0273-2289/04/118/0133/$25.00

Regulation and Role of Arachidonate Cascade During Changes in Life Cycle of Adipocytes

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Received May 9, 2003; Revised October 13, 2003; Accepted October 24, 2003

Abstract

Although some eicosanoids serve as potent natural ligands to activate peroxisome proliferator-activated receptor (PPARγ), the ability of adipocytes to produce eicosanoids and regulate PPARγ remains unclear. Here, adipogenic 3T3-L1 cells were employed to determine the gene expression of isoforms of biosynthetic enzymes in the arachidonate cyclooxygenase (COX) pathway and the synthesis of prostaglandins (PGs). The expression of COX-2 was induced transiently in a biphasic manner upon the triggering of the differentiation and maturation phases while COX-1 was constitutive. The exclusive expression of lipocalin-type PGD synthase occurred and gradually increased during the maturation process along with the stable expression of PPARγ. Moreover, we confirmed the formation of PGD_2 from arachidonic acid by the mature adipocytes, suggesting conversion into PGJ_2 derivatives. Even though cytosolic and membrane-associated subtypes of PGE synthase were expressed at relatively constant levels, the ability of preadipocytes to produce PGE_2 was greater than that of mature adipocytes in the cell response. The treatment of the mature adipocytes with exogenous PGD_2, 15-deoxy-$\Delta^{12,14}$-PGJ_2 and PGE_2, in the presence of aspirin, enhanced the adipogenesis. These findings imply the specific roles of prostanoids produced by the mature adipocytes in the maintenance of terminal differentiation through an autocrine control mechanism.

Index Entries: Adipocyte; life cycle; peroxisome proliferator-activated receptor γ; adipogenesis; arachidonate cascade; eicosanoid; cyclooxygenase; prostaglandin; isoforms.

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Introduction

The arachidonate cascade contains distinct enzymatic pathways such as cyclooxygenase (COX) and lipoxygenase pathways to catalyze the conversion of arachidonic acid released from the membrane phospholipids in response to a variety of stimuli into bioactive lipid mediators called eicosanoids *(1,2)*. The rate-limiting step of the arachidonate COX pathway is the reaction catalyzed by prostaglandin (PG) endoperoxide synthases to form PGH_2 through PGG_2 as an intermediate from free arachidonic acid. These enzymes are generally called COXs, which are known to exist in two isoforms, a constitutively expressed COX-1 and stimuli-inducible COX-2 *(3)*. Moreover, studies using knockout mice and specific inhibitors have clarified the unique physiologic and pathologic functions. These segregated roles of the COX isoforms have been considered to be owing to the difference in the gene expression, kinetic properties, subcellular localization, and preferential interaction with either phospholipases or PG synthases.

The resulting PGH_2 produced by the action of the COX isoforms is utilized as a substrate of the several terminal enzymes referred to as PG synthases for the production of the corresponding prostanoids. Molecular cloning studies have revealed the existence of distinct isoforms of PG synthases including prostaglandin D synthase (PGDS) and prostaglandin E synthase (PGES). PGD_2 is mainly formed in the central nervous system (CNS), and its production involves the action of lipocalin-type PGDS (L-PGDS). The produced PGD_2 is known to regulate the response of sleep and pain in the brain. Alternatively, the formation of this compound is produced by mast cells, basophils, and other blood cells. The latter reaction is catalyzed by another isoform called hematopoietic PGDS (H-PGDS). PGD_2 is known to be unstable under aqueous solution to give dehydration products such as PGJ_2, Δ^{12}-PGJ_2, and 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2) *(4,5)*, which are biologically the most active by exhibiting nuclear action *(6)*. On the other hand, PGE_2 is produced ubiquitously in the tissues of animals. In particular, this compound is well established to be responsible for inflammation, tumorigenesis, bone resorption, and protection of gastric ulcer. More recently, PGES has been identified to occur in multiple forms with different features, cytosolic PGES (cPGES) and membrane-associated PGES (mPGES). Murakami et al. *(7)* extensively studied the regulatory functions of PGE_2 biosynthesis by using transfectants of human embryonic kidney 293 cells and provided evidence for distinct functional coupling with upstream COX isoforms in the cultured cells.

Obesity with excess accumulation of adipose tissues is a well-known risk factor for the onset of serious diseases such as diabetes mellitus accompanying insulin resistance, hypertension, atherosclerosis, and even certain types of cancer. Obesity is caused by an increase in cell number or size of adipocytes, or both. The hypertrophic adipocytes are localized around internal organs and undergo changes in the life cycle of adipocytes leading

to insulin resistance. For example, hypertrophic adipocytes are known to release some cytokines, including tumor necrosis factor-α (TNF-α) and free fatty acids, that are different from the insulin-sensitive adipocytes in the quality of cells. Although the precise mechanism of the generation of insulin resistance remains unclear, peroxisome proliferator-activated receptor γ (PPARγ), a member of the nuclear receptor superfamily, plays a critical role in the changes in the life cycle of adipocytes *(8,9)*. PPARγ requires ligands to be activated to serve as transcription factors. At first, a class of antidiabetic drugs termed *thiazolidinediones* was found to be high-affinity ligands. Later, PGD_2-derived PGJ_2 derivatives from the arachidonate COX pathway *(10,11)*, as well as other arachidonate metabolites *(12)*, were shown to be potent natural ligands. In addition, of PGJ_2 derivatives, in particular, 15d-PGJ_2 was demonstrated to be the most active to promote adipocyte differentiation in cultured cells expressing PPARγ *(10,11)*. Thus, compared with the biologic action of eicosanoids, much less is known about the mechanism of regulation of the arachidonate cascade including gene expression of biosynthetic enzymes in adipogenesis and obesity. Whether the active endogenous eicosanoids are synthesized in adipocytes and related cells and serve as their own regulators of the life cycle of adipogenesis will therefore need to be determined. Alternatively, it is conceivable that the effective eicosanoids are formed elsewhere and transported to the target tissues in vivo.

In the present study, we investigated gene expression, regulation, and role of the arachidonate cascade during changes in the life cycle of adipocytes. Attention was especially focused on the expression of specific isoforms involved in the arachidonate COX pathway using the useful 3T3-L1 preadipogenic cell line, which was established from Swiss mouse embryo 3T3 fibroblasts *(13,14)*. 3T3-L1 cells are capable of undergoing the mitotic clonal expansion phase after being treated with culture medium supplemented with dexamethasone, 3-isobutyl-1-methyl xanthine (IBMX), and insulin. Exposure of the cultured cells to maturation medium containing insulin then generates the mature adipocytes of biochemical and spontaneous morphologic characteristics. Our results demonstrate the ability of 3T3-L1 adipocytes to produce distinct types or levels of prostanoids depending on the changes in the life cycle. Moreover, we provide evidence for the specific gene expression of isoformic enzymes in the COX pathway. Here, the endogenous prostanoids formed by the mature adipocytes are shown to control the progression of adipogenesis through an autocrine control mechanism.

Materials and Methods

Chemicals and Equipment

Dulbecco's modified Eagle's medium with 25 m*M* HEPES (DMEM-HEPES), phorbol-12-myristate-13-acetate (PMA), calcium ionophore A23187, and newborn bovine serum (NBS) were obtained from Sigma

(St. Louis, MO). Fetal bovine serum (FBS) was purchased from Biological Industries (Kibbutz Beth Haemek, Israel). Anti-mouse IgG–biotin conjugate was supplied by Seikagaku Kogyo (Tokyo, Japan). Ribonuclease inhibitor and TrueScript II reverse transcriptase (RT) were obtained from Sawady (Tokyo, Japan). Polymerase chain reaction (PCR) MasterMix was purchased from Promega (Madison, WI). LightCycler-FastStart DNA Master SYBR Green I (including FastStart *Taq* DNA polymerase) and the LightCycler instrument for real-time PCR were obtained from Roche (Mannheim, Germany); 96-well microplates (type E) for enzyme-linked immunosorbent assay (ELISA) were from Sumitomo Bakelite (Tokyo, Japan); plastic 60-mm Petri dishes and 24-well plates for tissue cultures were from Iwaki Glass (Tokyo, Japan); and authentic PGs, SC-560, NS-398, and aspirin were from Cayman (Ann Arbor, MI). The Triglyceride E-Test kit was supplied by Wako (Osaka, Japan). For use as a vehicle in the cell culture, PMA, A23187, PGD_2, and 15-PGJ_2 were dissolved in dimethyl sulfoxide, and others were dissolved in ethanol. All other chemicals used were of reagent or tissue culture grade.

Cell Culture and Adipocyte Differentiation

The mouse 3T3-L1 preadipogenic cell line (JCRB9014) was obtained from Health Science Research Resources Bank (Osaka, Japan). For subcultures, the cells were plated at 5×10^4 cells/mL in the growth medium consisting of DMEM-HEPES supplemented with 10% FBS and 200 µ*M* ascorbic acid at 37°C under 7% CO_2. To proceed with a series of the changes in the life cycle of 3T3-L1 cells, the cells were plated at 5×10^4 cells/mL in the growth medium and refed every 2 or 3 d until reaching 80% confluence *(15)*. After 2 d of the continued culture without changing the culture medium, the cells were exposed to the differentiation medium corresponding to the growth medium supplemented with 1 µ*M* dexamethasone, 0.5 m*M* IBMX, and 10 µg/mL of insulin for 45 h to induce mitotic clonal expansion *(9,16)*. Then, the culture medium was replaced with maturation medium prepared by using the growth medium and 5 µg/mL of insulin, and cultured every 2 d until about 10 d. The adipogenic actions were evaluated by staining the accumulated lipid droplets with Oil Red O as described previously *(17)*. In addition, the enzyme activity of *sn*-glycerol-3-phosphate dehydrogenase was determined for monitoring the adipocyte differentiation since this cytosolic enzyme is a rate-limiting enzyme for the synthesis of triacylglycerol from glucose through the glycolytic pathway *(18)*. The content of triacylglycerols during adipogenesis was determined with the Wako Triglyceride E-Test kit using *sn*-glycerol-3-phosphate oxidase *(19)*.

RT-PCR Analysis

Total RNA was extracted from cultured cells by the method of acid guanidinium thiocyanate/phenol/chloroform mixture *(20)* for analysis by RT-PCR. For the synthesis of cDNA by the RT reaction, the total reaction

Table 1
Oligonucleotides of Sense and Antisense Primers Used for RT-PCR[a]

Primer	Size (mer)	Sequence
5'-Primers		
PPARγ	20	5'-GAR-CCT-GCD-TCY-CCD-CCY-TA-3'
COX-1	20	5'-GGG-ACC-AAA-GGG-AAG-AAA-CA-3'
COX-2	20	5'-GAG-ATG-ATC-TAC-CCT-CCT-CA-3'
cPGES	20	5'-TGG-TAC-GAT-CGA-AGG-GAC-TA-3'
mPGES	20	5'-ATC-AAG-ATG-TAC-GCG-GTG-GC-3'
H-PGDS	20	5'-AAT-ATG-AGG-GGA-AGA-GCC-GA-3'
L-PGDS	20	5'-GAC-AAG-TTC-CTG-GGG-CGC-TG-3'
GAPDH	20	5'-GTC-TTC-ACC-ACC-ATG-GAG-AA-3'
3'-Primers		
PPARγ	20	5'-GAR-CTS-CAG-CTV-AAG-CTG-AA-3'
COX-1	21	5'-AAC-CCC-ATA-GTC-CAC-CAG-CAT-3'
COX-2	21	5'-CCC-CTT-CAC-ATT-ATT-GCA-GAT-3'
cPGES	20	5'-TC-TGC-TCC-ATC-TAC-TTC-TGG-3'
mPGES	20	5'-AC-CTC-CCA-GAG-GAT-CTG-CAG-3'
H-PGDS	20	5'-CA-GTA-GAA-GTC-TGC-CCA-GGT-3'
L-PGDS	20	5'-CT-ACT-GTA-GAG-GGT-GGC-CAT-3'
GAPDH	20	5'-TC-CAC-CAC-CCT-GTT-GCT-GTA-3'

[a]The sizes of amplified cDNA fragments using RNA from 3T3-L1 cells were as follows: PPARγ, 1032 bp; COX-1, 768 bp; COX-2, 909 bp; cPGES, 413 bp; mPGES, 371 bp; H-PGDS, 431 bp; L-PGDS, 344 bp; GAPDH, 677 bp.

mixture contained 1 µg of total RNA in 10 µL of 1× RT reaction buffer of 10 m*M* Tris-HCl buffer (pH 8.8), 50 m*M* KCl, 1.5 m*M* $MgCl_2$ 1 m*M* dNTPs, 0.5 µ*M* oligo $(dT)_{15}$ primer, 0.5 µ*M* random primer (9mer), 10 U of ribonuclase inhibitor, and 50 U of TrueScript II RT enzyme. The RT reaction was done for 1 h at 42°C followed by heating at 98°C for 5 min. The resulting 1-µL aliquot of the reaction mixture was subjected to PCR amplification in a total of 20 µL of 1× PCR Master Mix (Promega) containing a proprietary reaction buffer (pH 8.5), 1.5 m*M* $MgCl_2$, 200 µ*M* dNTPs, 1 µ*M* sense primer, 1 µ*M* antisense primer, and 25 U/mL of *Taq* DNA polymerase. After heating at 95°C for 2 min, amplification was performed for 30 cycles of denaturation at 95°C for 1 min, primer annealing at 55°C for 1 min, and extension at 72°C for 1 min. A 2-µL aliquot of the reaction mixture was used for analysis by electrophoretic separation on a 1.5% agarose gel. The sense and antisense primers used are provided in Table 1 *(21–28)*. The DNA sequence of the amplified DNA was confirmed with the ABI Prism 310 Genetic Analyzer and Dye Terminator Cycle Sequencing Ready reaction kit reagents (Applied Biosystems, Foster City, CA).

Quantitative Real-Time PCR

The RT reaction was done as described in the previous section, and a 2-µL aliquot of RT products was used for quantitative analysis using real-

time PCR with the LightCycler instrument (Roche). The reaction and the operation were performed using LightCycler-FastStart DNA Master SYBR Green I and FastStart *Taq* DNA polymerase according to the manufacturer's instructions *(29)*. The melting curves were analyzed for confirmation of the specificity of the amplified DNA fragments from the target transcripts. The transcript levels of each sample were normalized to those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

ELISA for PGD_2 and PGE_2

To determine the ability of cultured 3T3-L1 cells to form PGD_2 during the maturation phase, the cells were incubated with 10 µ*M* arachidonic acid for 1 h at 37°C in the maturation medium. The resulting culture medium was collected and subjected to determination of PGD_2 using a PGD_2-methoxime enzyme immunoassay kit (Cayman) after conversion of PGD_2 to a stable methoxime derivative. The calibration curve was generated by diluting the standards with the maturation medium. The assays were essentially done according to the manufacturer's instructions.

For determination of the stimulated synthesis of PGE_2 by cultured cells in response to PMA and A23187, ELISA for PGE_2 was carried out as described subsequently. To allow progression of the life cycle of adipocytes, 3T3-L1 cells were cultured and exposed to the differentiation and maturation media. At the indicated time, the culture medium was replaced with the DMEM-HEPES medium containing 2% NBS, and the cells were treated with 50 n*M* PMA and 100 n*M* A23187 for 24 h in a CO_2 incubator. The culture medium of cultured 3T3-L1 cells was centrifuged, and then the resulting supernatant was subjected to ELISA for PGE_2. Determination of PGE_2 was essentially carried out according to previous reports by our laboratory *(30,31)*.

Determination of Proteins

The amount of proteins from cultured cells was determined by the method of Lowry et al. *(32)* after precipitation with trichloroacetic acid *(33)*.

Analysis

Assays were carried out in triplicate unless otherwise indicated. Data are represented as the means ± SE from a representative experiment done at least three times.

Results

Life Cycle of 3T3-L1 Adipocytes

Mouse adipogenic 3T3-L1 cells were cultured in different types of culture media to induce a series of cell cycles including the proliferation, differentiation, and maturation phases during adipogenesis according to the standard method of Green and colleagues *(13–15)*. The cells grown until

reaching 80% confluence were maintained for 2 d to cause the resting state. The resulting confluent cells were exposed to the differentiation medium containing IBMX, dexamethasone, and insulin to trigger the clonal expansion phase, after which the maturation medium including insulin was refed every 2 d to promote terminal differentiation. Cell growth was determined by counting the cell number (Fig. 1A). After the gradual cell growth, the cell concentrations after 24 h in the maturation medium nearly doubled compared with the confluent cells after 45 h of culture in the differentiation medium, which is characteristic of clonal expansion phase, allowing the cells to trigger the conversion of 3T3-L1 cells to the adipocytes *(9,16)*. Thereafter, almost no further cell growth was detected during the maturation process.

To provide evidence that the cultured 3T3-L1 cells promoted adipogenesis during the maturation process, the cells were stained with the lipophilic dye Oil Red O and viewed macroscopically (Fig. 1B) or microscopically with a differential interference microscope (Fig. 1C). These analyses clearly showed the accumulation of lipid droplets on the inside of cells especially after 6 d of culture in the maturation phase. We also confirmed significant increases in the enzyme activity of glycerol-3-phosphate dehydrogenase, a rate-limiting enzyme for the biosynthesis of triacylglycerols *(18)*, as well as increased contents of lipid droplets as determined by the assay of triacylglycerols.

The nuclear receptor PPARγ is known to be found primarily in adipose tissue, where it plays a critical role in the change in the life cycle of adipocytes including the differentiation of preadipocytes into adipocytes *(8)* as well as adipocyte hypertrophy *(34)*. However, little is known about the time course of the gene expression of PPARγ in the life cycle of 3T3-L1 adipocytes. Hence, we determined the mRNA levels of PPARγ during changes in the adipogenesis by the method of RT-PCR (Fig. 2). The gene expression of PPARγ reflecting the transcriptional activation occurred in a slight and transient manner at 3 h after the differentiation phase, and then the significant increased levels became evident after 3 h of replacing with the maturation medium, which was maintained for 7 d of the adipogenesis (Fig. 2).

Gene Expression of Isoforms in Arachidonate COX Pathway

For the formation of prostanoids, arachidonate COX pathways are required. This biosynthetic pathway involves functional cooperation between PG endoperoxide synthases, alternatively called COX, and several types of PG synthases. For these biosynthetic enzymes, there are distinct types of isoforms with unique physiologic functions *(3)*. For the evaluation of gene expression of target enzymes, mostly we made use of RT-PCR, which has the advantages of higher sensitivity and the convenience of many samples even though the quantitative data are less accurate than with real-time PCR. First, we determined the specific gene expression of the isoforms of the COX pathway leading to the formation of PGE_2 (Fig. 3). The resulting

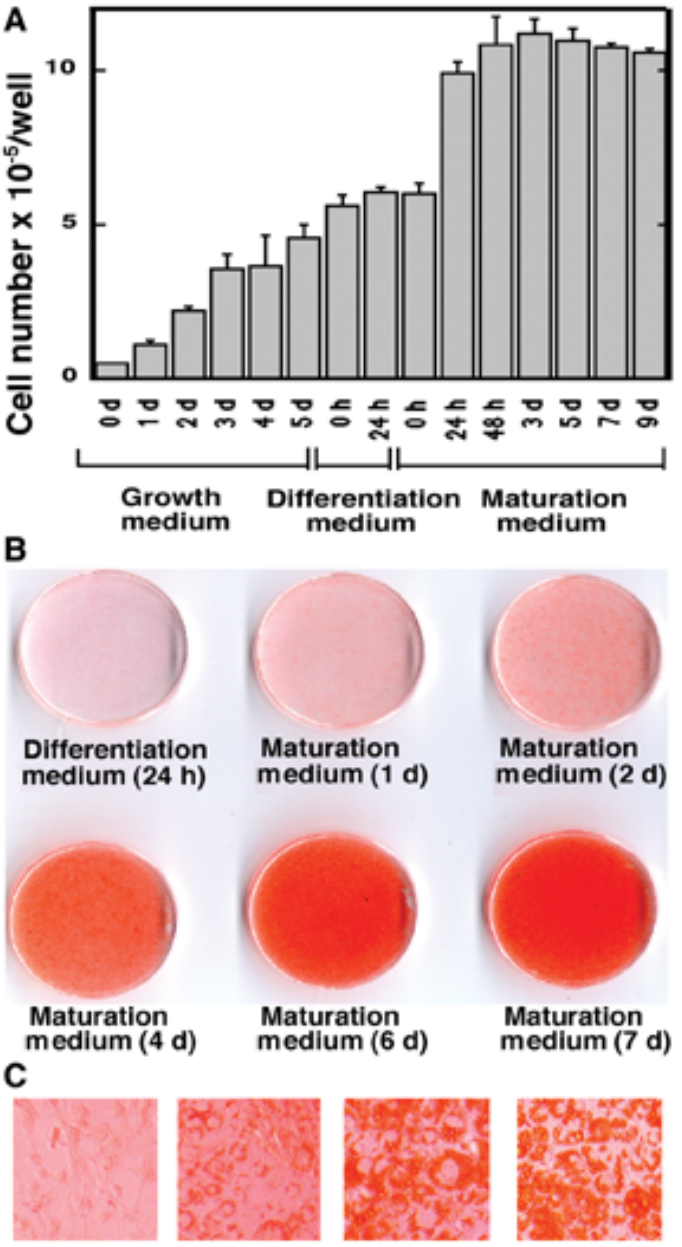

Fig. 1. **(A)** Cell growth in life cycle of 3T3-L1 cells. The cells were plated at 5×10^4 cells/mL in a 24-well dish containing 1 mL of growth medium. After changing the culture medium every 2 d, the cells were grown until reaching 80% confluence. After an additional 2 d of culture without changing the culture medium, the confluent cells were exposed to the differentiation medium for 45 h, after which the resulting cells were refed every 2 d with the maturation medium until 9 d. At the indicated time, the cell number was counted after treating with trypsin and EDTA. Data represent the means ± SE from five independent wells. **(B)** Macroscopic view of cell culture plates. The cells were cultured and induced into differentiation and maturation. At the indicated time, the cells were fixed and stained with Oil Red O to visualize the accumulated lipid droplets. **(C)** Microscopic view with a differential interference microscope at ×200 magnification.

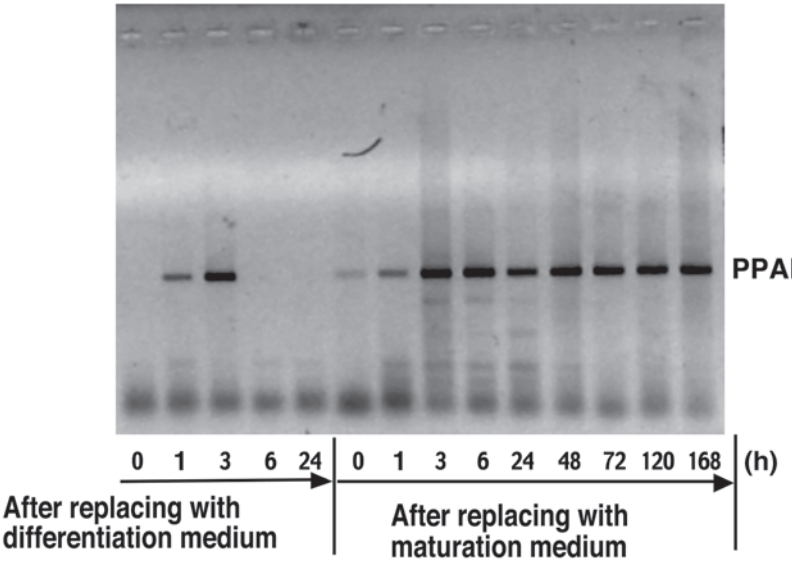

Fig. 2. Gene expression of nuclear receptor PPARγ. 3T3-L1 cells in 4 mL of culture medium were plated at 5×10^4 cells/mL, differentiated, and matured to terminal differentiation. At the indicated time, total RNA was extracted and subjected to analysis of the transcript of PPARγ by RT-PCR. Separation of the DNA fragments was done on 1.5% agarose gel electrophoresis. Other details are described under Materials and Methods.

data about isoforms of the COX enzymes are quantitatively represented in Fig 4. When the mRNA levels of COX isoforms were analyzed by RT-PCR, the transcriptional level of COX-1 was found to be almost constitutive, whereas the gene expression of COX-2 was inducible and increased in a transient and biphasic manner after 3 h of replacing with the differentiation medium and around several hours after treatment in the maturation medium. These changes in the culture medium reflected the stimuli for triggering the differentiation and maturation phases in adipocytes.

PGES utilizes PGH_2 derived from the reaction of COX enzymes as a substrate for the formation of PGE_2. Recent studies have revealed the occurrence of at least two isoforms: cPGES and mPGES *(7)*. These two types of isoforms need to couple with the upstream COXs in the adipose cells. To determine the gene expression of these two types of isoforms of PGES, we employed analysis by RT-PCR with specific primers for them (Fig. 3). Their transcript levels were quantitatively expressed by densitometric analysis (Fig. 5). These analyses revealed that both isoforms of PGES have similar and constitutive gene expression patterns even though a transient peak at 3 h was seen after induction of the maturation phase. Transcriptional activation continued until the later phase of the maturation phase.

PGD_2 is also synthesized by the action of PGDS in the COX pathway. Recent studies have shown the existence of two types of isoforms: L-PGDS and H-PGDS *(35)*. The PGD_2 dehydration product has been shown to give

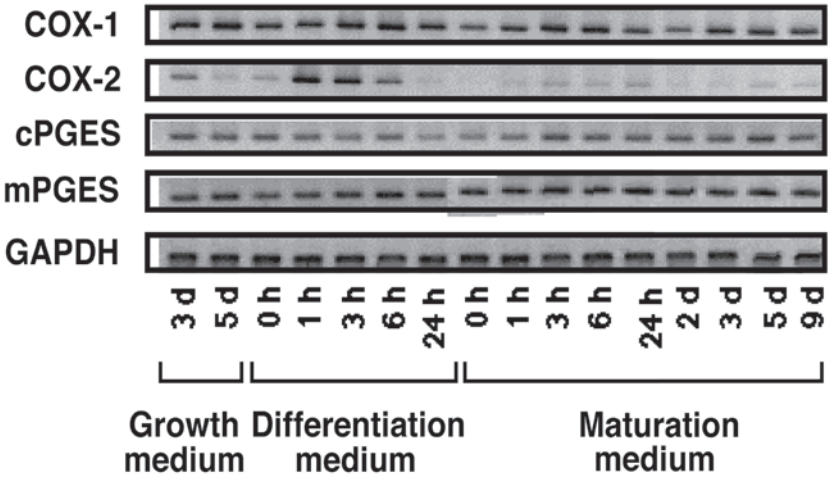

Fig. 3. Gene expression of isoforms COX-1, COX-2, cPGES, and mPGES. 3T3-L1 cells in 4 mL of culture medium were plated at 5×10^4 cells/mL, differentiated, and matured to terminal differentiation. At the indicated time, total RNA was extracted and subjected to analysis of the isoforms COX-1, COX-2, cPGES, and mPGES by RT-PCR. Other details are described under Materials and Methods.

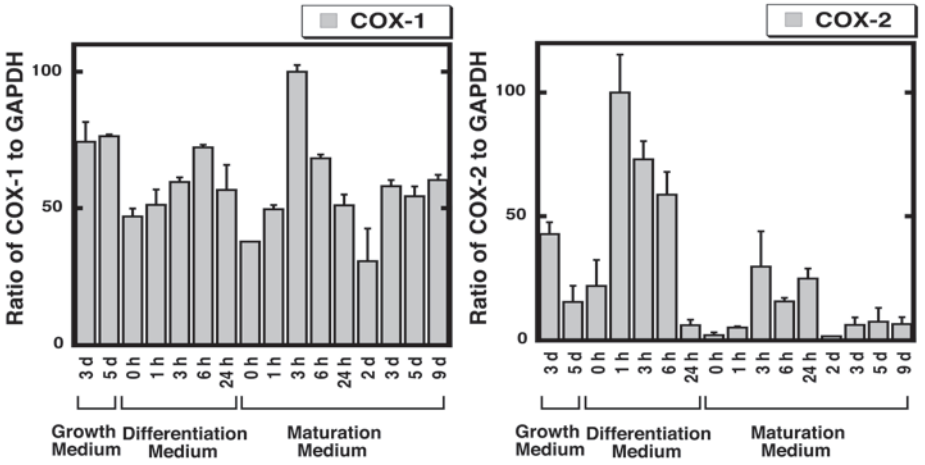

Fig. 4. Changes in transcript levels of COX-1 and COX-2. The data in Fig. 3 were expressed after the levels of COX-1 and COX-2 were normalized to those of the level of GAPDH as a housekeeping gene.

rise to 15d-PGJ_2, which is the most potent endogenous ligand for PPARγ yet discovered *(10,11)*. However, the gene expression of PGDS during the life cycle of adipocytes is unknown. Therefore, we tried to determine the levels of the transcripts for the isoforms of PGDS in 3T3-L1 adipocytes. As shown in Fig. 6, we only detected the mRNA level of L-PGDS, which increased gradually during the maturation process. We could not detect the other type of isoform, H-PGDS, in 3T3-L1 cells even though the trials were exten-

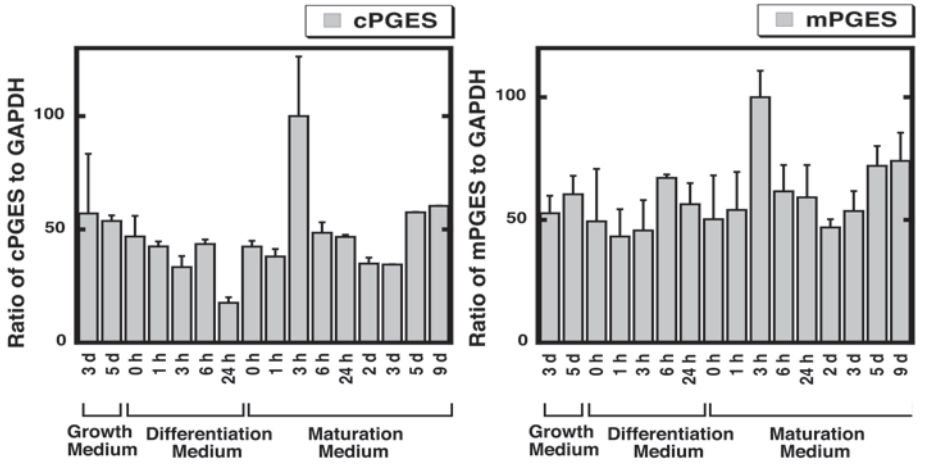

Fig. 5. Changes in transcript levels of cPGES and mPGES. The data in Fig. 3 were expressed after the levels of cPGES and mPGES were normalized to those of the level of GAPDH.

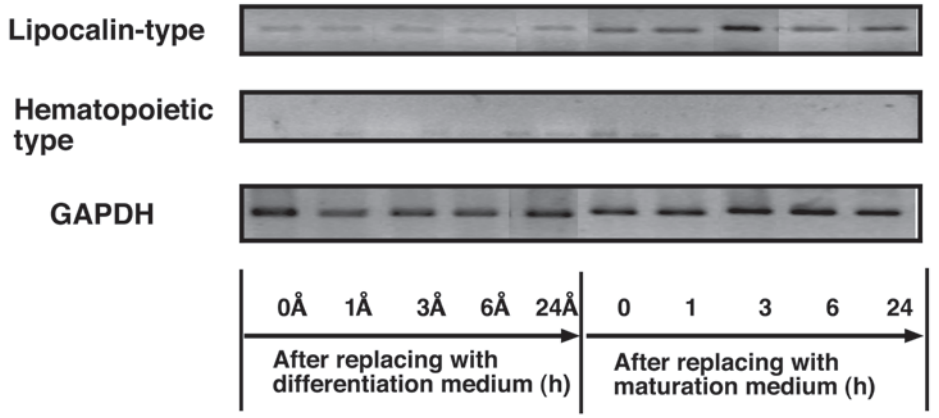

Fig. 6. Gene expression of isoforms of L-PGDS and H-PGDS. 3T3-L1 cells in 4 mL of culture medium were plated at 5×10^4 cells/mL, differentiated, and matured to terminal differentiation. At the indicated time, total RNA was extracted and subjected to the analysis for the expression of the isoforms by RT-PCR. Other details are as described in Materials and Methods.

sive. As a positive control, we confirmed that RT-PCR was successful with total RNA from mouse spleen cells with designed primers. When the cultured cells were exposed to the maturation medium over 24 h, the highest level seemed to occur at about 3 h of culture in the maturation phase. Furthermore, we attempted to monitor the gene expression for a longer term of several days (Fig. 7). Determination of the levels of the transcript for L-PGDS using quantitative real-time PCR revealed a more increased accumulation of mRNA levels of L-PGDS after 2 and 3 d of the maturation phase as compared with that after 3 h.

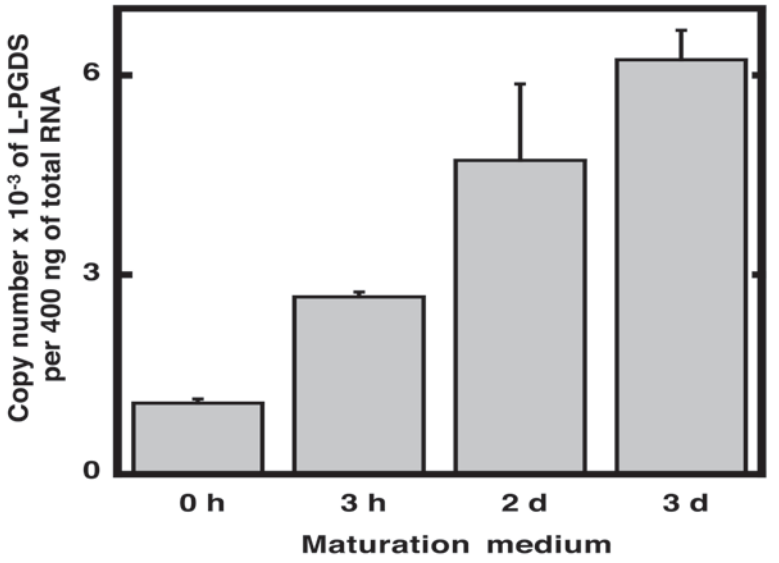

Fig. 7. Changes in transcript levels of L-PGDS during maturation process. 3T3-L1 cells in 4 mL of culture medium were plated at 5×10^4 cells/mL, differentiated, and matured to terminal differentiation. At the indicated time, total RNA was extracted and subjected to analysis of the mRNA level of L-PGDS by quantitative real-time PCR by normalizing to the level of GAPDH.

Regulation of Arachidonate Cascade and Role of Prostanoids in 3T3-L1 Adipocytes

To confirm the ability of 3T3-L1 adipocytes to form PGD_2 from exogenous arachidonic acid during the maturation phase, the cultured cells were incubated with 10 μ*M* arachidonic acid for 1 h. Analysis of the resulting medium showed that the mature adipocytes maintained the potency to form PGD_2 during the maturation process (Fig. 8). The formation of PGD_2 appeared to be linked to the increase in gene expression of L-PGDS.

When we incubated the enzyme extracts from different stages of the life cycle of 3T3-L1 adipocytes with [1-^{14}C]arachidonic acid, we confirmed the production of PGE_2, another COX product. To clarify the cell response of the arachidonate cascade during the change in the life cycle of 3T3-L1 cells, we determined the production of PGE_2 after a 24-h culture in the presence or absence of 50 n*M* PMA, an active phorbol diester, and 100 n*M* A23187, a calcium ionophore (Fig. 9). The cultured cells at the growing phase responded to treatment with the mixture of PMA and A23187, resulting in a significant increase in the synthesis of PGE_2. By contrast, the response of the confluent cells at the differentiation phase and adipocytes at the maturation phase was inferior to that of the preadipocytes at the growing phase.

To determine whether prostanoids formed by 3T3-L1 cells serve as regulators of the maturation process during adipogenesis, the cultured cells after triggering the differentiation process were exposed to one of the

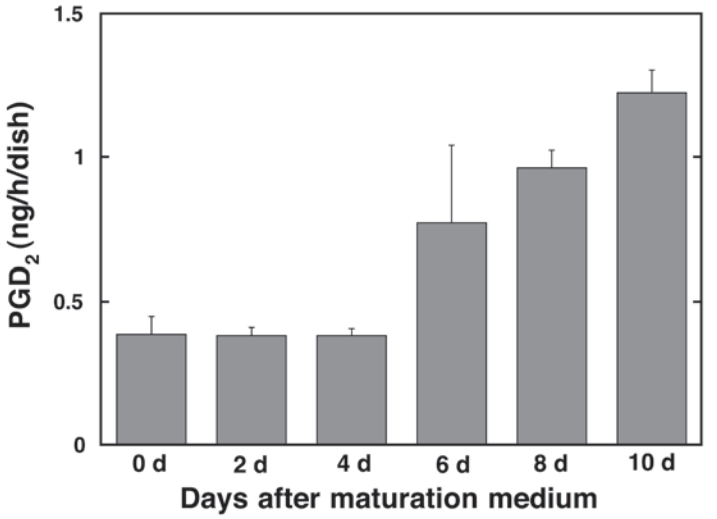

Fig. 8. Production of PGD_2 from exogenous arachidonic acid by adipocytes in maturation medium. 3T3-L1 cells in 4 mL of culture medium were plated at 5×10^4 cells/mL in a 60-mm dish, differentiated, and matured to terminal differentiation in the culture medium. At the indicated time of the maturation phase, the cultured cells were incubated with 10 μ*M* arachidonic acid for 1 h in the maturation medium. The resulting culture medium was subjected to determination of PGD_2-methoxime by enzyme immunoassay after PGD_2 was converted into a stable methoxime derivative as described in Materials and Methods.

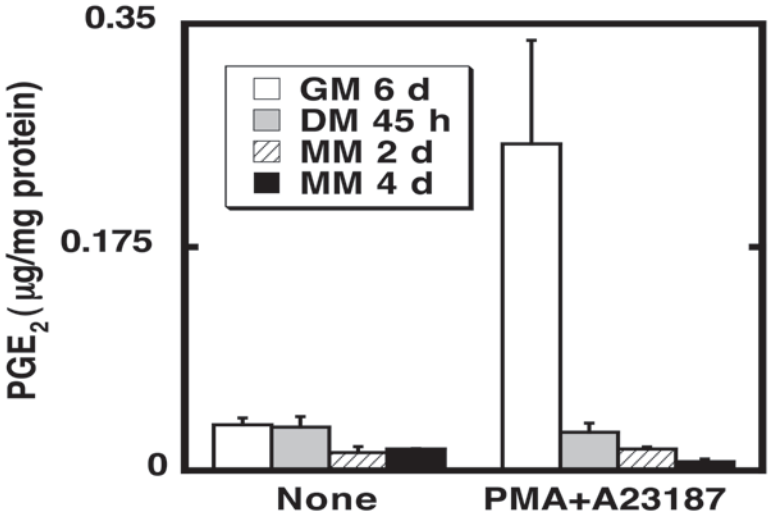

Fig. 9. Synthesis of PGE_2 by cultured 3T3-L1 cells in response to PMA and A23187. 3T3-L1 cells in 1 mL of culture medium were plated at 5×10^4 cells/mL in a 24-well dish, differentiated, and matured to terminal differentiation. At the indicated time, the cultured cells at the different phases were replaced with DMEM-HEPES medium supplemented with 2% NBS treated for 24 h with vehicle or a mixture of 50 n*M* PMA and 100 n*M* A23187. After the treatment, the culture medium was collected and subjected to ELISA of PGE_2 as described in Materials and Methods. (□) After 6 d of growth medium; (▨) after 45 h of differentiation medium; (▨) after 2 d of maturation medium; (■) after 4 d of maturation medium.

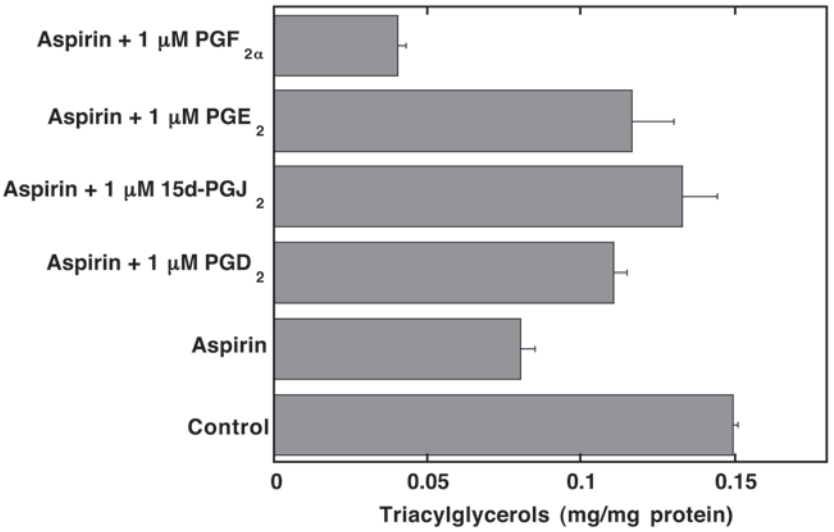

Fig. 10. Effect of exogenous prostanoids on accumulation of triacylglycerols during maturation phase in the presence of aspirin. 3T3-L1 cells in 2 mL of the culture medium were plated at 5×10^4 cells/mL in a 35-mm dish and differentiated for 45 h. Then, the cultured cells were refed every 2 d with the maturation medium supplemented with one of the prostanoid species in the presence of aspirin for a total of 6 d. The amount of triacylglycerols was determined as described in Materials and Methods.

prostanoid species in the presence of aspirin, a COX inhibitor, in the maturation medium for a total of 6 d, followed by measurement of triacylglycerols content (Fig. 10). The treatment of cultured cells with aspirin attenuated the accumulation of triacylglycerols. The addition of PGD_2, 15d-PGJ_2, or PGE_2 was efficacious at reversing the effect of aspirin. In sharp contrast, $PGF_{2\alpha}$ had an additional inhibitory effect on the accumulation of triacylglycerols in the presence of aspirin.

Discussion

Recent studies have shown that the nuclear hormone receptor PPARγ has a pivotal role in the life cycle and gene expression of adipocytes in the lipid metabolism. In addition, this nuclear receptor is known to be important in the changes in the quality of adipocytes leading to hypertrophy and insulin resistance in vitro and in vivo. For activation of PPARγ, the ligands need to be provided to the adipocytes. The effective exogenous ligands include a class of antidiabetic drugs such as thiazolidinediones with potent adipogenic properties. Not until recently were naturally occurring ligands for PPARs identified *(10,11)*. Those studies revealed the potency of a diverse group of lipids and related compounds to activate PPARγ. Of these, the PGD_2 dehydration product 15d-PGJ_2 has been shown to be a potent endogenous ligand. However, the regulation and role of the arachidonate cascade in the life cycle of the adipocytes and related cells remain unclear.

With this in mind, we studied the gene expression of the arachidonate COX pathway by focusing on the gene expression of isoforms for the biosynthetic enzymes, and the autocrine control by biosynthesized prostanoids of the changes in the life cycle of adipocytes. Our studies revealed the specific gene expression of isoforms for the arachidonate COX pathway in the life cycle of cultured 3T3-L1 cells, a useful model adipogenic cell line. Exposure of the confluent resting cells to the culture medium caused biphasic increases in the gene expression of the COX-2 isoform several hours after the initiation of the differentiation phase and maturation phase, whereas COX-1 was relatively constitutively expressed. Analysis of the gene expression of two PGES isoforms showed similar expression patterns with the continued expression from the growing phase to the maturation phase. As for the isoforms of PGDS responsible for the formation of PGDS and derived PGJ_2 derivatives, only L-PGDS was expressed without the expression of H-PGDS. The transcript levels of L-PGDS tended to increase gradually at the maturation phase. The formation of PGD_2 from arachidonic acid was confirmed during the maturation phase. These findings suggested the role of PGD_2 and related PGJ_2 derivatives in the continuous progression of the maturation process through the autocrine control. The formation of PGD_2 and its derivatives would occur by coupling of L-PGDS preferentially with the constitutive COX-1. Studies on the effects of prostanoids on adipocyte differentiation in the presence of aspirin indicated the role for endogenous PGD_2 and related substances in stimulating adipogenesis as a mechanism of autocrine control. We also found that the response of mature adipocytes to form PGE_2 was less efficient than for growing preadipocytes, reflecting the switch of the ability of 3T3-L1 cells to generate prostanoids during the change in the life cycle of adipocytes. Figure 11 is a schematic representation of the regulation and role of the arachidonate cascade in 3T3-L1 adipocytes.

Adipocytes play a central role in lipid homeostasis, maintenance of energy balance, and insulin resistance by storing lipids, as well as secretion of a number of cytokines such as TNF-α, adipsin, and leptin *(36)*. The life cycle of adipocytes includes the cell growth of preadipocytes, triggering of differentiation, and terminal differentiation. Obesity is characterized by excess accumulation of adipose tissues accompanying the storage of lipid droplets through increases in the number or size of adipocytes. In particular, hypertrophic adipocytes are known to show insulin resistance, which contributes to development of hypertrophic obesity. Thus, studies on the molecular and cellular events leading to the change in the life cycle of adipocytes will be important. We therefore performed studies in special reference to the gene expression and role of the arachidonate COX pathways.

Here, we used 3T3-L1 preadipocytes, a subclone of Swiss mouse embryo 3T3-L1 fibroblasts in culture. When 3T3-L1 cells are stimulated with the appropriate stimuli, conversion into adipocytes becomes evident as characterized by the phenotype similar to the normal adipose cells *(37)*.

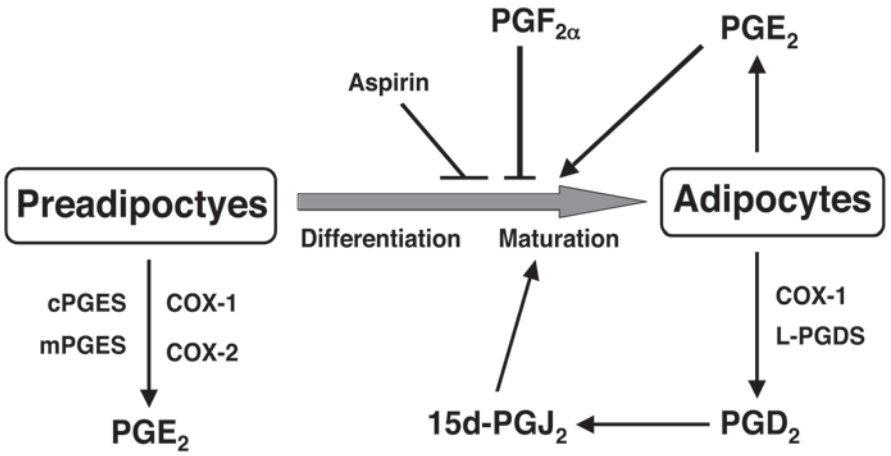

Fig. 11. Schematic representation of regulation and role of arachidonate cascade during changes in life cycle of 3T3-L1 adipocytes. Preadipocytes had a higher ability to form PGE_2 than the mature adipocytes in response to PMA and A23187. The formation of PGE_2 would involve the actions of both types of isoforms corresponding to COX and PGES, respectively. Mature adipocytes produced PGD_2 through the selective action of constitutive COX-1 and L-PGDS. Moreover, PGD_2 and its conversion product, 15d-PGJ_2, were efficacious in stimulating the accumulation of triacylglycerols during the maturation phase in the presence of aspirin. PGE_2 was also effective in promoting the storage of lipids. In sharp contrast, exogenous $PGF_{2\alpha}$ markedly attenuated the increase in lipid droplets. Thus, preadipocytes and mature adipocytes are able to produce distinct types and levels of prostanoids that regulate their own life cycle of 3T3-L1 cells in an autocrine-control manner.

Following hormone induction in the differentiation medium, we confirmed that confluent preadipocytes undergo mitogenic clonal expansion. As shown in Fig. 1A, the cell number increased by exactly twofold after 24 h of stimulation with the maturation medium following the differentiation phase. Then, the cell growth became arrested during the maturation phase while the accumulation of lipids proceeded as shown in Fig. 1B,C, suggesting the coordinate gene expression for adipogenesis. Consistent with the progression of the maturation of adipocytes, our result revealed that the nuclear hormone receptor PPARγ serving as a ligand-dependent transcription factor was not already present in the preadipocytes. Instead, the gene expression was strongly induced on the maturation process leading to terminal differentiation, and the expression was constantly maintained until 9 d (Fig. 3). This same expression pattern was also described previously *(9)*.

The critical role of PPARγ in triggering the adipocyte differentiation has been recognized by the finding that the ectopic expression of PPARγ in NIH-3T3 fibroblasts promoted adipocyte differentiation together with PPAR agonists *(38)*. The continued expression of PPARγ suggested the involvement of this nuclear receptor in the regulation of the biologic events in the mature adipocytes. Two isoforms of PPARγ are known, PPARγ1 and

PPARγ2, the latter being the predominant form in adipocytes with an extended amino terminus *(9)*. We used the common primers for detection of the mRNA level of PPARγ by RT-PCR, so we did not distinguish both isoforms. However, considering the major form in adipose tissues, the PPARγ2 form should have been detected.

As synthetic PPARγ agonists, thiazolidinediones are well known to be high-affinity ligands for this nuclear receptor. Recent extensive studies have revealed that 15-PGJ_2, a prostanoid derived from PGD_2, is the most potent natural ligand for the activation of PPARγ. Therefore, much attention has been paid to the action of this compound as a natural PPARγ ligand. Moreover, 15-PGJ_2 was also found to be the most active to promote adipogenesis of murine C3H10T1/2 fibroblasts *(11)* and NIH-3T3 cells expressing PPARγ *(10)* among the related prostanoids. However, as pointed out earlier, it remains unclear whether 15-PGJ_2 can be generated in cells expressing PPARγ or whether this compound was synthesized in other cells and transported to the target cells. In addition, it has not been determined whether this prostanoid is working in vivo, indicating no direct evidence for this prostanoid as the endogenous ligand in adipocytes.

Therefore, we examined the gene expression of biosynthetic enzymes responsible for the arachidonate COX pathway with special reference to the gene expression of isoforms of COX and PG synthases (Figs. 3–7). Our results provided several lines of evidence for the specific expression of isoforms of the COX pathway. From the data, we can infer the possible functional coupling between the COX isoforms and those of PG synthases. We found that COX-1 and isoforms of PGES were expressed constitutively during the life cycle from the growing preadipocytes to the mature adipocytes. On the other hand, the COX-2 isoform increased in a biphasic manner several hours after triggering of the differentiation and maturation phases. These transient increases are typical characteristics of the inducible COX-2 *(3)*. In our studies, the transcript level of COX-2 at the maturation phase was lower than at the differentiation phase (Fig. 4).

The gene expression of cPGES and mPGES (more recently referred to as MPGES-1) was similarly stimulated with the culture with maturation medium, with the slightly highest levels at about 3 h after commitment to the maturation phase. However, the expression of both PGESs appears to be totally constitutive in a whole cycle. These observations implied that the functional link should occur between COX and PGES isoforms to produce PGE_2 in preadipocytes and adipocytes (Figs. 3, 5, and 11). We determined the production of PGE_2 by the different types of life cycle of 3T3-L1 cells using immunoassay (Fig. 9). The present study clearly revealed that the mature adipocytes had a lower ability to form PGE_2 for 24 h in response to tumor promoter PMA and calcium ionophore A23187 compared with the growth phase of preadipocytes. Our determination was done to monitor the delayed synthesis of the formation of prostanoids. This delayed synthesis of PGs should accompany the gene expression of COX-2 and other PG synthases. Earlier, Hyman et al. *(41)* reported the decline in the acute for-

mation of PGs including PGE_2 as main products for 5 min in response to the addition of A231287. Taken together, these findings show that the rapidly growing preadipocytes are likely to have higher activity to synthesize the prostanoids than terminally differentiated adipocytes in the case of both the acute and delayed phases of the formation of PGs.

The detailed mechanism of the decline in the formation of PGs in adipocytes has yet to be determined in relation to the expression of subtypes of phospholipase A_2 (PLA_2) responsible for the release of arachidonic acid. As a preliminary study, we found the gene expression of cytosolic PLA_2 but failed to detect any secretory PLA_2 during the life cycle of 3T3-L1 cells (data not shown).

We also determined the gene expression of the isoforms of PGDS that contributes to the formation of PGD_2. This prostanoid has been shown to be spontaneously converted into PGJ_2, Δ^{12}-PGJ_2, and 15d-PGJ_2 in the presence of serum or albumin *(4)*. According to a more recent study, PGD_2 can be converted into 15d-PGJ_2 via an albumin-independent mechanism *(5)*. Our analyses with RT-PCR and quantitative real-time PCR revealed the exclusive gene expression of L-PGDS without the expression of H-PGDS, another isoform. Interestingly, the gene expression of L-PGDS increased gradually concomitantly with the progression of terminal differentiation at the maturation phase (Figs. 6 and 7). These findings indicate the endogenous synthesis of PGD_2 during the maturation process. Since PGD_2 is chemically unstable, optimal sampling time and conditions for the measurement of PGD_2 would be required for reliable determination of the level of PGD_2. In addition, the immunoassay of PGD_2 needs to be done using its methoxime derivative as described previously *(42)*. As shown in Fig. 8, we demonstrated that the mature adipocytes maintained the ability to form PGD_2. We are also in the process of developing an immunoassay for 15d-PGJ_2. The formation of 15-PGJ_2 was confirmed when the authentic PGD_2 was incubated with the maturation medium under standard culture conditions. Further study is required for the accurate determination of these prostanoids formed endogenously.

Nevertheless, the ability of the mature adipocytes to form PGD_2 was found to be evident because COX-1 was constitutively expressed and can be coupled with L-PGDS. The predominant expression of L-PGDS in the CNS is well established *(35)*. The present study provides evidence for the specific expression of L-PGDS, which would contribute to the basal synthesis of PGD_2 and its PGJ_2 derivatives. Furthermore, there will be the potential under special conditions to large amounts of PGD_2 in response to metabolic factors.

To investigate the role of endogenous prostanoids in adipocyte differentiation, we treated with specific inhibitors of the COX enzymes. As shown in Fig. 10, aspirin, a well-known nonsteroidal antiinflammatory drug and a specific inhibitor for both COX inhibitors, caused the efficacious inhibition of adipocyte differentiation, as determined by assay of the content of triacylglycerols. This observation suggested that the prostanoids endog-

enously produced by the mature adipocytes are helpful for the progression of adipogenesis. In addition to the activity to inhibit COX enzymes, higher concentrations of some COX inhibitors such as indomethacin and ibuprofen have been reported to activate the nuclear receptor PPARγ *(41)*. However, aspirin has been shown to be much less active for the activation of PPARγ compared with those inhibitors.

Our data also provided evidence showing the stimulation of adipocyte differentiation when cells after the differentiation phase were exposed to exogenous PGD_2, 15d-PGJ_2, and PGE_2 during the maturation phase. These findings are supportive of the role of endogenous prostanoids synthesized by mature adipocytes in the stimulation of maturation. As already stated, the present study revealed the gradual increase in gene expression of L-PDS and the continued expression of both isoforms of PGES. Therefore, the formation of PGD_2 or PGE_2 would require the functional cooperation of constitutive COX-1 and its corresponding PG synthases even though inducible COX-2 was responsible for the early stage after triggering the maturation phase. In particular, our data implicate a role of endogenous PGD_2 and related PGJ_2 derivatives in helping adipogenesis by a mechanism of autocrine control. A previous study showed that PGE_2 interacted with G-protein-linked EP3 receptors and caused the inhibition of lipolysis in rat isolated adipocytes *(42)*. This action of PGE_2 would account for the increased accumulation of triacylglycerols. In sharp contrast, treatment of the cells after differentiation with $PGF_{2\alpha}$ after 6 d of culture with maturation medium resulted in significant inhibition of adipocyte differentiation. In agreement with our results, the antiadipogenic effect of this prostanoid was also previously reported elsewhere *(43–45)*.

In summary, we demonstrated that 3T3-L1 cells were able to exhibit the specific gene expression of isoforms for the arachidonate COX pathway and allowed the shift of the COX metabolites, depending on the different stages of the life cycle, from preadipocytes to mature adipocytes. The exclusive gene expression of L-PGDS was remarkable at the late maturation phase. Our study indicated that both preadipocytes and mature adipocytes synthesize distinct prostanoids to partially regulate their own cellular function in an autocrine manner.

Acknowledgment

This work was supported by the Program for Promotion of Basic Research Activities for Innovative Biosciences.

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