Synergistic Suppression of Early Phase of Adipogenesis by Microsomal PGE Synthase-1 (PTGES1)-Produced PGE\textsubscript{2} and Aldo-Keto Reductase 1B3-Produced PGF\textsubscript{2}\textalpha

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Abstract

We recently reported that aldo-keto reductase 1B3-produced prostaglandin (PG) F\textsubscript{2\alpha} suppressed the early phase of adipogenesis. PGE\textsubscript{2} is also known to suppress adipogenesis. In this study, we found that microsomal PG\textsubscript{E2} synthase (PGES)-1 (mPGES-1; PTGES1) acted as the PGES in adipocytes and that PGE\textsubscript{2} and PGF\textsubscript{2\alpha} synergistically suppressed the early phase of adipogenesis. PGE\textsubscript{2} production was detected in preadipocytes and transiently enhanced at 3 h after the initiation of adipogenesis of mouse adipocytic 3T3-L1 cells, followed by a quick decrease; and its production profile was similar to the expression of the cyclooxygenase-2 (PTGS2) gene. When 3T3-L1 cells were transfected with siRNAs for any one of the three major PTGESs, i.e., PTGES1, PTGES2 (mPGES-2), and PTGES3 (cytosolic PGES), only PTGES1 siRNA suppressed PGE\textsubscript{2} production and enhanced the expression of adipogenic genes. AE1-329, a PTGER4 (EP4) receptor agonist, increased the expression of the Ptgs2 gene with a peak at 1 h after the initiation of adipogenesis. PGE\textsubscript{2}-mediated enhancement of the Ptgs2 expression was suppressed by the co-treatment with L-161982, a PTGER4 receptor antagonist. Moreover, AE1-329 enhanced the expression of the Ptgs2 gene by binding of the cyclic AMP response element (CRE)-binding protein to the CRE of the Ptgs2 promoter; and its binding was suppressed by co-treatment with L-161982, which was demonstrated by promoter luciferase and chromatin immunoprecipitation assays. Furthermore, when 3T3-L1 cells were caused to differentiate into adipocytes in medium containing both PGE\textsubscript{2} and PGF\textsubscript{2\alpha}, the expression of the adipogenic genes and the intracellular triglyceride level were decreased to a greater extent than in medium containing either of them, revealing that PGE\textsubscript{2} and PGF\textsubscript{2\alpha} independently suppressed adipogenesis. These results indicate that PGE\textsubscript{2} was synthesized by PTGES1 in adipocytes and synergistically suppressed the early phase of adipogenesis of 3T3-L1 cells in cooperation with PGF\textsubscript{2\alpha}, through receptor-mediated activation of PTGS2 expression.

Introduction

Obesity contributes to insulin resistance and type 2 diabetes mellitus [1,2]. As a major target of insulin action, adipose tissue plays a critical role in the regulation of whole body metabolism and glucose homeostasis [3,4]. Adipogenesis has been extensively studied, and several key transcription factors involved in the regulation of adipogenesis have been identified [5,6]. Peroxisome proliferator-activated receptor (PPAR)\textgamma\gamma plays a central role in this regulation [7,8]. Ligand-activated PPAR\textgamma\gamma regulates many genes involved in glucose and lipid homeostasis and is involved in the maintenance of insulin responsiveness [8,9,10].

Prostaglandins (PGs) and their metabolites are involved in the regulation of adipogenesis. PGD\textsubscript{2} [11] and its metabolite, D\textsuperscript{12}-PGF\textsubscript{3\alpha} [12], activate the middle-late phase of adipogenesis, and PGD\textsubscript{2}-overproducing mice become obese under the high-fat diet [13]. Moreover, prostacyclin (PGI\textsubscript{2}) enhances adipogenesis through PGI\textsubscript{2} receptor [14,15]. In contrast, PGF\textsubscript{2\alpha} is produced by aldo-keto reductase (AKR) 1B3 in adipocytes; and it suppresses the early phase of adipogenesis through PTGFR receptors [16,17]. PGF\textsubscript{2\alpha} promotes the production of anti-adipogenic PGF\textsubscript{2\alpha} and PGE\textsubscript{2} by enhancing the expression of cyclooxygenase-2 (PTGS2; COX-2) through PTGFR (FP) receptor-activated mitogen-activated protein kinase/extracellular signal-regulated kinase/extracellular signal-regulated kinase cascade and the binding of the cyclic AMP response element (CRE)-binding protein (CREB) to the CRE of the Ptgs2 promoter [18]. Moreover, PGE\textsubscript{2} is known to suppress adipogenesis by acting through the PTGER4 (EP4) receptor [19], and to increase the de novo synthesis of anti-adipogenic PGF\textsubscript{2\alpha} and PGE\textsubscript{2} in mouse embryonic fibroblasts [20]. These anti-adipogenic PGs repress the function of PPAR\textgamma\gamma via their specific PG receptors.

Several PG\textsubscript{E2} synthases (PTGESs) have been identified in various tissues [21,22]. Microsomal PG\textsubscript{E2} synthase-1 (mPGES-1; PTGES1) is a member of the membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG) protein family [23], and produces PGE\textsubscript{2} in response to various stimuli [24]. Microsomal PG\textsubscript{E2} synthase-2 (mPGES-2; PTGES2) has also been identified and its
expression is high in the heart and brain [25]. Cytosolic PGES (cPGES; PTGES3) is constitutively and ubiquitously expressed in various cells [26]. However, the PGE_2-producing enzyme in adipocytes has never been identified; and the mechanism causing suppression of the early-phase of adipogenesis by anti-adipogenic PGs such as PGE_2 and PGF_2a remains unclear.

In this study, we demonstrate that PTGES1 was expressed in preadipocytes and that its mRNA and protein levels were consistently detected during adipogenesis. PGE_2 production was detected in preadipocytes and increased during adipogenesis with a peak at 3 h after the initiation of adipogenesis, and PTGES1 was responsible for the production of PGE_2 in adipocytes. PGE_2 elevated the expression of anti-adipogenic PGF_2a and PGE_2 by enhancing the expression of PTGS2 by acting through the PTGER4 receptor, which action enhanced the binding of CREB to the Ptg2 promoter via activation of the PTGER4 receptor/CREB cascade in 3T3-L1 cells. Thus, PTGES1-produced PGE_2 and AKR1B3-synthesized PGF_2a synergistically suppressed the early phase of adipogenesis through elevation of PTGS2 expression in 3T3-L1 cells.

Materials and Methods

Cell Culture

Mouse 3T3-L1 cells (Health Science Research Resources Bank, Osaka, Japan) were maintained in Dulbecco’s Modified Eagles Medium (DMEM; Sigma, St. Louis, MO, USA) supplemented with 10% fetal calf serum and antibiotics. The cells were maintained in a humidified atmosphere of 3% CO_2 at 37°C.

Adipocyte differentiation of 3T3-L1 cells was initiated by incubation for 2 days in DMEM containing insulin (10 μg/ml; Sigma), 1 μM dexamethasone (Sigma), and 0.5 mM 3-isobutyl-1-methyloxanthine (Sigma). On day 2, the medium was replaced with DMEM containing insulin (10 μg/ml) and then changed every 2 days.

Oil Red O staining was carried out as described previously [11]. Spectrophotometric measurement for Oil Red O staining was performed by dissolving the stained lipid droplets in the cells with isopropl alcohol, and then the absorbance was measured at 520 nm.

RNA Preparation and Quantification of RNA

Total RNA was extracted with Sepasol-RNai (Nacalai Tesque, Kyoto, Japan), followed by further purification with an RNeasy Purification System (Qiagen, Hilden, Germany) [17]. The first-strand cDNAs were synthesized from 1 μg of total RNA with random hexamer and ReverTra Ace Reverse Transcriptase (Toyobo, Osaka, Japan) at 42°C for 60 min after initial denaturation at 72°C for 3 min, followed by heat-denaturation of the enzyme at 99°C for 5 min. The cDNAs were further utilized as the templates for quantitative PCR analyses.

Expression levels were quantified by using a LightCycler system (Roche Diagnostics, Mannheim, Germany) with THUNDER-BIRD qPCR Mix (Toyobo) and primer sets (Table 1). The expression level of the target genes was normalized to that of the TATA-binding protein (TBP).

Suppression by RNAi

PTGES1 Stealth siRNA (5'-UUCCUUCCGAGCGCUCGU-3') and Stealth Negative Control (N.C.) siRNA were obtained from Invitrogen (Carlsbad, CA, USA). siRNAs for PTGES2 (5'-CUGUAACUGCUCCUGCUACC-3'), PTGES3 (5'-GGUAGCUCGUAUGAAGCAGG-3') and MISSION siRNA Universal Negative Control were purchased from Sigma Genosys (Sapporo, Japan). Transfection with siRNA (20 nM) was performed by use of X-tremeGENE siRNA Transfection Reagent (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s instructions. Transfection efficiency of siRNA was measured by using FAM-labeled GAPDH siRNA (Ambion, Austin, TX, USA), and we found it approximately 50–60% in 3T3-L1 cells (data not shown).

For determination of the knockdown-efficiency of each siRNA, 3T3-L1 cells were transfected with each siRNA and cultured for 2 days.

For identification of the functions of PTGESs, 3T3-L1 cells were transfected with each siRNA, and caused to differentiate into adipocytes for 6 days. Transfection with siRNA was carried out every 2 days.

Western Blot Analysis

Cells were harvested and lysed in RIPA buffer containing 50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.1% w/v SDS, 0.5% w/v sodium deoxycholate, 1% w/v NP-40, and 1% w/v Triton X-100 with Protease Inhibitor cocktail (Nacalai Tesque) and phosphatase inhibitors; 50 μM Na_3VO_4, 1 mM NaF and 1 mM Na_2VO_4. The lysates were centrifuged for 20 min at 12,000 x g at 4°C to remove the cell debris. Protein concentrations were measured with a Pierce BCA Protein Assay Reagent (Thermo Scientific, Rockford, IL, USA). The proteins were separated on SDS-PAGE gels and transferred onto PVDF membranes (Immobilon P; Millipore, Bedford, MA, USA). The blots were incubated with a given antibody: anti-PTGES1 (1:1,000; Cayman Chemicals, Ann Arbor, MI, USA), anti-PTGES2 (1:1,000; Cayman Chemicals), anti-PTGES3 (1:1,000; Cayman Chemicals), anti-PTGES2 (C-20; 1:500; Santa Cruz Biotech., Santa Cruz, CA, USA), anti-PPARγ (H-100; 1:1,000; Santa Cruz Biotech.) or anti-stearoyl-CoA desaturase (SCD1; S-15; 1:1,000; Santa Cruz Biotech.) polyclonal antibody, or anti-fatty acid binding protein 4 (FABP4; EPR3579; 1:1,000; Epitomics, Burlingame, CA, USA) or anti-actin (AC-15; 1:2,000; Sigma) monoclonal antibody. After the blots had been washed with TBS-T (20 mM Tris, 137 mM NaCl, 0.1% w/v Tween-20, pH 7.6), they were incubated with anti-rabbit, anti-goat or anti-mouse IgG antibody conjugated with horseradish peroxidase (Santa Cruz Biotech.). Immunoreactive signals were detected with an Immobilon Western Detection Reagent (Millipore) by use of an LAS-3000 Image analyzer (Fujifilm, Tokyo, Japan) and analyzed with MultiGauge software (Fujifilm). Each expression level was normalized by that of actin.

Measurement of PGs

PGE_2 and PGF_2a levels were measured by use of their respective enzyme immunoassay kit (EIA; Cayman Chemical) as described previously [17]. In brief, cells were treated with A23187 (5 μM; Calbiochem, San Diego, CA, USA), a calcium ionophore, for 10 min at 37°C. Medium was collected, and centrifuged at 3,000 x g for 5 min to remove the cells. The resultant supernatant was then used for measurement of PGE_2 and PGF_2a by performing their respective EIA according to the manufacturer’s instructions.

Measurement of Triglyceride Level

Cells were washed with PBS, and lysed with PBS containing 5% w/v Triton-X-100, and then incubated at 90°C for 3 min. The supernatant was prepared by centrifugation to remove cell debris, and subsequently used for measurement of the intracellular triglyceride level by using a WAKO LabAssay Triglyceride Kit (Wako Pure Chemical, Osaka, Japan) according to the manufac-
The precipitated DNA level was estimated by the use of serially the same primers used in PCR analysis as described above. Briefly, to measure the anti-CREB antibody-precipitated DNA level by using the gels with ethidium bromide, amplified PCR products (expected size of 168-bp) were analyzed by performing agarose gel electrophoresis, followed by staining of the bands. The amplified DNA was reverse-precipitated and utilized for subsequent PCR amplification with KOD FX DNA Polymerase (Toyobo) with a primer set specific for the promoter: 5′-CAGAGAGGG-GAAAAGTGG-3′ and 5′-GAGCAGATGCTGACTGACTC-3′. PCR was conducted under the following conditions: initial denaturation at 94°C for 2 min, followed by 30 cycles of 98°C for 10 sec, 55°C for 20 sec, and 60°C for 20 sec. The amplified PCR products (expected size of 168-bp) were analyzed by performing agarose gel electrophoresis, followed by staining of the bands with ethidium bromide.

In addition, we performed the quantitative PCR analysis to measure the anti-CREB antibody-precipitated DNA level by using the same primers used in PCR analysis as described above. Briefly, the precipitated DNA level was estimated by the use of serially diluted concentration-known DNA including the Pgk2 promoter region as the standard.

### Luciferase Reporter Assay

The luciferase reporter vectors carrying the mouse Pgk2 promoter were generated previously [18]. 3T3-L1 cells were co-transfected with each construct (0.9 μg) and pRL-SV40 (0.1 μg, Promega, Madison, WI, USA) in 6-well plates, the latter plasmid carrying the Renilla luciferase gene under the control of the SV40 promoter: 5′-CATATCTGACCTGGTGTCTG-3′ and 5′-GGATTGAGACCGAAGATTG-3′. In this manner, the luciferase activity was calculated relative to that of pGL4.10[luc2] vector (Promega), which was defined as ‘1′. Data were obtained from three independent experiments, and each experiment was presented as the mean ± S.D.

### Chromatin Immunoprecipitation (ChIP) Assay

The ChIP assay was performed as described previously [11] by using anti-CREB polyclonal antibody (H-74; Santa Cruz Biotech.). Immunoprecipitated DNA-protein complexes were reverse-crosslinked, and the free DNAs were purified by ethanol-precipitation and utilized for subsequent PCR amplification with KOD FX DNA Polymerase (Toyobo) with a primer set specific for CRE at position -59 in the Pgk2 promoter: 5′-CAGAGAGGG-GAAAAGTGG-3′ and 5′-GAGCAGATGCTGACTGACTC-3′. PCR was conducted under the following conditions: initial denaturation at 94°C for 2 min, followed by 30 cycles of 98°C for 10 sec, 55°C for 20 sec, and 60°C for 20 sec. The amplified PCR products (expected size of 168-bp) were analyzed by performing agarose gel electrophoresis, followed by staining of the gels with ethidium bromide.

In addition, we performed the quantitative PCR analysis to measure the anti-CREB antibody-precipitated DNA level by using the same primers used in PCR analysis as described above. Briefly, the precipitated DNA level was estimated by the use of serially diluted concentration-known DNA including the Pgk2 promoter region as the standard.

### Table 1. Nucleotide sequence of primers used in this study.

| Gene   | Acc No. | Forward primer | Reverse primer |
|--------|---------|----------------|----------------|
| Pparγ  | NM_001127330 | 5′-CAAGATACCAAAAGTGCAATCA-3′ | 5′-AGGACGTCCTTTCAGAATAAAG-3′ |
| C/ebpα | NM_007678 | 5′-CTGAAAAGAGGCCACCTC-3′ | 5′-AAGAGAGGAACGCGTCC-3′ |
| Fabp4  | NM_024406 | 5′-CAGCCTTCTCACCCTGAAG-3′ | 5′-TGTTGGCAAACGCCCACCT-3′ |
| Scl1   | NM_009127 | 5′-TCTCTCTGGAGCTCCTAC-3′ | 5′-CTAGGGCCTGTCTTGTAGT-3′ |
| Pgenes1| NM_022415 | 5′-GCCAATCGTCTGTCATCAAG-3′ | 5′-ACGTTCAGCCGCATCTC-3′ |
| Pgenes2| NM_133783 | 5′-CCGAAGAGGACAGACGT-3′ | 5′-AGTAGTCTGGAGCCACAT-3′ |
| Pgenes3| NM_019766 | 5′-CGAATTTCGAGCTGTCTCG-3′ | 5′-TGAATCATCATCTGTCCATCT-3′ |
| Pgenes4| NM_011198 | 5′-GATCTCTGCGAGCTGTG-3′ | 5′-GGATTGGAACCGAAGATT-3′ |
| Ptger1 | NM_013641 | 5′-GAGCCAGGAGTCTGAG-3′ | 5′-GCTCATACATGCGCCAAGAG-3′ |
| Ptger4 | NM_00113607 | 5′-CCTAACCCACCTCAGGGT-3′ | 5′-AGAAAAGCGCCTGTACCTC-3′ |
| Tbp    | NM_013684 | 5′-GTGATGTGAAGTTCCCCATAAGG-3′ | 5′-CTACTGAAGCCTGGTGGTCA-3′ |

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Statistical Analysis

Comparison of 2 groups was analyzed by Student’s t-test. For comparison of more than 2 groups with comparable variances, one-way ANOVA and Tukey’s post-hoc test was carried out. p<0.05 was considered significant.

### Results

#### Identification of PGES in Adipocytes

At first, we investigated the suppression of adipogenesis by treatment with PGES. Mouse 3T3-L1 cells were caused to differentiate into adipocytes for 6 days in the presence of various concentrations of PGES. Oil Red O staining showed that intracellular lipid-droplets increased in size and number during adipogenesis, and these enhancements were repressed in a PGES-concentration-dependent manner (Fig. 1A and 1B). Moreover, the intracellular triglyceride level was also enhanced during adipogenesis, and when the cells were caused to differentiate into adipocytes in the presence of various concentrations of PGES2, its level was significantly repressed in a concentration-dependent manner (Fig. 1C). When the cells were cultured in medium containing various concentrations of PGES for 3 h, followed culture for 6 days in the absence of PGES2, suppression effect at 3 h was slightly weaker than those for 6 days (data not shown).

Furthermore, the expression level of adipogenic genes such as total Ppara (Ppara1 and Ppara2), CCAAT-enhancer binding protein (C/ebpα, Fabp4), and Scl1 in adipocytes was elevated approximately 2.8-, 3.6-, 59.2-, and 8.9-fold, respectively, as compared with each of those of the undifferentiated cells (Fig. 1D). Furthermore, the enhanced expression of these genes was suppressed by about 57, 74, 96, and 88%, respectively, of that of the vehicle-treated differentiated cells (Fig. 1D). These results indicate that PGES2 suppressed adipogenesis measured in terms of the expression of adipogenic genes in 3T3-L1 cells.

Next, we examined the expression of the Pgenes genes in 3T3-L1 cells. Cells were caused to differentiate into adipocytes for 6 days, and the gene expression of the three major PTGESs, i.e., PTGES1, PTGES2, and PTGES3, during adipogenesis was measured by performing quantitative PCR and Western blot analyses. All three PTGESs were expressed in preadipocytes and consistently so during adipogenesis (Fig. 2A). The protein levels of all three PTGESs, examined by Western blot analysis, well resembled those of their mRNA expression (Fig. 2A). However, the expression profiles of the Pgk2 gene and protein were quite
different from them; as it was transiently up-regulated at 3 h after the initiation of adipogenesis, and then decreased to the basal level (Fig. 2A). Then, we measured the PGE2 level during adipogenesis by EIA. PGE2 was produced in preadipocytes, and its production level rapidly increased to a peak at 3 h after initiation of adipogenesis and then quickly decreased to a level lower than that of the undifferentiated cells (Fig. 2B). This production pattern well resembled the expression profile in the PTGS2 (Fig. 2A).
Figure 2. Expression of PTGESs and PTGS2 in 3T3-L1 cells. A. Expression of three PTGESs and PTGS2 during the differentiation of 3T3-L1 cells. The expression level of each gene was measured by quantitative PCR. The data are presented as the mean ± S.D. from 3 independent experiments. *p<0.01 as compared with value for undifferentiated cells. Protein levels were detected by Western blot analysis by use of crude cell extracts (20 μg/lane). Band intensities were measured by using MultiGauge software, and normalized by actin level. Data are representative of 3 independent experiments and presented as the mean ± S.D. *p<0.01, as compared to undifferentiated cells. B. The PGE2 level was measured by EIA. *p<0.01 as compared to undifferentiated cells. doi:10.1371/journal.pone.0044698.g002
Figure 3. Adipogenesis in Ptges-knockdown cells. A, siRNA-mediated suppression of the expression of the Ptges genes. 3T3-L1 cells (V; vehicle) were transfected with an siRNA for the Ptges1 (m-1), Ptges2 (m-2) or Ptges3 (C) gene or with N.C. siRNA (N.C.), and caused to differentiate into adipocytes for 2 days. Each gene expression was measured by quantitative PCR. Data are presented as the mean ± S.D. from 3 independent experiments. *p<0.01 as compared with value for N.C. siRNA. Protein levels were detected by Western blot analysis using crude cell extracts (20 μg/lane). Band intensities were measured by using MultiGauge software, and normalized by actin level. Data are representative of 3 independent experiments and presented as the mean ± S.D. * p<0.01, as compared with N.C. siRNA-transfected cells. B. PGE2 production in Ptges-knockdown cells. 3T3-L1 cells were transfected with each siRNA described in the legend of Fig. 3A, and the PGE2 level was measured by EIA. *p<0.01, as compared with value for N.C. siRNA. C. Oil Red O staining of Ptges-knockdown cells. 3T3-L1 cells (undifferentiated cells: U) were transfected with siRNA and caused to differentiate into adipocytes for 6 days (D). Transfection was carried out every 2 days. Bar = 50 μm. D. Measurement of Oil Red O dye extracted from lipid droplet-laden cells. E. Intracellular triglyceride level in Ptges-knockdown cells. Cells were cultured as described in the legend of Fig. 3C. Data are presented as the mean ± S.D. from 3 independent experiments. *p<0.01, as indicated by the brackets.
These results reveal that all three PTGESs were consistently expressed during adipogenesis. PGE2 production was detected in preadipocytes and transiently enhanced at 3 h after the initiation of adipogenesis, whose pattern well resembled the expression of PTGS2 in 3T3-L1 cells.

PTGES1 is Responsible for the Production of PGE2 in Adipocytes

To identify the active PGES in adipocytes, we transfected 3T3-L1 cells separately with each of the PTGES siRNAs, and differentiated into adipocytes for 2 days. The mRNA levels of all three Ptges genes were significantly decreased more than 50% by their respective siRNAs, as compared with each of their levels when treated with N.C. siRNA (Fig. 3A). Almost the same results were obtained at the protein level by Western blot analysis; and the actin level, as the internal control, was almost the same in all samples (Fig. 3A). Each siRNA was specific for its PTGES, as it did not inhibit the expression of the other Ptges mRNAs (data not shown). Moreover, the siRNA for PTGES1 decreased the PGE2 production to about 61.4% of that with N.C. siRNA in 3T3-L1 cells (Fig. 3B). In contrast, siRNAs for PTGES2 and PTGES3 did not have any effect on the production of PGE2 (Fig. 3B); although the mRNA and protein levels of PTGES2 and PTGES3 were significantly decreased by their respective siRNAs (Fig. 3A).

To confirm that PTGES1 is the PGES suppressing adipocyte differentiation, we examined the role of PGES in the accumulation of intracellular lipids. As shown above, PGE2 inhibited the accumulation of intracellular lipids of 3T3-L1 cells (Fig. 1A and 1B). When the cells were transfected with any one of the three PTGES siRNAs, the intracellular lipid level demonstrated by Oil Red O staining was increased only in the PTGES1 siRNA-transfected cells (Fig. 3C and 3D). Whereas, there were no changes in PTGES2 or PTGES3 siRNA-transfected cells, which showed almost the same lipid accumulation as the vehicle-treated cells (Fig. 3C and 3D). In addition, the intracellular triglyceride level in PTGES1 siRNA-transfected cells was clearly increased as compared with that in the vehicle-treated or PTGES2 or PTGES3 siRNA-transfected cells (Fig. 3E), indicating that PTGES1 was

Figure 4. Expression of adipogenic genes in Ptges-knockdown cells. Cells were cultured as described in the legend of Fig. 3C, and mRNA levels were measured by quantitative PCR. The data are presented as the mean ± S.D. from 3 independent experiments. *p<0.01, as indicated by the brackets. Protein levels were detected by Western blot analysis using crude cell extracts (20 μg/lane). The data are the representative of 3 independent experiments. *p<0.01, as indicated by the brackets. doi:10.1371/journal.pone.0044698.g004
involved in the accumulation of the intracellular triglyceride level in adipocytes.

Next, we investigated the expression of adipogenic genes in PTGES siRNA-transfected cells. The transcription level of the adipogenic genes such as *Pparγ*, *Fabp4*, and *Sd1* was enhanced approximately 1.7-, 1.4-, and 1.6-fold, respectively, by transfection with PTGES1 siRNA, as compared with those levels for cells treated with vehicle or transfected with N.C. siRNA, PTGES2 siRNA or PTGES3 siRNA (Fig. 4). Protein levels of *Pparγ*, *FABP4*, and *SCD1* were also up-regulated by transfection of 3T3-L1 cells with PTGES1 siRNA, but not affected in N.C., PTGES2 or PTGES3 siRNA-transfected cells (Fig. 4). These results reveal that PTGES1 acted as the PGES in adipocytes and that PTGES1-produced PGE2 suppresses adipogenesis by reducing the expression of adipogenic genes in 3T3-L1 cells.

Involvement of PTGER4 Receptors in the Suppression of Adipogenesis

PGE2 exerts its action through interaction with four PGE2 receptor subtypes; PTGER1 (EP1), PTGER2 (EP2), PTGER3 (EP3), and PTGER4 (EP4) [27]. So next, we investigated the expression of the *Ptger* genes during adipocyte differentiation of 3T3-L1 cells. *Ptger1* mRNA was detected in preadipocytes and its expression level gradually increased after 1 day of the initiation of the adipocyte differentiation (Fig. 5). The *Ptger4* gene was expressed highly in preadipocytes, and its level decreased almost 50% after the initiation of adipogenesis (Fig. 5). Whereas, the expression of *Ptger2* and *Ptger3* receptor genes was under the detection limit of our experimental conditions (data not shown).

Next, we examined which PTGER receptor, PTGER1 or PTGER4 was involved in the PGE2-mediated suppression of adipogenesis. When 3T3-L1 cells were caused to differentiate into adipocytes for 6 days in medium containing a PTGER1 receptor agonist, DI-004, the accumulation of lipid-droplet in the cells was not changed as judged by Oil Red O staining (Fig. 6A and 6B). Whereas, the amount of lipid-droplets was clearly decreased by treatment with a PTGER4 receptor agonist, AE1-329, which decrease was almost the same as that seen in PGE2-treated cells (Fig. 6A and 6B). Moreover, PGE2-mediated suppression of lipid accumulation was cleared by co-treatment with an EP4 receptor antagonist, L-161892, but not with AH6809, a PTGER1 receptor antagonist (Fig. 6A and 6B). Next, we measured the intracellular triglyceride level when the cells were caused to differentiate into adipocytes in medium containing PGE2, DI-004 or AE1-329 or PGE2 with or without AH6809 or L-161892. Differentiation-mediated enhancement of the intracellular triglyceride level was suppressed by treatment with PGE2 or AE1-329, but not with DI-004 (Fig. 6C). Moreover, PGE2-mediated decrease in the intracellular triglyceride level was cleared by co-treatment with L-161892, but not AH6809 (Fig. 6C). When 3T3-L1 cells were cultured for 3 h by chemicals, followed by further cultured for 6 days without chemicals, almost the same results as those for 6 days were observed (data not shown).

Furthermore, when PTGES1 siRNA-transfected cells were caused to differentiate into adipocytes for 6 days with or without DI-004 or AE1-329, Oil Red O staining of the intracellular lipids was carried out and the intracellular triglyceride level was measured. Only AE1-329 could repress the PTGES1 siRNA-mediated enhancement of adipogenesis (Fig. 6D-F). Almost the same results were obtained when the PTGES1 siRNA-transfected cells were treated with PTGER1 or PTGER4 agonist for 3 h, followed by cultured for 6 days in the absence of PTGER1 or PTGER4 agonist (data not shown). These results, taken together, indicate that PTGES1-produced PGE2 suppressed adipogenesis by acting through PTGER4 receptors in 3T3-L1 cells.

Activation of PTGER4 Receptor Enhances PGE2 Production with Elevation of *Ptgs2* Expression in 3T3-L1 Cells

When 3T3-L1 cells were cultured with AE1-329, we also found that the production of PGE2 was increased approximately 2.8-fold, as compared with that obtained for the vehicle-treated cells (Fig. 7A). The expression of the *Ptgs2* gene was enhanced approximately 4.3-fold at 3 h after the initiation of adipogenesis, as compared with that in the preadipocytes (Fig. 7B). Moreover, when the cells were caused to differentiate into adipocytes in the presence of AE1-329, the expression of the *Ptgs2* gene was elevated about 1.5-fold at 3 h, as compared with that in vehicle-treated cells (Fig. 7B). Then the expression level quickly decreased to a level lower than that detected in preadipocytes (Fig. 7B). Furthermore, the AE1-329-mediated enhancement of the expression of the *Ptgs2* gene was repressed by co-treatment with L-161892 (Fig. 7C, *left panel*). In addition, PGE2 itself was able to elevate the expression of the *Ptgs2* gene in 3T3-L1 cells, and this enhancement was suppressed by co-treatment with L-161892 (Fig. 7C, *right panel*). These results indicate that PGE2 enhanced its own production by acting through the PTGER4 receptor to elevate the expression of the *Ptgs2* gene in an autocrine manner in 3T3-L1 cells.

Involvement of CREB in the PGE2-mediated Activation of *Ptgs2* Gene Expression

CREB has been identified as the activator for the transcription of the *Ptgs2* gene in 3T3-L1 cells [18]. So, we investigated whether the CREB was involved in the PGE2/PTGER4 receptor-elevated *Ptgs2* gene expression by performing a luciferase reporter assay. The transcription initiation site of the mouse *Ptgs2* gene has been determined [28]. When the construct carrying the promoter region from −300 to +124, named −300/+124, was used for the transfection, efficient reporter activity was detected (Fig. 8A). Moreover, when the −300/+124 construct-transfected cells were
Figure 6. PGE₂ suppresses adipogenesis through PTGER4 receptors. A. Accumulation of lipid-droplets through PTGER4 receptor action. 3T3-L1 cells (undifferentiated cells: U) were caused to differentiate into adipocytes (D) for 6 days in medium containing either PGE₂ (100 nM), DI-004 (PTGER1 receptor agonist; 1 μM; ONO Pharmaceutical) or AE1-329 (PTGER4 receptor agonist; 1 μM; ONO Pharmaceutical) or PGE₂ and AH6809 (PTGER1 receptor antagonist; 1 μM; Cayman Chemical) or L-161982 (PTGER4 receptor antagonist; 10 μM; Cayman Chemical). Bar = 50 μm. B. Measurement of Oil Red O dye extracted from lipid droplet-laden cells. C. Effects of PGE₂ and PTGER agonists/antagonists on the intracellular
treated with AE1-329, the luciferase reporter activity was enhanced to become approximately 151% (black column) of that of the vehicle (white column); and this AE1-329-activated Ptgs2 promoter activity was suppressed by the co-treatment with L-161982 (gray column) to become about 78% of the promoter activity of the AE1-329-treated cells (Fig. 8A). Furthermore, when the region from −300 to −50 was deleted, the luciferase reporter activity was significantly decreased, and the responses to AE1-329 and L-161982 disappeared (Fig. 8A). To confirm the importance of the CRE at position −59 in the PGE2-derived elevation of Ptgs2 gene expression in 3T3-L1 cells, we introduced a mutation at this position in the −300/+124 construct; −300/+124(mu) [18]. When the cells were transfected with this −300/+124(mu) construct, the responsiveness to AE1-329 and L-161982 was lost;
although the basal promoter activity was not altered (Fig. 8A). These results indicate that PGE2 activated \( \text{Ptgs2} \) gene expression through the CRE at position \(-59\) of the mouse \( \text{Ptgs2} \) promoter in 3T3-L1 cells.

Next, we examined the binding of CREB to the CRE at position \(-59\) of the \( \text{Ptgs2} \) promoter by performing a chromatin immunoprecipitation (ChIP) assay. The expected size (168-bp; Fig. 8B, left panel) of an amplicon containing the CRE at \(-59\) was detected in the formaldehyde-fixed DNA-protein complexes immunoprecipitated with anti-CREB antibody (Fig. 8B, right panel). Moreover, when the cells were treated with AE1-329, the binding efficiency was enhanced about 4.7-fold as compared with that of the untreated cells (Fig. 8B, right panel), and the AE1-329-derived increase in the efficiency of binding of CREB to the CRE was clearly suppressed by co-treatment with L-161982 (Fig. 8B, right panel). On the contrary, there was no detectable signal when rabbit normal IgG was added (Fig. 8B, right panel). These results indicate that PGE2-mediated upregulation of \( \text{Ptgs2} \) gene expression occurred by enhancing the binding of CREB to the CRE of the \( \text{Ptgs2} \) gene promoter in 3T3-L1 cells.
Figure 9. Synergistic suppression of adipogenesis by PGE$_2$ and PGF$_{2\alpha}$. A. Enhancement of PGE$_2$ and PGF$_{2\alpha}$ production in AE1-329-treated adipocytes. 3T3-L1 cells were incubated for 1 h in DMEM containing or not AE1-329 (1 μM) with or without L-161982 (10 μM). The medium was then removed and replaced with fresh DMEM containing AE1-329 and/or L-161982 and A23187 (5 μM), and the cells were further incubated for 10 min. The medium was collected for the measurement of PGE$_2$ and PGF$_{2\alpha}$ levels by performing the respective EIA. Data are expressed as the mean ± S.D. of 3 independent experiments. *p<0.01, as indicated by the brackets. B. Oil Red O staining. 3T3-L1 cells were caused to differentiate into adipocytes for 6 days in DMEM containing PGE$_2$ (100 nM) and/or PGF$_{2\alpha}$ (100 nM; Cayman Chemical). PGE$_2$ and PGF$_{2\alpha}$ were added every day. Bar = 50 μm. C. Measurement of Oil Red O dye extracted from lipid droplet-laden cells. D. Intracellular triglyceride level. 3T3-L1 cells were cultured as described in the legend of Fig. 9B. Data are presented as the mean ± S.D. from 3 independent experiments. *p<0.01, as indicated by the brackets. E. Expression of adipogenic genes. Cells were cultured as described in the legend of Fig. 9B. Transcription levels were measured by quantitative PCR. Data are the mean ± S.D. of 3 independent experiments. *p<0.01, as indicated by the brackets.

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Synergistic PGE<sub>2</sub> and PGF<sub>2α</sub>-mediated Suppression of Adipogenesis

PGE<sub>2α</sub> and PGE<sub>2</sub> suppress the progression of adipogenesis through their specific PG receptors, i.e., PTGFR and PTGER4, respectively [18,20]. Moreover, PGE<sub>2α</sub> induces the production of anti-adipogenic PGE<sub>2</sub> and PGF<sub>2α</sub> by triggering the PTGFR receptor/MEK/ERK cascade in 3T3-L1 cells [18]. PGE<sub>2</sub> also enhances the production of anti-adipogenic PGE<sub>2</sub> and PGF<sub>2α</sub> in mouse embryonic fibroblasts [20].

We examined whether PTGER4 receptor-mediated activation would enhance PGF<sub>2α</sub> production in 3T3-L1 cells. PGE<sub>2</sub> production was increased by the treatment with AE1-329, and this enhancement was lost by co-treatment with L-161982, (Fig. 9A). Furthermore, PGF<sub>2α</sub> production was also enhanced by treatment with AE1-329 and the co-treatment with L-161982 blocked this increase (Fig. 9A). These results reveal that AE1-329-derived activation of the PTGER4 receptor enhanced de novo synthesis of anti-adipogenic PGE<sub>2</sub> and PGF<sub>2α</sub> in 3T3-L1 cells.

As both PGE<sub>2</sub> and PGF<sub>2α</sub> act as anti-adipogenic PGs in adipocytes, we investigated their suppression effects on adipogenesis in 3T3-L1 cells. When the cells were caused to differentiate into adipocytes for 6 days in medium containing either PGF<sub>2α</sub> or PGE<sub>2</sub> along with NS-398, which is an inhibitor of PTGS2 and the enhanced triglyceride level was suppressed by co-treatment with either of PGE<sub>2</sub> and PGF<sub>2α</sub>. Moreover, when the cells were caused to differentiate into adipocytes in medium containing both PGE<sub>2</sub> and PGF<sub>2α</sub>, the intracellular triglyceride level was lower than that in PGE<sub>2</sub>- or PGF<sub>2α</sub>-treated cells (Fig. 9D).

Next, we measured the expression level of adipogenic genes in PGE<sub>2</sub>- and/or PGF<sub>2α</sub>-treated cells. When 3T3-L1 cells were caused to differentiate into adipocytes, the expression levels of Ppar<gamma>, Fabp4, and Scd1 genes were enhanced approximately 3.1-, 7.1-, and 3.3-fold, respectively, as compared with those in the undifferentiated cells (Fig. 9E). In addition, the expression levels of these genes were enhanced even more in adipocytes cultured in medium containing NS-398. When the cells were caused to differentiate into adipocytes in medium containing both PGE<sub>2</sub> and PGF<sub>2α</sub>, the expression levels of the genes were decreased to a greater extent than when the cells were cultured in medium containing PGE<sub>2</sub> or PGF<sub>2α</sub> (Fig. 9E). Furthermore, the suppression effect on adipogenesis by PGF<sub>2α</sub> was weaker than that by PGF<sub>2α</sub> (Fig. 9E). These results indicate that PGE<sub>2</sub> or PGF<sub>2α</sub> synergistically suppressed adipogenesis in 3T3-L1 cells.

**Discussion**

PGs are known to be involved in the regulation of adipogenesis. PGE<sub>2</sub> is synthesized by lipocalin-type PGD synthase in adipocytes and accelerates the mid-late phase of adipogenesis [11]. PGF<sub>2α</sub> is involved in the activation of preadipocytes to adipocytes through PTG<sub>1</sub> receptor [14,15]. In contrast, PGF<sub>2α</sub> and PGE<sub>2</sub> suppress the progression of adipogenesis [17,18,19,20]. PGF<sub>2α</sub> is synthesized by AKR1B3 in adipocytes and represses the early phase of adipogenesis by engaging the PTGFR receptor [17]. Moreover, PGF<sub>2α</sub> enhances the production of itself and PGE<sub>2</sub> by enhancing the expression of the COX-2 gene via activation of the PTGFR receptor-ERK/CREB cascade [18]. PGE<sub>2</sub> also acts as anti-adipogenic factor, by acting through the PTGER4 receptor [19]. A recent study demonstrated that PGE<sub>2</sub>-PTGER4 signaling suppresses adipocyte differentiation by negatively affecting Ppar<gamma> expression in an autocrine manner in adipocytes [20]. However, the PGE<sub>2</sub>-producing enzyme in adipocytes and the precise mechanism regulating the suppression of adipogenesis by PGE<sub>2</sub> have not been fully understood. Here, we found that PTGES1 synthesized PGE<sub>2</sub> in 3T3-L1 cells, which then suppressed the early phase of adipogenesis via the PTGER4 receptor. Moreover, PGE<sub>2</sub> enhanced Pgs2 gene expression through the positive feedback loop via PTGER4 receptor, and the elevated PGE<sub>2</sub> and PGF<sub>2α</sub> production. Furthermore, AKR1B3-produced PGF<sub>2α</sub> suppresses the early phase of adipogenesis through PTGFR receptor [17], and increased the expression of the Pgs2 gene [18], like PGE<sub>2</sub>. Thus, PTGES1-produced PGE<sub>2</sub> and AKR1B3-synthesized PGF<sub>2α</sub> synergistically suppress the progression of the early phase of adipogenesis (Fig. 10).

Until now, three major enzymes that catalyze the production of PGE<sub>2</sub> from PGH<sub>2</sub> have been identified [21,29]: PTGES1 [23], PTGES2 [25], and PTGES3 [26]. PTGES1 has been identified as the member of the MAPEG family [30]. These three PTGESs were constitutively expressed during adipocyte differentiation of 3T3-L1 cells (Fig. 2). When the expression of PTGES1 was suppressed by its siRNA, the PGE<sub>2</sub> level was significantly decreased (Fig. 3A and 3B), indicating that PTGES1 was the PGES active in adipocytes. There are two different papers in the literature concerning the expression of PTGES1 in adipocytes. Heut et al. reported that PTGES1 levels in obese fats are significantly lower than those in lean animals [31]. However, the other report by Xie et al. indicated that PTGES1 is enhanced during differentiation of 3T3-L1 cells [32], thus differing from our results. At the present time, there is no clear explanation for this discrepancy. Further precise studies of the in vitro and in vivo functions of PTGES1 in adipocytes are needed to solve this problem. In addition, we have to elucidate the effects of GST activity of PTGES1 in the regulation of adipogenesis, because PTGES1 also carries GST activity [33].

PG synthesis is coordinately regulated through the coupling of terminal PG synthases with each or both of PTGES1 (COX-1) and

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**Figure 10. Synergistic suppression of the early phase of adipogenesis by PGE<sub>2</sub> and PGF<sub>2α</sub> in 3T3-L1 cells.**

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PTGS2 [34]. Both PTGSs were expressed in the undifferentiated 3T3-L1 cells (data not shown), indicating that both PTGSs would probably have the ability to couple with PTGES1 for the production of PGE₂. PTGES1 is co-localized with both PTGS isozymes in the perinuclear region [35]. However, PTGES1 is functionally coupled with PTGS2 to produce PGE₂ [35]. In fact, the PGE₂ production profile well resembled the expression profile of the Ptg2 gene (Fig. 2). PGs are known to be associated with Ptg2 gene expression in an autocrine manner in a variety of cells including adipocytes [18,20,36,37]. PGE₂ in adipogenesis [19]. Anti-adipogenic PGF 2α decreases the production of PGE2 and PGF2 in 3T3-L1 cells [18]. PGE₂ also enhances the production of PGE₂ and PGF₂α through the PTGER₄ receptor in mouse embryonic fibroblasts [20] and suppresses the progression of adipogenesis [19]. Anti-adipogenic PGF₂α and PGE₂ increased themselves to enhance the suppression of adipogenesis in the early phase of adipogenesis. However, the suppression of adipogenesis by these anti-adipogenic PGs was terminated within several hours after the initiation of adipogenesis. Therefore, the molecular mechanism underlying this termination needs further elucidation.

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