Note

Plasmalogen Inhibits Body Weight Gain by Activating Brown Adipose Tissue and Improving White Adipose Tissue Metabolism

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Summary Plasmalogen, a phospholipid, exhibits preventive and therapeutic effects on dementia. Phospholipids improve fat metabolism, but it is unknown whether plasmalogen has an effect on fat metabolism. In this study, the effects of plasmalogen were determined by administering plasmalogen to KK-Ay mice. As a result, weight gain was significantly suppressed in the plasmalogen-treated group compared with the control group from 7 wk after the start of administration. In addition, plasmalogen administration increased uncoupling protein 1 (UCP1) expression in brown adipose tissue. The effect is thought to result from liver kinase B1 (LKB1)/AMP-activated protein kinase (AMPK)/PR domain containing 16 (PRDM16)/peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) pathway activation via adrenergic β3 receptors. Furthermore, the expression of the carnitine palmitoyltransferase-1 (CPT-1) gene associated with thermogenic factors and β-oxidation was increased. We investigated the browning of white adipose tissue, but no increase in UCP1 gene expression was observed in perirenal adipose tissue, epididymis adipose tissue, mesenteric adipose tissue and inguinal region white adipose tissue. In contrast, plasmalogen increased the activity of AMPK, which is a central enzyme in lipid metabolism, in perirenal adipose tissue. Furthermore, the activity of the protein kinase A (PKA)/LKB1/AMPK/acetyl-coenzyme A carboxylase (ACC), stearoyl-CoA desaturase-1 (SCD-1), and hormone-sensitive lipase (HSL) pathways was confirmed. Plasmalogen may inhibit weight gain by activating brown fat to increase heat production, inhibiting lipid synthesis, and promoting lipolysis in white fat.

Key Words plasmalogen, UCP1, AMPK, BAT, WAT

Plasmalogen (Pls) is a type of phospholipid, which includes choline Pls (C-Pls) and ethanolamine Pls (E-Pls), and is widely distributed in animals and some anaerobic microorganisms. Pls expression in humans accounts for approximately 18% of all phospholipids, but its content varies greatly depending on the organ or tissue. E-Pls accounts for nearly 70% of the ethanolamine glycerophospholipids present in the brain. Recent studies reported low levels of plasma E-Pls in patients with dementia. In addition, the effects of scallop-derived Pls have been reported in patients with moderate and severe dementia in open-label studies along with marked improvement in cognitive function. Therefore, Pls have attracted attention as a useful food ingredient in Japan. Pls is abundant in chicken and scallops, and it is extracted and sold as a health food. According to the Ministry of Agriculture, Forestry and Fisheries food supply and demand table, chicken is the most consumed meat in Japan, accounting for approximately 41.5% of total meat consumption in 2019. It has been reported that phospholipids affect lipid metabolism. Brown adipose tissue (BAT) specifically expresses uncoupling protein 1 (UCP1), which converts fatty acids to heat by modulating the proton gradient in mitochondria. In humans, BAT is abundant during the fetal and neonatal period and atrophies with growth. Thus, BAT is recognized as an organ that produces heat and maintains body temperature. Positron emission tomography, which is widely used for cancer diagnosis, has also confirmed the presence of BAT in adults. Furthermore, there is an inverse relationship between the activity of BAT and the degree of obesity. There are two types of BAT: primary BAT, which is often found in fetuses and newborns, and beige fat, which is white adipose tissue (WAT) that becomes BAT-like during cold stimulation, overeating, or drug stimulation. In recent years, beige fat and BAT activation have been postulated affect weight loss.

UCP1 expression is regulated by β-adrenergic receptors and various other factors. UCP1 is regulated by PR domain containing 16 (PRDM16), peroxisome proliferator-activated receptor γ (PPARγ), PPARγ coactivator 1α (PGC-1α), and fibroblast growth factor 21 (FGF-21). Furthermore, CCAAT/enhancer binding protein β (C/EBPβ) forms a conjugate with lipid metabolism.

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PRDM16 to induce PGC-1α expression and increase UCP1 expression (19). We also found that growth hormone was involved in UCP1 expression via the transcription and activator of transcription 5 (STAT5) pathway (20).

AMP-activated protein kinase (AMPK) is an important factor in lipid metabolism. AMPK is activated by sirtuin (Sirt) 1 and protein kinase A (PKA)/kinase B1 (LKB1) (21–23). Following activation, AMPK phosphorylates acetyl-coenzyme A carboxylase (ACC), which is involved in fatty acid synthesis, thereby inhibiting its action. Furthermore, AMPK inhibits the synthesis of fatty acid synthase (FAS) and steraryl-CoA desaturase-1 (SCD-1), which are involved in lipid synthesis, and activates hormone-sensitive lipase (HSL), which is involved in lipolysis (24). Thus, Pls is thought to suppress weight gain and improve lipid metabolism by promoting the activation of AMPK.

We have evaluated various herbal medicines and their components for their potential to treat lifestyle-related diseases. In the present study, we found that Pls activates BAT and improves WAT lipid metabolism.

Materials and Methods

Pls. The Pls (Lot No. 20190417) used in this study was provided by the MARUDAI FOOD Co., Ltd. (Osaka, Japan). Pls was extracted from chicken breast, mixed with cyclic oligosaccharides and sodium citrate, heated, and spray dried. The dry processed powder contained more than 3% Pls. This powder was dissolved in purified water and used for the experiments.

Animals and treatment. Ten-week-old male KK-Ay/Tajcl (KK-Ay) mice were obtained from CLEA Japan, Inc. (Tokyo, Japan). All mice were housed in a climate-controlled (temperature: 22–24°C, humidity: 40–60%), light-regulated room under a 12-h light/dark cycle. All mice were provided a normal chow (CR-2) diet during the experiments and were given access to water. The mice were divided into two groups and water was orally administered to the control group (n=9) and Pls (13.6 mg/kg/d) was orally administered to the Pls group (n=8) for 8 wk. Body weight was measured daily, whereas food and water intake were measured once a week. In addition, blood was collected every 4 wk. After treatment, fasting occurred for 12 h and blood was collected from the hearts of all mice under isoflurane sedation anesthesia prior to dissection. The collected organs (liver, kidney, heart, brain) and tissues (BAT, mesenteric adipose, epididymis adipose, perirenal adipose, inguinal region WAT) were measured (liver, kidney, heart, brain, epididymis adipose) and impregnated with RNAlater TM solution (Invitrogen, California, USA) and stored at −80°C for the subsequent analysis.

All procedures were carried out in accordance with the guiding principles of the care and use of animals in the field of physiological sciences established by the Physiological Society of Japan. The study was approved by the ethics committee of Laboratory Animals at Mukogawa Women’s University in Japan (P-06-2019-01-A).

Blood analysis. Serum triglycerides (TG), cholesterol (CHO), non-esterified fatty acid (NEFA), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were measured with a kit according to the manufacturer’s instructions (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan).

Western blot analysis. BAT, mesenteric adipose, epididymis adipose, and perirenal adipose protein was extracted with homogenization buffer (50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1% Nonidet-P40, 0.25% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS)), 1 mM ethylenediaminetetraacetic acid (EDTA)), 50 mM NaF, 2 mM Na3VO4, 30 mM sodium pyrophosphate, 2 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzoinde, 0.02 mg/mL trypsin inhibitor, 0.02 mg/mL leupeptin, and 0.02 mg/mL aprotinin). After centrifuging the lysates at 8,000 rpm for 10 min, the supernatants were collected and protein concentrations were measured using the DS protein assay (BIO-RAD, California, USA). The protein concentration was adjusted to 40 μg/lane and mixed with 0.5 mM Tris-HCl (pH 6.8), glycerol, 10% SDS, 0.1% bromophenol blue, and 2-mercaptoethanol, and heated at 95°C for 5 min. The proteins were electrophoresed using 10% SDS-polyacrylamide gel electrophoresis (PAGE) gel at 20 mA for 90 min. The separated proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Amersham Life Science, Inc., Buckinghamshire, UK). The membranes were blocked and incubated successively with primary (4°C for overnight) and secondary (room temperature for 1 h) antibodies. The primary antibodies were as follows at a dilution of 1:1,000 in Can Get Signal® Immunoreaction Enhancer Solution 1 (TOYOBO, Osaka, Japan): UCP1, Phosphor (P)-HSL Ser660, C/EBPβ, P-AMPK Thr172, AMPK, P-LKB1 Ser428, LKB1, P-ACC Ser79, FAS, P-PKA Thr197 (Cell Signaling Technology, Beverly, USA), and Actin (SIGMA, Saint Louis, USA). Actin was selected as an anti-mouse secondary antibody (1:10,000), and the other primary antibodies were selected by combining with anti-rabbit (1:2,000), both of which were diluted with Can Get Signal® Immunoreaction Enhancer Solution 2. Finally, the membranes were developed using Chemi-Lumi one super (Nacalai Tesque, Inc., Kyoto, Japan) and the images were analyzed using Image J software.

RNA extraction and reverse transcription polymerase chain reaction. RNA was extracted from 100 mg of BAT, perirenal adipose, mesenteric adipose, epididymis adipose and inguinal region WAT using Sepaol®-RNA I Super G (Nacalai Tesque). cDNA was synthesized from total RNA using ReverTra Ace® qPCR RT Master Mix with gDNA Remover (TOYOBO) using the following conditions: 35°C for 15 min, 50°C for 5 min, 98°C for 5 min, and 4°C until use. Real-time qPCR (RT-qPCR) was performed using THUNDERBIRD® Next SYBR® qPCR Mix (TOYOBO). The cycling conditions were as follows: 95°C for 30 s followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 1 min. The level of UCP1, cell death-inducing DNA fragmentation factor alpha-like effector A (Cidea), cytchrome c oxidase subunit (Cox) 7a, Cox8b, PGC-1α, PRDM16, FGF-21, PPARα, carnit-
tine palmitoyltransferase-1 (CPT-1) α, CPT-1β, β3 adrenergic receptor (Adrb3), monocyte chemotactic protein-1 (MCP-1), interleukin-6 (IL-6), tumor necrosis factor α (TNFα), and stearoyl-CoA desaturase 1 (SCD-1) expression were evaluated in various tissues and the housekeeping gene, β-actin, was used as an internal control. The primers are listed in Table 1. The results were analyzed using the ΔΔCt method.

**Table 1. Mouse primer sequences used for RT-qPCR.**

| Gene          | Sense primer (5′-3′)                          | Antisense primer (5′-3′)                          |
|---------------|-----------------------------------------------|--------------------------------------------------|
| UCP1          | GTGAAGGTCAGAATGCAAGC                         | AGGGCCCCCTTCATGAGTC                             |
| Cidea         | CTCGTTACCTGTGCTGCTGTA                       | TGCTTGCAAGACTGGGACATACTTAC                      |
| Cox7α         | AGGAGCCAAAATGAGGCC                         | TCTRTGGGGGAAAGGAG                              |
| Cox8b         | GGAGTGCGACCCCGAGAAT                        | CGGCGGAAGTGAGAGTTT                             |
| PG-C1α        | TTCAAGATCTGTGGTTACTA                        | ACCTGAAAAGTCACATTTCA                           |
| PGC-1α        | CACTGCCCCTGAGGATACGTA                       | ACAGACAAATGCGATGGGAGAGA                       |
| FGF-21        | AGATCAGGGAGAGGAGGAAACA                      | TCAGAAGGGACAGGATGAAAT                          |
| PPARα         | AGCGGAGTTCTTTAAGAAGAG                      | GTGCAATCTGGATGTTGCTCTT                          |
| CPT-1α        | GCCTCCATTGCTGGTGCTGTA                       | ATGATCGTCTGGCTGCTGCTAG                         |
| CPT-1β        | ATGTGCTGGGATGCTGCTGTA                       | TGCCCTGAGGTGCTGCTGCTGTA                       |
| Adrb3         | GCAGAGTCACCAGCTCAACA                       | CTGGGATCTCCAGGGCCACCTG                        |
| MCP-1         | TTGGCTCAAGCAGCTGAC                        | CTGGAGCTCTGAGGAGAGGAC                        |
| IL-6          | ATGAATTCTTCTTCAGGAGAG                      | ATGAGGGAAAGGGGAGGCTGAG                       |
| TNFα          | TCTCTTCAGGAAGGAAGCGCTG                     | ATGAGGGAAAGGGGAGGCTGAG                       |
| SCD-1         | TTTGCCAGTCCTTGTGGTGC                      | ACGATGAGGGGAAATACAGC                         |
| β-Actin       | CTTTGCAGTCCTTGTGGTGC                      | ACGATGAGGGGAAATACAGC                         |

**Fig. 1.** Pls suppresses body weight and blood TG levels in KK-Ay mice. The individual (A) body weight, (B) body weight gain, (C) food intake, and (D) water intake were measured daily and weekly, respectively. (E) The organ and tissue weight were measured at dissection. The data shown are the mean±SE (n=9 or 8). Compared with Control, *p<0.05, ***p<0.001. □: Control, △: Pls.

**Statistical analysis.** The results are shown as the mean±standard error of the mean. Statistical analysis of the data was performed using a Student’s t-test between the two groups. In addition, Fig. 1A–D also performed a two-way ANOVA. The analyses were performed using SPSS Statistics 27 software (IBM Japan, Tokyo, Japan). A p-value <0.05 was considered statistically significant.
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**Results**

Pls suppresses body weight gain and blood TG levels in KK-Ay mice

We examined the effects of Pls on a mouse model of lifestyle-related diseases. The results indicated that the body weight gain of KK-Ay mice treated with Pls for 8 wk was significantly suppressed from 7 wk of treatment compared with the control group (Fig. 1A). In addition, the change in body weight from the starting day of treatment was also significantly suppressed from week 6 (Fig. 1B). There was no difference in the amount of food and water consumed between the two groups.

**Table 2.** Effect of Pls on the blood serum.

|                  | 4 wk                  | 8 wk                  |
|------------------|-----------------------|-----------------------|
|                  | Control *(n=9)*       | Pls *(n=8)*           | Control *(n=9)* | Pls *(n=8)* |
| CHO (mg/dL)      | 87.43±5.18            | 86.61±3.28            | 91.85±4.07     | 78.10±12.06 |
| TG (mg/dL)       | 315.33±29.72          | 233.25±19.22*         | 119.00±4.80    | 77.13±16.13* |
| NEFA (mEq/L)     | 1.73±0.12             | 1.24±0.10*            | 0.96±0.02      | 0.88±0.07    |
| AST (IU/L)       | 70.35±8.44            | 61.39±5.22            | 96.74±11.29    | 111.07±17.92 |
| ALT (IU/L)       | 6.50±1.44             | 7.69±0.60             | 11.58±0.65     | 11.42±0.59   |

The date shown are the mean±SE. Compared with Control, *p<0.05.

**Fig. 2.** Pls activated BAT in KK-Ay mice. The expression of (A) UCP1 mRNA, (B) UCP1 protein, (C) PGC-1α, PRDM16, FGF-21, PPARα, Cidea, Cox7α, Cox8b, CPT-1α, CPT-1β, and Adrb3 mRNA, (D) C/EBPβ protein, (E) P-AMPK protein, and (F) P-LKB1 protein were examined by RT-PCR or Western blotting. The data shown are the mean±SE *(n=9 or 8). Compared with Control, *p<0.05, **p<0.01, ***p<0.001. □: Control, ■: Pls.
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No difference was observed between the weights of the organs and the tissues (Fig. 1E). With respect to blood factors, there was no difference in CHO, AST and ALT levels. In contrast, blood TG levels were significantly lower in the Pls group at 4 and 8 wk. In addition, blood NEFA levels were also significantly lower in the Pls group at 4 wk (Table 2).

**Pls activates BAT and promotes lipid burning**

To elucidate the mechanism of the inhibitory effect of Pls on weight gain, we confirmed expression changes of lipid metabolism-related factors in BAT. The results indicated that the expression levels of UCP1 mRNA and protein were significantly increased in the Pls group compared with the control group (Fig. 2A, B). In addition, factors involved in UCP1 expression (PGC-1α, PRDM16, FGF-21, PPARα, Adrb3), BAT-specific heat-producing factors (Cidea, Cox7a, Cox8b), and factors involved in β-oxidation (CPT-1α, CPT-1β) were also increased (Fig. 2C). Furthermore, C/EBPβ protein expression, which is involved in UCP1 expression, was increased (Fig. 2D). Fatty acid synthesis is important to β-oxidation. Activation of AMPK and LKB1, upstream factors of PRDM16, was observed (Fig. 2E, F).

**Pls activated AMPK in perirenal adipose tissue but were not involved in browning of WATs**

The effect of Pls on browning in vivo was determined. No increase in UCP1 expression was observed in any WAT (Fig. 3A). In contrast, the expression of the activated form of AMPK, a key enzyme in lipid metabolism, was significantly increased in the Pls group compared with the control group in perirenal adipose tissue (Fig. 3B), but no difference was observed in mesenteric or epididymis adipose tissue (Fig. 3C, D).

**Pls promoted lipolysis and suppressed synthesis in the perirenal adipose tissue of KK-Ay mice**

The Pls group showed decreased expression of the IL-6 gene, which is an inflammatory marker in the perirenal adipose tissue (Fig. 4A). Although there was no change in the expression levels of CPT-1α and CPT-1β mRNA (Fig. 4B) and FAS protein (Fig. 4C), whereas an increase in P-ACC (Fig. 4D) and P-HSL (Fig. 4E) was observed, and the expression of SCD-1 mRNA (Fig. 4B) decreased. Furthermore, the expression levels of P-LKB1 (Fig. 4F) and P-PKA (Fig. 4G) protein, upstream factors of AMPK, were increased in the perirenal adipose tissue.

**Discussion**

In the present study, we found that Pls had an inhibitory effect on weight gain in an animal model of obesity. The results suggest that Pls causes promote fatty acid oxidation by increasing UCP1 expression via Adrb3/LKB1/AMPK/PRDM16 and C/EBPβ/PGC-1α in BAT of KK-Ay mice (Fig. 5A). In addition, Pls promoted lipolysis and inhibited lipid synthesis via the PKA/LKB1/AMPK/ACC, HSL, and SCD-1 pathways in WAT.
Fig. 4. Pls affects lipid metabolic pathways in perirenal adipose tissue. (A) Inflammation markers in perirenal adipose tissue were examined using RT-PCR. The expression of (B) CPT-1α, CPT-1β, and SCD-1 mRNA, (C) FAS protein, (D) P-ACC protein, and (E) P-HSL protein were detected by RT-PCR or Western blotting. The data shown are the mean ± SE (n=9 or 8). Compared with Control, *p<0.05, **p<0.01. □: Control, ■: Pls.

Fig. 5. Schematic diagram of the action mechanism of Pls.
Pls intake improves and prevents dementia (25–27), but the effect of Pls on improving lipid metabolism is unknown. In the present study, Pls treatment reduced body weight, TG and NEFA and increased UCP1 expression in BAT in obese animals. NEFA is usually consumed as energy, but it is abundant in the blood when obese. In this study, blood NEFA levels were lower with Pls treatment at 4 wk. Therefore, it was suggested that Pls had no effect weak NEFA by long-term administration. On the other hand, blood AST and ALT levels, which indicate liver function, were not affected by Pls, so it is considered that there was no adverse effect.

UCP1 is known to be transcriptionally induced by PGC-1α (28–30). We also observed increased PGC-1α and increased expression of PRDM16 and C/EBPβ, which are PGC-1α transcriptional enhancers. PRDM16 and C/EBPβ are conjugated and bind to the promoter of PGC-1α to induce transcription (28, 31). C/EBPβ is thought to be a differentiation factor for adipocytes in adipose tissue, but it has also been reported to have a function in UCP1 expression. The detailed mechanism of the conflicting functions remains unknown. AMPK is a factor involved in the increased expression of PRDM16 (17). Our experiments demonstrated activation of AMPK and its activator, LKB1 in BAT. LKB1 is activated by PKA and Sirt1. PKA is, in turn, activated in response to intracellular cAMP elevation. Furthermore, it has been reported that activation of β-adrenergic receptors is involved in UCP1 expression (32–34). In the present study, the expression of the β-adrenergic receptor, Adrb3, was increased. Pls may increase UCP1 expression by activating the PKA/LKB1/AMPK pathway by modulating Adrb3 and increasing intracellular cAMP levels.

UCP1 is specifically expressed in BAT and is known to inhibit weight gain through heat production (7, 35). Therefore, induction of UCP1 expression is thought to be effective for improving obesity. In this study, we found that the expression of UCP1 increased in BAT. In addition, the factors involved in UCP1 expression were also increased by Pls administration.

Since the browning of WAT has been associated with the suppression of weight gain, we examined the browning of WAT. We observed no increase in UCP1 expression in WAT, suggesting that Pls does not exhibit a browning effect. In contrast, an increase in AMPK activity, which plays an important role in lipid metabolism, was observed in perirenal adipose tissue. Therefore, we checked for downstream factors and found an increase in P-ACC and P-HSL proteins and a decrease in SCD-1 mRNA. The activity of AMPK is regulated by various factors. Of these, Pls may be involved in P-AMPK protein upregulation via PKA/LKB1 in these experiments.

In summary, we found that Pls has an inhibitory effect on weight gain by activating BAT and improving lipid metabolism in WAT.

Authorship
Research conception and design: MH, MK, and MG; experiments: MH and MK; statistical analysis of data: MH; interpretation of the data: MH and MG; writing of the manuscript: MH.

Disclosure of state of COI
There are no conflicts of interest to declare.

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