# Roles of Prostaglandin EP4 Receptor in Adipocytes

# (脂肪細胞におけるプロスタグランジン EP4 受容体の機能解析)

2013

Tomoaki Inazumi

(稲住　知明)

# CONTENTS

## GENERAL INTRODUCTION

Prostanoids, including prostaglandin (PG) $D_2$, $PGE_2$, $PGF_{2\alpha}$, prostacyclin ($PGI_2$), and thromboxane (TX) $A_2$, are produced from arachidonic acid (AA) by the sequential actions of cyclooxygenase (COX) and respective synthases. Prostanoids are formed and released in response to various stimuli, and function in a paracrine or autocrine manner in the vicinity of the cells producing these mediators. Prostanoids act on their cognate receptors on the surface of target cells to exert their actions (Coleman et al. 1994; Narumiya et al. 1999). There are eight types and subtypes of prostanoid receptor, designated PGD receptor (DP), EP1, EP2, EP3, and EP4 subtype of PGE receptor, PGF receptor (FP), PGI receptor (IP), and $TXA_2$ receptor (TP), all of which are G protein-coupled receptors (GPCRs). Upon agonist stimulations, DP, EP2, EP4, and IP elicit a rise in the concentration of intracellular cyclic adenosine monophosphate

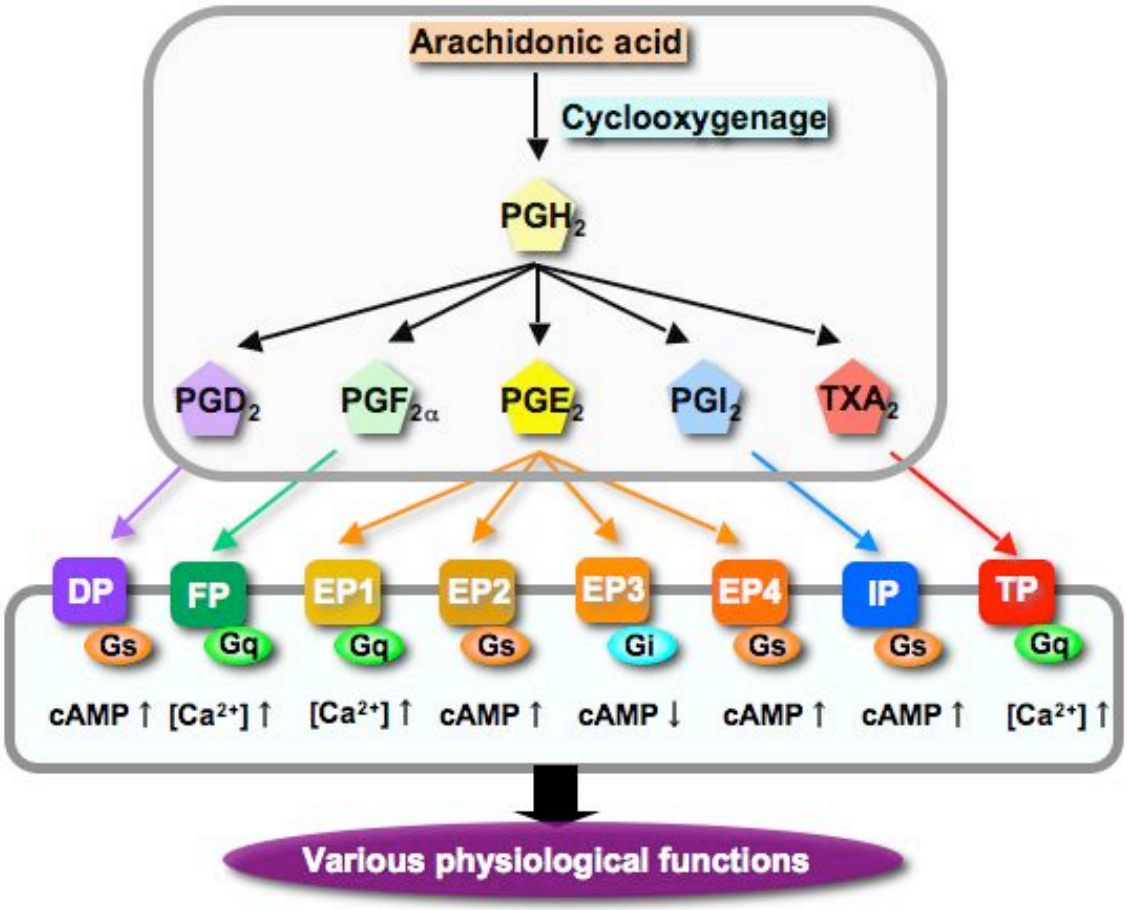


**Fig. 0-1. PG synthesis and cognate receptor signaling.**

(cAMP) via Gs, EP1, FP, and TP elicit a rise in the concentration of cytosolic free calcium ion via Gq, and EP3 elicits a decrease in the concentration of intracellular cAMP via Gi (Sugimoto et al. 1994) (Fig. 0-1).

Adipose tissue is a complex organ that regulates and coordinates energy homeostasis. It is primarily composed of adipocytes. In addition to adipocytes, adipose tissue contains the stromal vascular fraction (SVF) of cells including preadipocytes, fibroblasts, vascular endothelial cells, and immune cells (Cinti 2005). Although adipose tissue was originally thought to just be an energy storage site, recent studies have revealed that it carries out key endocrine functions. Therefore, dysfunction of the adipose compartment leads to the pathology associated with metabolic diseases such as obesity and type-2 diabetes.

There are two types of adipose tissue, white adipose tissue (WAT) and brown adipose tissue (BAT). WAT is characterized by adipocytes containing large unilocular lipid droplets. WAT is the main type of adipose tissue found in adult humans and is distributed throughout the body in subcutaneous regions, surrounding visceral organs and in the face. Despite their histological similarities, subcutaneous and visceral adipose tissues are thought to have distinct metabolic functions. Indeed, accumulation of visceral fat is correlated with pathologic inflammation and insulin resistance (Hamdy et al. 2006), whereas subcutaneous adipose tissue improves glucose tolerance (Tran et al. 2008).

By contrast, BAT participates in thermogenesis and is located mainly around the neck. BAT is histologically distinct from WAT; it is composed of multiloculated adipocytes that contain large number of mitochondria. For many years, BAT was thought to be absent in human adult. However recent studies have identified functional BAT in healthy adults (Virtanen et al. 2009; Cypess et al. 2009).

White and brown adipocytes, as well as osteoblasts, myocytes and chondrocytes, differentiate from mesenchymal stem cells (MSCs) (Gesta et al. 2007). Adipogenesis can be divided into two phases: commitment and terminal differentiation. The differentiation of committed preadipocytes into adipocytes requires the induction of the master adipogenic regulator, peroxisome proliferator-activated receptor γ (PPARγ). Differentiated adipocytes store lipids in the form of triglycerides, and function as mature adipocytes (Fig. 0-2).

It has been shown that COX products, such as $PGE_2$, are abundantly produced

by adipocytes and regulate differentiation of adipocytes and their functions (Curtis-Prior 1975). However, it remains unknown which or how PG receptor modulate adipocyte development and function.

In this study, I first employed an adipocyte differentiation system from mouse embryonic fibroblasts (MEFs), and found that $PGE_2$-EP4 signaling suppresses the early stage of adipocyte differentiation in an autocrine manner (Chapter 1). Next I examined appearance and function of adipose tissues in EP4-deficient mice and found that $PGE_2$-EP4 signaling negatively regulates lipid accumulation in adipocytes at least in part by suppressing insulin signaling (Chapter 2).

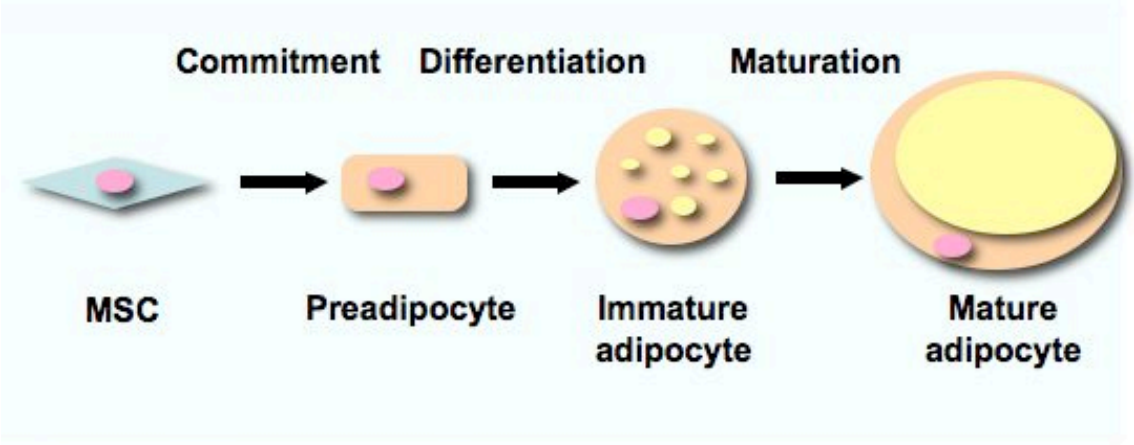


**Fig. 0-2. The course of adipogenesis.**

## ABBREVIATIONS

| | |
|---|---|
| AA | arachidonic acid |
| ATGL | adipose triglyceride lipase |
| BAT | brown adipose tissue |
| cAMP | cyclic adenosine monophosphate |
| cDNA | complementary deoxyribonucleic acid |
| COX | cyclooxygenase |
| DIC | differentiation-inducing cocktail |
| DMEM | Dulbecco's modified Eagle's medium |
| DP | prostaglandin D receptor |
| ELISA | enzyme-linked immunosorbent assay |
| EP | prostaglandin E receptor |
| EP1 | prostaglandin E receptor subtype 1 |
| EP2 | prostaglandin E receptor subtype 2 |
| EP3 | prostaglandin E receptor subtype 3 |
| EP4 | prostaglandin E receptor subtype 4 |
| FBS | fetal bovine serum |
| FFA | free fatty acid |
| FP | prostaglandin F receptor |
| GPCR | G protein-coupled receptor |
| HSL | hormone sensitive lipase |
| IBMX | isobutylmethylxanthine |
| IP | prostaglandin I receptor |
| IRF | interferon regulatory factor |
| KO | knock out |
| LPL | lipoprotein lipase |
| MEF | mouse embryonic fibroblast |
| mRNA | messenger ribonucleic acid |
| MSC | mesenchymal stem cell |
| NOS | nitric oxide synthase |
| NPR | natriuretic peptide receptor |
| PCR | polymerase chain reaction |

| | |
|---|---|
| PG | prostaglandin |
| PKA | protein kinase A |
| $PLA_2$ | phospholipase $A_2$ |
| PPARγ | peroxisome proliferator-activated receptor γ |
| PVDF | polyvinylidene difluoride |
| RT | reverse transcription |
| SDS | sodium dodecyl sulfate |
| SEM | standard error mean |
| SVF | stromal vascular fraction |
| TG | triglyceride |
| TP | thromboxane receptor |
| UCP | uncoupling protein |
| WAT | white adipose tissue |
| WT | wild-type |

## Chapter 1: Endogenous Prostaglandin $E_2$-EP4 Signaling Suppresses Adipocyte Differentiation in Mouse Embryonic Fibroblasts.


## Abstract

The prostaglandin (PG) receptors EP4 and FP have the potential to exert negative regulation of adipogenesis, but the exact contribution of endogenous PG-driven receptor signaling is not fully understood. In this study, I employed an adipocyte differentiation system from mouse embryonic fibroblasts (MEFs), and compared the effects of each PG receptor-deficiency on adipocyte differentiation. In wild-type (WT) MEFs, inhibition of endogenous PG synthesis by indomethacin augmented the differentiation, and exogenous $PGE_2$ as well as an FP-agonist reversed the effect of indomethacin. In EP4-deficient cells, basal differentiation was up-regulated to the levels in indomethacin-treated WT cells and indomethacin did not further enhance differentiation. Differentiation in FP-deficient cells was equivalent to WT and was still sensitive to indomethacin. $PGE_2$ or indomethacin treatment of WT MEFs for the first two days was enough to suppress or enhance differentiation. Differentiation stimuli induced COX-2 gene and protein expression, as well as $PGE_2$ production in WT MEFs. These results suggest that $PGE_2$-EP4 signaling suppresses the earliest stage of adipocyte differentiation in an autocrine manner, and FP-mediated inhibition is not directly involved in adipocyte differentiation in the MEF system.

## Introduction

Adipogenesis is a crucial aspect in controlling body fat mass (Gregorie et al. 1998; Rosen et al. 2000). Adipose tissue is not only important for energy storage but is also an endocrine organ that regulates energy homeostasis by secreting various adipokines, such as cytokines, chemokines, growth factors and lipid mediators (Matsuzawa 2006). The presence of receptors for adipokines in preadipocytes and adipocytes has been shown, suggesting that secreted adipokines have autocrine effects and regulate their own differentiation and functions (Karastergiou et al. 2010). Although it has been shown that a number of factors including adipokines regulate adipogenesis in various settings, most of the evidence comes from supra-physiological or pharmacological doses of these molecules to elicit a response. Hence, their physiological significance in local milieu has not been established.

It has been shown that COX products, such as $PGE_2$ and $PGF_{2\alpha}$, inhibit adipocyte development (Curis-Prior 1975; Casimir et al. 1996; Kim et al. 2000). Indeed, We previously identified that $PGE_2$-EP4 signaling suppresses adipocyte differentiation from 3T3-L1 preadipocytes (Tsuboi et al. 2004; Sugimoto et al. 2004). In contrast, $PGF_{2\alpha}$ has also been shown to suppress adipocyte differentiation from 3T3-L1 preadipocytes via the FP receptor (Casimir et al. 1996). Thus, both $PGF_{2\alpha}$ and $PGE_2$ have the potential to suppress adipogenesis through FP and EP4, respectively. However, it has not been fully examined as to whether $PGF_{2\alpha}$ and/or $PGE_2$ are produced in preadipocytes as a kind of adipokine and control adipocyte differentiation in an autocrine manner.

In this chapter, to elucidate the physiological roles of EP4- and FP-mediated regulation of adipocyte differentiation and maturation, I employed an adipocyte differentiation system from mouse embryonic fibroblasts (MEFs), and compared the effects of each receptor-deficiency on adipocyte differentiation.

## Results

*Indomethacin augments adipocyte differentiation from MEFs*

MEFs were primed with differentiation-inducing cocktail (DIC) containing insulin, dexamethasone and IBMX for 2 days followed by treatment with insulin for an additional 6 days. Their differentiation into adipocytes was monitored by Oil Red O staining, and their triglyceride (TG) content was measured as an index of differentiation. Indeed, the differentiated cells contained 336.9 ± 15.7 mg TG/plate ($2.0 \times 10^6$ cells / plate), but the MEFs cultured in the absence of the differentiation cocktail exhibited only 87.4 ± 6.1 mg TG/plate. When the differentiation program was performed in the presence of 10 μM of indomethacin, an inhibitor of COX, the TG content in the cells was increased to approximately 1.5-2.0 fold of the control level (Fig. 1-1A). To examine whether indomethacin affects the number of differentiated cells and/or the TG content per differentiated cell, I assessed the time-dependent changes in TG content and the number of cells containing fat droplets during differentiation program (Fig. 1-1, B-D). In both cell groups, TG was undetected on day 2, slight but significant levels of TG were detected on day 4, and then drastically increased on day 6 and 8. However, in every time point, the TG levels in indomethacin-treated cells were significantly higher by 2-fold than control cells (Fig. 1-1B). In both groups, the oil droplets became visible on day 4, but the number of TG-positive cells was constant until day 8 (Fig. 1-1C). Interestingly, indomethacin increased the TG-positive cell number by approximately 2-fold. Indeed, the TG levels per TG-positive cell were indistinguishable between the two groups (Fig. 1-1D). These results suggest that indomethacin promotes adipocyte differentiation but not maturation. When I examined expression of the gene (*Pparg2*) for PPARγ, a transcription factor playing a central role in adipocyte differentiation, its induction was observed upon DIC treatment, and such gene expression was augmented by indomethacin (Fig. 1-1E). Indeed, indomethacin accelerated the induction of lipogenic enzyme genes such as fatty acid synthase, *Fasn* (Fig. 1-1F) and of lipolytic enzyme genes such as hormone-sensitive lipase, *Lipe* (Fig. 1-1G). These results suggest that PG endogenously synthesized by MEFs suppresses adipocyte differentiation.

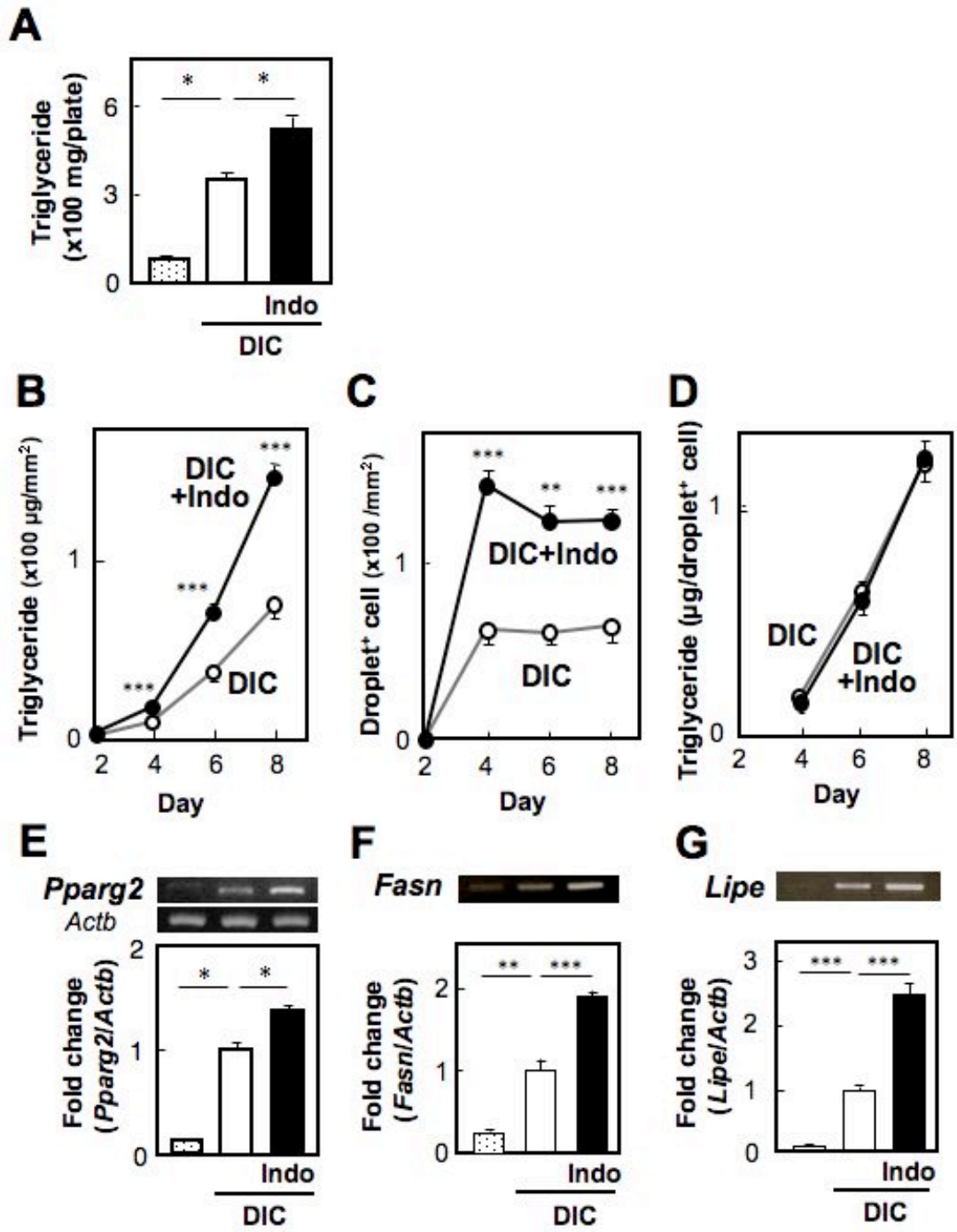
A
Triglyceride (x100 mg/plate)
Indo
DIC
B
Triglyceride (x100 μg/mm²)
DIC +Indo
DIC
Day
C
Droplet⁺ cell (x100 /mm²)
DIC+Indo
DIC
Day
D
Triglyceride (μg/droplet⁺ cell)
DIC
DIC +Indo
Day
E
Pparg2
Actb
Fold change (Pparg2/Actb)
F
Fasn
Fold change (Fasn/Actb)
G
Lipe
Fold change (Lipe/Actb)

**Fig. 1-1. Indomethacin facilitates adipocyte differentiation of MEFs.**
MEFs grown to confluency (~2 x $10^6$ cells/plate) were treated with a standard differentiation-inducing cocktail (DIC) in the presence or absence of indomethacin (10 μM, Indo). On day 8 (A, E-G) or the indicated days of the differentiation program (B), triglyceride (TG) content (A, B), or RNA expression level of *Pparg2* (E), *Lipe* (F), and *Fasn* (G) in the cells was measured as described in the Materials and Methods. On the indicated days of the differentiation program, cells were stained with oil red-O and visualized by bright-field light microscopy. The number of droplet-positive (droplet$^+$) cells was counted (C), and the average TG content per droplet$^+$ cell was calculated (D). The values are represented as the means ± SEM (n = 3). *$p < 0.05$, **$p < 0.01$.

*Expression of PG receptors in MEFs*

I next examined the mRNA expression of PG receptors in MEFs during the differentiation program (Fig. 1-2A). EP1 and EP4 mRNAs were expressed during the differentiation period. In addition, among the PGE receptor subtypes, significant expression of EP4 mRNA was detected throughout the differentiation process. I failed to detect a significant amount of EP2 receptor mRNA in these cells. Expression of FP and EP3 mRNA was undetectable in the untreated cells but FP mRNA could be detected in the cells on days 2 and 8, and EP3 mRNA could be detected in cells on day 8. To confirm the expression of functional PG receptors, I investigated whether each receptor agonist could induce signal transduction in undifferentiated (Fig. 1-2B) and differentiated cells (Fig. 1-2C). In undifferentiated MEFs at day 0, $PGE_2$ as well as an EP4 agonist ($10^{-7}$ M each) induced cAMP accumulation to a similar extent, but an EP1-, EP2-, and EP3-agonist failed to do so. These results indicate that the EP4 receptor is a Gs-coupled PGE receptor predominant in undifferentiated MEFs. In contrast, $PGE_2$, and any of the EP-specific agonists failed to induce intracellular $Ca^{2+}$ mobilization. Although RT-PCR analysis showed a faint band for EP1 gene expression in MEFs, EP1 may be expressed only in a very small population of undifferentiated MEFs. An FP agonist also failed to stimulate intracellular $Ca^{2+}$ mobilization, indicating that the FP receptor is not expressed in undifferentiated MEFs. In differentiated cells on day 8, $PGE_2$ as well as an EP4 agonist again induced cAMP accumulation, but an EP1-, EP3-agonist failed to do so. On the other hand, an FP agonist induced intracellular $Ca^{2+}$ mobilization, but $PGE_2$, and any of the EP-specific agonists failed to do so. These results suggest that at least functional FP and EP4 receptors are expressed in the differentiated cells.

*Both EP4- and FP-agonists suppress adipocyte differentiation from MEFs*

I next examined the effect of exogenously added PGs on adipocyte differentiation. $PGE_2$ as well as an FP-agonist, fluprostenol (1 μM each) significantly reduced the indomethacin-augmented TG content to the levels of the control group (Fig. 1-2D). Similar results were obtained regarding *Pparg2* gene expression (Fig. 1-2E). Thus, both EP4 and FP receptors have the potential to suppress differentiation. Since indomethacin treatment facilitates differentiation, either EP4 and/or FP signaling may endogenously suppress adipocyte differentiation in MEFs.

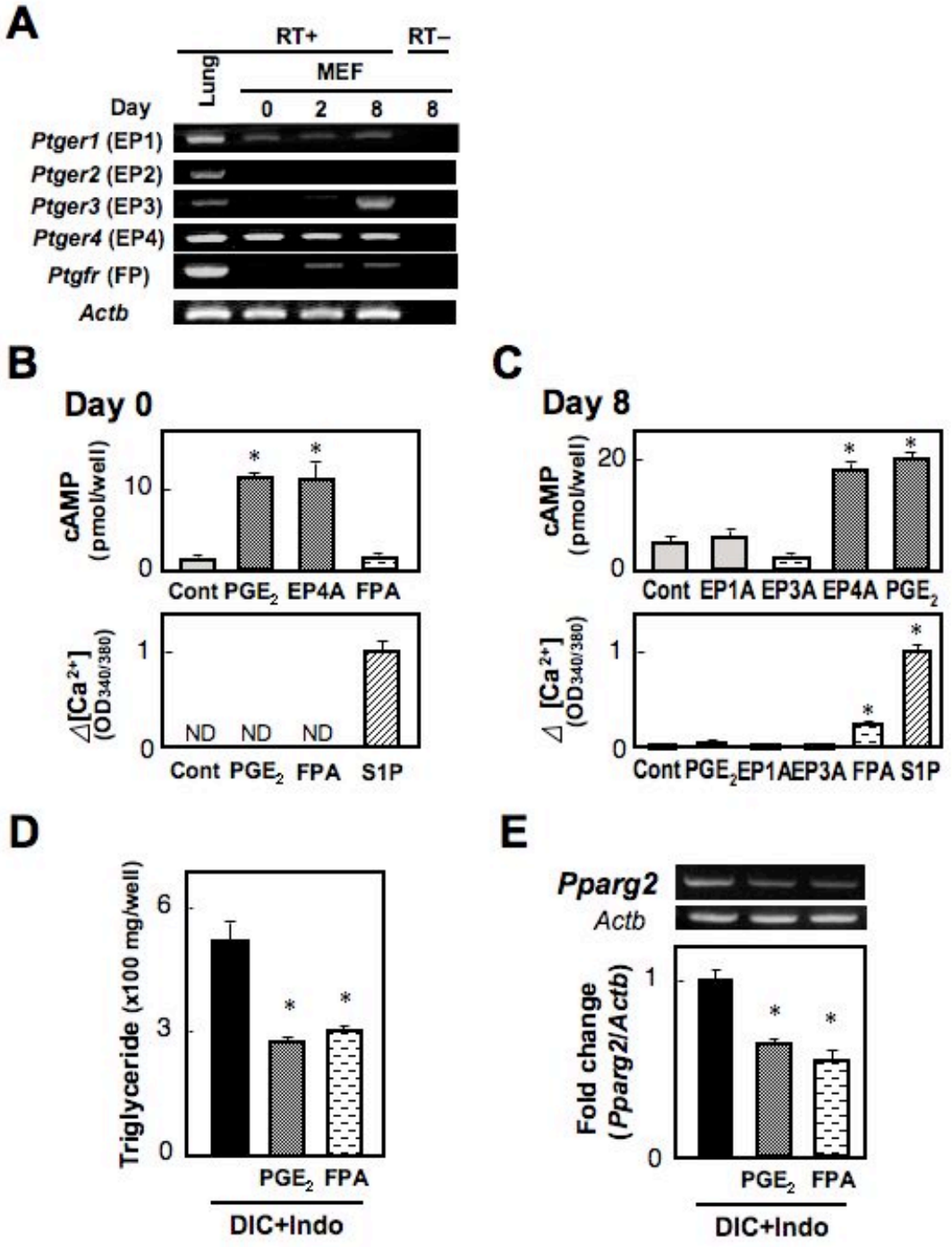
A
RT+
RT−
Lung
MEF
Day
0
2
8
8
Ptger1 (EP1)
Ptger2 (EP2)
Ptger3 (EP3)
Ptger4 (EP4)
Ptgfr (FP)
Actb
B
Day 0
cAMP (pmol/well)
10
0
Cont PGE2 EP4A FPA
Δ[Ca2+] (OD340/380)
1
0
ND ND ND
Cont PGE2 FPA S1P
C
Day 8
cAMP (pmol/well)
20
0
Cont EP1A EP3A EP4A PGE2
Δ [Ca2+] (OD340/380)
1
0
Cont PGE2 EP1A EP3A FPA S1P
D
Triglyceride (x100 mg/well)
6
3
0
PGE2 FPA
DIC+Indo
E
Pparg2
Actb
Fold change (Pparg2/Actb)
1
0
PGE2 FPA
DIC+Indo

**Fig. 1-2. Prostanoid EP4 and FP receptors have the potential to suppress adipocyte differentiation in MEFs.**

(A) Gene expression of PGE and PGF receptors in MEFs. MEFs grown to confluency were treated with DIC, and total RNA was extracted from untreated cells (day 0), cells on day 2 or cells on day 8. The total RNA was subjected to the reverse transcription reaction in the presence (RT+) or absence (RT-) of reverse transcriptase and subsequent PCR analysis. Mouse lung RNA was used as a positive control. (B, C) Undifferentiated (B, Day 0) or differentiated MEFs (C, Day 8) were subjected to the cAMP (top) or $Ca^{2+}$ assay (bottom). $PGE_2$ (0.1 μM), an EP1 agonist (0.1 μM, EP1A), an EP3 agonist (0.1 μM, EP3A), an EP4 agonist (0.1 μM, EP4A) and an FP agonist (0.1 μM, FPA) were used. In the $Ca^{2+}$ assay, sphingosine 1-phosphate (10 μM, S1P) was used as a positive control. (D, E) MEFs were treated with DIC supplemented with vehicle, $PGE_2$ (1 μM), or an FP agonist (1 μM, FPA) in the presence of indomethacin. Triglyceride content in the cells was measured on day 8 (D), and total RNA was extracted on day 8, and subjected to real time RT-PCR analysis (E). The *Pparg2* gene expression levels were normalized to the β-actin (*Actb*) mRNA levels. The values are represented as the means ± SEM ($n = 3$). *$p < 0.05$.

*EP4-deficiency but not FP-deficiency mimics the enhancing effect of indomethacin on adipocyte differentiation*

To examine which receptor signaling suppresses adipocyte differentiation from MEFs, I prepared MEFs isolated from *Ptger4*$^{-/-}$ and *Ptgfr*$^{-/-}$ mice, and examined the outcome of DIC-induced adipocyte differentiation. Interestingly, EP4-deficient cells revealed higher levels of TG content on day 8 than wild-type (WT) cells, and such levels were equivalent to those of indomethacin-treated WT cells (Fig. 1-3A). Similar results were obtained regarding *Pparg2* gene expression levels in the cells on day 8 (Fig. 1-3B). Moreover the number of droplet positive cells in EP4-deficient cells was higher than WT cells and similar to that of indomethacin-treated WT cells (Fig. 1-3C). Moreover, indomethacin did not further augment the total TG content, droplet positive number, or *Pparg2* expression levels in EP4-deficient cells (Fig. 1-3A-C). Moreover, an EP4 antagonist (1 μM), but not an EP1 or EP3 antagonists, mimicked the enhancing effect of indomethacin on differentiation (Fig. 1-3D). When I measured cAMP content on day 0, indomethacin as well as an EP4 antagonist attenuated cAMP levels, and $PGE_2$ reversed the indomethacin-suppressed cAMP levels (Fig. 1-3E). These results suggest that endogenous $PGE_2$-EP4 signaling suppresses adipocyte differentiation via cAMP pathway in WT cells. On the other hand, *Ptgfr*$^{-/-}$ cells on day 8 showed TG levels similar to those of WT cells, and indomethacin still increased the TG levels as observed in WT cells (Fig. 1-4A). FP gene deficiency essentially did not affect the *Pparg2* gene

expression levels in MEFs (Fig. 1-4B). These results indicate that the $PGF_{2\alpha}$-FP signaling is not involved in the suppression of adipocyte differentiation, although FP signaling has the potential to suppress it. Thus, endogenous $PGE_2$-EP4 signaling appears to suppress adipocyte differentiation in MEFs.

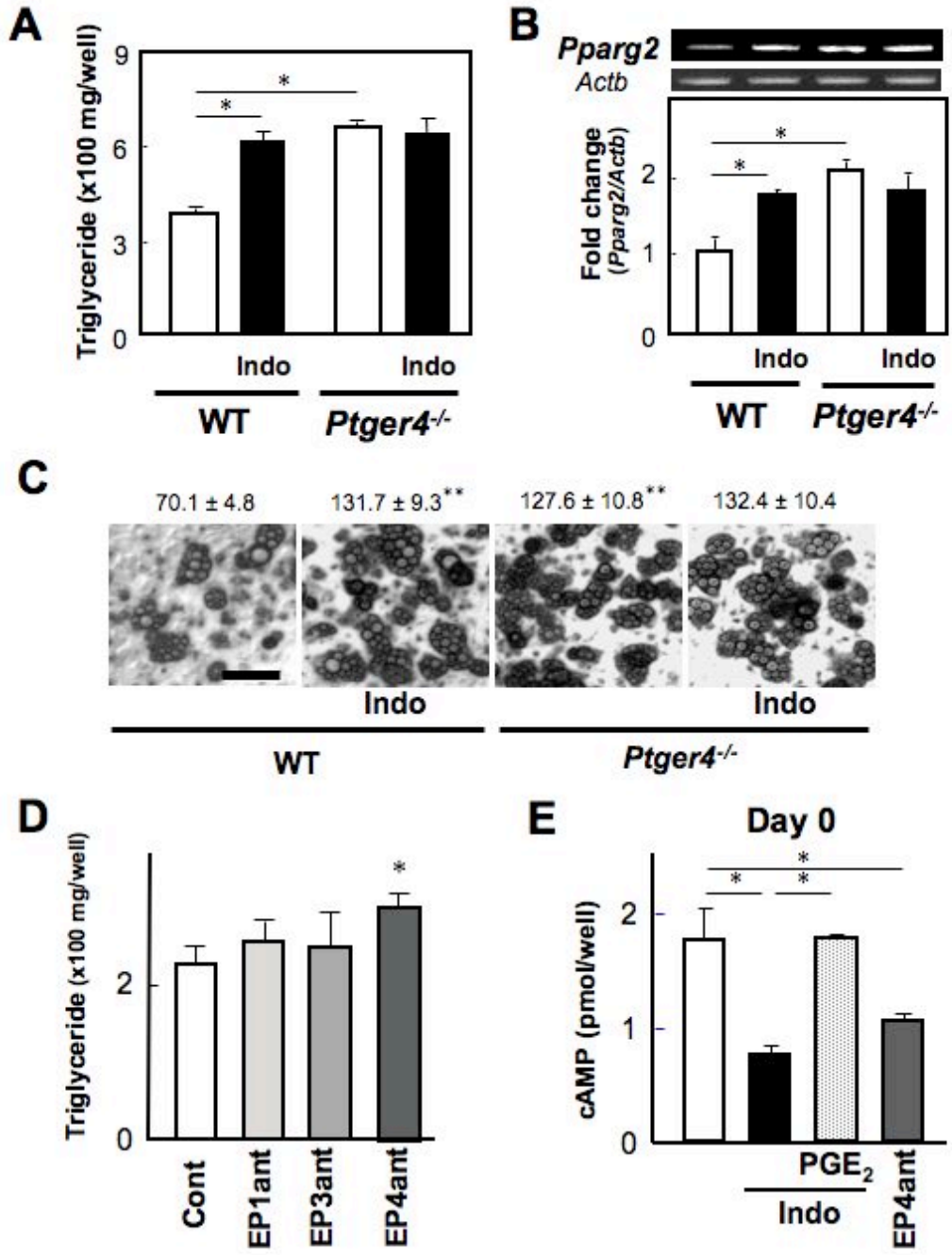

**Fig. 1-3. Endogenous $PGE_2$-EP4 signaling suppresses adipocyte differentiation in MEFs.**

MEFs from wild type (WT) and *Ptger4*$^{-/-}$ mice (A-C) grown to confluency were treated with DIC in the presence or absence of indomethacin (10 μM). Triglyceride content in the cells was measured on day 8 (A), and total RNA was extracted on day 8, and subjected to real time RT-PCR analysis (B). The *Pparg2* gene expression levels were normalized to the β-actin (*Actb*) mRNA levels. (C) WT and *Ptger4*$^{-/-}$ cells on day 8 were stained with oil red-O and visualized by bright-field light microscopy. (D) MEFs grown to confluency were treated with DIC in the presence of EP1-, EP3-, or EP4-antagonist (1 μM each). Triglyceride content in the cells was measured on day 8. (E) MEFs were treated with vehicle, an EP4-antagonist (1 μM) or indomethacin (10 μM) in the presence or absence of $PGE_2$ (1 μM) for 1 h, and subjected to cAMP assay. The values are represented as the means ± SEM ($n$ = 3). *$p$ < 0.05, **$p$ < 0.01.

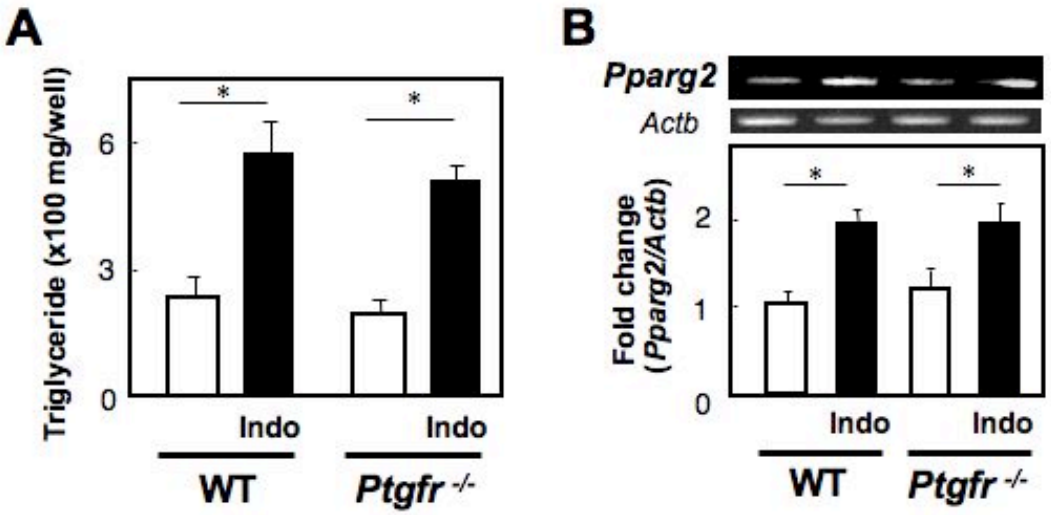


**Fig. 1-4. FP receptor deficiency fails to affect adipocyte differentiation in MEFs.**

MEFs from wild type (WT) and *Ptgfr*$^{-/-}$ mice grown to confluency were treated with DIC in the presence or absence of indomethacin (10 μM). Triglyceride content in the cells was measured on day 8 (A), and total RNA was extracted on day 8, and subjected to real time RT-PCR analysis (B). The *Pparg2* gene expression levels were normalized to the β-actin (*Actb*) mRNA levels. The values are represented as the means ± SEM ($n$ = 3). *$p$ < 0.05.

*Endogenous $PGE_2$-EP4 signaling suppresses transcription of* Pparg2 *gene on day 2 of adipocyte differentiation*

It has been considered that the destiny of each cell is determined during the first 2 days of adipocyte differentiation program, as a result of its chronic exposure to DIC, and that thereafter the committed cells gradually start to fulfill their function as adipocytes, which is adipocyte maturation. If endogenous EP4 signaling suppresses the differentiation stage, treatment of the cells with indomethacin only for the first 2 days may be enough to facilitate the differentiation. As expected, cells treated with indomethacin for the first two days showed TG levels and *Pparg2* expression levels as high as those in cells treated with indomethacin for eight days (Fig. 1-5, A and B). I then examined whether exposure of cells to exogenous PGs during the differentiation stage could reverse the effect of indomethacin. $PGE_2$ treatment for the first 2 days significantly suppressed the levels of TG content and *Pparg2* expression, but an FP agonist failed to alter these levels (Fig. 1-5, C and D). These results indicate that endogenous $PGE_2$-EP4 signaling suppresses the differentiation stage of adipogenesis. I next investigated the time course of induction of the *Pparg2* gene, which is a prerequisite for the commitment of individual cells to adipocyte differentiation in the MEF system (Fig. 1-5E). *Pparg2* expression was drastically induced by DIC treatment, reaching a peak level on day 2 until the DIC was removed, and then expression gradually decreased until day 8. If suppressive PG signaling dominates the fate of differentiation during the first two days, indomethacin may alter the peak level of *Pparg2* gene expression on day 2. As expected, indomethacin significantly augmented *Pparg2* gene expression on day 2. Moreover, $PGE_2$, but not an FP agonist, reversed the enhancing effect of indomethacin on *Pparg2* transcription (Fig. 1-5F). These results indicate that endogenous $PGE_2$-EP4 signaling suppresses adipocyte differentiation by attenuating transcription of the *Pparg2* gene.

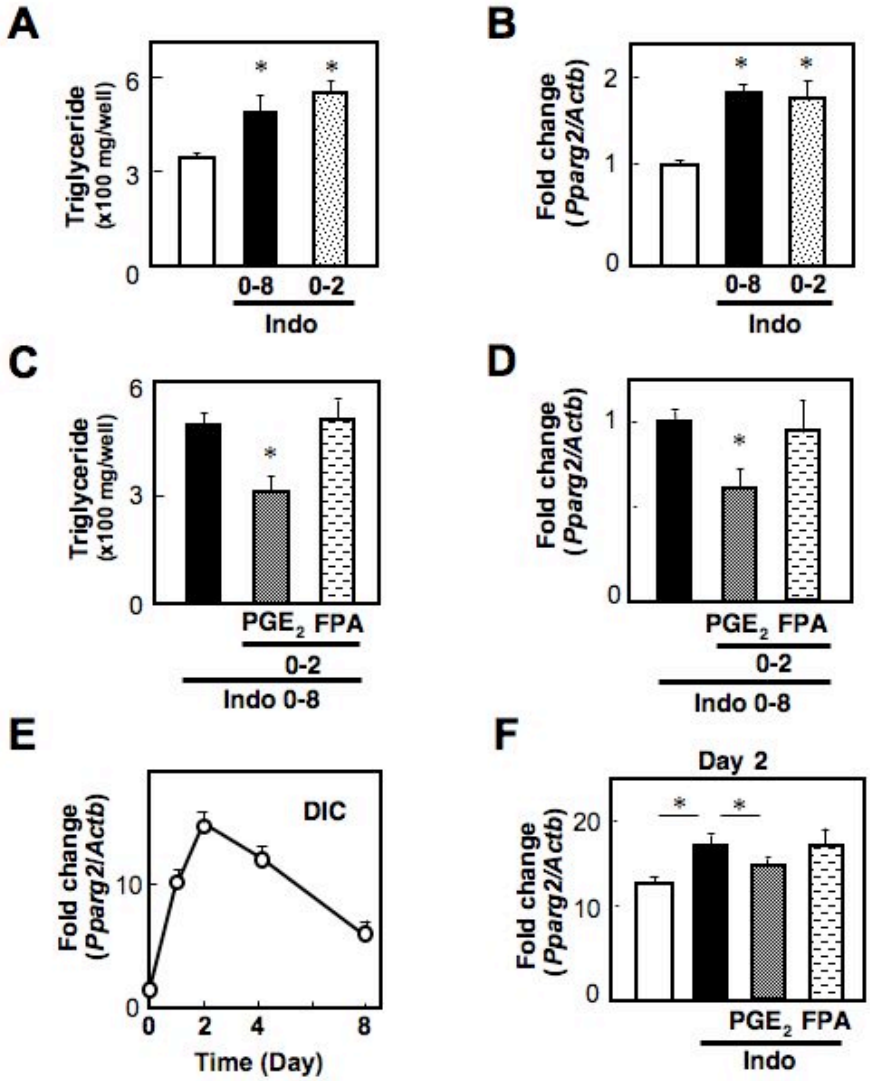


**Fig. 1-5. $PGE_2$-EP4 signaling suppresses transcription of *Pparg2* gene.**
(A, B) MEFs grown to confluency were treated with DIC in the presence (Indo) or absence of indomethacin. On day 2, DIC was replaced with media containing insulin in the presence (0-8) or absence of indomethacin (0-2). (C, D) MEFs were treated with DIC containing indomethacin supplemented with vehicle, $PGE_2$, or an FP agonist (FPA). On day 2, DIC was replaced with media containing insulin and indomethacin in the absence of PG receptor agonists. On day 8, triglyceride content in the cells was measured (A, C), and *Pparg2* gene expression in the cells was measured by real time RT-PCR analysis (B, D). (E) Time course of induction of *Pparg2* gene transcripts in MEFs. Cells were treated with DIC, harvested at the various time points of the differentiation program, and then subjected to *Pparg2* gene expression analysis. (F) MEFs were treated with DIC containing indomethacin supplemented with vehicle, $PGE_2$, or an FP agonist (FPA). On day 2, the cells were harvested and subjected to *Pparg2* gene expression analysis. The *Pparg2* gene expression levels were normalized to the β-actin (*Actb*) mRNA levels. The values are represented as the means ± SEM ($n$ = 3). *$p$ < 0.05.

*DIC treatment induces COX-2 expression and $PGE_2$ production*

The preceding experiments demonstrated that endogenous $PGE_2$-EP4 signaling reduces the peak level of *Pparg2* gene expression induced by DIC. Then, is $PGE_2$ really produced by MEFs in an indomethacin-sensitive manner? To assess this, I examined the time-course of $PGE_2$ production and gene expression of COX isozymes in MEFs just after the addition of DIC. DIC stimulated $PGE_2$ production, which reached a peak at 3 h and then gradually decreased. Such DIC-induced $PGE_2$ production was inhibited by indomethacin (Fig. 1-6A). In accordance with this, DIC transiently induced *Ptgs2* gene expression in MEFs, reaching a peak at 1 h. In contrast, *Ptgs1* gene expression was observed in MEFs irrespective of DIC treatment (Fig. 1-6B). Indeed, COX-1 protein expression was detected in MEFs before DIC treatment, and DIC did not alter its levels. COX-2 protein could barely be detected in MEFs before DIC treatment, but a faint and significant amount was detected at 1 and 3 h after the addition of DIC (Fig. 1-6C). Thus, DIC rapidly induces COX-2 and $PGE_2$ production in MEFs in an indomethacin-sensitive manner. If COX-2-derived $PGE_2$ is involved in the negative regulation of adipocyte differentiation, a selective inhibitor for COX-2 should mimic the facilitating effect of indomethacin. As expected, NS398, a COX-2 selective inhibitor augmented the levels of TG content, whereas SC560, a COX-1 selective inhibitor failed to do so (Fig. 1-6D). These results indicate that in MEFs the differentiation-inducing stimuli induce COX-2 expression and $PGE_2$ production, and the resultant $PGE_2$, via acting on the EP4 receptor negatively regulates adipocyte differentiation by reducing the peak level of *Pparg2* gene induction.

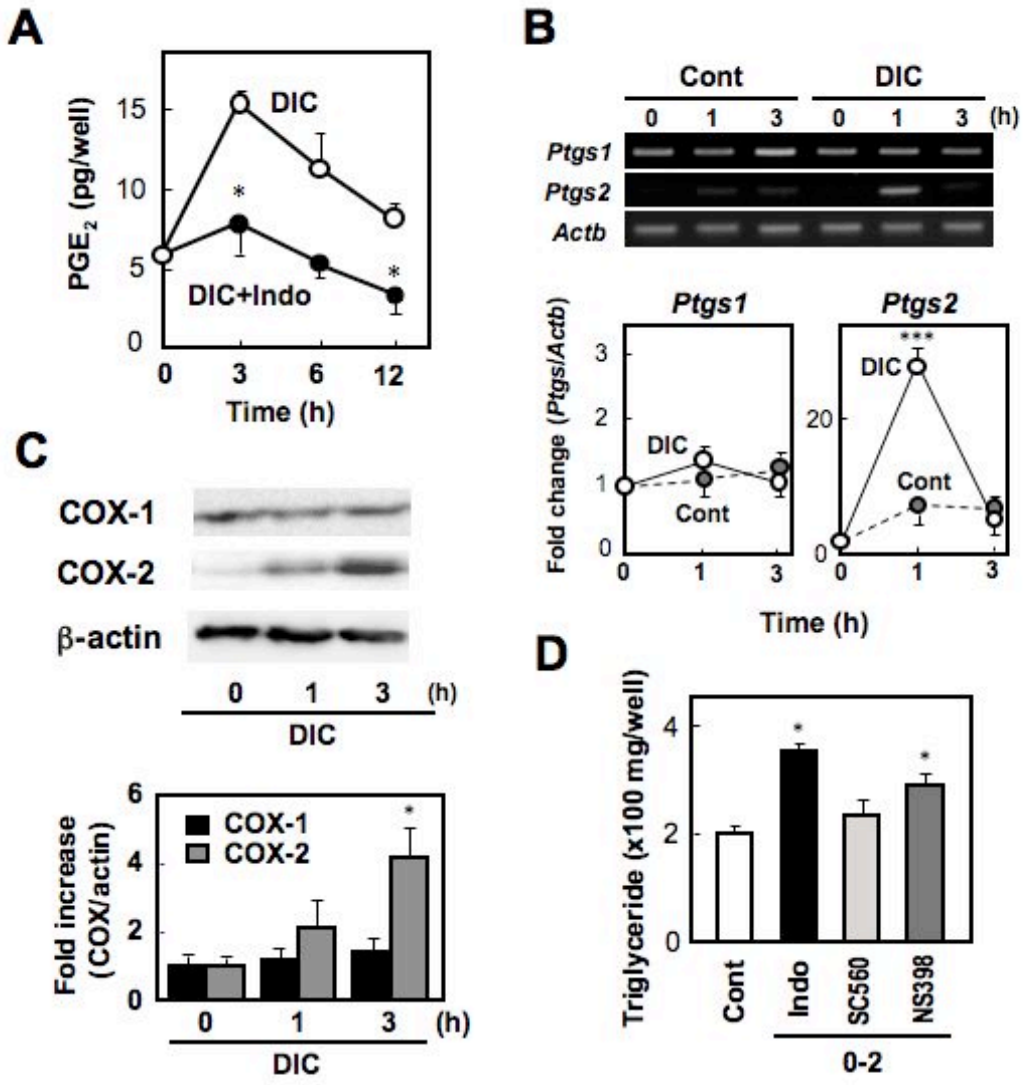


**Fig. 1-6. COX-2-derived $PGE_2$ suppresses adipocyte differentiation of MEFs.**
(A) $PGE_2$ production of MEFs treated with DIC for the indicated times in the presence (+Indo) or absence of indomethacin was measured. (B) MEFs were treated with DIC or control medium. Total RNA was isolated at the indicated times and subjected to real time RT-PCR analysis. The COX-1 (*Ptgs1*) and COX-2 (*Ptgs2*) mRNA levels were normalized to the β-actin (*Actb*) mRNA levels. Data are represented as a fold of the value at 0 h. (C) Whole cell lysate was prepared at the indicated times and subjected to SDS-PAGE followed by immunoblotting with anti-COX-1, anti-COX-2, or anti-β-actin as a control. The histogram (bottom) shows quantitative representations of COX levels normalized to β-actin levels. (D) MEFs grown to confluency were treated with DIC supplemented with vehicle (Cont), indomethacin (Indo), COX-1 selective inhibitor, SC560 or COX-2 selective inhibitor, NS398 (10 μM each). On day 2, the DIC was replaced with media containing insulin without COX inhibitors. On day 8, triglyceride content in the cells was measured. The values are represented as the means ± SEM ($n = 3$). *$p < 0.05$, ***$p < 0.001$.

## Discussion

*$PGE_2$-EP4 signaling suppresses an early stage of adipocyte differentiation*

PGs have long been thought to contribute to fat cell development, but the role of PGs in the regulation of adipocyte differentiation is complex and has remained unclear (Kim et al. 2000). One of the reasons for its complexity is that different classes of PGs exert opposing effects on adipocyte differentiation. For instance, both $PGI_2$ and $PGE_2$, the two PGs predominantly synthesized by fat cells, appear to have opposing effects on early adipogenesis; $PGI_2$ promotes adipocyte differentiation via the IP receptor (Vassaux et al. 1992), whereas $PGE_2$ inhibits differentiation via the EP4 receptor (Tsuboi et al. 2004; Sugimoto et al. 2004). $PGF_{2\alpha}$ also suppresses differentiation via the FP receptor (Casimir et al. 1996; Tsuboi et al. 2004). In the current study, I evaluated the contribution of each endogenous receptor signaling by using an adipocyte differentiation system from MEFs, where pharmacological actions on the PG receptor EP4 and FP signaling were reproduced as reported previously: exogenously added $PGE_2$ and an FP agonist suppressed adipocyte differentiation (Fig. 1-1, E and F). Inhibition of endogenous PG synthesis by indomethacin increased the number of TG-producing cells and transcription of *Pparg2* gene (Fig. 1-1, A and B), suggesting that suppressive PG signaling (EP4 or FP) dominates the fate of differentiation. EP4 deficiency mimicked the effect of indomethacin, and indomethacin no longer accelerated differentiation (Fig. 1-3A-C). On the other hand, FP deficiency failed to affect differentiation, and indomethacin was still effective (Fig. 1-3, D and E). $PGE_2$ treatment for only the first 2 days was enough to suppress differentiation (Fig. 1-5, C and D). These results indicate that $PGE_2$-EP4 signaling suppresses the earliest stage of adipocyte differentiation in MEFs (Fig. 1-7). $PGF_{2\alpha}$-FP signaling appears to have the potential to affect adipocyte differentiation, but FP signaling is not involved in the differentiation system in MEFs (Fig. 1-4). Considering that treatment of MEFs with an FP agonist for the first 2 days fails to alter both TG content and *Pparg2* gene expression (Fig. 1-5, C and D), $PGF_{2\alpha}$-FP signaling may suppress the maturation stages of adipocyte differentiation (Fig. 1-7).

*Differentiation stimuli-induced COX-2 gene expression and $PGE_2$ production in MEFs*

The current study demonstrates that COX-2 is responsible for $PGE_2$-elicited suppression of adipocyte differentiation in MEFs. There have been a number of reports regarding the contribution of COX isozymes to the regulation of adipocyte differentiation (Fajas et al. 2003; Yan et al. 2003; Chu et al. 2010). Yan *et al.* reported that both a COX-1- and COX-2-inhibitor enhances differentiation of 3T3-L1 cells, indicating that both COX isozymes participate in the negative regulation of adipogenesis (Yan et al. 2003). Interestingly, Yan *et al.* also demonstrated that COX-2 inhibitors, but not a COX-1 inhibitor, reversed TNF-α-induced inhibition of differentiation. A similar modulating effect of COX-2 has been shown in adiponectin-elicited inhibition of adipocyte differentiation from BMS2 cells (Yokota et al. 2002). Chu et al. recently established COX-2-knocked down 3T3-L1 cell lines; they found that these cell lines show augmented levels of adipocyte differentiation, and this phenotype was reversed by the addition of $PGE_2$ (Chu et al. 2010). Thus, the COX-2 pathway may work as a conserved negative regulator of adipocyte differentiation in broad types of preadipocytes.

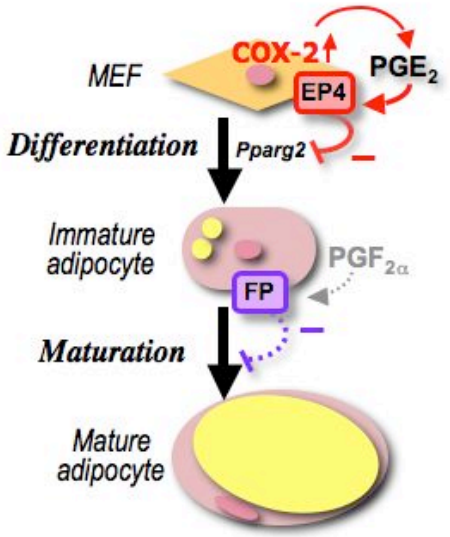


**Fig. 1-7. Schematic model representing the relationship among prostanoid receptor signaling events during adipocyte differentiation in MEFs.**
Adipocyte differentiation stimuli induce COX-2 gene expression and $PGE_2$ production, and the resultant $PGE_2$ suppresses the peak level of *Pparg2* expression via the EP4 receptor-cAMP pathway in an autocrine manner. The functional FP receptor is induced during differentiation and has the potential to exert negative regulation, but is not directly involved in adipocyte differentiation in the MEF system.

## Chapter 2: Prostaglandin $E_2$-EP4 Signaling Maintains Lipid Homeostasis by Regulating Insulin Signaling and Lipolysis in Adipose Tissue.


## Abstract

I examined the physiological role of EP4 receptor signaling in adipocyte function *in vivo* using EP4-deficient mice. Under standard diet-fed conditions, EP4-deficient mice exhibited higher body weight and white adipose tissue (WAT) mass than WT mice. In EP4-deficient mice, adipocyte cell size, but not cell number, was larger than in WT mice. Interestingly, EP4-deficient mice failed to show alterations in serum resistin and adiponectin levels and in expression levels of macrophage marker genes in WAT. An EP4 agonist stimulated the free fatty acid (FFA) release (lipolysis) in WT WAT, and the basal lipolytic activity was lowered in EP4-deficient WAT. In association with this, both serum FFA levels and hepatic TG accumulation were attenuated in EP4-deficient mice. These results suggest that $PGE_2$-EP4 signaling contributes to stimulation of lipolysis in adipocytes under physiological conditions. In EP4-deficient WAT, expression levels of the genes up-regulated by insulin were increased, and those of the genes down-regulated by insulin were decreased. An EP4 agonist significantly suppressed insulin-induced Akt phosphorylation in WAT, but not in liver and skeletal muscle in WT mice. These results suggest that $PGE_2$-EP4 signaling negatively regulates lipid accumulation in adipocytes at least partly by suppression of insulin signaling.

## Introduction

Fat and glucose derived from food intake are utilized as energy sources in skeletal muscle and many other organs, and the excess energy is stored mainly to white adipose tissue (WAT). Obesity is typically characterized by adipocyte hypertrophy when triglyceride synthesis (lipogenesis) exceeds breakdown (lipolysis), resulting in elevated TG storage (Jaworski et al., 2007).

In addition to its role in energy storage, WAT is also important as an endocrine organ that regulates energy homeostasis by secreting various adipokines (Kershew et al., 2004). Therefore, excessive or defective functions of adipose tissue may generate metabolic disorders, such as liver steatosis, insulin resistance, type 2 diabetes, and cardiovascular diseases. Therefore, uncovering the physiological modulator of fat mass is very important for understanding the establishment of obesity.

It has been shown that COX products, such as $PGE_2$, regulate adipocyte functions. A recent study suggested that COX-2 may be involved in body fat regulation (Fain et al., 2001). Mice heterozygous for the COX-2 gene showed approximately 30% increased body weight, with 2-3-fold larger fat pads compared with those of WT animals. $PGE_2$ production in adipose tissue from COX-2 null mice was only 20% of that of WT mice. These results suggest that COX-2 as well as $PGE_2$ participates in physiological regulation of adipose tissue functions. However, it has not been fully examined as to which EP receptor signaling is involved in adipose tissue functions and what mechanisms exist therein.

In the previous chapter, using MEF culture system, I found that endogenous $PGE_2$-EP4 signaling suppresses early phase of adipocyte differentiation, but it remains unknown whether $PGE_2$-EP4 signaling plays a similar role in physiological adipogenesis *in vivo*.

In this chapter, to investigate the physiological role of EP4 receptor in adipogenesis and/or adipose tissue function for energy metabolism, I carefully investigated appearance and function of adipose tissues in EP4-deficient mice. Finally I found that ablation of EP4 receptor increases adiposity due to attenuated physiological lipolysis under regular diet-fed conditions. Based on the results in this study, I conclude that $PGE_2$-EP4 signaling negatively regulates lipid accumulation in adipocytes at least

partly by suppression of insulin signaling.

## Results

*COX-1, COX-2 and EP4 receptor genes are expressed in WAT of WT mice under physiological conditions.*

To begin with in vivo analysis of *Ptger4*$^{-/-}$ female mice, I first examined the mRNA expression levels of EP4 receptor and two COX isozymes in various tissues of WT mice fed with standard diet. In WT mice, *Ptger4* mRNA was detected in various tissues that are important for energy metabolism such as WAT, BAT, and skeletal muscle. In contrast, EP4 mRNA expression was undetectable in liver. Both COX-1 (*Ptgs1*) and COX-2 (*Ptgs2*) mRNAs were expressed in WAT (Fig. 2-1). These results suggest that not only EP4 receptor but also both COX isozymes are present in WAT of WT mice under physiological conditions.

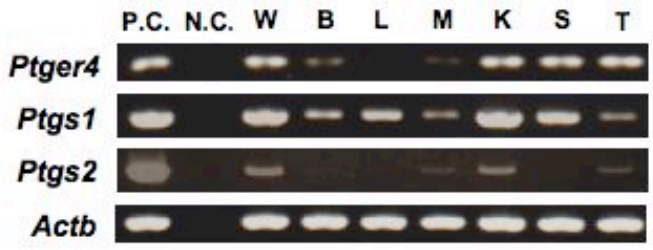


**Fig. 2-1. RNA expression of EP4 receptor and cyclooxygenases in various tissues of WT mice.**

RT-PCR analysis of *Ptger4*, *Ptgs1, Ptgs2, and Actb* mRNA expression in various tissues from WT and EP4 KO mice. P.C., positive control; N.C., negative control; W., WAT; B., BAT; L., liver; M., muscle; K., kidney; S., spleen; T., thymus.

*EP4 receptor ablation results in increased adiposity under standard diet conditions*

Under standard diet-fed conditions, the averaged body weight of *Ptger4*$^{-/-}$ female mice was significantly greater than that of wild-type (WT) mice from 5 weeks old (Fig. 2-2A). Eight-week-old *Ptger4*$^{-/-}$ mice exhibited a significant excess of visceral (e.g., perigonadal, mesenteric, and perirenal) and subcutaneous (e.g., inguinal) WAT depots (Fig. 2-2, B and C). In contrast, the averaged weights of other organs, including liver, kidney, and heart, was not remarkably altered, compared with those of WT mice (Fig. 2-2D). Histological analysis and cell size measurement of WAT showed significantly larger adipocytes in *Ptger4*$^{-/-}$ mice than in WT mice (Fig. 2-3, A-C). In contrast, there was no alteration in the cell number in WT and *Ptger4*$^{-/-}$ WAT (Fig. 2-3C). Increased body weight, increased WAT weight and larger adipocyte size were also observed in *Ptger4*$^{-/-}$ male mice (Fig. 2-4, A-F). These results suggest that EP4 receptor physiologically suppresses lipid storage in adipocytes and does not affect adipogenesis. In agreement with this, mRNA levels of the adipogenesis marker genes, such as *Pparg2* and *Fabp4*, were not altered in perigonadal WAT of *Ptger4*$^{-/-}$ mice (Fig. 2-3D).

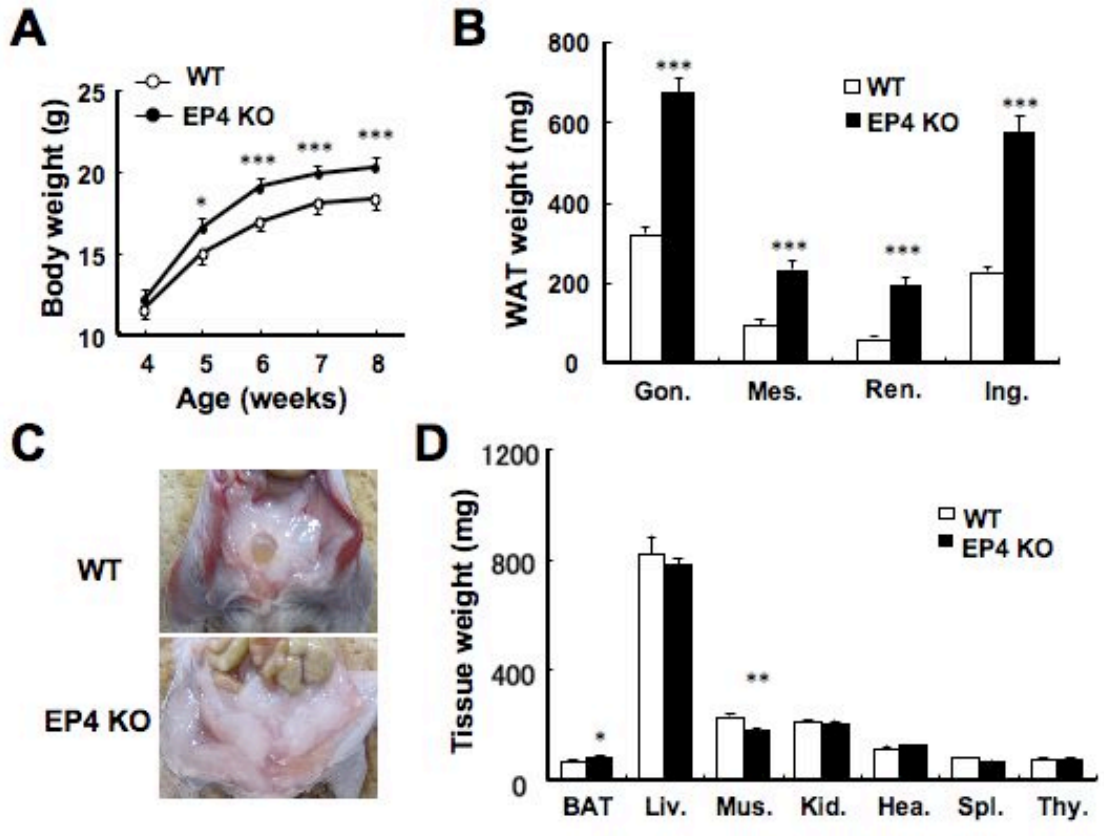


**Fig. 2-2. EP4 receptor ablation increases weights of white adipose tissues in female mice.**

**A,** Body weight of wild-type (WT) or *Ptger4*$^{-/-}$ (EP4 KO) female mice fed with standard diet ($n$ = 14-17). **B,** Weight of various WATs from WT or EP4 KO mice ($n$ = 6-8). Gon., perigonadal; Mes., mesenteric; Ren., perirenal; Ing., inguinal. **C,** Representative appearance of abdominal WAT of WT or EP4 KO mice. **D,** Weight of various tissues from 8-week-old WT or EP4 KO female mice ($n$ = 6-8). The values are represented as mean ± SEM. *$P$ < 0.05, **$P$ < 0.01, ***$P$ < 0.005.

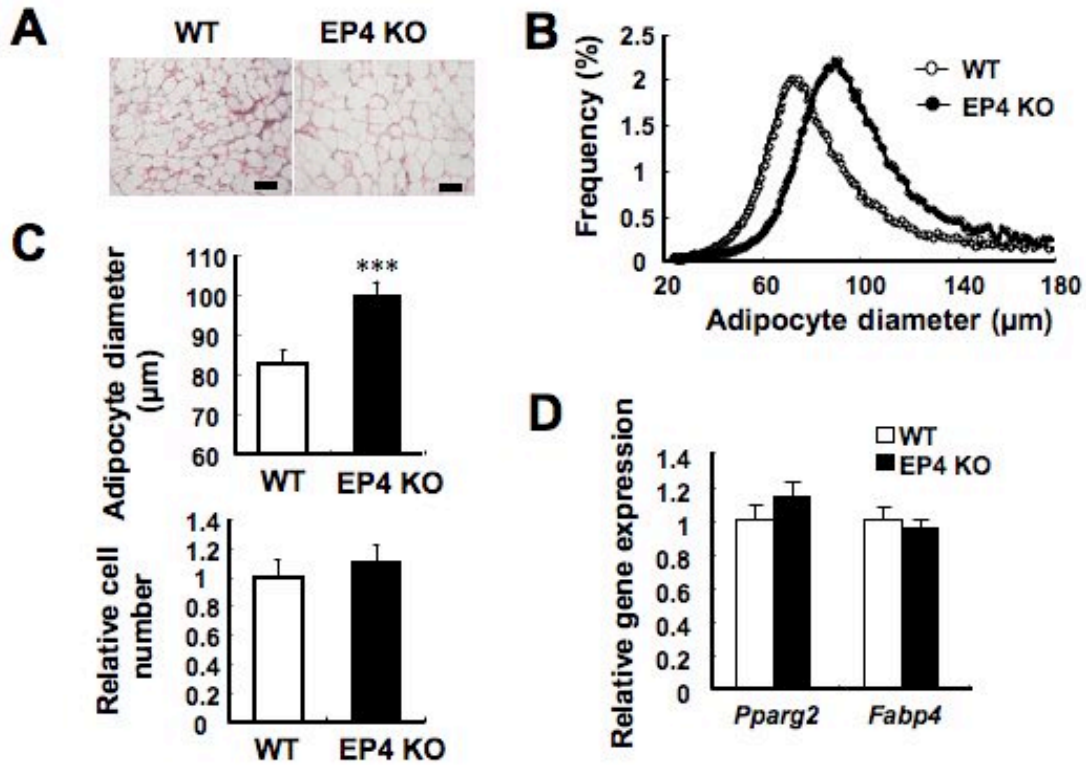


**Fig. 2-3. Adipocyte size, but not cell number is increased in female EP4-deficient mice.**

**A,** Hematoxylin and eosin staining of perigonadal WAT from 8-week-old WT or EP4 KO mice. Scale bars, 100 μm. **B,** Distribution of adipocyte size in perigonadal WAT from WT or EP4 KO female mice ($n$ = 8-9). **C,** Mean adipocyte diameter (upper panel) and relative cell number (lower panel) from WT or EP4 KO mice ($n$ = 8-9). **D,** Real-time RT-PCR analysis of adipogenic genes in perigonadal WAT from WT or EP4 KO mice ($n$ = 4). The values are represented as mean ± SEM. ***$P < 0.005$.

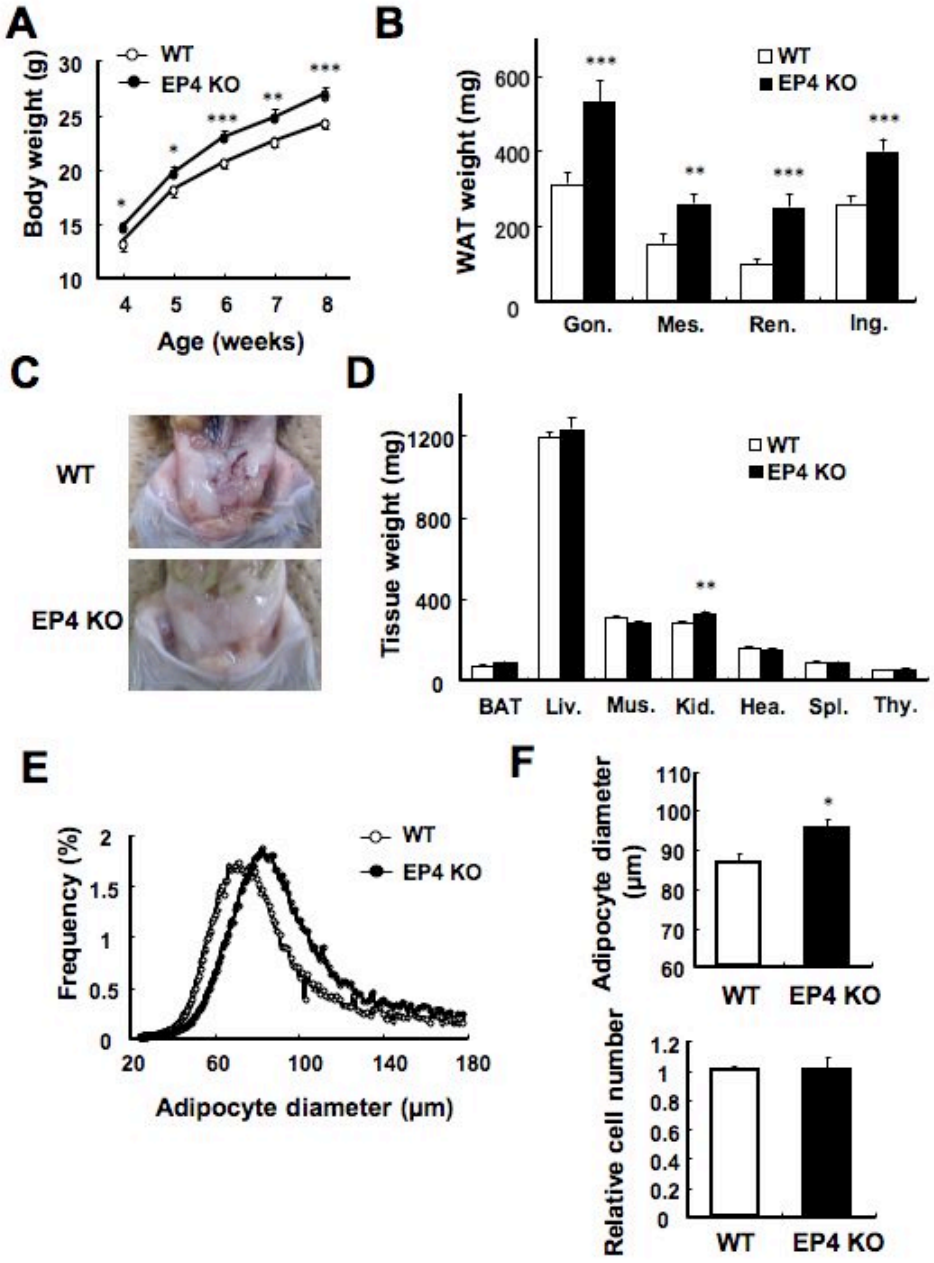


**Fig. 2-4. EP4 deficiency increased adipocyte size also in male mice.**

**A,** Body weight of wild-type (WT) or *Ptger4*$^{-/-}$ (EP4 KO) male mice fed with standard diet (*n* = 14-17). **B,** Weight of various WATs from WT or EP4 KO male mice (*n* = 6-8). Gon., perigonadal; Mes., mesenteric; Ren., perirenal; Ing., inguinal. **C,** Representative appearance of abdominal WAT of WT or EP4 KO mice. **D,** Weight of various tissues from 8-week-old WT or EP4 KO male mice (n=6-8). Liv., liver; Mus., soleus muscle; Kid., kidney; Hea., heart; Spl., spleen; Thy., thymus. **E,** Distribution of adipocyte size in perigonadal WAT from WT or EP4 KO mice (*n* = 8-9). **F,** Mean adipocyte diameter (upper panel) and relative cell number (lower panel) from WT or EP4 KO mice (*n* = 8-9). The values are represented as mean ± SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.005.

To examine whether increased WAT mass is due to loss of EP4 receptor in adipocytes, I generated fat-specific EP4 knockout (EP4 FKO) mice by crossing *Fabp4*-cre mice to *Ptger4*$^{flox/flox}$ mice. These mutant mice showed remarkable suppression of *Ptger4* mRNA specifically in WAT and BAT (Fig. 2-5A). There was no obvious difference in body weight between Flox and EP4 FKO mice (Fig. 2-5B). However, it should be noted that EP4 FKO mice again exhibited increased adiposity (Fig. 2-5, C and D). These results demonstrate that EP4 receptor in adipose tissue contributes to the suppression of lipid storage.

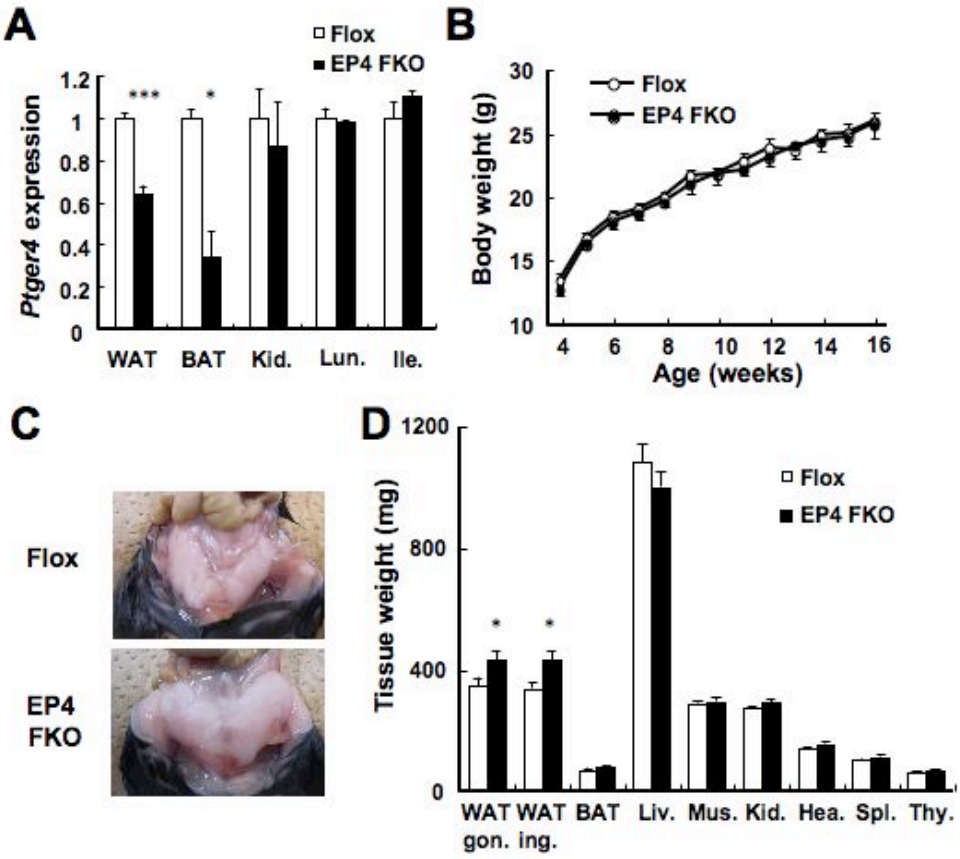


**Fig. 2-5. Fat-specific EP4 receptor gene suppression also leads to increased adiposity in female mice.**

**A,** Real-time quantitative RT-PCR analysis of *Ptger4* mRNA in tissues from *Ptger4*$^{flox/flox}$ (Flox) or fat-specific EP4 knockout (EP4 FKO) mice ($n$ = 3-9). Kid., kidney; Lun., lung; Ile., ileum. **B,** Body weight of Flox or EP4 FKO female mice fed with standard diet ($n$ =7-18). **C,** Representative appearance of abdominal WAT of 16-week-old Flox or EP4 FKO female mice. **D,** Weight of various tissues from 16-week-old Flox or EP4 FKO female mice ($n$ = 5-16). The values are represented as mean ± SEM. *$P < 0.05$.

*Ptger4*$^{-/-}$ mice also showed increased weight (Fig. 2-2D) of intrascapular brown adipose tissue (BAT). In order to investigate whether BAT function is affected by EP4 deficiency, I then examined mRNA expression levels of genes related to thermogenesis (also known as BAT marker genes) in WT and *Ptger4*$^{-/-}$ BAT. However, I failed to detect any differences in mRNA levels of the thermogenesis gene, *Ucp1,* and β-oxidation genes, such as *Ppargc1a*, *Ppara* and *Cpt1b*, between WT and *Ptger4*$^{-/-}$ BAT (Fig. 2-6B). These results suggest that thermogenesis function in the BAT appears normal even in the absence of EP4 receptor.

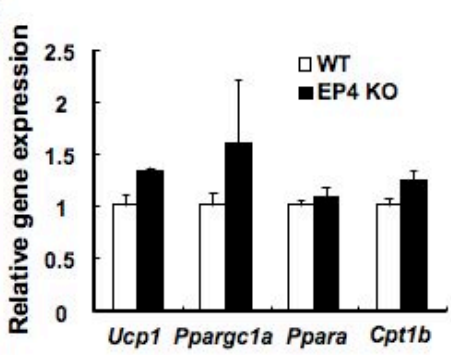


**Fig. 2-6. BAT appears to function normally in EP4-deficient mice.**
Real-time RT-PCR analysis of expression of BAT marker genes in intrascapular BAT from WT or EP4 KO mice ($n$ = 5).

*EP4-deficient mice are not in chronic inflammatory state despite increased adiposity*

In diet-induced obesity models and many genetic obesity models (e.g., *ob*/*ob* or *db*/*db* mice), inflammatory cells including macrophages are recruited to WAT (Xu et al., 2003), and these cells cause an imbalance in the secretion of adipocytokines including adiponectin and resistin (Hu et al., 1996; Steppan et al., 2001) and contribute to obesity-associated insulin resistance. Then, I next investigated whether *Ptger4*$^{-/-}$ mice were in the morbid obesity state as mentioned above. I measured serum adipokine levels

to examine the inflammation state within WAT in the mutant mice (Fig. 2-7, A-C). Serum leptin levels in *Ptger4*$^{-/-}$ mice were higher than in WT (Fig. 2-7A), which is presumably due to the augmented WAT depots. In contrast, serum adiponectin and resistin levels were not different (Fig. 2-7, B and C). Moreover, there were no alterations in the expression levels of macrophage marker genes between WT and *Ptger4*$^{-/-}$ WAT (Fig. 2-7D). These results indicate that *Ptger4*$^{-/-}$ mice are not in the morbid obesity state.

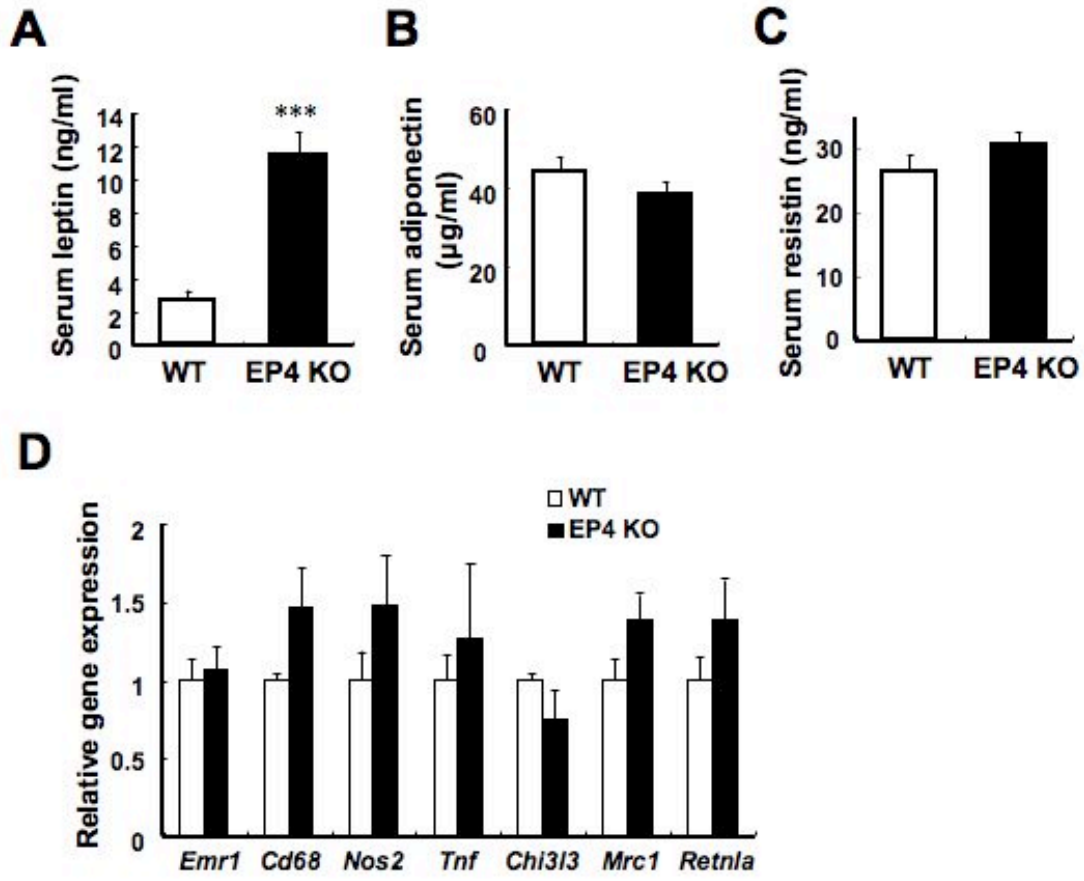


**Fig. 2-7. Chronic inflammation markers are not detected in EP4-deficient mice.** **A-C,** Serum leptin (A), adiponectin (B), and resistin (C) concentrations of 8-week-old WT or EP4 KO mice fed *ad libitum* with standard diet ($n$ = 14-16). **D,** Real-time RT-PCR analysis of macrophage marker genes in perigonadal WAT from 8-week-old WT or EP4 KO mice ($n$ = 4). *Emr1* and *Cd68*, pan-macrophage markers; *Nos2* and *Tnf*, inflammatory M1 macrophage markers; *Chi3l3*, *Mrc1*, and *Retnla*, tissue-resident M2 macrophage markers. The values are represented as mean ± SEM. ***$P$ < 0.005.

*EP4-deficient mice exhibit diminished WAT lipolysis and decreased liver fat accumulation*

Next, I investigated the mechanism of increased lipid storage in *Ptger4*$^{-/-}$ WAT. First I investigated the energy intake, but failed to detect any differences in food intake between WT and *Ptger4*$^{-/-}$ mice (Fig. 2-8A). The lipid storage in WAT is defined by a balance between lipogenesis and lipolysis. I therefore investigated expression levels of lipogenic and lipolytic genes in WT and *Ptger4*$^{-/-}$ perigonadal WAT (Fig. 2-8B). However, I failed to detect any differences in the RNA expression levels of lipogenic genes, such as *Srebf1, Scd1, Fasn, and Acaca*, and the glucose transporter gene, *Slc2a4* between WT and *Ptger4*$^{-/-}$ mice. These results suggest that $PGE_2$-EP4 signaling is not involved in lipogenesis under physiological conditions. In adipocytes, lipolysis takes place as follows: TG is hydrolyzed into glycerol and FFA by sequential actions of three lipases, adipose triglyceride lipase (ATGL, *Pnpla2*), hormone sensitive lipase (HSL, *Lipe*), and monoglyceride lipase (MGL, *Mgll*). It has been known that HSL is activated by phosphorylation by PKA. I therefore examined the expression and activation level of these lipases. EP4 deficiency attenuated the expression level of *Pnpla2*, but failed to alter the levels of other lipase genes including *Lipe* and *Mgll* (Fig. 2-8C). Moreover, I found that the activation (phosphorylated) levels of HSL were markedly attenuated by EP4 deficiency (Fig. 2-8D). These results suggest that EP4 signaling promotes lipolysis in WAT by inducing *Pnpla2* expression and HSL activation. In order to investigate whether EP4 activation stimulates lipolysis in WAT, I examined the effect of an EP4 agonist on free fatty acid (FFA) release from WAT explants. As expected, an EP4 agonist ($10^{-7}$ M) augmented lipolytic activity of isolated WT WAT explants (Fig. 2-8E). These results indicate that EP4 signaling has an ability to stimulate lipolysis in adipocytes. Moreover, in *Ptger4*$^{-/-}$ WAT, basal lipolytic activity was significantly attenuated compared with WT WAT (Fig. 2-8F). These results suggest that EP4 signaling contributes to stimulation of lipolysis in adipocytes under physiological condition.

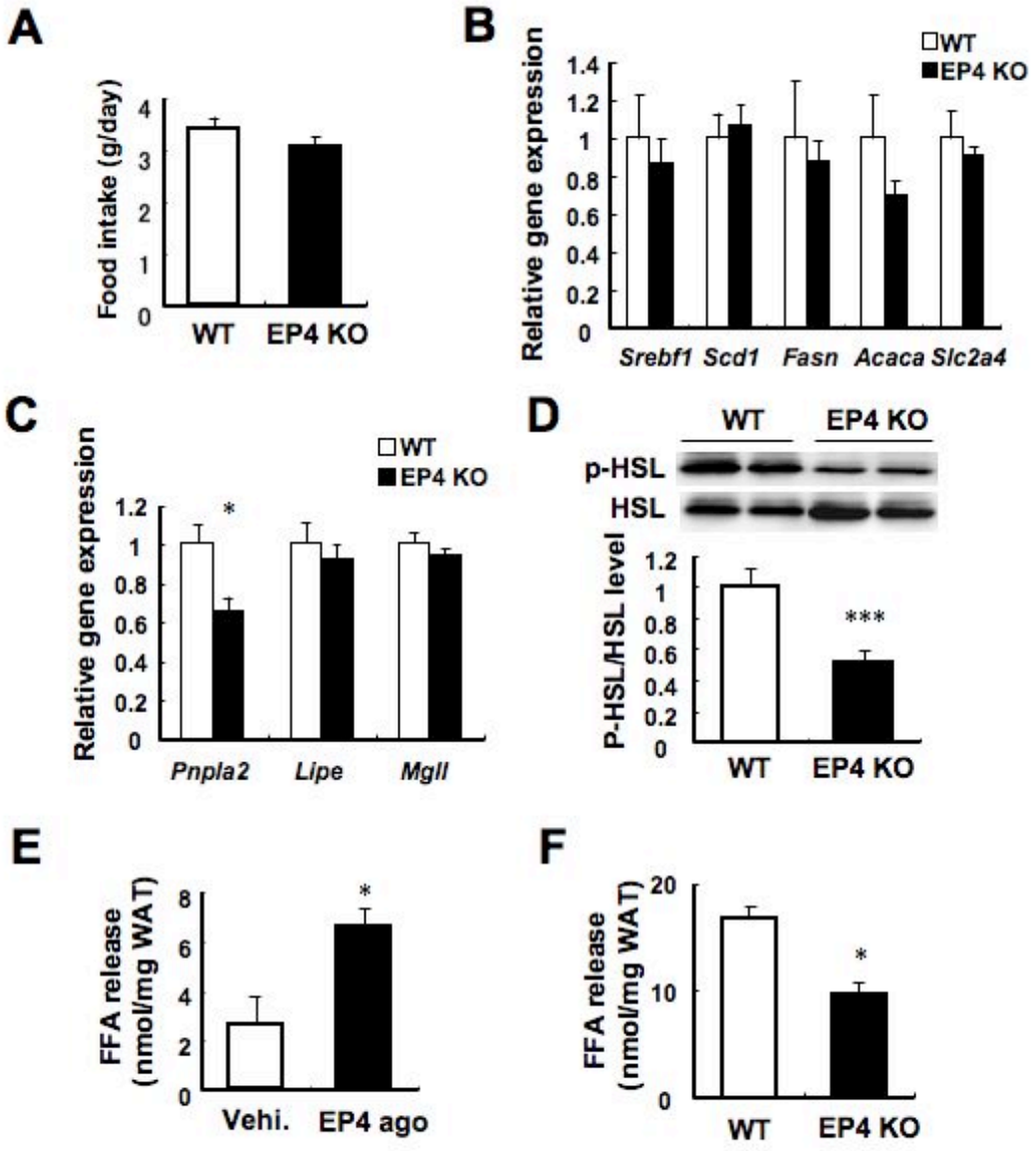


**Fig. 2-8. EP4 signaling promotes lipolysis.**

**A,** Daily food intake of 8-week-old WT or EP4 KO mice ($n$ = 5-6). **B,** Real-time RT-PCR analysis of lipogenic genes in WAT from 8-week-old WT or EP4 KO mice ($n$ = 4). **C,** Real-time RT-PCR analysis of lipolysis-related genes in perigonadal WAT from 8-week-old WT or EP4 KO mice ($n$ = 4). **D,** Phosphorylation of hormone sensitive lipase (HSL) in WAT from WT or EP4 KO mice fasted for 5 h. *Upper*, Western blot analysis of phospho-HSL (P-HSL) and total HSL levels in perigonadal WAT from WT or EP4 KO mice. *Lower*, quantifications of P-HSL normalized to total HSL ($n$ = 6). **E,** *Ex vivo* lipolysis. FFA release from WT WAT explants in the absence or presence of 0.1 μM of EP4 agonist for 4h ($n$ = 6). **F,** FFAs released from WT or EP4 KO WAT explants were measured ($n$ = 3). The values are represented as mean ± SEM. *$P < 0.05$, ***$P < 0.005$.

I found that excessive lipid storage in *Ptger4*$^{-/-}$ mice is due to attenuated lipolysis in adipocytes. If this is true, FFA release into the bloodstream and fat accumulation in liver could be suppressed in *Ptger4*$^{-/-}$ mice. Indeed, *Ptger4*$^{-/-}$ mice showed markedly lower serum FFA levels than WT mice (Fig. 2-9A). Moreover, I found that the hepatic fat accumulation was attenuated in *Ptger4*$^{-/-}$ mice by both histological analysis and TG measurement (Fig. 2-9, B and C). In *Ptger4*$^{-/-}$ liver, I failed to detect any alterations in the mRNA levels of genes related to lipid metabolism, such as *Fabp4, Srebf1, Scd1, Fasn*, *Acaca* (lipogenesis) and *Ppara* (fatty acid oxidation), compared with WT liver (Fig. 2-9D). These results suggest that less fat accumulation in *Ptger4*$^{-/-}$ liver is not due to attenuated lipogenesis within liver, but due to the attenuated FFA release from WAT and the resultant lowered uptake of FFA from blood.

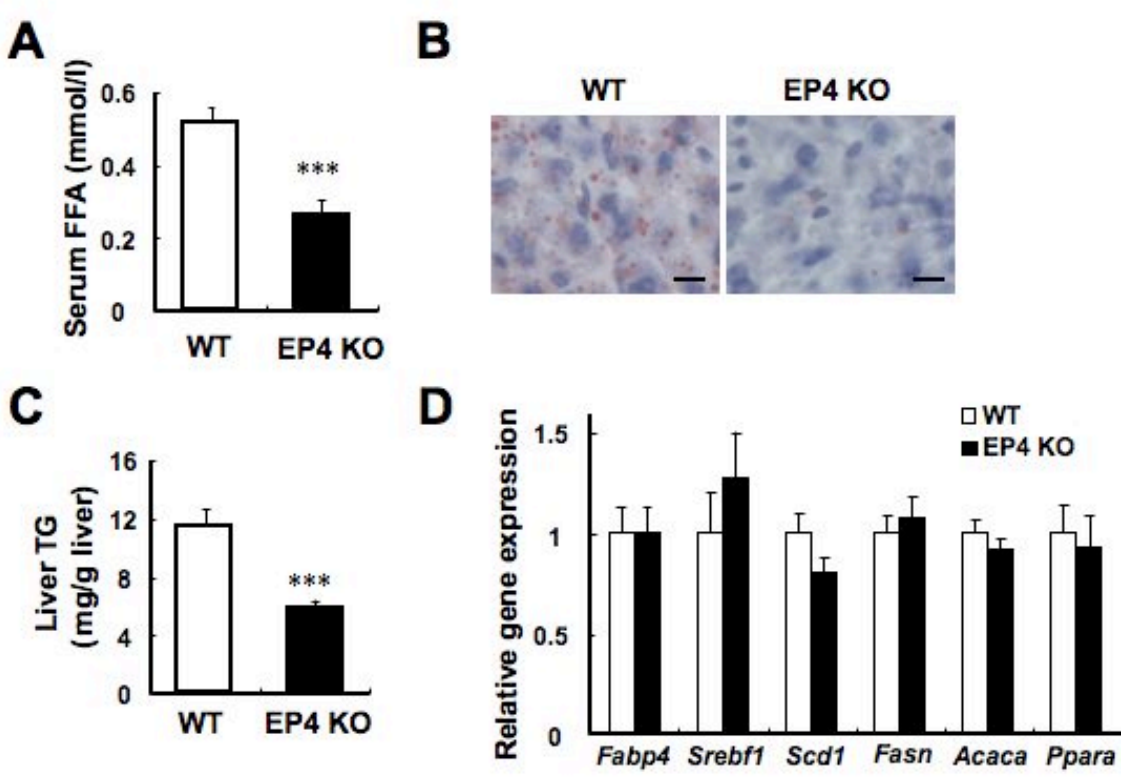


**Fig. 2-9. Hepatic fat accumulation is suppressed in EP4-deficient mice.**
**A,** Serum FFA levels of 8-week-old WT or EP4 KO mice fed *ad libitum* with standard diet ($n$ = 6). **B,** Representative photomicrograph of oil red O- and hematoxylin-stained liver sections from WT or EP4 KO mice. Scale bars, 10 μm. **C,** Hepatic triglyceride content of 8-week-old WT or EP4 KO mice ($n$ = 9-11). **D,** Real-time RT-PCR analysis of lipogenic genes in liver from WT or EP4 KO mice ($n$ = 4). The values are represented as mean ± SEM. ***$P < 0.005$.

*Enhanced insulin sensitivity in EP4-deficient mice*

In contrast to EP4 signaling, insulin is known to inhibit lipolysis in WAT by suppressing *Pnpla2* expression and HSL phosphorylation by PKA (Kitamura et al., 1999; Kershaw et al., 2006). I therefore investigated whether EP4 signaling counteracts insulin function in systemic levels. In both non-fasted and fasted conditions, blood glucose levels in *Ptger4*$^{-/-}$ mice were similar to those in WT mice (Fig. 2-10A). Indeed, I failed to detect any differences in glucose tolerance test between WT and mutant mice (Fig. 2-10B). In contrast, *Ptger4*$^{-/-}$ mice exhibited a marked reduction in blood glucose levels upon insulin injection (Fig. 2-10C). These results suggest that EP4 signaling in particular tissue could counteract the effect of insulin *in vivo*.

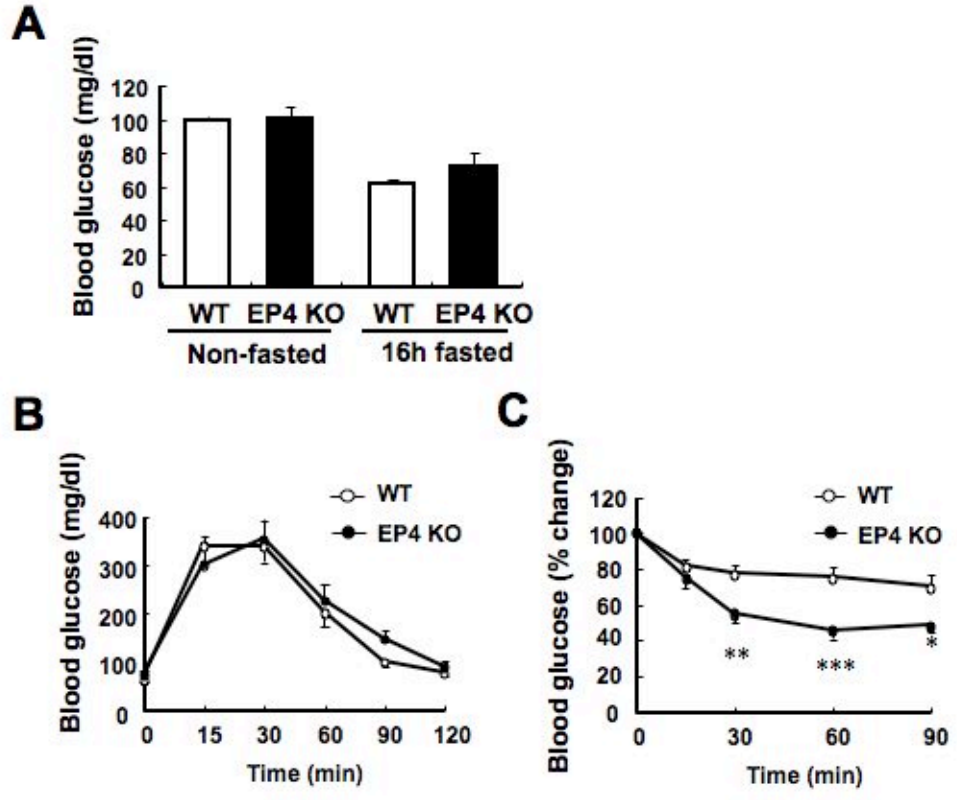


**Fig. 2-10. Enhanced insulin sensitivity in EP4-deficient mice.**
**A,** Blood glucose levels of WT or EP4 KO mice non-fasted or fasted for 16 h ($n$ = 5-6). **B,** Glucose tolerance test. Blood glucose concentrations in WT or EP4 KO mice fasted overnight were measured at the indicated time points after intraperitoneal injection of glucose ($n$ = 5-6). **C,** Insulin tolerance test. Blood glucose concentrations in WT or EP4 KO mice fasted for 5 h were measured at the indicated time points after intraperitoneal injection of insulin ($n$ = 5-6). The values are represented as mean ± SEM. *$P$ < 0.05, **$P$ < 0.01, ***$P$ < 0.005.

*An EP4 agonist suppresses insulin signaling in WAT*

In what tissue, does EP4 signaling counteract insulin? Since fat-specific EP4KO also showed excessive lipid storage (Fig. 2-5), I hypothesized that $PGE_2$-EP4 signaling counteracts the effects of insulin in adipocytes. Indeed, it is well-known that WAT also takes part in glucose uptake from blood, although its relative contribution to systemic glucose uptake is not so high (Abel et al., 2001). To access this hypothesis, I investigated the expression patterns of insulin-regulated genes in the perigonadal WAT of WT and *Ptger4*$^{-/-}$ mice (Fig. 2-11). In *Ptger4*$^{-/-}$ WAT, expression levels of the genes positively regulated by insulin such as *Lpl*, *Npr3*, and *Lep* were significantly higher than WT, and the levels of the genes negatively regulated by insulin, such as *Slc27a1* and *Irf4*, were lower than WT; the effect of insulin on gene expression profile in WAT was likely to be enhanced by the EP4 deficiency. These results suggest that $PGE_2$-EP4 signaling could counteract endogenous insulin signaling in WAT.

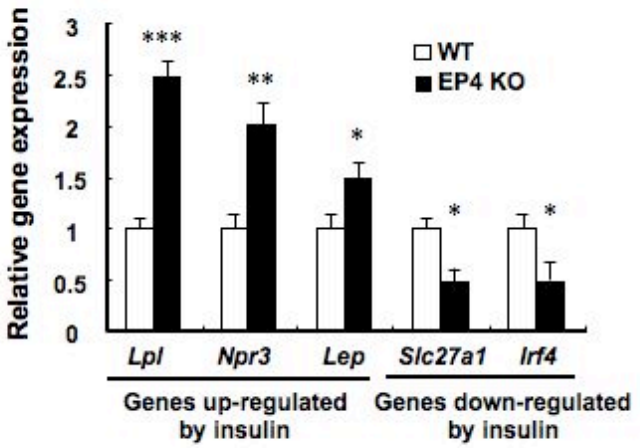


**Fig. 2-11. Effect of insulin on gene expression profile is enhanced in EP4-deficient WAT.**

Quantitative expression analysis of genes up-regulated or down-regulated by insulin in perigonadal WAT from 8-week-old WT or EP4 KO mice ($n$ = 4) by real-time RT-PCR. *Lpl*, lipoprotein lipase; *Npr3*, natriuretic receptor 3; *Lep*, leptin; *Slc27a1*, fatty acid transport protein 1; *Irf4*, interferon regulatory factor 4. The values are represented as mean ± SEM. *$P$ < 0.05, **$P$ < 0.01, ***$P$ < 0.005.

I further examined the direct effect of exogenously-added EP4 agonist on insulin-induced Akt phosphorylation in WAT, in addition to liver, and skeletal muscle *in vivo*. Intraperitoneal administration of an EP4 agonist significantly suppressed insulin-induced Akt phosphorylation in WAT, whereas the EP4 agonist failed to affect insulin-induced signaling both in liver and skeletal muscle (Fig. 2-12, A-C). Suppressive effect of the EP4 agonist on insulin-induced Akt phosphorylation was abolished in *Ptger4*$^{-/-}$ WAT (Fig. 2-12D). These results demonstrate that EP4 receptor signaling has an ability to suppress insulin function in WAT *in vivo*. Based on these results, I conclude that EP4 signaling promotes lipolysis, at least in part, by suppressing the anti-lipolytic actions of insulin in WAT.

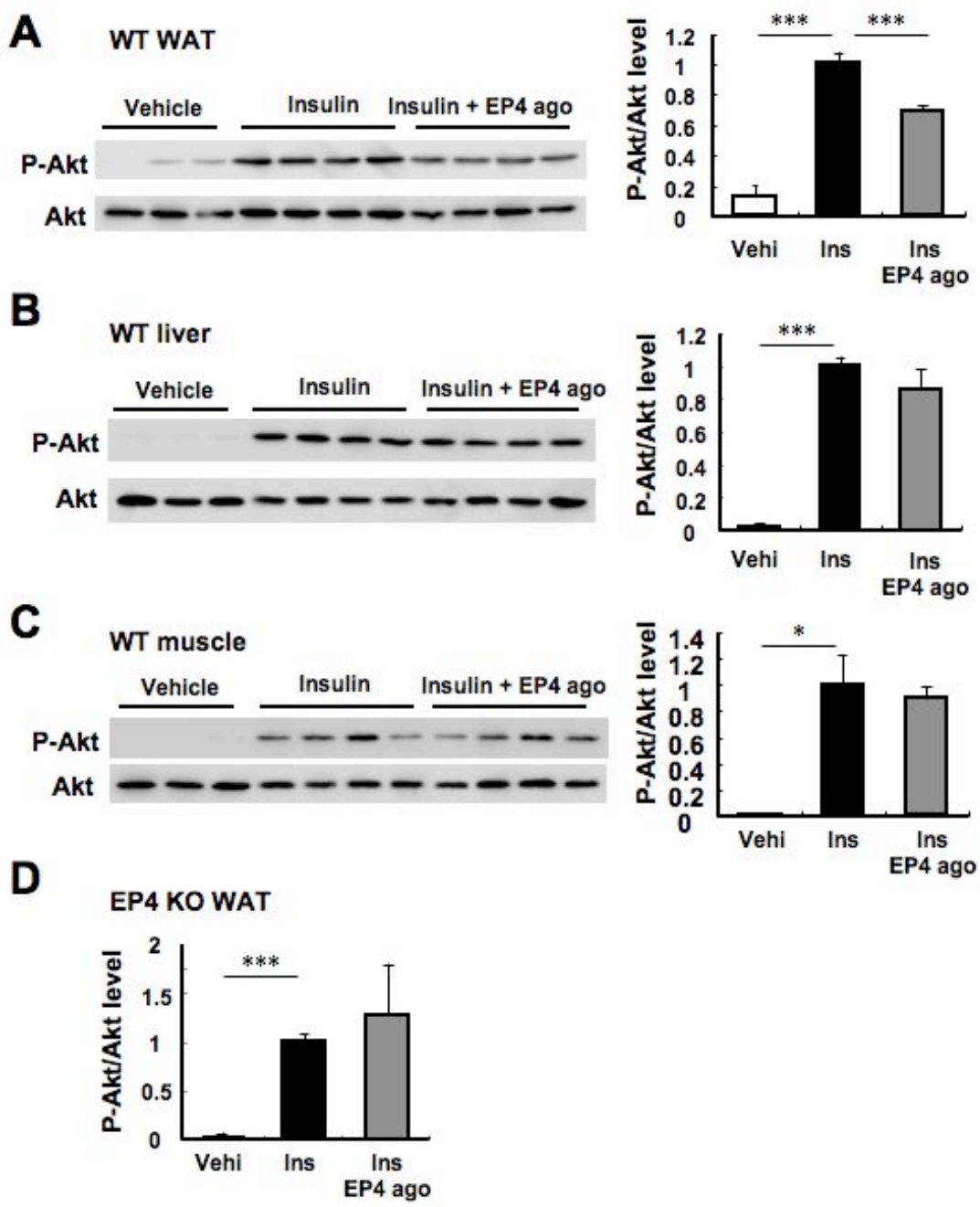


**Fig. 2-12. EP4 receptor signaling suppresses insulin signaling in WAT but not in liver or skeletal muscle.**

**A-C,** Effect of an EP4 agonist on insulin-induced Akt phosphorylation. Insulin (3 U/kg) with or without an EP4 agonist (300 μg/kg) were administered intraperitoneally into WT mice fasted for 5 h. *Left*, Western blot analysis of phospho-Akt (P-Akt) at Ser473 and total Akt levels in WAT (A), liver (B), and skeletal muscle (C) at 15 min after insulin injection. *Right*, quantifications of P-Akt normalized to total Akt ($n$ = 3-4). **D,** Effect of an EP4 agonist on insulin-induced Akt phosphorylation in EP4 KO WAT. The values are represented as mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$.

## Discussion

*$PGE_2$-EP4 signaling is unlikely to be involved in physiological adipogenesis* in vivo

In chapter 1, I found that endogenous $PGE_2$-EP4 receptor signaling negatively regulates differentiation of preadipocytes into adipocytes. However, the current study demonstrate that EP4-deficient mice showed an increase in adipocyte cell size, but not in cell number, compared with WT mice, even though the EP4-deficient mice showed increased adiposity. These results indicate that $PGE_2$-EP4 signaling is unlikely to be involved in physiological adipogenesis *in vivo*. However, recent studies have revealed that under pathological conditions, such as high fat diet-induced chronic inflammation state, adipogenesis occurs during adulthood (Tchoukalova et al., 2010). Since macrophages within chronic inflammation sites have been shown to produce $PGE_2$ (Wu et al., 2013), contribution of EP4 signaling to adipogenesis in such pathological conditions is an interesting issue to be examined.

*$PGE_2$-EP4 signaling negatively regulates lipid storage of adipocyte* in vivo

$PGE_2$-EP4 receptor signaling has been known as an important modulator working in many pathological contexts, including colitis, Alzheimer's disease and multiple sclerosis (Kabashima et al., 2002; Hoshino et al., 2012; Yao et al., 2009). Here I investigated the physiological function of EP4 receptor by employing knockout mouse model. My results show for the first time that EP4 receptor signaling contributes to physiological stimulation of lipolysis of adipocytes *in vivo*, at least partly by suppressing the anti-lipolytic actions of insulin, leading to stimulation of FFA flow from WAT to liver.

*When does $PGE_2$-EP4 signaling function in WAT?*

In contrast to the results in the current study, $PGE_2$ has been shown to inhibit lipolysis via EP3 receptor by suppression of cAMP production (Jaworski et al., 2009). There is a question in what situation these two PG receptors work respectively. In *Ptger4*$^{-/-}$ mice, body weight was higher in normal diet-fed conditions, and this

phenotype can be observed from young age. In contrast, EP3 receptor contributes to incidence of obesity under high fat diet-fed conditions (Jaworski et al., 2009). Therefore, EP4 receptor may work in physiological adipocyte maturation phase, while EP3 receptor may work mainly in pathological adipocyte hypertrophy stage. In agreement with this hypothesis, I found that EP3 receptor is expressed only in mature adipocytes while EP4 receptor is expressed in all differentiation stages in adipocyte culture system (Fig. 1-2A). Then, when and how is $PGE_2$ produced in adipose tissue? Jaworski *et al.* showed that the expression of AdPLA, the major $PLA_2$ isozyme in adipose tissue, is induced after feeding or insulin administration (Jaworski et al., 2009). Therefore, it is possible that after feeding and insulin release from islet, $PGE_2$ production is enhanced in adipose tissue, and $PGE_2$ –EP4 receptor signaling may function in a negative feed back loop of an insulin signaling. In my laboratory, a trial to detect the $PGE_2$ production in adipose tissue after these stimuli is now in progress.

*Perspective on an EP4 agonist as an anti-metabolic disease agent*

In this study, I found that EP4 signaling promotes physiological lipolysis and negatively regulates lipid accumulation in WAT. Therefore, EP4 agonists may have a potential for an anti-obesity agent. However, increased lipolysis might lead to high FFA levels in peripheral blood. Elevated blood FFA levels are known to correlate strongly with insulin resistance and obesity-linked diabetes (Borden, 2011). To evaluate EP4 agonist as an anti-obesity agent, further examinations to test its ability to reduce fat mass and regulate insulin sensitivity by using obesity animal model will be required in the future.

In summary, the current study demonstrates that $PGE_2$-EP4 signaling maintains lipid homeostasis by regulating insulin signaling and lipolysis in adipose tissue (Fig. 2-13). Therefore, regulation of EP4 receptor signaling might serve as a potential therapeutic strategy for obesity and diabetes.

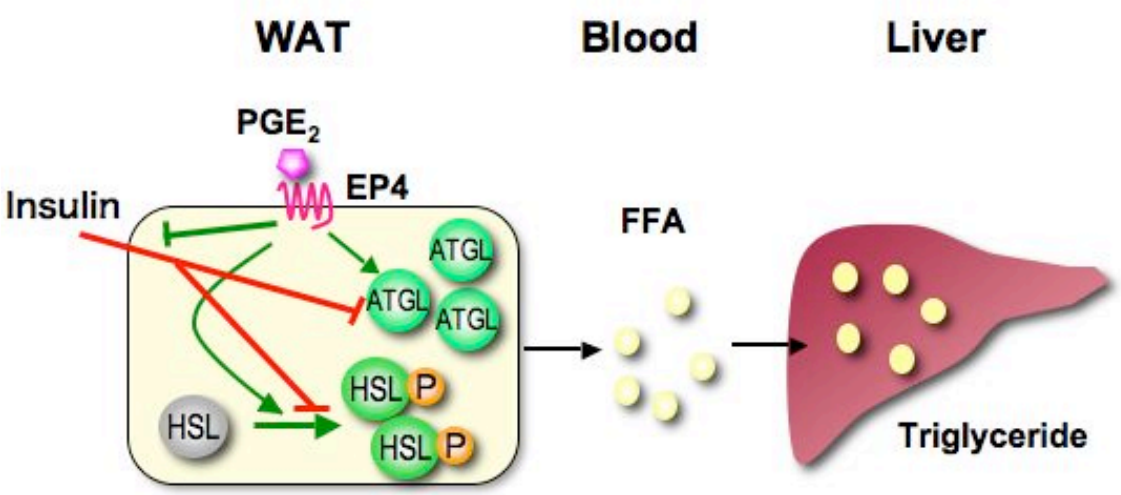


**Fig. 2-13. A schematic model showing a mechanism underlying stimulation of lipolysis through $PGE_2$-EP4 signaling in adipocytes under physiological conditions.**

$PGE_2$ stimulates lipolysis by acting on EP4 receptor in adipocytes at least partly via suppression of insulin signaling. As a result of $PGE_2$ actions, free fatty acid is released into blood stream and accumulated in other organs, such as liver, in the form of triglyceride.

# CONCLUSIONS

The summary of the results presented in this study as follows:

Chapter 1

1. Endogenous $PGE_2$-EP4 signaling suppresses early phase of adipocyte differentiation in MEFs.
2. Differentiation stimuli induce COX-2 gene expression and $PGE_2$ production in MEFs.

Chapter 2

1. $PGE_2$-EP4 signaling suppresses lipid storage in adipose tissue.
2. $PGE_2$-EP4 signaling directly promotes lipolysis in adipose tissue.
3. $PGE_2$-EP4 signaling suppresses insulin signaling in adipose tissue.

# MATERIALS AND METHODS

## Chapter 1

### *Mice*

Specific-pathogen-free, C57BL/6 mice were obtained from Japan SLC (Hamamatsu, Japan). Mice were maintained on a 12-h light, 12-h dark cycle under specific-pathogen-free conditions. *Ptger4*$^{-/-}$ mice with a mixed background of 129SV and C57BL/6 and *Ptgfr*$^{-/-}$ mice with a genetic background of C57BL/6 were generated as described (Segi et al. 1998; Sugimoto et al. 1997; Kabshima et al. 2002). Mice with the same background were used for the controls. All experimental procedures were approved by the Committees of Animal Research of Kyoto University Faculty of Pharmaceutical Sciences and Kumamoto University.

### *Reagents*

$PGE_2$, fluprostenol, SC560 and NS398 were purchased from Cayman Chemical (Ann Arbor, MI). The EP-specific agonists, ONO-DI-004 (EP1), ONO-AE1-259 (EP2), ONO-AE-248 (EP3) and ONO-AE1-329 (EP4) were generous gifts from Ono Pharmaceutical Company (Osaka, Japan). Indomethacin was purchased from Sigma (St. Louis, MO). Mouse polyclonal anti-COX-1 antibody and mouse polyclonal anti-COX-2 antibody were purchased from Cayman Chemical. Mouse monoclonal anti-actin antibody was purchased from Chemicon. Intracellular cyclic AMP was measured using a radioimmunoassay kit (Yamasa, Choshi, Japan), and $PGE_2$ was quantified using an enzyme immunoassay kit (Cayman Chemical).

### *Culturing of mouse embryonic fibroblast (MEF) cells, adipocyte differentiation and measurement of triglyceride content*

Mouse embryos at embryonic day 14.5 were harvested from WT, *Ptger4*$^{-/-}$, or *Ptgfr*$^{-/-}$ mice. Embryos were minced and filtrated through a 95 μm nylon mesh and washed, and then MEFs were prepared. MEFs were grown to confluency (2 x $10^6$ cells / 60 mm dish) in Dulbecco's modified Eagle's medium (DMEM) high glucose supplemented with 10% calf serum. Differentiation was initiated by culturing the cells

in differentiation-inducing cocktail containing 10% fetal bovine serum (FBS), 0.5 mM isobutylmethylxanthine (IBMX), 0.25 μM dexamethasone, and 0.2 μM insulin. After two days, the culture medium was changed to adipocyte growth medium containing 10% FBS, 0.2 μM insulin and exchanged every two days for an additional six days. MEFs grown in a 60 mm dish were harvested in 1 ml of 2-propanol, sonicated, and triglyceride levels in the cell lysate were measured using the Triglyceride E test kit according to the manufacturer's instructions (Wako, Tokyo, Japan).

***RNA isolation and real time RT-PCR***

Total cellular RNA was isolated from MEFs on the indicated days of the differentiation program with the RNeasy mini kit (QIAGEN), and subjected to the RT reaction with a Superscript Ⅱ First-strand Synthesis Kit, and subjected to real time PCR with a LightCycler (Roche Applied Science) using Fast Start DNA Master SYBR Green I as reported previously (Segi et al. 2003). Crossing point values were acquired by using the second derivative maximum method. The expression level of each gene was quantified using external standardized dilutions. Relative expression levels of target genes between samples were normalized by those of β-actin (*Actb*). Primer sequences for each gene are shown in **Table 1**. The specificity of each primer set was confirmed by checking the product size by gel electrophoresis.

***Measurement of $PGE_2$ production, cAMP formation and $Ca^{2+}$ mobilization***

$PGE_2$ levels were measured using the prostaglandin $E_2$ EIA kit according to the manufacturer's instructions (Cayman Chemical). Cyclic AMP levels in MEFs were determined as reported previously (Tsuboi et al., 2004). $Ca^{2+}$ mobilization was analyzed by FlexStation (Molecular Devices) as follows: MEFs were loaded with 4 μM Fura-2/AM and fluorescence was measured by illuminating the cells with alternating 340/380 nm light every 3 s, and fluorescence intensity was measured at 510 nm. Changes in intracellular $Ca^{2+}$ concentration were presented as the change in the ratio of fluorescence intensity for excitation at 340 and 380 nm.

***Immunoblot analysis***

MEFs grown in a 100 mm dish were harvested at the indicated hours of the differentiation program in SDS sample buffer and sonicated. Aliquots (30 μg protein)

were then subjected to polyacrylamide gel electrophoresis (10%), and the separated proteins were transferred to a PVDF membrane. The membrane was incubated with anti-COX-1 (1:1000), anti-COX-2 (1:1000), or anti-actin (1:3000) antibody and bands were visualized with the ECL reagent (GE Healthcare).

***Statistical analysis***

Each experiment was independently repeated three times, and the mean value ± SEM were shown. Comparison of two groups was analyzed by the Student's *t* test. For comparison of more than two groups with comparable variances, one-way ANOVA was performed first. Then, either the Dunnet's or Tukey's test was used to evaluate the pairwise group difference. *P* values < 0.05 were considered to indicate a significant difference.

## Chapter 2

***Mice***

Specific-pathogen-free, C57BL/6 mice were obtained from Japan SLC (Hamamatsu, Japan). Mice were maintained on a 12-h light, 12-h dark cycle under specific-pathogen-free conditions. *Ptger4*$^{-/-}$ with the mixed background of 129SV and C57BL/6 were generated as described (Segi et al. 1998; Kabashima et al. 2002). Mice with the same background were used for control. For generating fat specific EP4 KO mice, *Fabp4*-cre (The Jackson Laboratory) mice were mated with *Ptger4*$^{flox/flox}$ mice (Schneider et al. 2004). *Fabp4*-cre/ *Ptger4*$^{flox/flox}$ (EP4 FKO) mice were compared with control *Ptger4*$^{flox/flox}$ littermates of same generation. All experimental procedures were approved by Committees of Animal Research of Kyoto University Faculty of Pharmaceutical Sciences and Kumamoto University.

***Reagents***

The EP4-specific agonist ONO-AE1-329 was generous gifts from Ono Pharmaceutical Company (Osaka, Japan). Isoproterenol was purchased from

Sigma-Aldrich (St. Louis, MO). Rabbit monoclonal anti-phospho-Akt antibody, rabbit monoclonal anti-total-Akt antibody, and rabbit monoclonal anti-phospho-HSL antibody were purchased from Cell Signaling. Rabbit polyclonal anti-total-HSL antibody was purchased from Santa Cruz (Dallas, TX).

***Determination of adipocyte size and number***

We isolated perigonadal WAT and fixed it with osmium tetroxide (Nakalai Tesque, Kyoto, Japan). The distribution of adipocyte size in WAT was measured by a coulter counter (Multisizer III, Coulter Electronics) as described previously (Hosooka et al. 2008). Theoretical adipocyte number was calculated by using WAT weight and average cell size.

***RNA isolation and real time RT-PCR***

Total RNA was extracted from tissues with Sepasol-RNA I Super G (Nacalai Tesque), and reverse transcribed to cDNA using PrimeScript RT reagent Kit (Takara Bio, Otsu, Japan) according to the manufacture's instructions. Real-time quantitative RT-PCR was performed in LightCycler system (Roche Applied Science) using Fast Start DNA Master SYBR Green I. Crossing point values were acquired by using the second derivative maximum method. The expression level of each gene was quantified using external standardized dilutions. The relative expression levels of target genes between samples were normalized by those of β-actin (*Actb*). Primer sequences for each gene are shown in **Table 2**. The specificity of each primer set was confirmed by checking the product size by gel electrophoresis.

***Immunoblot analysis***

Tissue lysates were prepared with RIPA buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% NP-40, 0.1% SDS, and 0.5% sodium deoxycholate) supplemented with a protease inhibitor cocktail (Nacalai Tesque), 10 mM sodium pyrophosphate, 1 mM $Na_3VO_4$, and 10 mM NaF. For Western blot analysis, 20 μg of total protein was subjected to SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked and probed with antibodies against phospho-Akt (Ser473) (1:1000), total-Akt, phospho-HSL (Ser563) (1:1000), (Cell Signaling), or total-HSL

(1:200). Then bands were visualized with the ECL reagent (GE Healthcare).

***Glucose and insulin tolerance test***

For glucose tolerance test, mice were fasted for 16 hr and injected intraperitoneally with D-glucose (2 g/kg). For insulin tolerance test, mice were fasted for 5 hr and injected intraperitoneally with insulin (0.25 U/kg, Nacalai Tesque). The glucose concentrations in the collected blood samples were measured with a glucometer (Glutest Ace R, Sanwa Kagaku, Nagoya, Japan).

***Serum analysis***

Serum free fatty acid was analysed with NEFA C Kit (Wako, Tokyo, Japan). Serum leptin (Morinaga, Yokohama, Japan), adiponectin (Otsuka pharmaceutical, Tokushima, Japan), and resistin (R&D, Minneapolis, MN) levels were determined by ELISA according to the manufacture's instructions.

***Ex vivo lipolysis assay***

Perigonadal fat pads were cut into 20 mg samples and incubated at 37$^{o}$C in 500 µl of Krebs-Ringer buffer (12 mM Hepes (pH 7.4), 121 mM NaCl, 4.9 mM KCl, 0.33 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 0.1% D-glucose) containing 3.5% fatty acid-free BSA. Fatty acid level in incubation buffer was measured with NEFA C Kit (Wako, Tokyo, Japan).

***Hepatic triglyceride measurement***

Liver was cut into 100 mg sample and homogenized in isopropanol. The homogenate was centrifuged at 20,000 x g for 5 min, and triglyceride level in the supernatant was measured with Triglyceride E Kit (Wako, Tokyo, Japan).

***Statistical analysis***

Statistically significant differences between two groups were assessed by Student's *t* test. Differences between multiple groups were assessed by one-way ANOVA with Tukey's test. $p < 0.05$ was considered statistically significant.

**Table 1. Primer sequences used for real-time RT-PCR in Chapter 1**

| Gene | Forward | Reverse |
|---|---|---|
| *Ptger1* | 5'-cgtcgctctcgacgattccgaaagaccgca-3' | 5'-cgatggccaacaccaccaacaccagcaggg -3' |
| *Ptger2* | 5'-ttcatattcaagaaaccagaccctggtggc-3' | 5'-agggaagaggtttcatccatgtaggcaaag -3' |
| *Ptger3* | 5'-atcctcgtgtacctgtcacagcgacgctgg -3' | 5'-tgctcaaccgacatctgattgaagatcatt-3' |
| *Ptger4* | 5'-ttccgctcgtggtgcgagtgttc -3' | 5'-gaggtggtgtctgcttgggtacg -3' |
| *Ptgdr* | 5'-aaaggaactgctgcctgcctcaggcaatca -3' | 5'-gttctcaagtttaaaggctccatagtacgc -3' |
| *Ptgfr* | 5'-gcatagctgtctttgtatatgcttgtgata-3' | 5'-gtgtcgtttcacaggtcactggggaattat-3' |
| *Ptgs1* | 5'-tgcatgtggctgtggatgtcatcaa-3' | 5'-cactaagacagacccgtcatctcca-3' |
| *Ptgs2* | 5'- agtgtgcgacatactca-3' | 5'-gcgtttgcggtactca-3' |
| *Pparg* | 5'- tctccagcatttctgctccacactatgaag-3' | 5'- cggcagttaagatcacacctatcataaata-3' |
| *Fasn* | 5'- ggcttctaaccgcaaaagt-3' | 5'- gtctcgttgcgtttgtagt-3' |
| *Lipe* | 5'- ctatggattacccaagcgg-3' | 5'- agtgttcgttcctcgg-3' |
| *Actb* | 5'- cctgtatgcctctggtcgta-3' | 5'-ccatctcctgctcgaagtct-3' |

**Table 2. Primer sequences used for real-time RT-PCR in Chapter 2**

| gene | forward | reverse |
|---|---|---|
| *Ptger4* | 5'-tgctccattccgctcgt-3' | 5'-gcacagtcttccgaagaagg-3' |
| *Ptgs1* | 5'-tgcatgtggctgtggatgtcatcaa-3' | 5'-cactaagacagacccgtcatctcca-3' |
| *Ptgs2* | 5'-agtgtgcgacatactca-3' | 5'-gcgtttgcggtactca-3' |
| *Pparg* | 5'-tctccagcatttctgctccacactatgaag-3' | 5'-cggcagttaagatcacacctatcataaata-3' |
| *Fabp4* | 5'-tgggaacctggaagcttgtctc-3' | 5'-gctgatgatcatgttgggcttg-3' |
| *Lpl* | 5'-ctgtacggcacagtgg-3' | 5'-tcctctcgatgacgaagc-3' |
| *Npr3* | 5'-tcctcctctacgttctggctt-3' | 5'-gtgtcagtcatggcaaccac-3' |
| *Lep* | 5'-tgtcggttcctgtggct-3' | 5'-ccctctgcttggcggatac-3' |
| *Slc27a1* | 5'-ctgctttggtttctgggactt-3' | 5'-gctctagccgaacacgaatca-3' |
| *Irf4* | 5'-ctcagagacagaggaagctcatc-3' | 5'-ggagggagcggtggtaatc-3' |
| *Pnpla2* | 5'-ctcattcgctggctgc-3' | 5'-ctgaaacacgagtcaggg-3' |
| *Lipe* | 5'-ggcttctaaccgcaaaagt-3' | 5'-gtctcgttgcgtttgtagt-3' |
| *Mgll* | 5'-caagagtggagcgagcaat-3' | 5'-aggacgtgataggcaccttcata-3' |
| *Ppara* | 5'-gtggtgcatttgggcgtatc-3' | 5'-caggccacagagcgcta-3' |
| *Ppargc1a* | 5'-gcccaggtacgacagcta-3' | 5'-accaacgtaaatcacacggc-3' |
| *Cpt1b* | 5'-tgggaagaatatgtctacctccgaa-3' | 5'-tagtgttgaacatcctctccatctg-3' |
| *Cpt2* | 5'-gaacggcattgggaaggag-3' | 5'-cggattgaatgccatgaatgga-3' |
| *Acadl* | 5'-tggcttcagcctccactca-3' | 5'-ggcgttcgttcttactcctt-3' |
| *Pdk4* | 5'-ttcaccacatgctcttcgaact-3' | 5'-taatcctcagaggaacaccgc-3' |
| *Slc2a4* | 5'-gacgacggacactcca-3' | 5'-cccatagcatccgcaac-3' |
| *Ucp1* | 5'-ctggcctctccagtggat-3' | 5'-ggacccgagtcgcagaaa-3' |
| *Emr1* | 5'-tgtacctgtcaaccaggcttt-3' | 5'-cctcctccactagattcaagtcc-3' |
| *Cd68* | 5'-ccagggaggttgtgacg-3' | 5'-atgtccactgtgctgcc-3' |
| *Nos2* | 5'-tctgcatggaccagtataaggc-3' | 5'-gcatacctgaaggtgtggttga-3' |
| *Tnf* | 5'-ggggccaccacgctcttctgtc-3' | 5'-tgggctacaggcttgtcactcg-3' |
| *Chi3l3* | 5'-cagaaccgtcagatattcattc-3' | 5'-ctttctccacagattcttcct-3' |
| *Mrc1* | 5'-tggaaccaccactgactacg-3' | 5'-ccttgcctgatgccaggtta-3' |
| *Retnla* | 5'-cgtggagaataaggtcaaggaac-3' | 5'-tctcccaagatccacaggcaaa-3' |

**Table 2. Primer sequences used for real-time RT-PCR in Chapter 2 (continued)**

| gene | forward | reverse |
|---|---|---|
| *Srebf1* | 5'-gcaaggccatcgactacatcc-3' | 5'-ttcgggtttcatgccctccata-3' |
| *Scd1* | 5'-catgtctgacctgaaagccga-3' | 5'-cgcaagaaggtgctaacga-3' |
| *Fasn* | 5'-ctatggattacccaagcgg-3' | 5'-agtgttcgttcctcgg-3' |
| *Acaca* | 5'-caccaacgctaaagtggc-3' | 5'-ggcggtgttgtacgct-3' |
| *Actb* | 5'-cctgtatgcctctggtcgta-3' | 5'-ccatctcctgctcgaagtct-3' |

## ACKNOWLEDGEMENTS

I would like to appreciate gratefully to Professor Yukihiko Sugimoto Graduate School of Pharmaceutical Sciences, Kumamoto University, and Professor Kazuhisa Nakayama, Graduate School of Pharmaceutical Sciences, Kyoto University for their supports through the course of this work.

I would like to express my appreciation to Professor Hiroshi Takeshima, Graduate School of Pharmaceutical Sciences, and Professor Manabu Negishi, Graduate School of Biostudies, Kyoto University for their peer-reviewing of the thesis.

My heartfelt appreciation is given to Associate Professor Eri Segi-Nishida, Graduate School of Pharmaceutical Sciences, Kyoto University and Assistant Professor Soken Tsuchiya, Graduate School of Pharmaceutical Sciences, Kumamoto University, for their excellent direction and fruitful suggestions.

I would like to thank Associate Professor Hiroshi Sakaue, Graduate School of Health Biosciences, Tokushima University, Professor Shuh Narumiya, Faculty of Medicine, Kyoto University for helpful discussions and technical supports.

I would like to express my appreciation to Dr. Hye-Won Shin, Dr. Yohei Katoh, Dr. Hiroyuki Takatsu, Dr. Senye Takahashi, Dr. Shogo Misumi, Dr. Nobutoki Takamune, Dr. Akihiko Kuniyasu, Dr. Kohichi Kawahara, Dr. Akina Saitoh, Dr. Kazushi Morimoto, Dr. Michita Suenobu, Ms. Toshiko Sugimoto, Mr. Naritoshi Shirata, Ms. Miki Yoshioka, Mr. Kenji Kuroiwa, Ms. Yukiko Kawasaki, Ms. Mari Sakaida, Mr. Naoki Egashira, Mr. Yuki Imoto, Ms. Hiroko Matsunaga, Mr. kyoshiro Tsuge, Mr. Hironori Hohjoh, Ms. Mayuho Watanabe, Mr. Ryo Iwasaki, Mr. Kazunori Muraki, Mr. Yutaro Shimadzu, Mr. Yuji Suzuki, Ms. Xiao Yan Ma, Mr. Yukiomi Eguchi for their technical advice and helpful discussion.

I also thank the members of Department of Physiological Chemistry, Graduate School of Pharmaceutical Sciences, Kyoto University and Department of Pharmaceutical Biochemistry, Graduate School of Pharmaceutical Sciences, Kumamoto University for their continuous encouragements, helpful discussions and technical supports.

Finally, I would like to thank my family for persistent understanding and support for many things.

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