Gene Deletion of Calcium-Independent Phospholipase A$_{2}\gamma$ (iPLA$_{2}\gamma$) Suppresses Adipogenic Differentiation of Mouse Embryonic Fibroblasts

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Adipogenic differentiation is a complex process by which fibroblast-like undifferentiated cells are converted into cells that accumulate lipid droplets. We here investigated the effect of gene deletion of calcium-independent phospholipase A$_{2}\gamma$ (iPLA$_{2}\gamma$), a membrane-bound PLA$_{2}$ enzyme, on adipogenic differentiation in mice. Since iPLA$_{2}\gamma$ knockout (KO) mice showed reduced fat volume and weight, we prepared mouse embryonic fibroblasts (MEF) from wild-type (WT) and iPLA$_{2}\gamma$ KO mice and examined the effect of iPLA$_{2}\gamma$ deletion on in vitro adipogenic differentiation. iPLA$_{2}\gamma$ increased during adipogenic differentiation in WT mouse-derived MEFs, and the differentiation was partially abolished in iPLA$_{2}\gamma$ KO-derived MEFs. In KO-derived MEFs, the inductions of peroxisome proliferator activator receptor $\gamma$ (PPAR$\gamma$) and CAAT/enhancer-binding protein $\alpha$ (C/EBP$\alpha$) were also reduced during adipogenic differentiation, and the reductions in PPAR$\gamma$ and C/EBP$\alpha$ expressions and the defect in adipogenesis were restored by treatment with troglitazone, a PPAR$\gamma$ ligand. These results indicate that iPLA$_{2}\gamma$ might play a critical role in adipogenic differentiation by regulating PPAR$\gamma$ expression.

Key words phospholipase A$_2$; adipogenesis; adipose differentiation; peroxisome proliferator activator receptor $\gamma$

INTRODUCTION

Adipocytes play important roles in the control of lipid homeostasis and energy balance by storing and mobilizing triacylglycerol. Increasing the number and size of adipocytes can result in an expansion of the white adipose tissue (WAT) mass. The adipocyte maturation process is called adipogenesis, and involves the differentiation of fibroblast-like preadipocytes into mature adipocytes. Adipogenic differentiation is a complex process associated with coordinated changes in gene expression, cell morphology, and hormone sensitivity. Several key transcription factors involved in this process have been identified: peroxisome proliferator activator receptor $\gamma$ (PPAR$\gamma$), CAAT/enhancer-binding proteins (C/EBPs), and sterol regulatory element-binding protein (SREBP) all play central roles in adipogenic differentiation. Bioactive lipid mediators such as prostaglandins (PGs) and lysophosphatidic acid (LPA) are also involved in the regulation of adipogenic differentiation. PGE$_{2}$, PGF$_{2\alpha}$, and LPA inhibit adipogenesis, whereas PGD$_{2}$ and its metabolite, 15-deoxy-\(\Delta^{12,14}\)-PGJ$_{2}$, bind to PPAR$\gamma$ as a ligand and promote adipogenesis. Prostacyclin (PGI$_{2}$) affects the induction of adipogenic differentiation. Phospholipase A$_{2}$ (PLA$_{2}$) catalyzes the cleavage of the sn-2 position of glycerophospholipids to yield free fatty acids and lysophospholipids, and it has an important role in membrane remodeling as well as lipid mediator signaling. To date, approximately 55 types of PLA$_{2}$ enzymes have been identified and divided into several families: secretory PLA$_{2}$ (sPLA$_{2}$), calcium-dependent cytosolic PLA$_{2}$ (cPLA$_{2}$), calcium-independent PLA$_{2}$ (iPLA$_{2}$), platelet-activating factor acetyl hydrolases (PAF-AHs), lysosomal PLA$_{2}$ and adipose PLA$_{2}$s. The iPLA$_{2}$ family is also referred to as the patatin-like phospholipase domain-containing protein (PNPLA) family, since its members share homology with patatin, a lipid acyl hydrolase in plants.

Among the iPLA$_{2}$ isozymes, independent phospholipase A$_{2}\gamma$ (iPLA$_{2}\gamma$/PNPLA8) is a membrane-associated protein that contains four methionine residues that can potentially encode 88, 77, 74 and 63 kDa polypeptides. iPLA$_{2}\gamma$ exhibits reduced lipid accumulation in adipose tissue even when fed a normal-fat diet. Moreover, Su et al. reported that knockout of iPLA$_{2}\gamma$ in 3T3-L1 fibroblast cells inhibited hormone-induced adipogenic differentiation. However, the involvement of iPLA$_{2}\gamma$ in adipogenesis has not been fully elucidated.

In the present study, we investigated the effects of iPLA$_{2}\gamma$ gene deletion on the adiposity of mice in vivo, and we then isolated mouse embryonic fibroblasts (MEFs) from iPLA$_{2}\gamma$-KO mice and examined their phenotypes, including in vitro adipogenic differentiation. The results revealed that the gene deletion of iPLA$_{2}\gamma$ suppressed the hormone-induced adipogenic differentiation of MEFs and their lipid accumulation. The inductions of C/EBP$\alpha$, C/EBP$\beta$, C/EB$\delta$ and PPAR$\gamma$, which are adipogenic differentiation markers, were attenuated in iPLA$_{2}\gamma$-KO MEFs. These results indicated that iPLA$_{2}\gamma$ might play a critical role in adipogenic differentiation by regulating the expression of PPAR$\gamma$. © 2020 The Pharmaceutical Society of Japan
MATERIALS AND METHODS

Mice The detail of the construction of the pnpla8−/− (iPLA2γ-KO) mice were described previously.17 All mice were housed in climate-controlled (21°C) specific pathogen-free facilities with a 12-h light-dark cycle, with free access to standard laboratory food (Picolab mouse diet 20; Laboratory Diet, Brentwood, MO, U.S.A.) and water. All procedures involving animals were performed under approved institutional guidance.

Computed Tomography (CT) Analysis The fat volume was analyzed in the 2- to 4-month-old wild-type (WT) and iPLA2γ KO mice using a CT system (eXplore Locus, GE Healthcare, London, ON, Canada). Mice were anesthetized with 2% isoflurane (Dainippon Sumitomo Pharmaceutical, Osaka, Japan) and scanned for 10 min under the following conditions: resolution power, 93 µm; view number, 400; voltage, 80 kVp; and electric current, 450 µA. CT images were analyzed using MicroView 2.0 software (GE Healthcare, U.S.A.).

Histopathology For histopathology, tissue sections were fixed in 10% formalin, embedded in paraffin, and stained with hematoxylin-eosin by a standard method. For Oil Red O staining, the fixed cells were treated with 60% isopropyl alcohol (Wako) for 1 min and then stained with 0.3% Oil Red O in 60% isopropyl alcohol solution for 10 min. The stained adipocytes were extracted with 1 mL of 4% Igepal (Axis-Shield) and left to dry. We first evaluated Oil Red O staining with an (ESI)-MS/MS spectrometer (QTRAP5500, AB Sciex, Framingham, MA, U.S.A.). The quantitation of prostanoids was performed using LC-MS/MS via multiple-reaction monitoring in negative-ion mode, as described previously.23

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Immunoblotting Tissue homogenates or cell lysates (10 µg protein equivalents) were subjected to SDS-PAGE using 7.5, 12 or 15% gels under reducing conditions. The separated proteins were electroblotted onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH, U.S.A.) with a semidy blotter (Bio-Rad Laboratories, Hercules, CA, U.S.A.) according to the manufacturer’s instructions. After blocking with 5% (w/v) skim milk in 10 mM Tris–HCl, pH 7.4, containing 150 mM NaCl and 0.05% Tween 20, the membranes were probed with the respective antibodies (1:5000 dilution) for 2 h, followed by incubation with horseradish peroxidase-conjugated anti-mouse immunoglobulin G (G) (IQG (1:5000 for PPARγ) and anti-rabbit IgG (1:5000 for C/EBPα, C/EBPβ, C/EBPδ, and iPLA2γ). After being washed, the membranes were visualized with Western Lightning Chemiluminescence Reagent Plus (PerkinElmer, Inc. Life Sciences, Boston, MA, U.S.A.). Antibodies against PPARγ, C/EBPα, C/EBPβ and C/EBPδ were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Rabbit polyclonal antibody against human iPLA2γ were prepared as described previously.17

Quantitative RT-PCR (Q-PCR) RNA extraction, cDNA synthesis and Q-PCR were carried out according to standard protocols in our laboratory, as previously described.17 The primer pairs were 5′-GCCACCAAACTTGGGAATC-3′ and 5′-TGCAAGTTGCTTCCAATCAAC-3′ for mouse Tgpp; 5′-GAGCTCTCATGCTCTGTGGAAAGA-3′ and 5′-CTCGTCTACAGCAATCTC-3′ for mouse mcd36; 5′-CGGGCCCAATCTTACCTC-3′ and 5′-AGGTTGGAGTGCTGAAGCAAA-3′ for mouse ap2; and 5′-TTCGTTATTTGGCAGGTAGA-3′ and 5′-CTTGGCTCTGGTTTTT-3′ for mouse i8s ribosomal RNA.

Statistics The data were statistically evaluated by unpaired Student’s t-test or Tukey’s test, and p-values < 0.05 were accepted as significant.

RESULTS AND DISCUSSION

The iPLA2γ-Deficient Mice Showed Reduced Adiposity Since our previous research demonstrated that iPLA2γ-KO mice exhibited lower body weights than WT mice, we examined the abdominal fat volume by performing a CT scan analysis. The results revealed that the total fat volume and the volumes of visceral fat and subcutaneous fat in the KO mice were significantly lower than those in their WT littermates (Fig. 1A). The weights of white and brown fat tissues were also reduced in the KO mice (Fig. 1B). Hematoxylin-eosin staining and Oil Red O staining of fat tissues demonstrated that the sizes of the white and brown adipocytes in the
Fig. 1. Reduction of the Fat Amount in iPLA\textsubscript{2}\textgreek{g}-Deficient Mice

\textbf{A}: CT images of abdominal fat in WT (a) and iPLA\textsubscript{2}\textgreek{g} KO (b) mice at 4 months of age. \textit{Yellow areas} represent visceral fat. (c) Visceral and subcutaneous fat volumes of WT (white bars) and KO mice (black bars) revealed by a CT scan analysis. Quantitative data are means ± standard deviation (S.D.). * \(p < 0.05\) vs. WT (\(n = 6\)).

\textbf{B}: Weights of white adipose tissue (WAT) and brown adipose tissue (BAT) in WT (white bar) and iPLA\textsubscript{2}\textgreek{g} KO (black bar) mice (left). The ratio of WAT or BAT weight to total body weight (right). Quantitative data are means ± S.D. * \(p < 0.05\) vs. WT (\(n = 6\)).

\textbf{C}: Histological analysis of WAT and BAT from WT and iPLA\textsubscript{2}\textgreek{g} KO mice. a, b, e, f: Hematoxylin eosin staining. c, d, g, h: Oil Red O staining. Scale bar: 100\,\mu m.

Fig. 2. Suppression of Adipogenic Differentiation in iPLA\textsubscript{2}\textgreek{g}-Deficient MEFs

\textbf{A}: Staining of cells induced by an adipogenic inducer with Oil Red O to visualize the extent of adipose conversion. (a) Representative whole well images of Oil Red O staining. (b) Representative magnified images. The scale bar is 100\,\mu m.

\textbf{B}: (a) The number of Oil Red O positive cells per field of view (\(n = 3\)). (b) Quantification of Oil Red O staining (\(n = 4\)). Data are means ± S.D. * \(p < 0.05\).
iPLA₂γ-KO mice were reduced by more than 60% relative to those in the WT mice (Fig. 1C). These results suggested that iPLA₂γ might be involved in adipose differentiation.

**iPLA₂γ Deficiency Prevented the Adipogenic Differentiation of MEFs**

To investigate the involvement of iPLA₂γ in adipogenesis, we next employed an adipogenic differentiation system from MEFs in vitro and examined the effects of iPLA₂γ deficiency on the differentiation. In our system, MEFs were prepared from 13.5-d embryos of WT or iPLA₂γ-KO mice, and primed with a differentiation-inducing cocktail (containing insulin, dexamethasone and IBMX) for 2 d, followed by treatment with insulin for an additional 7 d. The adipose differentiation status was confirmed by staining with Oil Red O (Fig. 2A).

As shown in Fig. 3A, the level of iPLA₂γ protein was somewhat down-regulated by the induction of adipogenic differentiation, and then began to increase 4 d after the induction. iPLA₂γ was increased during adipogenic differentiation and was markedly expressed in the differentiated adipocytes. We further observed that the in vitro adipogenic differentiation of MEFs was suppressed by iPLA₂γ deficiency (Fig. 2A).

The number of Oil Red O-stained cells differentiated from KO mouse-derived MEFs was significantly lower than that from WT MEFs (Fig. 2B). Su et al. also reported that iPLA₂γ expression was induced during a hormone-induced differentiation of 3T3-L1 cells into adipocytes, and that the knockdown of iPLA₂γ inhibited this adipogenic differentiation. Taken together, these results indicate that iPLA₂γ might be critical for the differentiation of fibroblasts into adipocytes after hormone stimulation.

Several prostanoids, such as PGE₂, PGF₂α, PGD₂ and PGI₂, are known to be involved in the regulation of adipogenic differentiation. We further investigated whether iPLA₂γ gene deletion affected the production of prostanoids from MEFs. As shown in Fig. 3B, the amounts of PGE₂, PGF₂α and PGD₂ in culture media from MEFs were increased during adipogenic differentiation. In addition, the amount of 6-ketoPGF₁α (a stable metabolite of PGI₂) was down-regulated by the induction and then began to increase 4 d after the induction. The alteration in PGI₂ production was correlated with that in iPLA₂γ expression during adipogenic differentiation. However, these prostanoid productions were not affected by...
iPLA2γ gene deletion.

**iPLA2γ Deficiency Inhibited the PPARγ Expression and Activation during the Differentiation of MEFs into Adipocytes**

We further examined the effects of iPLA2γ deficiency on the expression of adipocyte-related genes during adipogenesis. The expressions of the adipocyte-related genes, aP2 and CD36, were also suppressed in iPLA2γ-KO mouse-derived cells (Fig. 4A). Since the expressions of both aP2 and CD36 is regulated by the transcriptional factor PPARγ,24,25) iPLA2γ might be involved in the regulation of PPARγ transcriptional activity during adipogenic differentiation. In fact, we observed that the expression levels of PPARγ were also reduced in the iPLA2γ KO mice (Figs. 3A, 4A).

It has been reported that during the adipogenic differentiation of fibroblasts, the expression of C/EBPβ and C/EBPδ is induced at the early stage of adipogenesis, and then C/EBPβ and C/EBPδ elicit the expression of PPARγ, which in turn leads to the induction of C/EBPα.24) Therefore, we next investigated the effect of iPLA2γ deficiency on the expressions of these C/EBPs. In WT mouse-derived MEFs, the C/EBPβ and C/EBPδ levels were up-regulated along with the induction of iPLA2γ, and then C/EBPα and PPARγ were increased. The induction of C/EBPβ and C/EBPδ at the early stage of adipogenic differentiation was not affected in the iPLA2γ KO mouse-derived MEFs, but the induction of C/EBPα and PPARγ at the late stage was reduced in the KO cells (Fig. 3A). These results suggest that iPLA2γ is involved in a late stage of the differentiation.

To further reveal whether the suppressive effect of iPLA2γ deficiency on adipogenic differentiation was caused by a suppression of PPARγ activity, we next examined the effects of two PPARγ agonists, troglitazone and rosiglitazone, on the adipogenesis of iPLA2γ KO MEFs. In this experiment, MEFs prepared from iPLA2γ KO embryos were primed with...
a differentiation-inducing cocktail and the PPARγ agonists, troglitazone or rosiglitazone for 2 d, and then the retention of Oil Red O was quantified. As shown in Fig. 4B, treatment with troglitazone or rosiglitazone rescued the adipogenic differentiation of iPLAγ-deficient MEFs. The expressions of the PPARγ target genes aP2 and CD36 in the KO MEFs were also up-regulated by troglitazone treatment.

We next investigated the effect of troglitazone treatment on the expression levels of transcriptional factors involved in adipogenesis in iPLAγ KO mouse-derived MEFs. The induction of C/EBPβ and C/EBPδ at the early stage of adipogenesis was not affected by troglitazone, but the expression of C/EBPα was increased. The induction of C/EBPα might thus be mediated by PPARγ activation. On the other hand, the PPARγ expression was not changed by the troglitazone treatment. These results indicated that iPLAγ might be involved in PPARγ activation as well as in PPARγ-independent PPARγ expression. Our findings also suggested that in iPLAγ KO MEFs, the defect of PPARγ activation might be critical for its impairment in adipogenic differentiation.

In the present study, we showed that gene deletion of iPLAγ suppressed the adipogenic differentiation of MEFs. As described above, Su et al. also reported that the knockdown of iPLAγ expression inhibited a hormone-induced differentiation of 3T3-L1 cells into adipocytes.20) It was further shown that the suppression of the adipogenic differentiation of both MEFs and 3T3-L1 cells was rescued by the addition of PPARγ agonists. These results indicated that iPLAγ produces certain lipid mediators that act as active ligands of PPARγ to induce adipogenic differentiation. Several bioactive lipids have been described as PPARγ ligands. It was shown that PGD2 and its metabolite, 15-deoxy-Δ12,14-PGJ2, bind to PPARγ as a ligand and promote adipogenesis.8,9) Fujimori et al. reported that lipocalin-type PGD synthase (L-PGDS), which is involved in PGD2 synthesis, was increased at a late stage of the adipogenic differentiation of 3T3-L1 cells and L-PGDS knockdown suppressed the differentiation of these cells into adipocytes.26)

However, as shown in Fig. 3B, PGD2 production during the adipogenic differentiation of MEFs was not affected by iPLAγ gene deletion. Although the possibility that an iPLAγ-mediated production of PGD2 and its metabolite in an intracellular specific site of MEFs plays an important role in adipogenic differentiation cannot be excluded, other bioactive lipids the productions of which are mediated via iPLAγ might be critical for adipogenesis. iPLAγ has been shown to mediate various types of lipid mediators other than prostanoids.14) It was reported that iPLAγ is involved in the production of atypical eicosanoid-lysolipids.27) Such novel types of lipids might regulate adipogenesis. Further studies are needed to clarify the involvement of iPLAγ in adipogenic differentiation.

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Conflict of Interest The authors declare no conflict of interest.

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