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)

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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

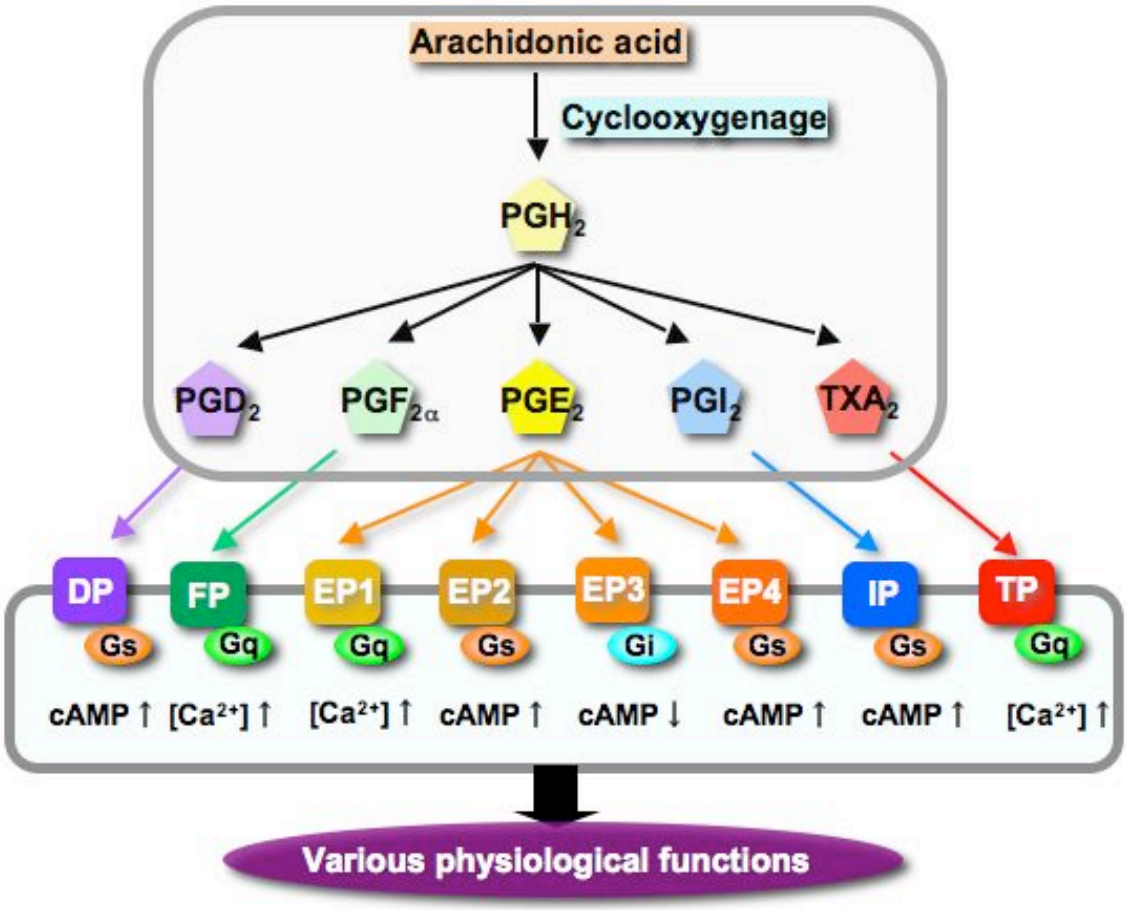

Fig. 0-1. PG synthesis and cognate receptor signaling.

(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 adipocyte 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 modulates particular function of adipocytes without affecting adipogenesis *in vivo* (Chapter 2).

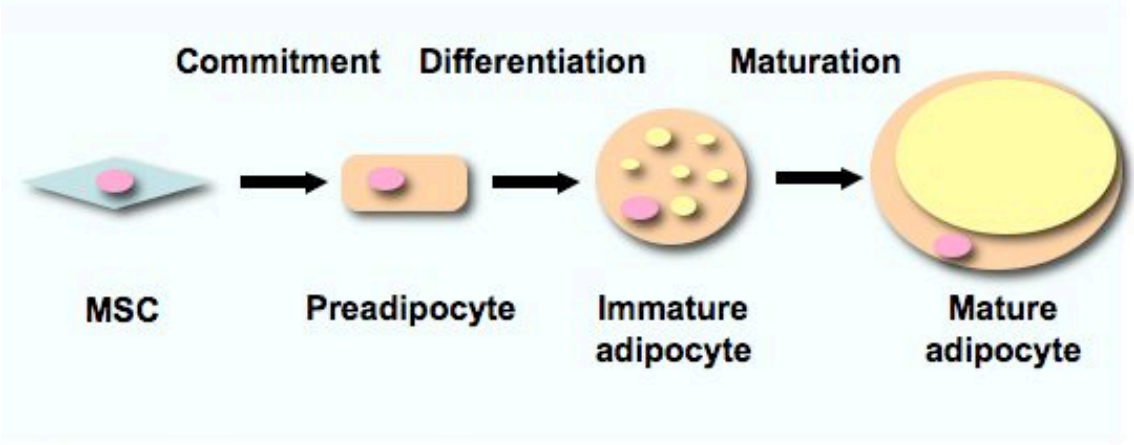

Fig. 0-2. The course of adipogenesis.

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 (2.0×10^6 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.

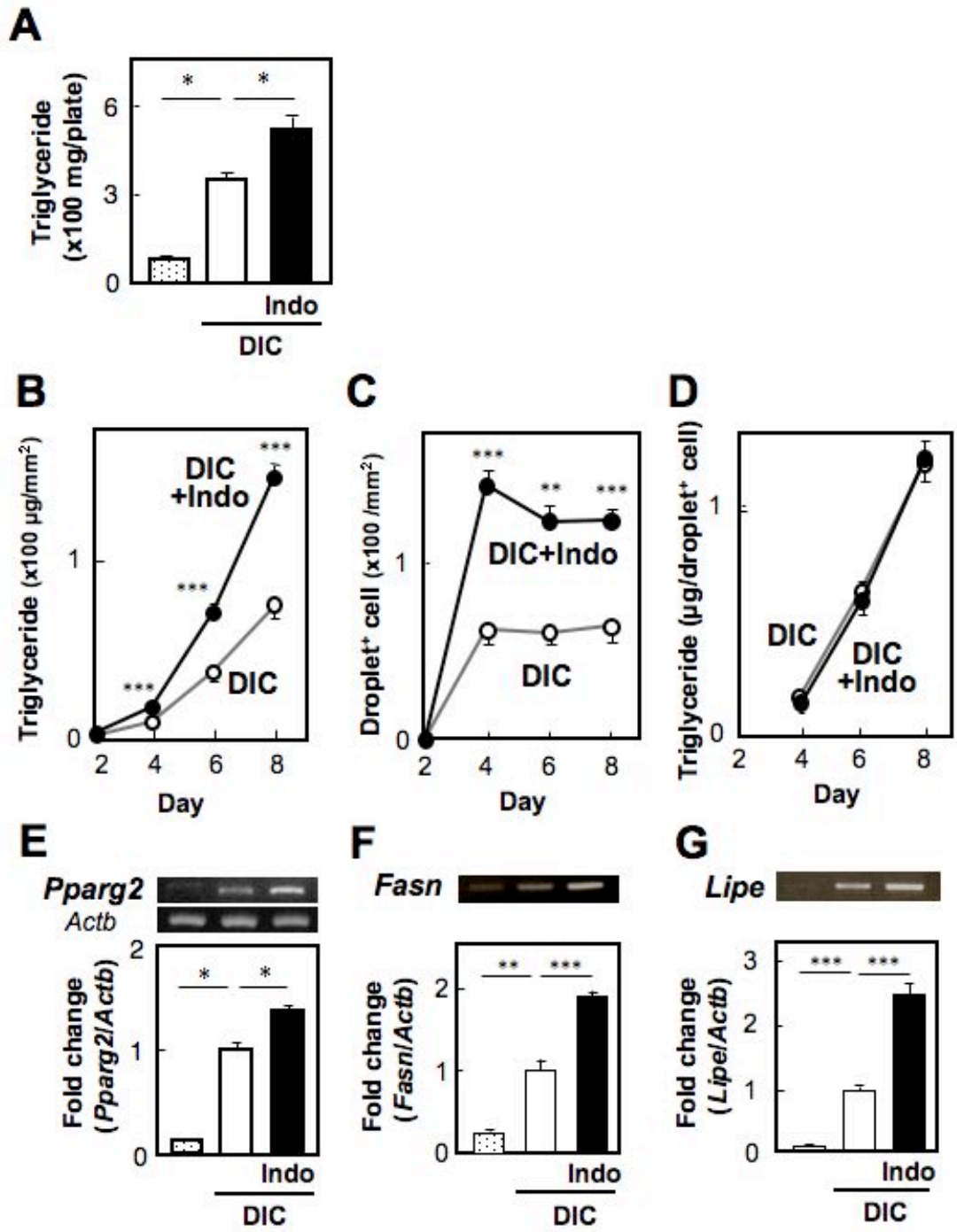
A
Triglyceride (x100 mg/plate)
Indo
DIC
B
Triglyceride (x100 μg/mm²)
DIC +Indo
DIC
Day
C
Droplet⁺ cell (x100 /mm²)
DIC+Indo
DIC
Day
D
Triglyceride (μg/droplet⁺ cell)
DIC
DIC +Indo
Day
E
Pparg2
Actb
Fold change (Pparg2/Actb)
F
Fasn
Fold change (Fasn/Actb)
G
Lipe
Fold change (Lipe/Actb)

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.

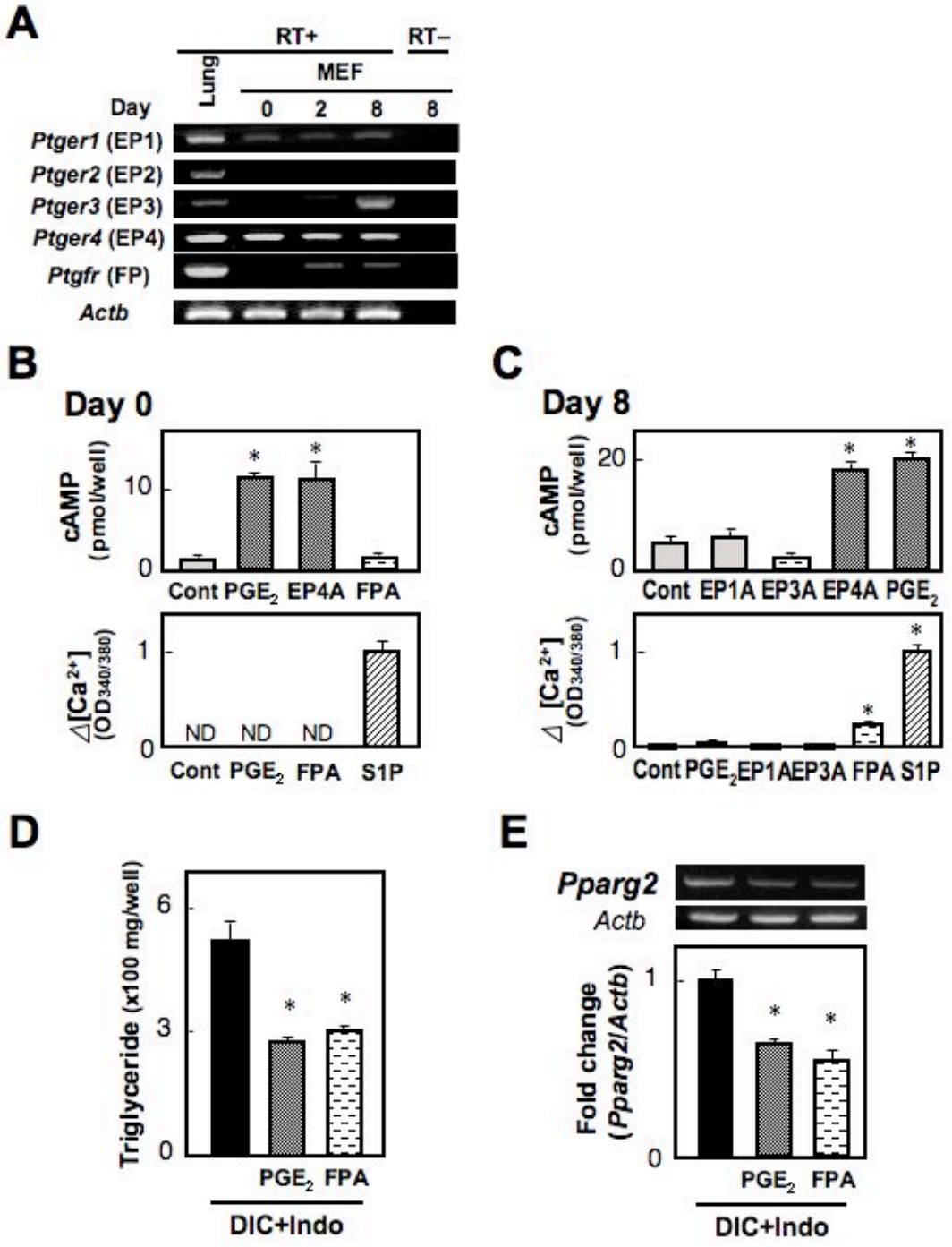
A
RT+
RT−
Lung
MEF
Day
0
2
8
8
Ptger1 (EP1)
Ptger2 (EP2)
Ptger3 (EP3)
Ptger4 (EP4)
Ptgfr (FP)
Actb
B
Day 0
cAMP (pmol/well)
10
0
Cont PGE2 EP4A FPA
Δ[Ca2+] (OD340/380)
1
0
ND ND ND
Cont PGE2 FPA S1P
C
Day 8
cAMP (pmol/well)
20
0
Cont EP1A EP3A EP4A PGE2
Δ [Ca2+] (OD340/380)
1
0
Cont PGE2 EP1A EP3A FPA 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.

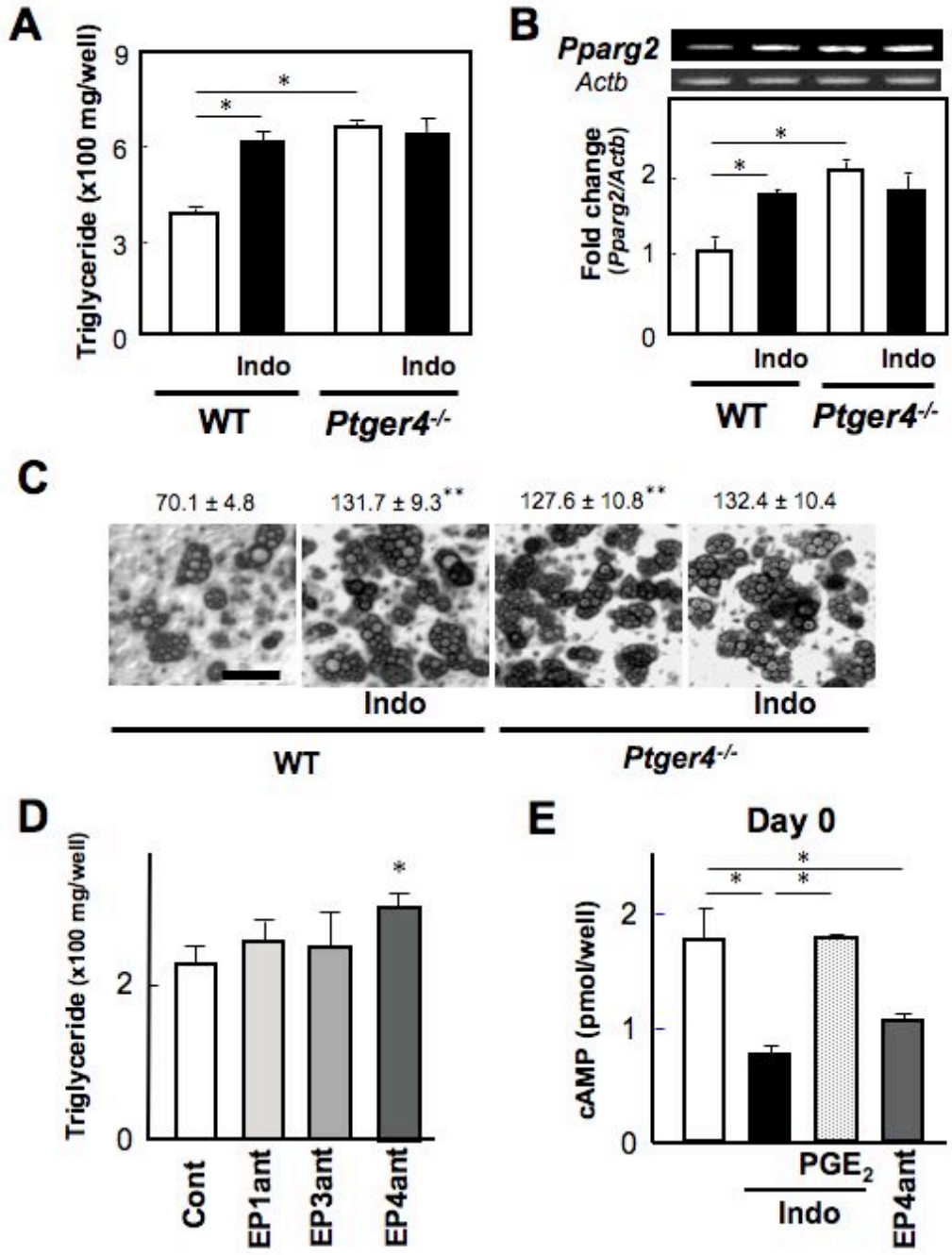

Fig. 1-3. Endogenous PGE_2-EP4 signaling suppresses adipocyte differentiation in MEFs.

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.

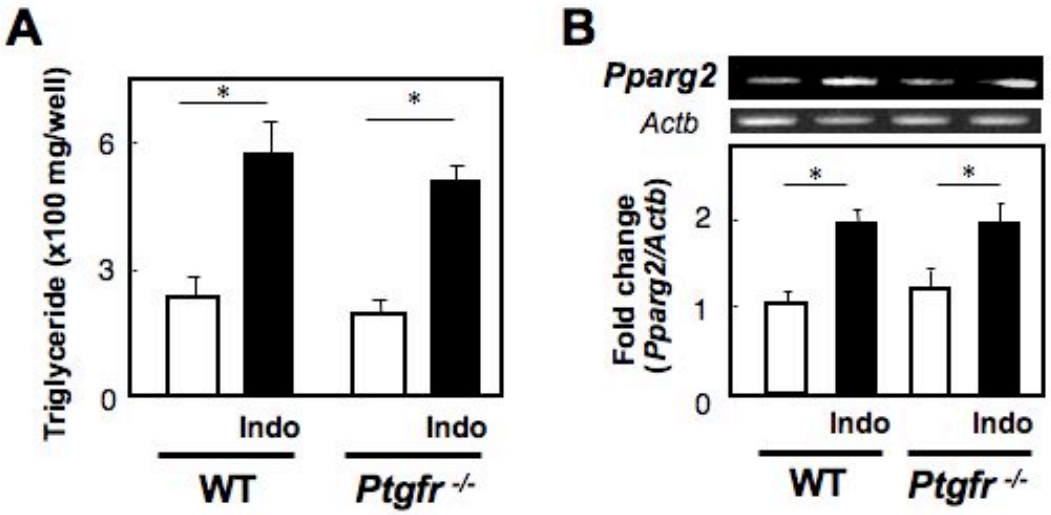

Fig. 1-4. FP receptor deficiency fails to affect adipocyte differentiation in MEFs.

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.

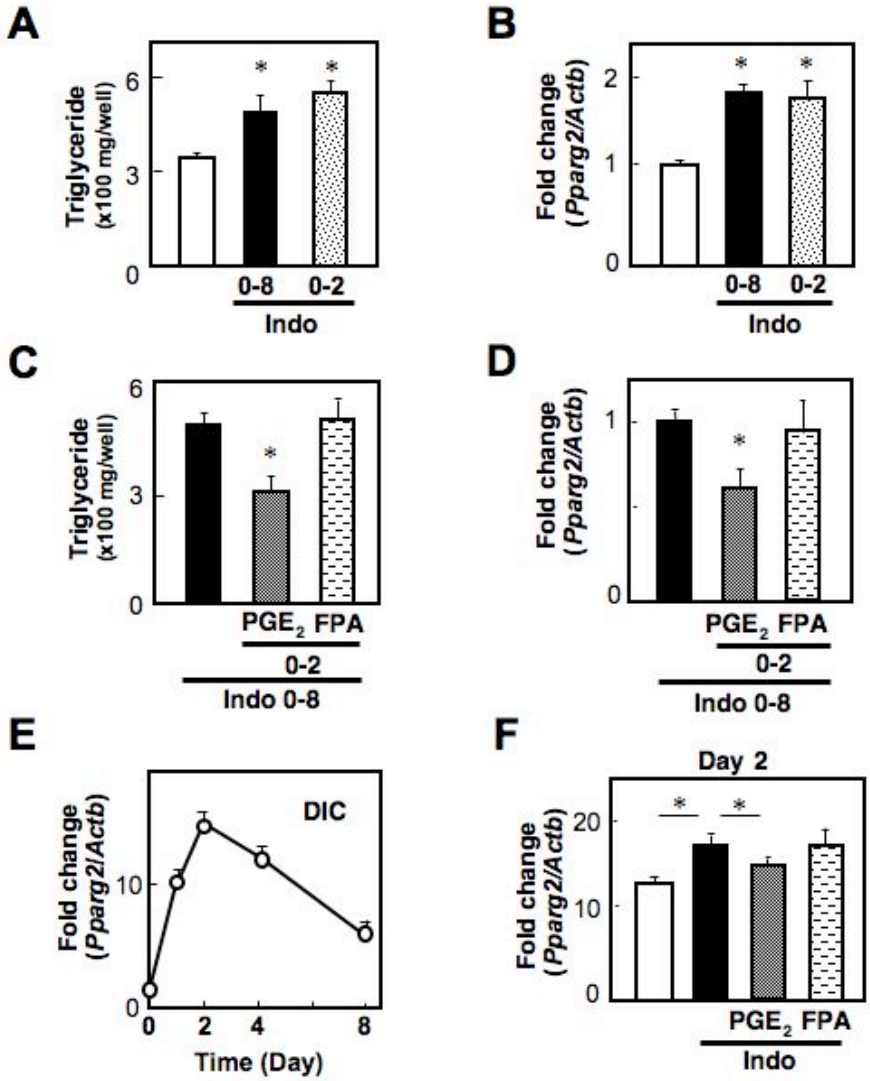

Fig. 1-5. PGE_2-EP4 signaling suppresses transcription of *Pparg2* gene.
(A, B) MEFs grown to confluency were treated with DIC in the presence (Indo) or absence of indomethacin. On day 2, DIC was replaced with media containing insulin in the presence (0-8) or absence of indomethacin (0-2). (C, D) MEFs 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.

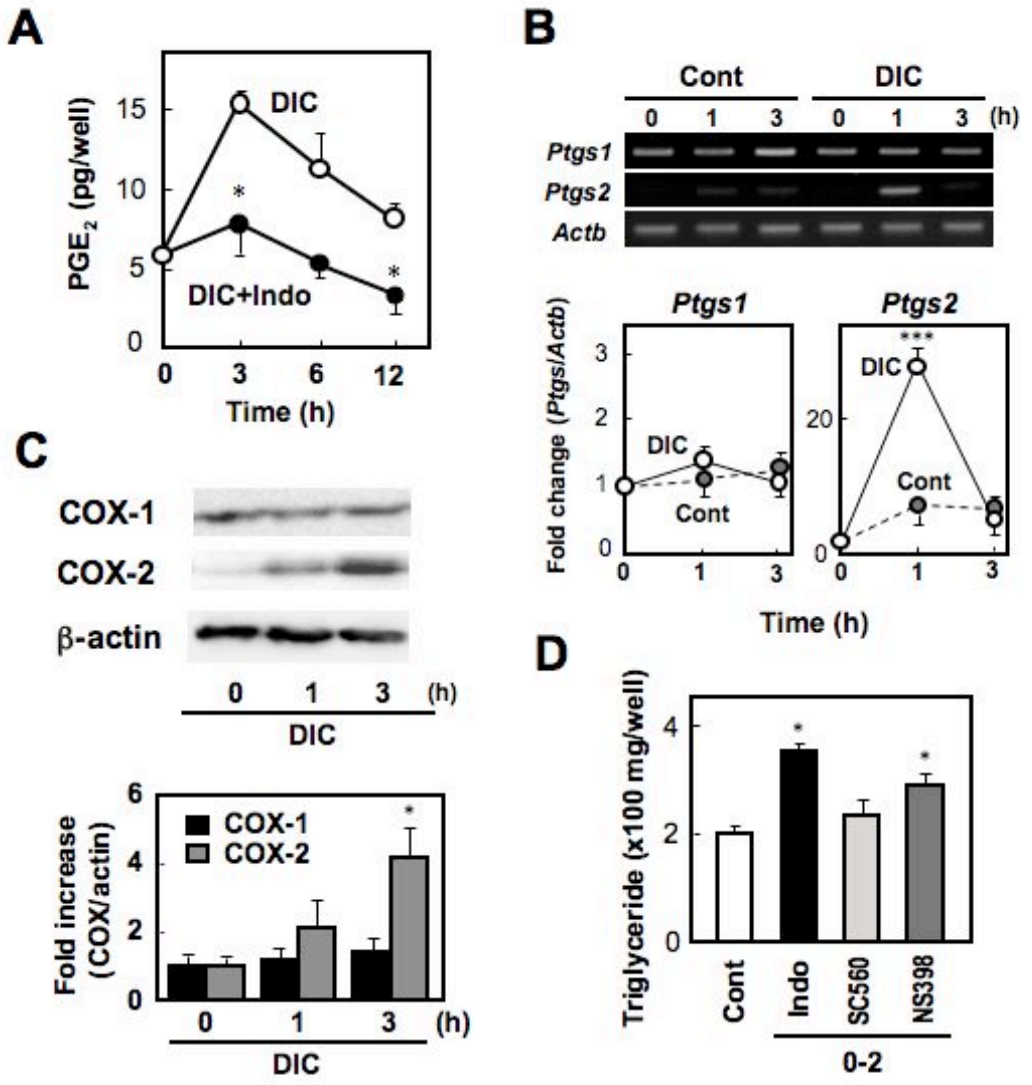

Fig. 1-6. COX-2-derived PGE_2 suppresses adipocyte differentiation of MEFs.
(A) PGE_2 production of MEFs treated with DIC for the indicated times in the presence (+Indo) or absence of indomethacin was measured. (B) MEFs were treated with DIC or control medium. Total RNA was isolated at the indicated 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.

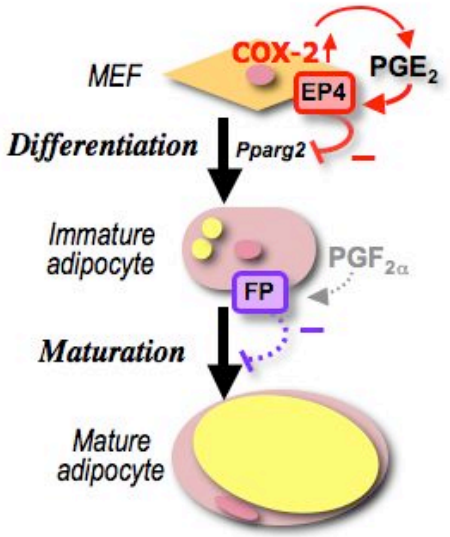

Fig. 1-7. Schematic model representing the relationship among prostanoid receptor signaling events during adipocyte differentiation in MEFs.
Adipocyte differentiation stimuli induce COX-2 gene expression and PGE_2 production, and the resultant PGE_2 suppresses the peak level of *Pparg2* expression via 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 (Excerpted version): Prostaglandin E_2-EP4 Signaling Modulates Particular Adipocyte Functions without Affecting Adipogenesis *in vivo*.

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 function. 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 the 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.

Results and discussion

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 (data not shown). Eight-week-old *Ptger4*$^{-/-}$ mice exhibited a significant excess of visceral (e.g., perigonadal, mesenteric, and perirenal) and subcutaneous (e.g., inguinal) WAT depots (data not shown). In contrast, the averaged weights of other organs including liver, kidney, and heart was not remarkably altered, compared with those of WT mice (data not shown). Histological analysis and cell size measurement of WAT showed significantly larger adipocytes in *Ptger4*$^{-/-}$ mice than in WT mice (data not shown). In contrast, there was no alteration in the cell number in WT and *Ptger4*$^{-/-}$ WAT (data not shown). Increased body weight, increased WAT weight and larger adipocyte size were also observed in *Ptger4*$^{-/-}$ male mice (data not shown).

In summary, these results suggest that EP4 receptor physiologically suppresses lipid storage in adipocytes and does not affect adipogenesis. Further investigations to assess how EP4 signaling suppresses fat mass, such as regulating lipogenesis, lipolysis, energy intake, energy expenditure or the other metabolic processes are now in progress. Understanding the mechanism underlying regulation of lipid homeostasis by EP4 receptor signaling might contribute to a potential therapeutic strategy for metabolic disorders such as obesity and diabetes.

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.

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. All experimental procedures were approved by Committees of Animal Research of Kyoto University Faculty of Pharmaceutical Sciences and Kumamoto University.

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*). 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. 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).

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'

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