Metabolomics reveal 1-palmitoyl lysophosphatidylcholine production by peroxisome proliferator-activated receptor α

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Abstract PPARα is well known as a master regulator of lipid metabolism. PPARα activation enhances fatty acid oxidation and decreases the levels of circulating and cellular lipids in obese diabetic patients. Although PPARα target genes are widely known, little is known about the alteration of plasma and liver metabolites during PPARα activation. Here, we report that metabolome analysis-implicated up-regulation of many plasma lysoglycerophospholipid species during bezafibrate (PPARα agonist) treatment. In particular, 1-palmitoyl lysophosphatidylcholine (LPC(16:0)) is increased by bezafibrate treatment in both plasma and liver. In mouse primary hepatocytes, the secretion of LPC(16:0) increased on PPARα activation, and this effect was attenuated by PPARα antagonist treatment. We demonstrated that Pla2g7 gene expression levels in the murine hepatocytes were increased by PPARα activation and that the up-regulation of Pla2g7 expression was suppressed by Pla2g7 siRNA treatment. Interestingly, LPC(16:0) activates PPARα and induces the expression of PPARα target genes in hepatocytes. Furthermore, we showed that LPC(16:0) has the ability to recover glucose uptake in adipocytes induced insulin resistance. These results reveal that LPC(16:0) is induced by PPARα activation in hepatocytes; LPC(16:0) contributes to the up-regulation of PPARα target genes in hepatocytes and the recovery of glucose uptake in insulin-resistant adipocytes.—Takahashi, H., T. Goto, Y. Yamazaki, K. Kamakari, M. Hirata, H. Suzuki, D. Shibata, R. Nakata, H. Inoue, N. Takahashi, and T. Kawada. Metabolomics reveal 1-palmitoyl lysophosphatidylcholine production by peroxisome proliferator-activated receptor α. J. Lipid Res. 2015. 56: 254–265.

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Obesity is recognized to have major adverse health effects and is a risk factor for serious chronic disorders (1–4), including diabetes and cardiovascular diseases. Dyslipidemia and hyperglycemia, in particular, result from obesity. Therefore, it is important to prevent dyslipidemia and hyperglycemia to avoid serious diseases.

PPARs are ligand-activated transcription factors and members of the nuclear hormone receptor superfamily (5–9). The family of PPARs comprises three isoforms: PPARα, PPARβ/δ, and PPARγ. PPARα is a very important factor in the regulation of lipid metabolism (5–7) and is expressed at high levels in the liver where it promotes β-oxidation, ketogenesis, and lipid transport (10, 11). During PPARα activation, transcription of PPARα-regulated genes [e.g., carnitine-O-palmitoyltransferase 1 (CPT1) and acyl-CoA oxidase (ACO)] is stimulated and β-oxidation is activated (12–14). This activation and increased PPARα sensing in the liver result in increased energy burning and reduced fat storage (15). It has been reported that PPARα activation enhances fatty acid oxidation and decreases the levels of circulating and cellular lipids in obese diabetic patients (9, 16). Regulating PPARα activity is therefore one of the most important means of managing chronic diseases related to the dysfunction of lipid metabolism.

PPARα has the capability to accommodate and bind to a variety of natural and synthetic lipophilic acids (15). It is well known that fibrates are widely prescribed hypolipidemic drugs that act in a PPARα-dependent mechanism. Bezafibrate is one of the fibrate drugs and improves dyslipidemia,
Each group was maintained on a 60% kcal high-fat diet (HFD), then divided into two groups of similar average body weights. Old mice were maintained for 7 days on a standard diet (SD) and maintained under a constant 12 h light/dark cycle. Male KK-Ay mice were purchased from CLEA Japan. Mice were housed in individual cages in a temperature-controlled room at 23 ± 1°C and maintained under a constant 12 h light/dark cycle. Male C57BL/6J mice were purchased from CLEA Japan. Mice were randomly divided into four groups (n = 4/group) and fed SD (16% kcal fat) or HFD for 8 weeks. The mice were then fed SD, HFD, SD plus fenofibrate, and HFD plus fenofibrate (200 mg/kg body weight/day, oral administration) for 2 weeks. At the end of the treatment period, anesthetized mice were euthanized by cervical dislocation after overnight fasting, and blood and organs samples were collected. Plasma TG and glucose levels were determined by the TG E-test and glucose CII-test (Wako), respectively.

### Extraction of mouse plasma, tissues, and cell culture sample

Mouse heparin-blood samples were centrifuged at 10,000 rpm for 10 min at 4°C. After centrifugation, plasma samples (5 µl) were dissolved in 95 µl of extraction solvent (80% methanol containing HMF as an internal standard). Mouse liver samples (10 mg) were homogenized in 1 ml of extraction solvent. The partial cell culture medium (300 µl/well) was evaporated to dryness and redissolved in 100 µl of extraction solvent. The hepatocyte extraction was washed with PBS and dissolved in extraction solvent (200 µl/well). The hepatocyte extraction was ultrasonically fragmented.

After centrifugation (15,000 rpm, 10 min, 4°C), the supernatant was collected as extract. The extracts were filtered through a 0.2 µm pore polycrylilene difluoride membrane (Whatman, Brentford, UK), and the filtrate was used in LC/MS.

### Metabolomic analysis by HPLC-Orbitrap MS

LC/MS for metabolomics was performed using an HPLC system (Agilent) coupled to an LTQ Orbitrap XL-MS system (Thermo Fisher Scientific Inc., San Jose, CA), equipped with an electrospray source operating in the positive- and negative-ion modes. The spray voltage and capillary temperature were 4 kV and 250°C, respectively. This analysis consists of two scan events. Scan Event 1 is full mass type (Analyzer; FTMS, Resolution; 60,000). Scan Event 2 is MS/MS type (Analyzer; Ion Trap MS, Act Type; collision-induced dissociation, Normalized Collision Energy; 35.0). An aliquot of the extracted sample (10 µl) was injected into an Inertisil ODS-4 reversed-phase column (column size, 3.0 × 250 mm; particle size, 3.0 µm; GL Sciences Inc., Tokyo, Japan). The column temperature was set at 30°C. Mobile phases A (0.1% formic acid) and B (acetonitrile including 0.1% formic acid) were used. The buffer gradient consisted of 30.0% to 90.0% B for 0 to 30 min, 95.0% B for 30 to 60.0 min, 95.0% to 30.0% B for 60.0 to 60.1 min, and 30.0% B for 24.9 min before the next injection, at a flow rate of 250 µl/min. These data were acquired with X-Calibur software (Thermo Fisher Scientific Inc.) and PowerGet software (Kazusa DNA Research Institute, Japan) using a previously described methods (22; http://www.kazusa.or.jp/komics/software/PowerGet). Briefly, PowerGet is a Java software package for detection, alignment, and annotation of metabolite features from data obtained using LC/high-resolution MS. The peak area was divided by the area of the internal standard. This value was used to calculate the rate of change for the control group. Differences between groups were compared with the Student’s t-test.

### Quantification of lysoGPs by ultraperformance liquid chromatography quadrupole time-of-flight mass spectrometry (UPLC-QTOF-MS)

LC/MS for target metabolites analysis was performed using an Acquity UPLC system coupled to a Xevo QTOF-MS system (Waters, Milford, MA), equipped with an electrospray source in the positive-ion mode operating with a lock-spray interface for accurate mass measurement. Leucine enkephalin was used as the lock mass compound. It was infused straight into the MS system at a flow rate of 20 µl/min at 200 pg/µl (in 50% acetonitrile, 0.1% formic acid) and redissolved in 100 µl of extraction solvent. The partial cell culture medium (300 µl/well) was evaporated to dryness and redissolved in 100 µl of extraction solvent. The hepatocyte extraction was washed with PBS and dissolved in extraction solvent (200 µl/well). The hepatocyte extraction was ultrasonically fragmented.

### Materials and Methods

**Materials**

Sigma (St. Louis, MO) supplied us with bezafibrate, LPC(16:0), GW7647, and GW6471. Fenofibrate were purchased from Tokyo Chemical Industry (Tokyo, Japan). Leucine enkephalin and insulin were purchased from Wako (Osaka, Japan). Dexamethasone, troglitazone, and 1-methyl-5-isobutylxanthine (IBMX) were purchased from Nacalai Tesque (Kyoto, Japan). Extrasynthese (Lyon, France) supplied us with 7-hydroxy-5-methylflavone (HMF). All the LC/MS solvents used in this study were purchased from Wako (Osaka, Japan). The LC/MS buffer (acetonitrile, ultrapure water, formic acid, and methanol) was LC/MS grade.

### Animal experiments

All the animal experiments were approved by the Kyoto University Animal Care Committee. The mice were kept in individual cages in a temperature-controlled room at 23 ± 1°C and maintained under a constant 12 h light/dark cycle. Male KK-Ay mice were purchased from CLEA Japan (Tokyo, Japan). The 4-week-old mice were maintained for 7 days on a standard diet (SD) and then divided into two groups of similar average body weights. Each group was maintained on a 60% kcal high-fat diet (HFD) and HFD containing 0.2% (w/w) bezafibrate for 4 weeks. The energy intake of all the mice was adjusted by pair feeding. Male C57BL/6J mice were purchased from CLEA Japan. Mice were

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formic acid). During hysOG analysis, the capillary, sampling cone, and extraction cone voltages were set at 3,500, 35, and 3.0 V, respectively. The source and desolvation temperatures were 120°C and 450°C, respectively. The cone and desolvation gas flow rates were set at 50 l/h and 800 l/h, respectively. Scan event is MS mode (scan range: 100–1,000 Da; scan time: 0.2 s). An aliquot of the extracted sample (3 ml) was injected into an Acquity UPLC BEH C18 reversed-phase column (column size, 2.1 × 100 mm; particle size, 1.7 μm). Mobile phases A and B were used. The column temperature was set at 40°C. The buffer gradient of hysOG analysis consisted of 5.0% to 99.0% B for 0 to 10 min, 99.0% B for 10 to 15 min, 99.0% to 5.0% B for 15 to 15.5 min, and 5.0% B for 4.5 min before the next injection, at a flow rate of 300 μl/min. These data were acquired with the MassLynx software (Waters). The amount of LPC(16:0) was estimated from calibration curves obtained using analytical-grade standard compound. The peak area of [M-H] ± 0.05 Da was divided by the area of the internal standard. This value was used to generate the calibration curves.

Preparation of mouse primary hepatocytes

Mouse hepatocytes were prepared as described previously (23). Briefly, C57BL/6j male mice (wild type and PPARα−/−) were anesthetized with intraperitoneal administration of pentobarbital, and the liver was perfused with liver perfusion medium (Life Technologies Japan Ltd., Tokyo, Japan), followed by liver digestion medium (Life Technologies Japan Ltd.). After filtration through a 100 μm nylon mesh filter, hepatocytes were isolated by repeated centrifugation at 50 g for 3 min (three times). The isolated hepatocytes were cultured in type-1 collagen-coated 12-well plates at a cell density of 2.0 × 10⁶ cells/well. After 3 h incubation at 37°C in 5% CO₂ atmosphere, hepatocytes were cultured in serum-free DMEM with or without bezafibrate, fenofibrate, GW7647, or GW6471 for 24 h. The hepatocytes were used for mRNA quantification and LC/MS assay.

siRNA experiments

Mouse siRNA was chemically synthesized by Qiagen (Tokyo, Japan). Negative control siRNA (Block-it NC siRNA) and lipofectamine 2000 were purchased from Life Technologies Japan Ltd. The primary hepatocytes were seeded in 12-well plates and transfected with 40 nmol/well synthesized siRNA targeting mouse Pla2g7. The primary hepatocytes were transfected with siRNA-lipofectamine complexes and incubated for 12 h at 37°C in 5% CO₂ atmosphere and then used in the experiments.

Cell culture

FAO cell lines from rat liver were cultured in growth medium, DMEM with 5% (v/v) fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO₂ atmosphere. The cells were cultured in serum-free DMEM with or without bezafibrate, fenofibrate, GW7647, or GW6471 for 24 h. The hepatoctyes were used for real-time quantitative RT-PCR analysis. The medium was replaced with serum-free DMEM for 18 h before glucose-uptake experiments.

RAW264.7 macrophage (RAW) cell lines were cultured in a growth medium, DMEM with 10% (v/v) fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO₂ atmosphere. RAW cells were seeded on 12-well plates at a cell density of 3.0 × 10⁶ cells/well for 24 h in growth medium and then serum-free DMEM containing 0.5% BSA for 24 h. The medium was used as RAW-conditioned medium (RAW-CM).

Quantification of mRNA expression levels

Total RNA was prepared from primary hepatocytes, as well as liver and skeletal muscle, using Sepasol (Nacalai Tesque Inc., Kyoto, Japan), according to the manufacturer’s protocols. Using M-MLV reverse transcriptase (Life Technologies Japan Ltd.), total RNA was reverse-transcribed using a thermal cycler (Takara PCR Thermal Cycler SP; Takara Bio Inc., Shiga, Japan). To determine mRNA expression levels, real-time quantitative RT-PCR analysis was performed with a Light Cycler System (Roche Diagnostics) using SYBR green fluorescence signals, as described previously (25, 26). The oligonucleotide primer sets of mouse 36B4 and PPARα target genes were designed using a PCR primer selection program at the website of the Virtual Genomic Center from the GenBank database as follows: mouse Cpt1a (Fwd: 5′-CT-CAG-TGGGAGGCGACTCTCAA′-3′; Rev: 5′-GGGCTCTGTGATTACA-GGAAA′-3′), mouse Cpt1b (Fwd: 5′-CCTGATTGGCCACG-TGGAA′-3′; Rev: 5′-CTGTCAGGCTAGCGGTATCAT′-3′), mouse Aco (Fwd: 5′-GACCACTTCCGCTATGATA′-3′; Rev: 5′-ACGGCATA-TGTACAGACGTAATGTG′-3′), mouse Pla2g6 (Fwd: 5′-CGTGCTTGAAACCTGTGAGCTG′-3′; Rev: 5′-CTGATAAGGCTTTGACGGTA′-3′), mouse Pla2g7 (Fwd: 5′-ATTTCTTTGGAAACCTGTAATGTG′-3′; Rev: 5′-GAAGATTTCTATGCTTTTGAGTA′-3′), mouse Pla2g12b (Fwd: 5′-ATCCGAAGCTGAGGAAAGGTA′-3′; Rev: 5′-TCATGCCTTCTTCTCTCTCC′-3′), and mouse 36B4 as an internal control (Fwd: 5′-TCCCTTCAGAGCTTGCTTGG′-3′; Rev: 5′-GACACCACTCAGAAAGCGA′-3′). All data indicating mRNA expression levels are presented as a ratio relative to a control in each experiment.

Induction of insulin resistance

Two different insulin-resistant adipocyte models were established as previously described with modifications (27, 28). One of these used TNF-α. On the sixth day after induction, the fully differentiated 3T3-L1 adipocytes were treated with serum-free DMEM containing 10 ng/ml recombinant mouse TNF-α (Peprotech, Rocky Hill, NJ) for 18 h. In another method used, RAW-CM 3T3-L1 adipocytes were incubated with control medium (basal medium of serum-free DMEM containing 0.5% BSA) or basal medium conditioned by RAW-CM for 18 h. The 3T3-L1 adipocytes exposed to TNF-α or RAW-CM became insulin resistant, as assessed by the ability of insulin to stimulate glucose uptake. The level of uptake of 2-deoxyglucose (1,2-3H) ([1,2-3H]-2DG) was measured, as previously described (29).

Luciferase assay

 Luciferase assay were performed as previously described, using a GAL4/PPAR chimera system (25, 30). We transfected p4x-UASg-k-luc (a reporter plasmid), pMhPPARα (an expression plasmid for a chimera protein for the GAL4 DNA-binding domain and each human PPAR-ligand-binding domain), and pRL-CMV (an internal control for normalizing transfection efficiency) into monkey CV1 kidney cells by using Lipofectamine (Life Technologies Japan Ltd.), according to the manufacturer’s protocol. Luciferase activity was assayed using the dual luciferase system (Promega, MO) according to the manufacturer’s protocol.
Statistical analyses

Data are presented as the mean ± SEM. Differences between groups were compared with the Student’s t-test (for two groups), Pearson correlation coefficient, and one-way ANOVA, followed by least-significant multiple comparison methods. Values of P < 0.05 were considered statistically significant.

RESULTS

LPC in plasma was increased by PPARα activation

We confirmed that plasma TG was decreased (to ~70% vs. control; Table 1), and the expression of PPARα target genes in the liver was increased by bezafibrate treatment for 4 weeks (Table 1). These data suggest that bezafibrate treatment for 4 weeks was sufficient to activate hepatic PPARα in KK-Ay mice.

To identify the metabolites that were influenced by PPARα activation, we investigated the plasma metabolites profile in mice treated with bezafibrate for 4 weeks by metabolome analysis based on LC/MS and by using metabolite databases, including the Kyoto Encyclopedia of Genes and Genomes and Lipid Maps. We detected 887 peaks of metabolites in mice plasma (data not shown). Metabolome analysis was used to elucidate the patterns of plasma metabolic change in the control versus bezafibrate-fed mice. Differences between these groups were compared with the statistical analysis. This analysis revealed that 27 metabolites including lysoGP species influenced by PPARα activation (Fig. 1A; supplementary Table 1). Metabolome analysis showed that LPC(16:0) was one of the metabolites that was predicted to be upregulated by PPARα activation (C24H50N1O7P1; Fig. 1A; supplementary Table 1). A previous study reported that LPC(16:0) increased adipocyte glucose uptake (31). We confirmed that plasma glucose was decreased (to ~60% vs. control) by bezafibrate treatment (Table 1).

We identified LPC(16:0) using LC/MS (supplementary Fig. 1A–D). In the MS/MS data, we confirmed the fragment ion (m/z 184), which has been reported to be characteristic of LPC (31, 32). We also quantified plasma LPC(16:0) and showed that plasma LPC(16:0) was increased by bezafibrate treatment (control group: 192.45 ± 12.85 µM; bezafibrate: 437.16 ± 29.63 µM; P < 0.001; Fig. 1B). These data suggest that LPC(16:0) in plasma was increased by bezafibrate treatment.

| TABLE 1. Effect of bezafibrate on plasma TG, glucose, and PPARα target gene expression in liver |
|-----------------------------------------------|
| **Group** | **Control** | **Bezafibrate** |
| Body weight (g) | 45.97 ± 0.93 | 44.2 ± 0.59 |
| Food intake (g/day) | 4.09 ± 0.007 | 4.10 ± 0.006 |
| Plasma TG (mg/dl) | 172.11 ± 11.54 | 123.16 ± 7.45 |
| Plasma glucose (mg/dl) | 568.49 ± 33.10 | 349.95 ± 18.73 |
| Cpt1a (% of control) | 100.00 ± 9.91 | 136.56 ± 10.29 |
| Cpt1b (% of control) | 100.00 ± 18.27 | 2,778.23 ± 316.81 |
| Aco (% of control) | 100.00 ± 24.35 | 679.17 ± 95.81 |

Data are mean ± SEM (n = 5–7). Control: HFD; bezafibrate: 0.2% bezafibrate + HFD.

*P < 0.05, versus control.

**P < 0.001 versus control.

Fig. 1. High plasma LPC(16:0) levels in mice activated with PPARα. A: Metabolome analysis of mice plasma treated with bezafibrate for 4 weeks. The values are mean fold-change relative to control. B: Quantitative analysis of plasma LPC(16:0). Data are mean ± SEM (n = 5). *** P < 0.001 versus control. Beza, bezafibrate; Cont, control.

Secretion of LPC(16:0) was increased by PPARα activation in liver

It is well known that PPARα is highly expressed in liver and skeletal muscle (10, 11). To elucidate the source of plasma LPC(16:0) in mice treated with bezafibrate, we quantified LPC(16:0) in these tissues.

Quantitative analysis of LPC(16:0) in control mice tissue revealed that the high concentration of LPC(16:0) was
Fig. 2. Mouse liver induced by PPARα activation produces LPC(16:0). A: Comparative analysis of LPC(16:0) content in mouse liver and skeletal muscle (Sm) treated with or without bezafibrate for 4 weeks (n = 4–7). B: Metabolome analysis of mouse liver treated with bezafibrate for 4 weeks (n = 5). The values are mean fold-change relative to control. C: Comparative analysis of LPC(16:0) content in mouse liver treated with or without fenofibrate for 2 weeks (n = 4). Data are mean ± SEM. * P < 0.05 versus control. Feno; fenofibrate.
in the liver where PPARα is highly expressed (202.61 ± 24.60 ng/mg liver weight, 41.66 ± 2.70 ng/mg skeletal muscle weight; Fig. 2A). We also demonstrated that the concentration of LPC(16:0) was markedly increased by PPARα activation in the liver, compared with skeletal muscle (control group: 202.61 ± 24.60 ng/mg liver weight; bezafibrate: 326.13 ± 5.57 ng/mg liver weight; P < 0.05; Fig. 2A). The metabolome analysis revealed that the concentrations of many lysoGPs in the liver, including LPC(16:0), were increased by bezafibrate treatment (Fig. 2B; supplementary Table 2). We also measured the LPC(16:0) level in mice liver receiving fenofibrate (specific PPARα activator) under SD or HFD eating conditions. These data showed that liver LPC(16:0) was increased by fenofibrate treatment under both SD (control group: 475.87 ± 20.11 ng/mg liver weight; fenofibrate group: 751.51 ± 68.40 ng/mg liver weight; feno = 0.05; Fig. 2C) and HFD (control group: 273.37 ± 37.98 ng/mg liver weight; fenofibrate group: 528.73 ± 28.26 ng/mg liver weight; P < 0.05; Fig. 2C) conditions. We also showed that the amount of liver LPC(16:0) is lower under HFD condition than SD condition (SD group: 475.87 ± 20.11 ng/mg liver weight; HFD group: 273.37 ± 37.98 ng/mg liver weight P < 0.05; Fig. 2C).

To further elucidate the mechanism of regulation of LPC(16:0) production, we analyzed the content of LPC(16:0) in primary hepatocytes treated with bezafibrate for 24 h. The content of LPC(16:0) in primary hepatocytes and culture medium increased in a dose-dependent manner (Fig. 3A, B). The concentration of LPC(16:0) in primary hepatocyte medium increased with GW7647 and fenofibrate (PPARα-specific agonist, respectively) in a dose-dependent manner (Fig. 3C, D). We also confirmed this effect in FAO rat hepatocytes treated with bezafibrate (Fig. 3E). Furthermore, the increase in LPC(16:0) secretion in primary hepatocytes treated with bezafibrate was diminished by GW6471 (PPARα antagonist) treatment (Fig. 3F). These findings show that production and secretion of LPC(16:0) were induced by PPARα activation in hepatocytes.

**Pla2g7 gene expression levels in the hepatocytes were increased by PPARα activation, and the secretion of LPC(16:0) was suppressed by Pla2g7 siRNA treatment**

LPC(16:0) is derived from phosphatidylcholine because of the action of phospholipase A2 (PLA2), which has many subtypes. To determine the main PLA2 subtype contributing to LPC(16:0) production in hepatocytes treated with bezafibrate, we analyzed the mRNA expression of PLA2 subtype in the liver. We determined that Pla2g6, Pla2g7, and Pla2g12b were mainly expressed in the liver and that the mRNA expression of Pla2g7 was markedly elevated (to ~1,500% of the control) by bezafibrate treatment (Fig. 4A). The mRNA expression of Pla2g7 levels in mice liver under SD and HFD eating conditions were also increased (to ~750% and 5,000% of the control group, respectively) by fenofibrate treatment (Fig. 4B). The mRNA expression of Pla2g7 was also increased by PPARα activation in murine primary hepatocytes, and the increase in mRNA expression of Pla2g7 was diminished by GW6471 treatment (Fig. 4C). These findings show that PPARα is involved in the expression level of Pla2g7.

To investigate the contribution of the expression of Pla2g7 to LPC(16:0) production, we analyzed LPC(16:0) production in murine primary hepatocytes treated with control siRNA or Pla2g7 siRNA. LPC(16:0) secretion in hepatocytes was attenuated by Pla2g7 siRNA treatment (control siRNA group: 39.57 ± 1.65 nM; Pla2g7 siRNA group: 29.71 ± 2.76 nM; P < 0.05; Fig. 4D). These data indicate that Pla2g7 contributes to the production and secretion of LPC(16:0) in hepatocytes.
**LPC(16:0) activates PPARα and induces mRNA expressions of PPARα target genes in hepatocytes**

To investigate the effect of *Pla*₈ on PPARα target genes expression, we analyzed the expression of PPARα target genes in murine primary hepatocytes treated with control siRNA or *Pla*₈ siRNA. We confirmed that control siRNA transfection has no effect on the expression of *Pla*₈ (supplementary Fig. 2). The mRNA expressions of *Ac* and *Cp* were attenuated by *Pla*₈ siRNA treatment (to ~60% and 70% vs. control siRNA group, respectively, *P* < 0.05; Fig. 5B, C). In murine primary hepatocytes treated with bezafibrate and *Pla*₈ siRNA, we also observed that upregulated expression of PPARα target gene mRNA induced by bezafibrate treatment was suppressed on *Pla*₈ siRNA treatment (Fig. 5E, F).

We hypothesized that PPARα activation is influenced by the LPC(16:0). To elucidate the function of LPC(16:0) on PPARα in hepatocytes, we investigated whether LPC(16:0) activated PPARα in a luciferase ligand assay. Compared with POPC, which is one of the precursors of LPC(16:0), LPC(16:0) increased luciferase activity remarkably (Fig. 6A). This activity was inhibited by GW6471 treatment (Fig. 6B).

We also demonstrated that the mRNA expression levels in primary hepatocytes of PPARα target genes, such as *Cp* and *Aco*, were increased by LPC(16:0) treatment in a dose-dependent manner (Fig. 6C–E). Furthermore, the effect of LPC(16:0) on the expression of PPARα target gene disappeared in PPARα KO primary hepatocytes (Fig. 6F–H). These findings indicate that LPC(16:0) has the ability to reinforce PPARα activation.

**LPC(16:0) stimulates glucose uptake in insulin-resistant 3T3-L1 adipocytes**

It is well recognized that LPC(16:0) increases adipocyte glucose uptake (31). We also confirmed the LPC(16:0) capacity to promote glucose uptake in 3T3-L1 adipocytes in a dose-dependent manner and LPC(16:0) had an additive effect increasing glucose uptake in 3T3-L1 adipocytes cotreated with insulin and LPC(16:0) (data not shown). We hypothesized that LPC(16:0) has the ability to promote glucose uptake under conditions of insulin resistance. To elucidate the function of LPC(16:0), we investigated whether it stimulates glucose uptake in insulin-resistant adipocytes. We revealed that LPC(16:0) has the ability to recover glucose uptake in insulin-resistant adipocytes, which were induced by TNF-α or RAW-CM (Fig. 8A, B). Furthermore, we analyzed a correlation between the concentration of plasma glucose and LPC(16:0) in mice treated with or without bezafibrate for 4 weeks. The results showed...
strong correlation between plasma glucose and LPC(16:0) \((r = -0.8335, P = 0.0027; \text{Fig. 8C})\). These findings suggest that LPC(16:0) is capable of recovering glucose uptake in insulin-resistant adipocytes, which raises the possibility that it contributes to the decrease in the plasma glucose level.

**DISCUSSION**

In this study, metabolome analysis revealed that many types of plasma lysoglycerophosphocholines were increased by PPARα activation. Lysoglycerophosphocholines have been found in a wide range of tissues and cell types (33). It has also been reported that lysoglycerophosphocholines play an important role in many physiological and pathophysiological processes (33–36). Lysoglycerophosphocholines are well known to be lipid mediators (autacoid) that have topical effects on cells. However, our data showed that lysoglycerophosphocholines have not only topical but also whole-body effects throughout the bloodstream. LPC(16:0) was one of the lysoglycerophosphocholines that was increased by PPARα activation in the plasma. Previous studies have demonstrated that LPC(16:0) is a major component of lysoglycerophosphocholines in plasma and activates glucose uptake in adipose tissue (31, 37). Therefore, we focused here on LPC(16:0) and investigated the relationship between LPC(16:0) and PPARα activation.

Liver tissue is important in lipid metabolism. Although the lipidomics approach revealed in detail the hepatic lipid profiling including that of TGs, phospholipids, and cholesterol esters (21, 38), little is known about the relationship between PPARα activation and LPC(16:0) production. In this study, we demonstrated that the activation of PPARα induced the upregulation of many types of lysoglycerophosphocholine including LPC(16:0) production. Quantitative analysis of LPC(16:0) showed that LPC(16:0) was markedly increased in the liver, which expresses PPARα to a great extent. In the murine primary hepatocytes, secretion of LPC(16:0) was induced by PPARα agonist (bezafibrate, fenofibrate, and GW7647) treatment and suppressed by PPARα antagonist. These findings suggest that synthesis of LPC(16:0) in hepatocytes is induced by PPARα activation and that secretion of LPC(16:0) from hepatocytes contributes to elevated plasma LPC(16:0) concentration. Our metabolomic data also showed that not only LPC(16:0) but also many other lysoglycerophosphocholines were changed by bezafibrate treatment. In particular, lysophosphatidylethanolamine (LPE) (20:4), LPC(18:2), LPC(20:4), LPE(16:0), LPE(18:1), and LPC(18:1) were changed both plasma and liver. These findings suggest that the source of these lysoglycerophosphocholines in plasma is liver.

LPCs are mainly generated from PLA2-catalyzed hydrolysis of phosphatidylcholine (37, 39). The various subtypes of PLA2, which have different localizations, individually regulate synthesis of LPC (37). In the present study, we showed that the mRNA expression of Pla2g7 in murine liver or primary hepatocytes was elevated by PPARα activation. Furthermore, we also showed that LPC(16:0) secretion in hepatocytes was attenuated by Pla2g7 siRNA treatment. We demonstrated for the first time that LPC(16:0) is induced by PPARα activation via Pla2g7-dependent pathway in the liver. Interestingly, the present study revealed that the expression of PPARα target genes was suppressed by Pla2g7 siRNA treatment and that LPC(16:0) is not only induced by PPARα activation but also has the ability to activate PPARα and upregulates the mRNA expression of PPARα target genes. These findings raise the possibility...
is metabolized in cells. These findings raise the possibility that LPC(16:0) activates PPARα both directly and indirectly.

Adipose tissue plays a key role in glucose homeostasis (40). A preceding study has indicated that LPC(16:0) stimulates glucose uptake in 3T3-L1 adipocytes (31). It is well recognized that obesity is characterized by chronic inflammation of adipose tissues and this inflammation contributes to the development of insulin resistance and type 2 diabetes (41, 42). Notably, macrophages contribute significantly to inflammation in adipose tissue (43). TNF-α is the major proinflammatory cytokine (44). Previous studies have reported that TNF-α, which is secreted by macrophages, induces additional infiltration of macrophages into adipose tissues (44–47). LPC(16:0) can increase glucose uptake in an insulin-independent manner (31). We hypothesize that LPC(16:0) has an ability to promote glucose uptake under conditions of insulin resistance. We revealed that LPC(16:0) has the ability to improve glucose uptake in insulin-resistant adipocytes induced by RAW-CM or TNF-α. We demonstrated for the first time that the ability of LPC(16:0) to induce glucose uptake is unaffected by

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**Fig. 6.** LPC(16:0) activated PPARα and PPARγ target gene expression. A, B: Evaluation of PPARα activity in luciferase reporter assay (n = 5). Effect of LPC(16:0) on *Cpt1a* (C), *Cpt1b* (D), and *Aco* (E) PPARα target gene expression in wild-type primary hepatocytes (n = 3–4). F–H: Effect of LPC(16:0) on PPARα target gene expression in PPARα−/− primary hepatocytes (n = 6). Data are mean ± SEM. LPC, LPC(16:0).

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**Fig. 7.** The quantification analysis of LPC(16:0) in the medium and cell-associated LPC(16:0) at 24 h after addition of LPC(16:0). A: The concentration of LPC(16:0) in the medium including (black line) or not including hepatocytes (broken line). B: The cell-associated LPC(16:0) treated with or without LPC(16:0). Data are mean ± SEM (n = 6). *** P < 0.001 versus LPC medium only or control.
insulin resistance. This effect raises the possibility that LPC(16:0) contributes to ameliorating hyperglycemia. Many previous studies indicate that the levels of LPCs and inflammation have crucial relevance. LPCs have been demonstrated to have both proinflammatory (48, 49) and anti-inflammatory (35, 50) effects. Therefore, additional work is required to completely determine the relationship between LPCs and inflammation.

It is interesting to note that fibrates, including bezafibrate, contribute to the improvement of not only dyslipidemia but also glucose metabolism disorder (18–20). However, the molecular basis of this effect remains unexplained. Excessive free fatty acids, particularly stearic acid (SA) and palmitic acid (PA), are also proinflammatory factors and induce insulin resistance (51, 52). Bezafibrate greatly facilitates lipid metabolism, thereby anticipating the reduction of SA or PA. However, our previous study showed that bezafibrate has no apparent effect on plasma SA and PA (53). The evidence suggested that SA or PA were not involved in the improvement of hyperglycemia in KK-Ay mice treated with bezafibrate. We hypothesize that the ability of LPC(16:0) to increase glucose uptake in adipocytes improves hyperglycemia during treatment with bezafibrate. To validate this hypothesis, we analyzed a correlation between plasma LPC(16:0) and plasma glucose in mice treated with or without bezafibrate for 4 weeks. The previous studies showed that plasma glucose level is reduced by fenofibrate (55) or Wy-14,643 [PPARα specific activator, (56)] treatment. The results of the previous studies support our findings. These data suggested that LPC(16:0) produced in liver by PPARα activation participates in the regulation of plasma glucose level.

In conclusion, metabolomics has revealed the upregulated LPC(16:0) in mice plasma and liver following bezafibrate treatment. PPARα activation induces the expression of PPARα target genes in hepatocytes. We also showed that LPC(16:0) has the ability to recover glucose uptake in insulin-resistant adipocytes. The data presented herein suggested that LPC(16:0) induced by PPARα activation improved dyslipidemia and hyperglycemia.

Note added in proof

The authors Rieko Nakata and Hiroyasu Inoue were inadvertently left out of the author list of the accepted version of this article. All other authors and the Journal’s Editors-in-Chief approved the addition after the article was in proof stage. Drs. Nakata and Inoue will appear as authors in all forms of the article except in the originally accepted Paper in Press.

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