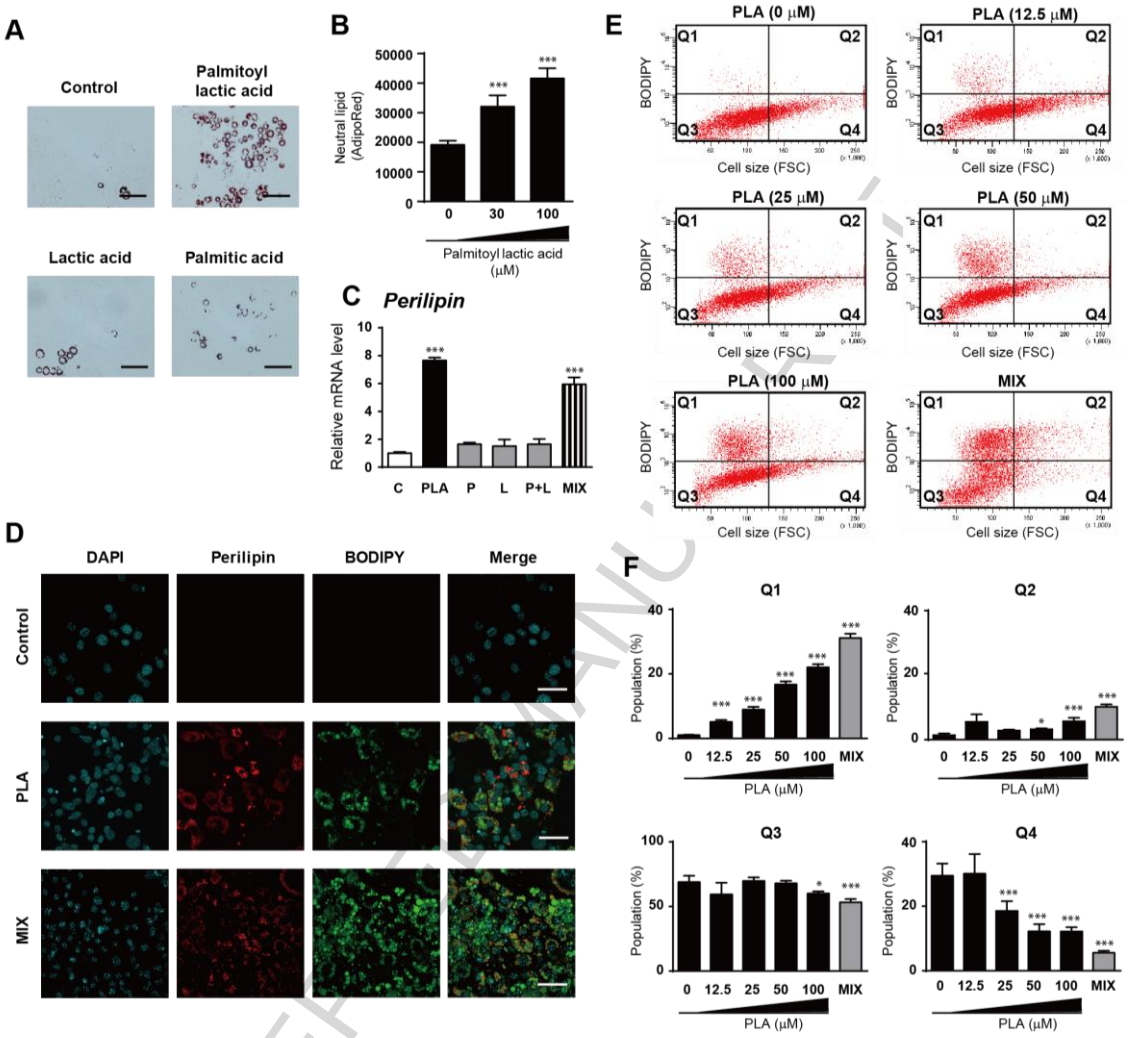

Figure 2. Effect of PLA on adipogenesis of 3T3-L1 preadipocytes

3.3. Like the PPARγ agonist, PLA promoted adipogenesis in the presence of dexamethasone

Dexamethasone, IBMX, and insulin, are insufficient to cause differentiation independently, although the mixture of these regents accelerate the differentiation of 3T3-L1 cells (Fig. 4A) [27]. Previous reports demonstrated that a supply of an exogenous PPARγ-agonist accelerated differentiation in combination with dexamethasone [28]. The promotion of adipogenesis with dexamethasone is a unique action of the PPARγ-agonist [28]. The identification of agents that accelerate differentiation, provides insights to the biochemical pathways that may function during adipocyte differentiation [27]. In this study, PLA significantly enhanced the expression of PPARγ at both the mRNA and protein levels, and PLA-enhanced PPARγ accumulated in the nucleus (Fig. 3A–D). Thus, we next tested the ability of PLA and the PPARγ-agonists pioglitazone and rosiglitazone to induce adipocyte differentiation in the system. As demonstrated in Fig. 4A, neutral lipid accumulation was increased by 1.9-fold by treatment with 100 μM PLA alone, while accumulation was increased by 5.4-fold by 100 μM PLA in the presence of dexamethasone. Furthermore, the population of adipocytes was analyzed by FACS experiment. When treated with PLA in the presence of dexamethasone, the population of differentiated adipocytes increased by 1.9-fold, compared with treatment with PLA alone (Fig. 4C). The PLA-induced adipogenesis in the presence of dexamethasone was phenotypically similar to 3T3-L1

cells induced by pioglitazone or rosiglitazone (Fig. 4A–C). Moreover, neutral lipid accumulation was not changed by PLA or the PPARγ-agonists in the presence of IBMX but was increased by both PLA and PPARγ-ligand in the presence of insulin (Fig. 4A and B). When treated with both PLA and the PPARγ-agonist in the presence of dexamethasone, the expression of FABP4, a typical adipocyte marker, significantly increased, compared with alone treatment (Fig. 4D). PPARγ agonists, such as thiazolidinediones (TZDs), are known to promote adipogenesis and induce browning *in vivo* [29-31]. Thus, we next examined whether PLA affects the brown fat-like phenotype in 3T3-L1 cells.

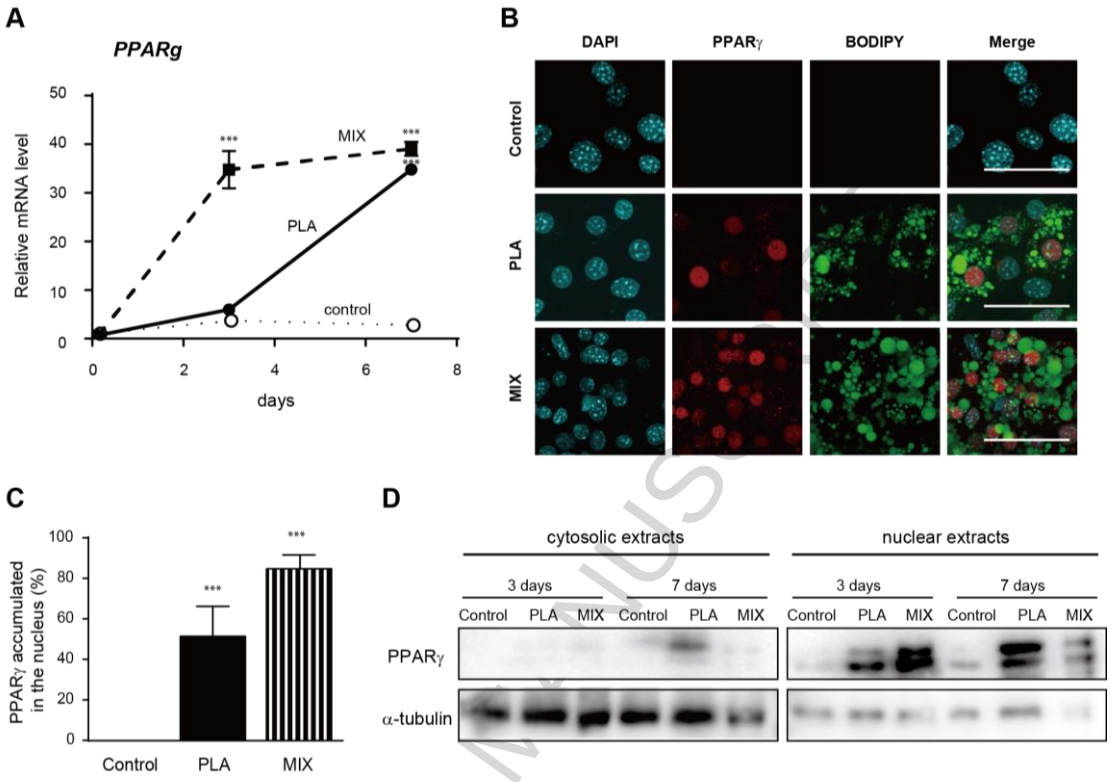

Figure 3. PLA significantly increased PPARγ

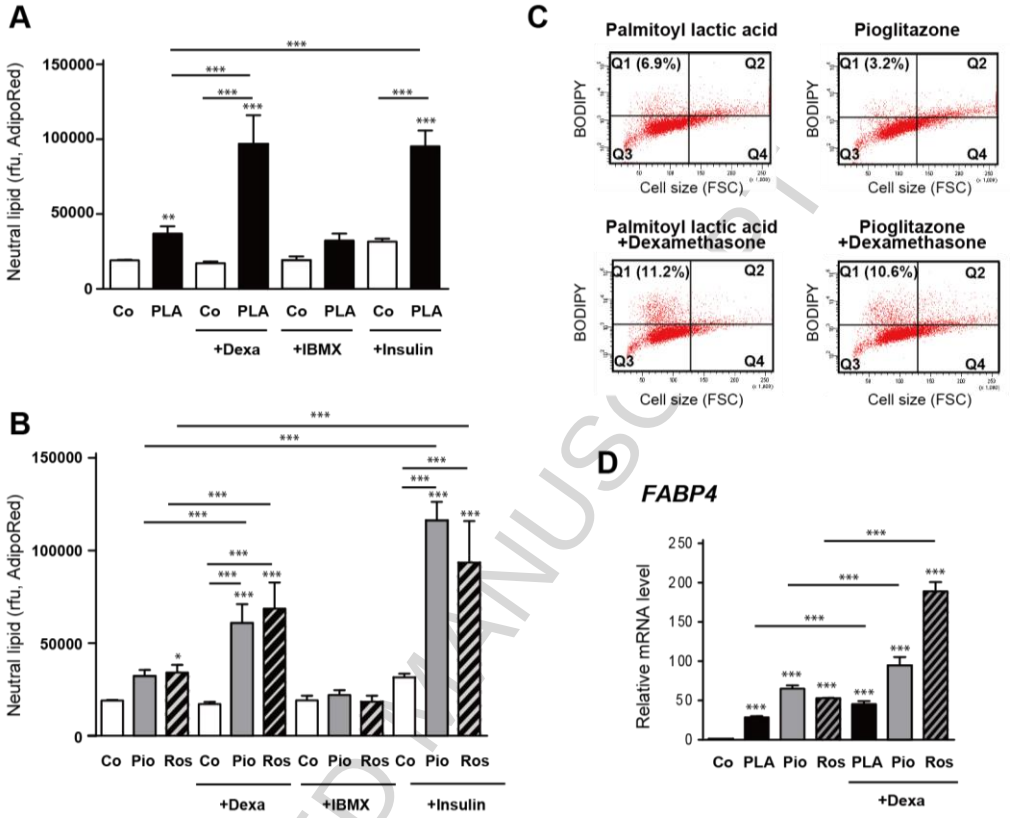

Figure 4. PLA significantly increased the adipogenesis in the presence of dexamethasone

3.4. PLA-induced a brown fat-like phenotype in 3T3-L1 adipocytes

We next conducted a comparative analysis of the expression levels of nine brown/beige cell-specific genes in 3T3-L1 adipocytes treated with PLA and TZDs. As shown in Fig

5A, PLA significantly increased the key brown fat markers (PRDM16, PGC-1α, and UCP1) at the mRNA level in 3T3-L1 adipocytes treated with PLA for 7 days, compared with untreated control cells. Treatment of cells with 100 μM PLA produced a 32.8-fold induction of *Prdm16*, a 3.5-fold induction of *Pgc1a*, and a 1.7-fold induction of *Ucp1*. TZDs also increased the expression levels of the brown fat markers *Prdm16* (93.0-fold for pioglitazone and 79.9-fold for rosiglitazone), *Pgc1a* (6.7-fold for pioglitazone and 5.9-fold for rosiglitazone), and *Ucp1* (1.5-fold for pioglitazone, 1.3-fold for rosiglitazone) in 3T3-L1 adipocytes. In contrast, none of these three brown fat markers were increased in 3T3-L1 white adipocytes induced by MIX (data not shown). PLA enhanced expression of other key brown/beige cell-specific genes, *Cox7a1*, *Cited1*, *Fgf21*, *Cidea*, and *Tmem26* (Fig. 5A). The PLA-induced expression profile of the brown/beige adipocyte-specific genes was similar to that by the TZDs except for *Tmem26* in 3T3-L1 adipocytes (Fig. 5A). These findings have raised the possibility that PLA promotes the brown fat-like phenotype in 3T3-L1 preadipocytes, like the PPARγ agonist. This notion is supported by the enhanced expression of the key brown cell marker proteins PRDM16, PGC-1α and UCP1 upon PLA treatment, which was further confirmed at the cellular level by immunoblotting and immunostaining (Fig. 5B and C).

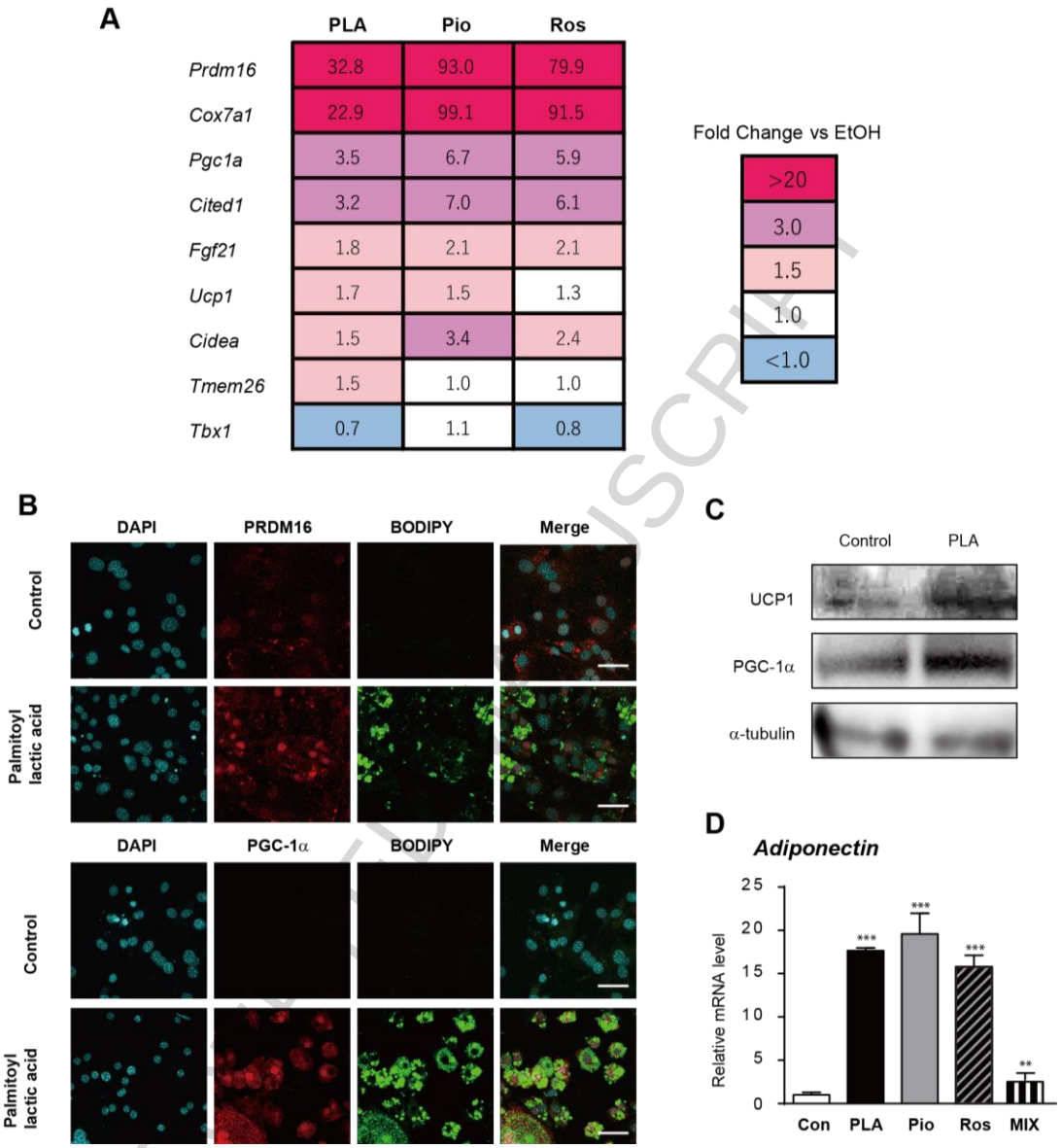

Figure 5. Effect of PLA on expression of brown/beige cell-specific genes in 3T3-L1 cells

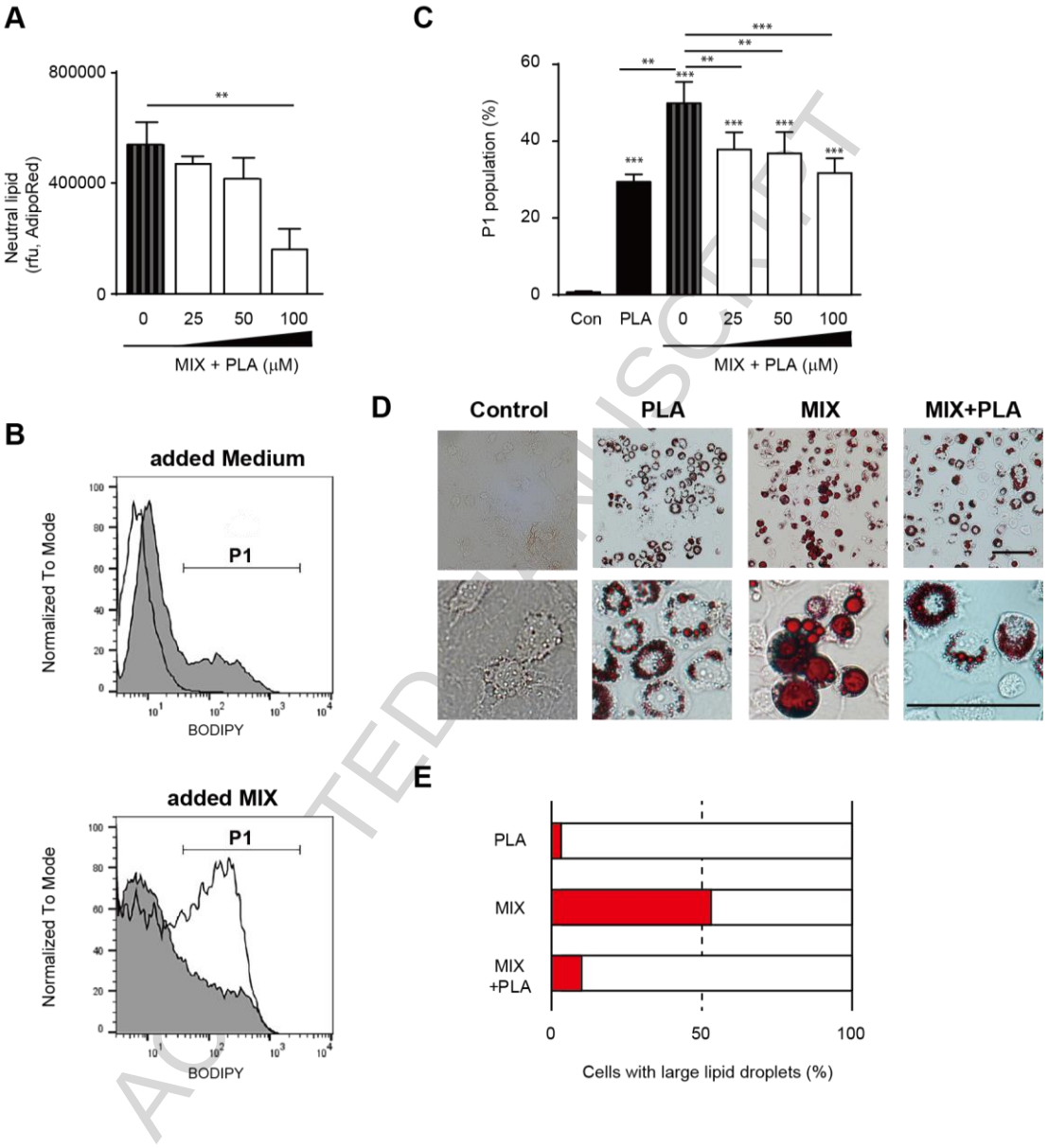

Figure 6. PLA induces lipid accumulation in the different manner from MIX and works antagonistically with MIX in 3T3-L1 cells

3.5. PLA promoted adiponectin expression and reduced lipid accumulation

PPARγ/retinoid X receptors have been shown to directly bind to a functional PPAR-responsive element in the human adiponectin promoter and to increase promoter activity [29]. Furthermore, PPARγ agonists, such as TZDs, increased adiponectin synthesis in cultured 3T3-L1 adipocytes, which was associated with increases in mtDNA content and fatty acid oxidation [33]. In a more recent study of krill oil, serum adiponectin was increased in animals fed krill oil [34]. Thus, we next examined the effect of PLA on adiponectin expression at the mRNA level in 3T3-L1 cells. After treatment of the cells with 100 μM PLA for 7 days, *Adiponectin* expression was significantly increased (17.7-fold in PLA-treated cells vs. 2.8-fold in MIX-treated cells). TZDs also increased *Adiponectin* expression by 19.6-fold in pioglitazone-treated cells and 15.8-fold in rosiglitazone-treated cells (Fig. 5D). Moreover, total lipid content significantly decreased in cells treated with both MIX and PLA (MIX-plus-PLA cells), as compared with those treated MIX only (Fig. 6A). FACS analysis also showed that the population of cells with high fat content was decreased by treatment with MIX plus PLA, as compared to the MIX alone, although single PLA treatment increased the

population of cells with high-fat content, as compared to the mock treated cells (Fig. 6B and C). Further imaging analysis revealed that the PLA-induced phenotype was remarkably different from the MIX-induced phenotype in the size of lipid droplet (LD) (Fig. 6D and E). The PLA-induced adipocytes contained numerous LDs per cell, but each LD was small. On the contrary, MIX-induced adipocytes had only a small number of LDs per cell, but each LD was large. The LD morphology of the MIX-plus-PLA cells were similar to those of the PLA-induced adipocytes rather than those of the MIX-induced adipocytes (Fig. 6D and E). These results suggest that PLA did not work with MIX additionally but antagonistically. Under these experimental conditions, the expression levels of intracellular neutral lipases hydrolyzing triacylglycerols, such as adipocyte triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) in the MIX-plus-PLA cells, were higher than those in the control cells, but lower than those in the MIX-induced adipocytes (Supplementary Fig. 1B). This suggests that the lipases may not be main contributors to accumulating smaller LDs in the PLA-induced adipocytes.

4. Discussion

Dietary supplementation of krill oil persistently improved hypertriglyceride content

without weight loss in human and animal model studies [18-24,34]. The biologically active components, such as TZDs, indomethacin, nobiletin, and melatonin, which expectedly exerted insulin sensitivity, as well as antiobesity and anti-inflammatory effects, induced adipogenesis of 3T3-L1 cells [28,29,35-37]. Therefore, adipogenesis inducers contained in krill oil were screened using 3T3-L1 cells and successfully identified PLA. PLA induced adipogenesis in a dose-dependent manner and significantly promoted adipogenesis in the presence of dexamethasone, compared with PLA treatment alone. The promotion of adipogenesis with dexamethasone is unique action of PLA, which has never been observed by treatment with arachidonic acid, palmitic acid, oleic acid, erucic acid, linoleic acid, EPA, or DHA in the presence of dexamethasone (data not shown) [28]. It is known that dexamethasone itself or even in the presence of a PPARα agonist, such as WY-14643 [38], does not induce differentiation but rather induces differentiation in the presence of a PPARγ agonist [28] (Fig. 4B). These facts led us to postulate that PLA acts as an agonist of PPARγ. Indeed, PPARγ was accumulated in the nuclei of PLA-treated 3T3-L1 cells during the differentiation process (Fig. 3B–D).

PPARγ agonists, such as TZDs, are known to promote adipogenesis and induce browning [28-31]. The induction of browning of white adipose tissue is considered an

effective therapeutic tool against diet-induced obesity [2,9]. In line with this finding, many pharmacological and dietary agents, such as capsaicin, berberine, irisin, monoterpene limonene, curcumin, and chrysin, have been reported to promote browning of white adipose cells and tissues [9,12-17]. We successfully demonstrated that PLA induced a brown fat-like phenotype through significant elevation of brown/beige cell-specific genes, such as *Prdm16*, *Cox7a1*, *Pgc1a*, *Cited1*, *Fgf21*, *Ucp1*, *Cidea*, and *Tmem26*, as well as the proteins PRDM16, PGC-1α, and UCP1 (Fig. 5A–C). UCP1 is a hallmark protein of brown adipocytes, at both the mRNA and protein levels [2,7,10,39]. The most fluctuating gene by PLA treatment was *Prdm16*. In addition to PRDM16, PGC1α was also up-regulated at both the mRNA and protein levels by PLA treatment. These proteins reportedly activate the brown fat gene program *in vivo* [2,10,39,40]. Sharp *et al.* identified *Cited1* and *Fgf21* as beige adipocyte-specific genes in rosiglitazone-induced beige adipocytes [39]. In this study, the gene expression levels of *Cited1* and *Fgf21* were also significantly elevated in PLA-treated cells, compared with the control cells. Thus, the expression profile of brown/beige cell-specific genes induced by PLA was similar to that by TZDs in 3T3-L1 adipocytes in this study. These findings provide significant opportunities for the development of PLA to promote browning *in vivo*.

In this study, PLA was identified as a new inducer of adipocyte differentiation, although the phenotype of PLA-induced adipocytes was different from that of MIX-induced adipocytes. The PLA-induced adipocytes contained numerous LDs per cell, but each LD was small, whereas the MIX-induced adipocytes often contained a large LD in each cell. The difference in the morphology of LD must be reflected from their different functions. White adipocytes usually contain a single giant LD occupying most of the cytoplasm (unilocular), while brown adipocytes, which are rich in mitochondria, were filled with a number of smaller LDs (multilocular) [41]. Thus, our observation shows that PLA worked as an inducer of the brown fat-like phenotype in 3T3-L1 cells. And also it suggests that the mechanisms by which PLA induces the lipid accumulation is different from that by which MIX does. Furthermore, when PLA was used with MIX, the LD morphology of the MIX-plus-PLA cells was similar to that of the PLA-induced adipocytes rather than the MIX-induced adipocytes. The MIX-plus-PLA cells also contained a large number of small LDs. Under these conditions, ATGL and HSL seem not to participate in reducing the LD size in the PLA-plus-MIX cells. This suggests the unidentified mechanism without the involvement of lipases for the smaller LDs accumulation in the PLA-induced adipocytes. Hence, further studies are warranted to elucidate the effects of PLA on lipid

accumulation in adipocytes.

In conclusion, the novel finding of the present study is that PLA was identified as an adipogenic component of krill oil that induced brown fat-like phenotype in 3T3-L1 preadipocytes via enhanced gene expression of adiponectin and reduced lipid accumulation in 3T3-L1 adipocytes differentiated by MIX. PLA is used as a food additive and emulsifying agent, but there is no report on its physiological function. To the best of our knowledge, this study is the first to investigate the biological activity of PLA in 3T3-L1 adipocytes. Thus, our *in vitro* data suggest that possibility of an antiobesity effect by PLA although an *in vivo* study is required to solidify its role.

Author contributions

YU and TW designed and conducted the experiments, performed data analysis, and wrote the manuscript. HY, ST, and YS assisted in the experiments. YS, TK, KY, and YO have supervised the exercise intervention. YO reviewed and edited the manuscript.

Conflict of Interest

The authors declared no conflicts of interest

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Figure legends

Figure 1. Adipogenic activity of each fraction and palmitoyl lactic acid

(A) Microscopic images (×280 magnification) of 3T3-L1 cells treated with the fractions L2–L9 eluted in the part by Sephadex LH-20 column chromatography. The 3T3-L1 cells were incubated for 7 days with 0.07 mg/mL of each fraction (L2–L9). Cultures of 3T3-Ll cells stained with Oil Red-O to show accumulated cytoplasmic lipid. Scale bar, 100 μm. (B) Structure of PLA.

Figure 2. Effect of PLA in adipogenesis of 3T3-L1 preadipocytes

(A) Microscopic images of 3T3-L1 cells treated with 0.1% ethanol (EtOH), as a control, 100 μM PLA, 100 μM palmitic acid, or 100 μM lactic acid for 7 days *via* Oil Red O staining to visualize lipid content. Scale bar, 100 μm. (B) Lipid content was measured with the AdipoRed™ assay. (C) qRT-PCR analyses to determine the mRNA expression levels of perilipin. The cells were treated with 100 μM PLA, 100 μM palmitic acid (P), 100 μM lactic acid (L), or a mixture of 100 μM palmitic acid and 100 μM lactic acid (P+L) for 7 days. The cells were treated with MIX for 3 days and then further incubated in fresh medium without MIX (MIX). Perilipin expression is presented as the ratio of

viable perilipin in cells treated with the different components versus cells treated with 0.1% ethanol (C). (D) 3T3-L1 cells after PLA treatment for 7 days were fixed with 4% *p*-formaldehyde and then stained with DAPI (blue), anti-perilipin antibody (red), and BODIPY® 493/503 (green). Images are maximum intensity projections of Z stacks obtained using an Olympus FV10i confocal microscope. Scale bar, 50 μm. (E) The results of the FACS experiment. 3T3-L1 cells incubated for 7 days in fresh medium containing 12.5, 25, 50, or 100 μM PLA. The neutral lipid in the cells was stained with BODIPY® 493/503. The cell size distribution was determined with an FSC detector. (F) Enumeration of cell populations in areas Q1–Q4. All experiments were performed in triplicate and the results are presented as the mean ± SEM. $*P < 0.05$, $***P < 0.005$ (compared with 3T3-L1 cells with EtOH treatment).

Figure 3. PLA significantly increased PPARγ

(A) mRNA expression levels of PPARγ. (B and C) Immunofluorescence using confocal microscopy was performed to detect PPARγ expression in 3T3-L1 cells after 0.1% ethanol (control), 100 μM PLA (for 7 days) or MIX (for 3 days) treatment (B). All cells with PPARγ positive could confirm a strong signal in the nucleus, and the percentage of cells exhibiting PPARγ nuclear expression was quantitated by immunofluorescence (C).

At least 50 cells were counted for quantification. DAPI (blue), anti-PPARγ antibody (red), and BODIPY® 493/503 (green). Scale bar, 50 μm. (D) Immunoblotting assay of PPARγ using nuclear and cytosolic extracts of 3T3-L1 cells treated with PLA. α-Tubulin was used as a loading control. All experiments were performed in triplicates and the results are presented as the mean ± SEM. ***$P < 0.005$ (compared with 3T3-L1 cells with EtOH treatment).

Figure 4. PLA significantly increased the adipogenesis in the presence of dexamethasone

(A and B) Lipid content was measured with the AdipoRed™ assay. 3T3-L1 cells were incubated for 7 days in fresh medium containing 0.1% EtOH (Co), 100 μM PLA, 3 μM pioglitazone (Pio), or 3 μM rosiglitazone (Ros) in the absence or presence of 0.25 μg/ml dexamethasone (Dexa), 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), or 10 μg/ml insulin. (C) The results of the FACS experiment. 3T3-L1 cells incubated for 4 days in fresh medium containing 100 μM PLA or 3 μM pioglitazone in the absence or presence of 0.25 μg/ml dexamethasone. The neutral lipids in the cells were stained with BODIPY® 493/503. The cell size distribution was determined with an FSC detector. (D) mRNA expression levels of FABP4. All experiments were performed in triplicate

and the results are presented as the mean ± SEM. *P < 0.05, ***P < 0.005 (compared with 3T3-L1 cells with EtOH treatment).

Figure 5. Effect of PLA on expression of brown/beige cell-specific genes in 3T3-L1 cells

(A) Expression pattern of brown/beige cell-specific genes. The 3T3-L1 cells were treated with 100 μM PLA, 3 μM pioglitazone (Pio), or 3 μM rosiglitazone (Ros) for 7 days. (B) 3T3-L1 cells after treatment with 100 μM PLA were fixed with 4% *p*-formaldehyde and then stained with DAPI (blue), anti PRDM16 antibody (red), anti PGC-1α antibody (red), and BODIPY® 493/503 (green). Scale bar, 50 μm. (C) Immunoblotting assay of UCP1 and PGC-1α using whole lysates of 3T3-L1 cells treated with PLA. α-Tubulin was used as a loading control. (D) mRNA expression levels of adiponectin. 3T3-L1 cells incubated for 7 days (MIX; for 3 days) in fresh medium containing 0.1% EtOH (Con), 100 μM PLA, 3 μM pioglitazone (Pio), 3 μM rosiglitazone (Ros), or MIX. All experiments were performed in triplicate and the results are presented as the mean ± SEM. **P < 0.01, ***P < 0.005 (compared with 3T3-L1 cells with EtOH treatment).

Figure 6. PLA induces lipid accumulation in the different manner from MIX and works antagonistically with MIX in 3T3-L1 cells

3T3-L1 cells were incubated for 3 days in fresh medium containing MIX in the presence or absence of PLA, and then, the cells were further incubated for 4 days in fresh medium in the presence or absence of PLA. (A) Lipid content was measured by the AdipoRed™ assay. (B) The results of the FACS experiment. Neutral lipids in the cells were stained with BODIPY® 493/503. The fluorescence intensity of cells incubated in fresh medium containing PLA in the presence (bottom) or absence (top) of MIX was analyzed and plotted as a histogram. The white filled area depicts the control, untreated with PLA. (C) Enumeration of cell populations in area P1. (D and E) Microscopic images of 3T3-L1 cells treated with 0.1% ethanol (control), 100 μM PLA (PLA), MIX plus 0.1% ethanol (MIX), or MIX plus 100 μM PLA (MIX+PLA), *via* Oil Red O staining to visualize lipid content. Scale bar, 100 μm. The colors correspond to the population (%) of cells with the large LD phenotype (LD >250 μm^2) shown in E. At least 50 cells were counted for quantification. All experiments were performed in triplicate, and the results are presented as the mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.005$ (compared with 3T3-L1 cells with EtOH treatment).

Highlights

1. Palmitoyl lactic acid was identified as an adipogenic component of krill oil

2. Palmitoyl lactic acid induced brown fat-like phenotype in 3T3-L1 preadipocytes

3. The mode of action of palmitoyl lactic acid was similar to PPARγ agonists