Prostaglandin E₂-EP4 signaling suppresses adipocyte differentiation in mouse embryonic fibroblasts via an autocrine mechanism

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Abstract

The prostaglandin (PG) receptors EP4 and FP have the potential to exert negative effects on adipogenesis, but the exact contribution of endogenous PG-driven receptor signaling to this process is not fully understood. In this study, we 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 transcription of the $Pparg2$ gene as well as the subsequent differentiation, respectively. 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 adipocyte differentiation by affecting $Pparg2$ expression in an autocrine manner, and FP-mediated inhibition is not directly involved in adipocyte differentiation in the MEF system.

Keywords

prostanoid, receptor subtypes, adipogenesis, fat cell, aspirin-like drugs
Introduction

Adipogenesis is a crucial aspect in controlling body fat mass (1, 2). Acquisition of the mature adipocyte phenotype is a highly regulated process in which mesenchymal stem cells (MSCs) undergo differentiation, resulting in both an increase in size and number of mature adipocytes in adipose tissue. 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 (3). 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 (4). 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.

Prostaglandins (PGs) are arachidonate metabolites synthesized by the action of cyclooxygenase (COX) as the rate-limiting enzyme. COX has been shown to exist as two isomers, COX-1 and COX-2. PGs exert a wide range of actions through their binding to plasma membrane receptors (5, 6). For instance, PGF\(_2\alpha\) exerts its actions via specific interactions with the prostanoid FP receptor, which activates phospholipase C, resulting in phosphatidylinositol breakdown (7). In contrast, PGE\(_2\) exerts its actions through its interaction with four PGE\(_2\) receptor subtypes (EPs; EP1, EP2, EP3 and EP4). The EP subtypes differ in their signal transduction pathways; EP1 is coupled to the
mobilization of intracellular [Ca\(^{2+}\)], EP2 and EP4 are coupled to the stimulation of adenylyl cyclase and phosphoinositide 3-kinase (PI-3 kinase) (8), and EP3 is mainly coupled to the inhibition of adenylyl cyclase. The diverse actions of PGE\(_2\) can be explained by the existence of these multiple EP subtypes with different signal transduction pathways (6, 9). It has been shown that COX products such as PGE\(_2\) and PGF\(_{2\alpha}\) inhibit adipocyte development (10-12). A recent study suggested that COX-2 may be involved in body fat regulation (13). Mice heterozygous for the COX-2 gene showed approximately 30% increased body weight, with 2-3-fold larger fat pads compared with those of wild-type animals. PGE\(_2\) production in adipose tissue from COX-2 null mice was only 20% of that of wild-type mice. These results suggest that COX-2 as well as PGE\(_2\) participates in the negative regulation of adipocyte differentiation. Indeed, we previously identified that PGE\(_2\)-EP4 signaling suppresses adipocyte differentiation from 3T3-L1 preadipocytes (14, 15). In contrast, PGF\(_{2\alpha}\) has also been shown to suppress adipocyte differentiation from 3T3-L1 preadipocytes via the FP receptor (11). 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.

As a first step to elucidate the physiological roles of EP4- and FP-mediated regulation of adipocyte differentiation and maturation, we employed an adipocyte differentiation system from mouse embryonic fibroblasts (MEFs), and compared the effects of each receptor-deficiency on adipocyte differentiation.
Materials and Methods

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<sup>-/-</sup> and their control wild-type (WT) mice with a mixed background of 129SV and C57BL/6 were littermates of offspring from heterozygote crosses (16). Ptgfr<sup>-/-</sup> mice with a genetic background of C57BL/6 were generated as described (17, 18), and C57BL/6 mice were used as WT controls. All experimental procedures were approved by the Committee of Animal Research of Kyoto University Faculty of Pharmaceutical Sciences and Kumamoto University.

Reagents. PGE<sub>2</sub>, 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), and the EP-specific antagonists, ONO-8713 (EP1), ONO-AE3-240 (EP3) and ONO-AE3-208 (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 (Temecula, CA). Intracellular cyclic AMP was measured using a radioimmunoassay kit (Yamasa, Choshi, Japan), and PGE<sub>2</sub> 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 MEF cells were prepared. MEF cells 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. MEF cells 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). MEF cells were fixed with 4% paraformaldehyde and incubated in Oil Red O solution (0.05 g of Oil Red O, 6 ml of isopropanol and 4 ml of water), and the number of oil droplet-positive cells was counted.

RNA isolation and real time RT-PCR. Total RNA was isolated from MEF cells on the indicated days of the differentiation program with the RNeasy mini kit (QIAGEN, Venlo, Netherlands), 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, Penzberg, Germany) using Fast Start DNA Master SYBR Green I as reported previously (19). 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 and their melting temperatures. RT-PCR for the detection of mRNAs for mouse EP3 isoforms (α, β and γ) was performed as reported previously (20, 21).

Measurement of PGE₂ production, cAMP formation and Ca²⁺ mobilization. PGE₂ levels were measured using the prostaglandin E₂ EIA kit according to the manufacturer’s instructions (Cayman Chemical). Cyclic AMP levels in MEF cells were determined as reported previously (14). Briefly, the MEF cells were washed with HEPES-buffered saline containing 140 mM NaCl, 4.7 mM KCl, 2.2 mM CaCl₂, 1.2 mM MgCl₂, 1.2 mM KH₂PO₄, 11 mM glucose, and 15 mM HEPES, pH7.4, and preincubated for 10 min. Reactions were started by the addition of test reagents along with 100 µM Ro-20-1724. After incubation for 10 min (Fig. 2, B and C) or 1 h (Fig. 3E) at 37°C, reactions were terminated by the addition of 10% trichloroacetic acid. The content of cAMP in the cells was measured by radioimmunoassay with a cAMP assay system. Ca²⁺ mobilization was analyzed by FlexStation (Molecular Devices, Sunnyvale, CA) as follows: MEF cells 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.** MEF cells 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, Little Chalfont, UK).

**Statistical analysis.** Experiments were independently repeated three times, and their mean value ± SEM are 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.

**Results**

*Indomethacin augments adipocyte differentiation of MEF cells*

MEF cells 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 x 10^6 cells / plate), but the MEF cells 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. 1A). In order to examine whether indomethacin affects the number of differentiated cells and/or the TG content per differentiated cell, we assessed the time dependent changes in TG content and the number of cells containing fat droplets (droplet^+ cells) during the differentiation program (Fig. 1, B-D). In both cell groups, TGs were undetected on day 2, slight but significant levels of TGs were detected on day 4, and then levels drastically increased on day 6 and 8. However, at every time point, the TG levels in indomethacin-treated cells were significantly higher by 2-fold than control cells (Fig. 1B). In both groups, the oil droplets became visible on day 4, but the number of droplet^+ cells were constant until day 8 (Fig. 1C). Interestingly, indomethacin increased the droplet^+ cell number by approximately 2-fold. Indeed, the TG levels per droplet^+ cell were indistinguishable between the two groups (Fig. 1D). These results suggest that indomethacin promotes adipocyte differentiation but not maturation. When we examined gene expression of 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. 1E). Indeed, indomethacin accelerated
the induction of lipogenic enzyme genes such as fatty acid synthase, Fasn (Fig. 1G) and
of lipolytic enzyme genes such as hormone-sensitive lipase, Lipe (Fig. 1F). These
results suggest that PG endogenously synthesized by MEF cells suppresses adipocyte
differentiation.

Expression of PG receptors in MEF cells

We next examined the mRNA expression of PG receptors in MEF cells
during the differentiation program (Fig. 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. We 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. Since we previously identified the existence of multiple EP3 isoforms in
mouse, namely EP3α, EP3β, and EP3γ, which differ in their selectivity of G protein
coupling (20, 21), we investigated which EP3 isoforms are induced upon adipocyte
differentiation by competitive RT-PCR (Supplementary Fig. 1). As a result, all three
EP3 isoforms were equally detected on day 8, but not on day 0. To confirm the
expression of functional PG receptors, we investigated whether each receptor agonist
could induce signal transduction in undifferentiated (Fig. 2B) and differentiated cells
(Fig. 2C). In undifferentiated MEF cells on day 0, PGE₂ 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 the dominant Gs-coupled PGE receptor in undifferentiated MEF cells. In contrast, PGE$_2$, and all of the EP-specific agonists failed to induce intracellular Ca$^{2+}$ mobilization. Since RT-PCR analysis showed a faint band for EP1 gene expression in MEF cells, EP1 may be expressed only in a very small population of undifferentiated MEF cells. An FP agonist also failed to stimulate intracellular Ca$^{2+}$ mobilization, indicating that the FP receptor is not expressed in undifferentiated MEF cells. In differentiated cells on day 8, PGE$_2$ as well as an EP4 agonist again induced cAMP accumulation, but an EP1- and EP3-agonist failed to do so. Although we detected mRNAs for EP3$\gamma$, an isoform coupled to Gs, as well as EP3$\alpha$ and EP3$\beta$ in the cells on day 8, an EP3 agonist did not increase basal cAMP levels, but rather decreased basal cAMP levels. The EP3 receptor appears to be mainly coupled to inhibition of adenylyl cyclase in differentiated adipocytes. 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, EP3 and EP4 receptors are expressed in differentiated adipocytes.

Both EP4- and FP-agonists suppress adipocyte differentiation from MEF cells

We next examined the effect of exogenously added PGs on adipocyte differentiation. PGE$_2$ as well as an FP-agonist, fluprosteno (1 µM each) significantly reduced the indomethacin-augmented TG content to the levels of the control group (Fig. 2D). In contrast, both an EP1 agonist and an EP3 agonist failed to affect TG content.
(data not shown). Similar results were obtained regarding Pparg2 gene expression (Fig. 2D). 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 MEF cells.

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

To examine which receptor signaling suppresses adipocyte differentiation from MEF cells, we prepared MEF cells isolated from Ptger4−/− and Ptgrf−/− 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. 3A). Similar results were obtained regarding Pparg2 gene expression levels in the cells on day 8 (Fig. 3B). Moreover, the number of droplet+ cells in EP4-deficient cells was higher than WT cells and similar to that of indomethacin-treated WT cells (Fig. 3C). Indeed, EP4 deficiency increased the total expression levels of lipolytic (Lipe) and lipogenic (Fasn) genes and did not affect TG content per cell as observed in indomethacin-treated WT cells (data not shown). Indomethacin did not further augment the total TG content, droplet+ number, and Pparg2 expression levels in EP4-deficient cells (Fig. 3. A-C). Moreover, an EP4 antagonist (1 µM) but not an EP1 or EP3 antagonist mimicked the enhancing effect of indomethacin on differentiation (Fig. 3D). When we measured cAMP content in the cells on day 0, indomethacin as well as an EP4
antagonist attenuated cAMP levels, and PGE$_2$ reversed the indomethacin-suppressed cAMP levels (Fig. 3E). These results suggest that endogenous PGE$_2$-EP4 signaling suppresses adipocyte differentiation via the 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 (Supplementary Fig. 2A). FP gene deficiency essentially did not affect the Pparg2 gene expression levels in MEF cells (Supplementary Fig. 2B). These results indicate that PGF$_2\alpha$-FP signaling is not involved in the suppression of adipocyte differentiation, although FP signaling has the potential to be involved. Thus, endogenous PGE$_2$-EP4 signaling appears to suppress adipocyte differentiation in MEF cells.

*Endogenous PGE$_2$-EP4 signaling suppresses transcription of the 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 the adipocyte differentiation program, as a result of their chronic exposure to DIC, and 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 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 2 days showed TG levels and Pparg2 expression levels as high as those in cells treated with indomethacin for 8 days (Fig. 4, A and B). We 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. 4, C and D). These results indicate that endogenous PGE$_2$-EP4 signaling suppresses the differentiation stage of adipogenesis.

We 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. 4E). $Pparg2$ expression was drastically induced by DIC treatment, reaching a peak level on day 2 until the DIC was removed, and then gradually decreased until day 8. If suppressive PG signaling dominates the fate of differentiation during the first 2 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. 4F). These results indicate that endogenous PGE$_2$-EP4 signaling suppresses adipocyte differentiation by attenuating transcription of the $Pparg2$ gene.

**DIC treatment induces COX-2 expression and PGE$_2$ production**

The above studies 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 MEF cells in an indomethacin-sensitive manner? To assess this, we examined the time-course of PGE$_2$ production and gene expression of COX isozymes in MEF cells just after the addition of DIC. PGE$_2$ production was stimulated by DIC,
which reached a peak at 3 h and then gradually decreased. Such DIC-induced PGE$_2$ production was inhibited by indomethacin (Fig. 5A). In good accordance with this, DIC transiently induced $Ptgs2$ gene expression in MEF cell reaching a peak at 1 h. In contrast, $Ptgs1$ gene expression was observed in MEF cells irrespective of DIC treatment (Fig. 5B). Indeed, COX-1 protein expression was detected in MEF cells before DIC treatment, and DIC did not alter its levels. COX-2 protein could barely be detected in MEF cells before DIC treatment, but a faint and significant amount was detected at 1 and 3 h after the addition of DIC, respectively (Fig. 5C). Thus, DIC rapidly induces COX-2 and PGE$_2$ production in MEF cells 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. 5D). These results indicate that in MEF cells 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.

Discussion

$PGE_2$-EP4 signaling endogenously suppresses adipocyte differentiation from MEF cells

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 (12). One of the reasons for its complexity is that different classes of PGs exert opposing effects on adipocyte differentiation. For instance, both PGI\textsubscript{2} and PGE\textsubscript{2}, the two PGs predominantly synthesized by fat cells, appear to have opposing effects on early adipogenesis; PGI\textsubscript{2} promotes adipocyte differentiation via the IP receptor (22, 23), whereas PGE\textsubscript{2} inhibits differentiation via the EP4 receptor (14, 15). PGF\textsubscript{2\alpha} also suppresses differentiation via the FP receptor (11, 14). In the current study, we 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\textsubscript{2} and an FP agonist suppressed adipocyte differentiation (Fig. 2, D and E). Inhibition of endogenous PG synthesis by indomethacin increased the number of TG-producing cells and transcription of the \textit{Pparg2} gene (Fig. 1, C and E), 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 the differentiation (Fig. 3, A-C), whereas FP deficiency failed to affect differentiation, and indomethacin was still effective (Supplementary Fig. 2). PGE\textsubscript{2} treatment for 2 days was enough to suppress differentiation (Fig. 4., C and D). Indeed, indomethacin increased and PGE\textsubscript{2} suppressed the peak level of \textit{Pparg2} gene transcription on day 2, which is critical for the commitment of individual cells to differentiation (Fig. 4F). These results indicate that PGE\textsubscript{2}-EP4 signaling suppresses transcription of the \textit{Pparg2} gene and thus the adipocyte differentiation of MEFs (Supplementary Fig. 3). PGF\textsubscript{2\alpha}-FP signaling appears to have the potential to affect adipocyte differentiation, but FP signaling is not involved
in the differentiation system of MEFs (Supplementary Fig. 2). 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. 3, C and D), PGF$\text{2}_\alpha$-FP signaling may work as a compensatory negative regulator of the maturation stage of adipocyte differentiation (Supplementary Fig. 3).

**Differentiation stimuli-induced COX-2 gene expression and PGE$_2$ production in MEF cells**

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 (24-26). 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 (25). Interestingly, Yan et al. also demonstrated that COX-2 inhibitors, but not a COX-1 inhibitor, reversed TNF-$\alpha$-induced inhibition of differentiation. A similar modulating effect of COX-2 has been shown in adiponectin-elicited inhibition of adipocyte differentiation from BMS2 cells (27). Chu et al. recently established COX-2-knocked down 3T3-L1 cell lines and found that these cell lines show augmented levels of adipocyte differentiation, and this phenotype was reversed by the addition of PGE$_2$ (26). Thus, the COX-2-PGE$_2$-EP4 pathway may work as a conserved negative regulator of adipocyte differentiation in broad types of preadipocytes.
**EP3 receptor gene expression is induced upon adipocyte differentiation**

One of the interesting findings in this study is differentiation-dependent induction of EP3 gene expression; the expression of three isoforms (α, β and γ) of EP3 mRNA was equally induced when MEF cells were differentiated into mature adipocytes. Similar results were previously reported; mRNA of the three isoforms of EP3 were expressed exclusively in mature adipocytes isolated from mouse adipose tissue (28). Although the EP3γ isoform may have the potential to activate the Gs/adenylyl cyclase pathway (21), activation of the EP3 receptor resulted in inhibition of cAMP production in adipocyte-differentiated MEFs (Fig. 2C). Indeed, PGE₂-EP3 signaling has been shown to inhibit lipolysis by suppression of cAMP production (29). Since the adipocyte-specific phospholipase A₂ (Pla2g16 product) works as a trigger for PGE₂ production in mature adipocytes, and since the Pla2g16 gene expression also depends on adipocyte differentiation (30), it is possible that the Pla2g16 and Ptger3 genes share common mechanisms in their regulation of expression during adipocyte differentiation.

**PGE₂-EP4 signaling may elicit reciprocal actions on adipogenesis and osteogenesis from mesenchymal stem cells**

Adipocytes and osteoblasts represent two distinct cell types that develop from a common progenitor cell, bone marrow-derived mesenchymal stem cells (MSCs) (31). The number of osteoblasts in bone marrow is known to decrease during age-related bone loss (osteoporosis), and the number of adipocytes has been found to increase in
negative association with that of osteoblasts. (32, 33). Importantly, a reciprocal relationship exists between the number of adipocytes and osteoblasts generated from the marrow MSC pool (34, 35). Factors which enhance osteogenic differentiation are suspected to negatively impact adipogenesis. In this respect, PGE$_2$-EP4 signaling may not only suppress an early stage of adipocyte differentiation, but may also promote osteoblast formation. Indeed, we demonstrated that exogenous PGE$_2$ as well as an EP4 agonist show bone-forming activity by sensitizing Runx2 (Cbfa1) gene expression both in vivo and in vitro (36). Thus, PGE$_2$-EP4 signaling may play a physiological role in the reciprocal regulation of osteogenesis and adipogenesis within bone marrow. Such a possibility that EP4 signaling plays a role in the commitment of MSCs to adipocytes and osteoblasts is an interesting issue to be examined in the future.

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Figure Legends

Fig. 1. Indomethacin facilitates adipocyte differentiation of MEFs. MEF cells 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 of the cells (A and B) or RNA expression level of Pparg2 (E), Lipe (F), and Fasn (G) 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). Values represent the means ± SEM of three independent experiments (n = 3). *p < 0.05, **p < 0.01.

Fig. 2. Prostanoid EP4 and FP receptors have the potential to suppress adipocyte differentiation of MEFs. (A) Gene expression of PGE and PGF receptors in MEF cells. MEF cells grown to confluency were treated with DIC, and total RNA was extracted from untreated cells (day 0), cells on day 2 (day 2) or cells on day 8 (day 8). Total RNA was subjected to the reverse transcription reaction in the presence (RT+) or absence of reverse transcriptase (RT-) and subsequent PCR analysis. Mouse lung RNA was used as a positive control. (B and C) Undifferentiated (B, Day 0) or differentiated MEF cells (C, Day 8) were subjected to the cAMP (top) and Ca²⁺ assay (bottom). PGE₂ (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 Ca²⁺ assay, sphingosine
1-phosphate (10 µM, S1P) was used as a positive control. (D and E) MEF cells were treated with DIC supplemented with vehicle, PGE\(_2\) (1 µM), or an FP agonist (1 µM, FPA) in the presence of indomethacin (+Indo). Triglyceride content of 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. Values represent the means ± SEM of three independent experiments \((n = 3)\). *\(p < 0.05\).

**Fig. 3.** Endogenous PGE\(_2\)-EP4 signaling suppresses adipocyte differentiation in MEFs. MEF cells 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, Indo). Triglyceride content of 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. The number of droplet-positive cells is shown at the top. (D) MEF cells grown to confluency were treated with DIC in the presence of an EP1-, EP3-, or EP4-antagonist (1 µM each, EP1ant, EP3ant or EP4ant). Triglyceride content of the cells was measured on day 8. (E) MEF cells were treated with vehicle, an EP4-antagonist (1 µM, EP4ant) or indomethacin (10 µM, Indo) in the presence or absence of PGE\(_2\) (1 µM) for 1 h, and subjected to the cAMP assay. Values represent the means ± SEM of three independent experiments \((n = 3)\). *\(p < 0.05\). **\(p < 0.01\).
Fig. 4. PGE\textsubscript{2}-EP4 signaling suppresses transcription of the \textit{Pparg2} gene. (A and B) MEF cells 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). On day 8, triglyceride content of the cells was measured (A) and \textit{Pparg2} gene expression in the cells was measured by real time RT-PCR (B). (C and D) MEF cells were treated with DIC containing indomethacin (Indo) supplemented with vehicle, PGE\textsubscript{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 (C) and \textit{Pparg2} gene expression was measured (D). (E) Time course of induction of \textit{Pparg2} gene transcripts in MEF cells. Cells were treated with DIC, and harvested at the various time points (0, 9 h, day 2, 4 and 8) of the differentiation program, and subjected to \textit{Pparg2} gene expression analysis. (F) MEF cells were treated with DIC containing indomethacin (Indo) supplemented with vehicle, PGE\textsubscript{2}, or an FP agonist (FPA). On day 2, the cells were harvested and subjected to \textit{Pparg2} gene expression analysis. The \textit{Pparg2} gene expression levels were normalized to the \(\beta\)-actin (\textit{Actb}) mRNA levels. Values represent the means ± SEM of three independent experiments \((n = 3)\). *\(p < 0.05\).

Fig. 5. COX-2-derived PGE\textsubscript{2} suppresses adipocyte differentiation in MEFs. (A) PGE\textsubscript{2} production of MEFs treated with DIC for the indicated times in the presence (+Indo) or absence of indomethacin was measured. (B) MEF cells were treated with
DIC or control (Cont) 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 and the 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) MEF cells grown to confluency were treated with DIC supplemented with vehicle, 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 was measured. Values represent the means ± SEM of three independent experiments (*n* = 3). *p < 0.05. **p < 0.01.
<table>
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Inazumi et al. Fig. 1
Inazumi et al. Fig. 2

A

Day

Lung

MEF

0

2

8

8

Ptger1 (EP1)

Ptger2 (EP2)

Ptger3 (EP3)

Ptger4 (EP4)

Ptgfr (FP)

Actb

B

Day 0

PGE₂

EP4A

FPA

Cont

* * *

Δ[Ca²⁺](OD₃₄₀/₃₈₀)

ND ND ND

Cont PGE₂ FPA S1P

C

Day 8

PGE₂

EP3A

FPA

Cont

* *

Δ[Ca²⁺](OD₃₄₀/₃₈₀)

Cont PGE₂ EP1A EP3A FPA S1P

D

PGE₂

FPA

DIC+Indo

* *

Triglyceride (x100 mg/well)

E

Pparg2

Actb

Fold change (Pparg2/Actb)

DIC+Indo

PGE₂

FPA

DIC+Indo

PGE₂

FPA

DIC+Indo

Inazumi et al. Fig. 2
Inazumi et al. Fig. 3

A

Triglyceride (x100 mg/well)

WT

Ptger4^-/-

Indo

Indo

0

1

2

3

4

5

6

7

8

9

*

*

B

Pparg2

Actb

Fold change (Pparg2/Actb)

WT

Ptger4^-/-

Indo

Indo

0

1

2

3

4

5

6

7

8

9

*

*

*

*

C

Triglyceride (x100 mg/well)

WT

Ptger4^-/-

Indo

Indo

70.1 ± 4.8

131.7 ± 9.3**

127.6 ± 10.8**

132.4 ± 10.4

D

Triglyceride (x100 mg/well)

Cont

EP1ant

EP3ant

EP4ant

0

1

2

3

4

5

6

7

8

9

* 

E

cAMP (pmol/well)

Day 0

PGE2

Indo

EP4ant

0

1

2

0

1

2

* 

* 

* 

* 

Inazumi et al. Fig. 3