Endogenous synthesis of prostacyclin was positively regulated during the maturation phase of cultured adipocytes

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Received: 25 May 2013 / Accepted: 6 July 2013 / Published online: 25 July 2013
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Abstract Prostacyclin alternatively called prostaglandin (PG) I_2 is an unstable metabolite synthesized by the arachidonate cyclooxygenase pathway. Earlier studies have suggested that prostacyclin analogues can act as a potent effector of adipose differentiation. However, biosynthesis of PGI_2 has not been determined comprehensively at different life stages of adipocytes. PGI_2 is rapidly hydrolyzed to the stable product, 6-keto- $PGF_{1\alpha}$, in biological fluids. Therefore, the generation of PGI_2 can be quantified as the amount of 6-keto- $PGF_{1\alpha}$. In this study, we attempted to develop a solid-phase enzyme-linked immunosorbent assay (ELISA) using a mouse antiserum specific for 6-keto- $PGF_{1\alpha}$. According to the typical calibration curve of our ELISA, 6-keto- $PGF_{1\alpha}$ can be quantified

from 0.8 pg to 7.7 ng in an assay. The evaluation of our ELISA revealed the higher specificity of our antiserum without the cross-reaction with other related prostanoids while it exhibited only the cross-reaction of 1.5 % with $PGF_{2\alpha}$. The resulting ELISA was applied to the quantification of 6-keto- $PGF_{1\alpha}$ generated endogenously by cultured 3T3-L1 cells at different stages. The cultured cells showed the highest capability to generate 6-keto- $PGF_{1\alpha}$ during the maturation phase of 4–6 days, which was consistent with the coordinated changes in the gene expression of PGI synthase and the IP receptor for PGI_2 . Following these events, the accumulation of fats was continuously promoted up to 14 days. Thus, our immunological assay specific for 6-keto- $PGF_{1\alpha}$ is useful for monitoring the endogenous levels of the unstable parent PGI_2 at different life stages of adipogenesis and for further studies on the potential association with the up-regulation of adipogenesis in cultured adipocytes.

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Keywords Prostacyclin · Prostaglandin I_2 ·
6-Keto- $PGF_{1\alpha}$ · Enzyme-linked immunosorbent
assay · Adipocyte · 3T3-L1 cells · Adipogenesis

Abbreviations

PG	Prostaglandin
ELISA	Enzyme-linked immunosorbent assay
PPAR γ	Peroxisome proliferator-activated receptor γ
COX	Cyclooxygenase
PGIS	Prostaglandin I synthase

DME-HEPES	Dulbecco's modified Eagle medium with 25 mM HEPES
FBS	Fetal bovine serum
RT	Reverse transcriptase
PCR	Polymerase chain reaction
IBMX	3-Isobutyl-1-methylxanthine
GM	Growth medium
DM	Differentiation medium
MM	Maturation medium
AA	Arachidonic acid
TXB ₂	Thromboxane B ₂

Introduction

Adipose tissue serves as fat depot for the storage and mobilization of fats depending on the necessity of energy requirement. Excess accumulation of fats is well known to generate obesity characterized by the increases in the number and size of mature adipocytes due to the enhanced energy intake and the reduced energy expenditure. Therefore, the body mass is controlled by adipogenesis through the differentiation of adipocytes from undifferentiated preadipocytes in adipose tissue *in vivo*. As well, adipocytes function as an endocrine organ to secrete a variety of bioactive adipocytokines, such as leptin and adiponectin, and are involved in the suppression of food intake and the maintenance of insulin sensitivity, respectively (Antuna-Puente et al. 2008). By contrast, hypertrophic adipocytes in obese tissues have been shown to secrete other types of adipocytokines including monocyte chemoattractant protein-1, interleukin-6, and tumor necrosis factor- α , which are inflammatory factors to promote macrophage infiltration into adipose tissue with obesity and generate adipocyte inflammation and insulin resistance (Sethi and Hotamisligi 1999; Sartipy and Loskutoff 2003; Permana et al. 2006). For these functional changes and the control of adipogenesis, peroxisome proliferator-activated receptor γ (PPAR γ), a nuclear hormone receptor and ligand-activated transcription factor, functions as a master regulator (Tontonoz et al. 1994; Chawla et al. 1994). Since prostaglandins (PGs) of the J₂ series, a member of bioactive eicosanoids, have been shown to be the potent natural ligand for the activation of PPAR γ

(Forman et al. 1995; Kliewer et al. 1995), much attention has been paid to the role of PGs in the control of adipocyte function. Prostaglandins are generally regarded as local hormones or autacoids that exert their effects on the neighboring cells in an autocrine or paracrine manner.

Adipocytes and the precursor cells can synthesize different types of prostanoids with opposite effects on adipogenesis through the arachidonate cyclooxygenase (COX) pathway (Hopkins and Gorman 1981; Hyman et al. 1982; Lu et al. 2004; Xu et al. 2006). In addition, the biosynthesis of individual PG species is specifically regulated at different stages leading to adipocytes from undifferentiated preadipocytes. Mouse preadipogenic 3T3-L1 cells, an established cell line, have been employed as a useful model system for the study on adipogenesis (Green and Kehinde 1974, 1975). The cells can be induced to undergo spontaneous morphologic and biochemical differentiation into adipocytes in culture under the controlled culture conditions involving the growth, differentiation, and maturation phases. Prostaglandins of the J₂ series including 15-deoxy- $\Delta^{12,14}$ -PGJ₂ and Δ^{12} -PGJ₂ formed through non-enzymatic dehydration of PGD₂ serve as members of pro-adipogenic factors because of their capability of activating PPAR γ and the effectiveness of exogenous PGJ₂ derivatives to promote adipogenesis (Forman et al. 1995; Kliewer et al. 1995; Mazid et al. 2006; Hossain et al. 2011). We have recently shown that those PGs of the J₂ series can be synthesized endogenously during the maturation phase of cultured 3T3-L1 cells and contribute to up-regulation of adipogenesis through the activation of PPAR γ (Mazid et al. 2006; Hossain et al. 2011). Moreover, our studies have revealed that parent PGD₂ for PGJ₂ derivatives can rescue the storage of fats reduced in the presence of COX inhibitors after the maturation phase. On the other hand, another types of prostanoids exhibit anti-adipogenic effects through the cell-surface membrane receptors. For example, PGE₂ inhibited adipocyte differentiation of cultured 3T3-L1 cells through the mediation of the EP4 receptor, one of the PGE₂ receptor subtypes when PGE₂ was added to both the differentiation and maturation phases (Tsuboi et al. 2004). Alternatively, PGF_{2 α} has been reported to inhibit the differentiation of preadipocytes into adipocytes through the specific FP receptor, which transmits the cellular signal to phosphorylate PPAR γ by mitogen-activated protein kinase (Reginato et al. 1998).

Earlier studies have reported that carbaprostacyclin, a stable analogue of prostacyclin also called PGI₂, stimulates terminal differentiation of Ob1771 mouse pre-adipose cells in serum-free hormone-supplemented medium (Negrel et al. 1989; Catalioto et al. 1991). Furthermore, under the same conditions, they recognized the pro-adipogenic effect of arachidonic acid, which was blocked by COX inhibitors. According to the ability of those cells to generate 6-keto-PGF_{1 α} , a stable hydrolysis product of unstable parent PGI₂, and PGF_{2 α} , those prostanoids are considered as autocrine mediators in the process of adipose conversion. Nevertheless, the pro-adipogenic action of endogenous PGI₂ remains obscure due to the instability of PGI₂ and its uncertainty of effective concentration of the parent form. Biologically active PGI₂ is rapidly hydrolyzed to inactive 6-keto-PGF_{1 α} in most biological fluids (Kelton and Blajchman 1980). It should be noted that different cell types and alterations in the culture conditions of cultured adipocytes and precursor cells would give contradictory results on adipogenesis. For example, PGF_{2 α} serves as anti-adipogenic factor in cultured 3T3-L1 cells in contrast to the stimulatory effect on the adipocyte differentiation of cultured Ob1771 pre-adipose cells as described above. In addition, a previous study observed that the repeated addition of exogenous PGI₂ to cultured 3T3-L1 cells inhibited insulin- and indomethacin-mediated adipocyte differentiation (Hopkins and Gorman 1981). Earlier, cultured 3T3-L1 cells have been described to generate PGI₂ as an immediate response to calcium ionophore A23187 for 5 min or by the incubation with extracellular arachidonic acid (Hyman et al. 1982). However, until now the biosynthesis of PGI₂, which is usually quantified as the levels of stable 6-keto-PGF_{1 α} , has not been monitored comprehensively at different life stages of adipocytes through the growth, differentiation, and maturation phases.

In this study, we obtained an antiserum specific for 6-keto-PGF_{1 α} and used it for the development of a sensitive, convenient solid-phase enzyme-linked immunosorbent assay for the quantification of 6-keto-PGF_{1 α} . After the confirmation of the precision and accuracy of our ELISA, this immunological assay was applied to the determination of 6-keto-PGF_{1 α} reflecting the endogenous levels of PGI₂ synthesized by cultured 3T3-L1 cells at different life stages of adipogenesis. We here provided the evidence that cultured adipocytes at the maturation phase have the highest ability to generate endogenous PGI₂ as determined by 6-keto-PGF_{1 α} ,

which was accompanied by the coordinated gene expression of PGI synthase (PGIS) and the IP receptor for PGI₂ and then followed by the continuous promotion of adipogenesis.

Materials and methods

Materials

Dulbecco's modified Eagle medium with 25 mM HEPES (DMEM-HEPES), penicillin G potassium salt, streptomycin sulfate, dexamethasone, recombinant human insulin, and ExtrAvidin-peroxidase conjugate were supplied by Sigma (St. Louis, MO, USA). Biotin-conjugated rabbit anti-mouse IgG antibody was purchased from Jackson Immuno Research Laboratories (West Grove, PA, USA). Fetal bovine serum (FBS) was obtained from MP Biomedicals (Solon, OH, USA). Authentic PGs were purchased from Cayman Chemical (Ann Arbor, MI, USA). M-MLV reverse transcriptase (RT) (Ribonuclease H minus, point mutant) and polymerase chain reaction (PCR) Master Mix were supplied by Promega (Madison, WI, USA). 3-Isobutyl-1-methylxanthine (IBMX), and Triglyceride E-Test Kit were purchased from Wako (Osaka, Japan). Oligonucleotides used for the PCR reaction were provided by Sigma Genosys Japan (Ishikari, Japan). 96-Well microplates for ELISA were purchased from BD Falcon (Durham, NC, USA), and other Petri dishes and multiwell plates with the Iwaki brand for tissue culture were from Asahi Glass (Tokyo, Japan). All other chemicals used are of reagent or tissue culture grade. Other materials and apparatus used for the experiments of cell culture, ELISA and gene expression were obtained as described previously (Xu et al. 2006; Mazid et al. 2006; Hossain et al. 2011).

Cell culture of 3T3-L1 cells and adipocyte differentiation

Mouse preadipogenic 3T3-L1 cells (JCRB9014) were plated at 5×10^4 cells/ml in growth medium (GM) containing DMEM-HEPES, 10 % FBS, 100 U/ml penicillin G, 100 μ g/ml streptomycin sulfate, and 200 μ M ascorbic acid, and then cultured at 37 °C under 7 % CO₂. After growth until confluence, the monolayer cells were exposed to differentiation medium (DM) composed of

GM supplemented with 1 μ M dexamethasone, 0.5 mM IBMX, and 10 μ g/ml insulin for 45 h. Then, DM was replaced by maturation medium (MM) consisting of GM and 5 μ g/ml of insulin, and refed every 2 days with fresh MM as described earlier (Green and Kehinde 1974, 1975; Lu et al. 2004; Xu et al. 2006; Mazid et al. 2006). At the indicated life stage, the resulting culture medium was collected and used for the determination of 6-keto-PGF_{1 α} by ELISA, whereas the cells were harvested for determination of triacylglycerol and cellular proteins as well as the staining with Oil Red O as described below.

Solid-phase ELISA for 6-keto PGF_{1 α}

A conjugate of 6-keto-PGF_{1 α} and bovine serum albumin was formed chemically and employed for the immunization of female BALB/c mice to prepare antisera specific for 6-keto-PGF_{1 α} according to our previous methods regarding the generation of antibodies for other prostanoids (Shono et al. 1988; Yokota et al. 1996; Syeda et al. 2012). For conducting solid-phase ELISA for 6-keto-PGF_{1 α} , another conjugate of 6-keto-PGF_{1 α} and bovine γ -globulin was prepared and used as an immobilized antigen attached on the surface of the bottom in a 96-well ELISA microplate as described earlier for other PGs (Yokota et al. 1996; Syeda et al. 2012). The resulting immobilized antigen was then allowed to react competitively with 50 μ l of a 2×10^5 -fold diluted solution of a mouse antiserum specific for 6-keto-PGF_{1 α} and 50 μ l of a solution containing various amounts of standard 6-keto-PGF_{1 α} or samples to be tested. The immunocomplex was furthermore incubated with biotin-conjugated rabbit anti-mouse IgG antibody followed by ExtrAvidin-peroxidase conjugate after washing at each step. The newly formed immunocomplex was finally detected spectrophotometrically by the measuring peroxidase activity using *o*-phenylenediamine as a substrate as detailed previously (Shono et al. 1988; Yokota et al. 1996; Syeda et al. 2012).

For validation of the precision in our developed ELISA for 6-keto-PGF_{1 α} , the intraassay coefficients of variation were determined in ten replicate 96-wells for each assay of samples containing 0, 0.01, 0.1, 1, and 10 ng of 6-keto-PGF_{1 α} . Additionally, the interassay coefficients of variation were assessed for five different times over 3 weeks using five replicate 96-wells containing the same amounts of 6-keto-PGF_{1 α} as described above.

Application of ELISA to the determination of endogenous synthesis of 6-keto-PGF_{1 α}

To determine 6-keto-PGF_{1 α} in different culture media used for the growth, differentiation and maturation phases from undifferentiated preadipocytes to mature adipocytes, the calibration curve was generated separately using each of GM, DM, and MM. Namely, authentic 6-keto-PGF_{1 α} was serially diluted using an equal volume of each fresh culture medium and the ELISA buffer containing phosphate-buffered saline without Ca²⁺ and Mg²⁺ ions, 0.5 % bovine serum albumin and 0.02 % NaN₃. For the analysis of samples, the corresponding culture medium was collected at the indicated time of cell cultures and diluted twofold with the ELISA buffer followed by further dilution by fourfold and eightfold with an equal volume of the respective fresh culture medium and the ELISA buffer. The resulting diluted samples were placed individually in 96-well ELISA microplates at three different dilutions in duplicate and processed for the quantification of 6-keto-PGF_{1 α} by the specific ELISA. To obtain the final concentration of 6-keto-PGF_{1 α} in the corresponding culture medium for an unknown sample, the values read within the range of 10–90 % of the binding from the calibration curve were used for calculation of the average value of each sample after the multiplication by dilution folds.

For the evaluation of the accuracy of our ELISA in the quantification of 6-keto-PGF_{1 α} in the respective culture medium, each of fresh GM, DM, and MM was fortified with known amounts of authentic 6-keto-PGF_{1 α} , and then subjected to ELISA for 6-keto-PGF_{1 α} after serial dilutions (twofold, fourfold, and eightfold) to give an equal-volume mixture of the same culture medium and the ELISA buffer as described above. The resulting samples were subjected to the quantification of 6-keto-PGF_{1 α} by ELISA and the subsequent regression analysis between added values and measured ones.

Gene expression analysis

Total RNA was extracted at different stages of adipocytes using a mixture of acid guanidium thiocyanate, phenol and chloroform (Xu et al. 2006; Mazid et al. 2006; Hossain et al. 2011). For the analysis of gene expression of a target gene, total RNA (1 μ g) was subjected to reverse transcription and amplification of

each desired DNA fragment by RT-PCR with M-MLV reverse transcriptase (Ribonuclease H minus, point mutant) and $1\times$ PCR Master Mix as reported previously (Xu et al. 2006; Chu et al. 2009; Hossain et al. 2011). In order to perform the RT reaction for producing single stranded cDNA, oligo-(dT)₁₅ and a random 9 mer (Promega) were used as primers. The cDNA fragments for the target genes were amplified by PCR in a semi-quantitative manner. Oligonucleotides used for detecting mRNA levels of PGIS were 5'-ATG AAGCCGACGCTCATGCAC-3' as 5'-primer and 5'-GAAACAGGCTGCTCTGCTTG-C-3' as 3'-primer. The transcription levels of IP receptor were detected using 5'-TGAGCCCTGCAGTGTGTTGTGG-3' as 5'-primer and 5'-GAAGCCTCGGATCATGAGAGG-3' as 3'-primer. The amplified DNA fragments were separated by electrophoresis on a 1.5 % agarose gel and stained with ethidium bromide. For confirming the target gene, the DNA sequences of the PCR products were determined using ABI Prism 3100 Genetic Analyzer following the reaction with BigDye Terminator v.1.1 Cycle Sequence Kit (Applied Biosystems, Foster City, CA, USA) as reported earlier (Xu et al. 2006; Chu et al. 2009).

Other procedures

For quantification of the amounts of triacylglycerols in cultured adipocytes, the homogenates from the cells harvested at different stages were subjected to the assay of triacylglycerols using Triglyceride E-Test Kit as reported earlier (Xu et al. 2006; Mazid et al. 2006; Chu et al. 2009). Cellular proteins were determined after precipitating with cold trichloroacetic acid to remove interfering substance according to the method reported previously (Lu et al. 2004; Xu et al. 2006; Chu et al. 2009). The accumulated lipid droplets were observed as macroscopic and microscopic views by staining cultured cells with Oil Red O as described previously (Lu et al. 2004; Xu et al. 2006; Mazid et al. 2006; Hossain et al. 2011).

Results

Development of a solid-phase ELISA specific for 6-keto-PGF_{1 α}

PGI₂ is known to undergo rapid conversion to 6-keto-PGF_{1 α} as the stable hydrolysis product in neutral

Fig. 1 Calibration curve of solid-phase ELISA for 6-keto-PGF_{1 α} . Binding percentage of the immobilized antigen is plotted against increasing amounts of authentic 6-keto-PGF_{1 α}

solutions (Moncada et al. 1976; Stehle 1982). Therefore, to monitor specifically the endogenous synthesis of PGI₂ in cultured cells, the target for the measurement should be 6-keto-PGF_{1 α} . Here, we attempted to develop a sensitive solid-phase ELISA for 6-keto-PGF_{1 α} using the immobilized antigen in 96-well microplates. Among several mice used for the immunization with the BSA conjugate of 6-keto-PGF_{1 α} , a mouse polyclonal antiserum highly reactive with the immobilized antigen was obtained and used for the development of the ELISA for 6-keto-PGF_{1 α} . After extensive optimization of the conditions of the ELISA procedures, a standard calibration curve was established allowing us to determine the amounts of 6-keto-PGF_{1 α} from 0.8 pg to 7.7 ng in an assay corresponding to 90 and 10 % of the maximal binding of the immobilized antigen, respectively (Fig. 1). This assay also gave a standard value of 38 pg at the 50 % displacement in the typical calibration curve.

To validate the precision of our solid-phase ELISA for 6-keto-PGF_{1 α} , we determined the intraassay and interassay coefficients of variation under the established standard assay conditions. The analysis of the intraassay coefficients of variation provided values ranging from 2.2 to 8.6 % with five groups of ten replicate assays for each group in the range of 0–10 ng/well of authentic 6-keto-PGF_{1 α} . In addition, the interassay coefficients of variations were evaluated by repeating the measurements five times using five

Fig. 2 Chemical structures of prostanoids and arachidonic acid used for determining the cross-reaction with them of mouse antiserum raised for 6-keto-PGF_{1α}

groups of five replicate assays for each group containing the range of 0–10 ng/well, giving values ranging from 2.2 to 7.2 %. The specificity of mouse polyclonal antibody used for the current ELISA was examined by the immunoreaction with increasing amounts of other prostanoids and free arachidonic acid (Figs. 2, 3). Our ELISA for 6-keto-PGF_{1α} exhibited almost no cross-reaction, with values of less than 0.1 % with other prostanoids except PGF_{2α}, with a cross-reaction of 1.5 % (Table 1). The findings indicated that our antiserum used for the development of our solid-phase ELISA was specific for 6-keto-PGF_{1α} and useful for the quantification of the target molecule.

Endogenous synthesis of 6-keto-PGF_{1α} and fat storage at different life stages of adipocytes

To monitor the endogenous synthesis of 6-keto-PGF_{1α} reflecting the biosynthesis of PGI₂ at different life stages of adipocytes—the growth, differentiation, and maturation phases, our solid-phase ELISA specific for 6-keto-PGF_{1α} was applied to the quantification of 6-keto-PGF_{1α} in the corresponding culture medium of each phase. Initially, we evaluated the accuracy for the determination of 6-keto-PGF_{1α} by our solid-phase ELISA by fortifying with increasing concentrations of authentic 6-keto-PGF_{1α} in the fresh culture medium using either GM, DM, or MM. The resulting

immunological assays of all of the different culture media showed a satisfactory, linear proportionality between added amounts and read values. Accordingly, the least-regression analysis of the MM supplemented known amounts of 6-keto-PGF_{1α} in a range of 0–200 pg/ml revealed the relationship with a calculated recovery of 103 % ($y = 1.03x - 0.0965$; Fig. 4). The resulting methods were employed for the determination of endogenous synthesis of 6-keto-PGF_{1α} at different life stages of cultured 3T3-L1 cells from undifferentiated preadipocytes to the adipocytes after 14 days of the maturation phase (Fig. 5). The ability of cultured 3T3-L1 cells to generate endogenous 6-keto-PGF_{1α} was found to be much higher during the maturation phase of adipocytes than other growth and differentiation phases. The highest levels were observed in the MM harvested during the periods of 4–6 days after the maturation phase of adipocytes.

When we monitored the progression of adipogenesis at different life stages of adipocytes under the same culture conditions as described above, the storage of fats was found to increase gradually up to the 14th day of the maturation phase as revealed by the increased amounts of triacylglycerol (Fig. 6a) and by the marked staining of lipid droplets with Oil Red O at microscopic and macroscopic views (Fig. 6b). The observation raised the possibility that the endogenous synthesis of parent PGI₂ as determined by the levels of

Fig. 3 Cross-reaction of mouse antiserum for 6-keto-PGF_{1α} with other prostanooids and arachidonic acid. A fixed amount of immobilized antigen in each 96-well ELISA microplate was incubated with a 2×10^5 -fold diluted solution of a mouse antiserum for 6-keto-PGF_{1α} in a total volume of 100 μ l together with each of increasing amounts of each authentic prostanooid and free arachidonic acid under the established condition. The values of the binding percentage were subjected to the logit transformation in the ordinate to give the linearized straight lines. All data are plotted in duplicates. AA arachidonic acid, TXB₂ thromboxane B₂

Table 1 Cross-reaction of mouse antiserum for 6-keto-PGF_{1α} with other prostanooids and arachidonic acid

Compound	Cross-reaction (%) ^a
6-Keto-PGF _{1α}	100
PGF _{2α}	1.5
PGE ₂	0.1
PGA ₂	<0.1
PGB ₂	<0.1
PGD ₂	<0.1
11-β-PGF _{2α}	<0.1
8-iso-PGF _{2α}	<0.1
Arachidonic acid	<0.1
Thromboxane B ₂	<0.1

^a ELISA was performed under the standard conditions for 6-keto-PGF_{1α} in the presence of increasing amounts of prostanooids and free arachidonic acid. The amount of each compound to be tested for 50 % binding was compared with that of standard 6-keto-PGF_{1α}

6-keto-PGF_{1α} might contribute to the promotion of adipogenesis of adipocytes during the maturation phase since the production of 6-keto-PGF_{1α} preceded

Fig. 4 The accuracy for the quantification of 6-keto-PGF_{1α} in the maturation medium of cultured adipocytes. The fresh maturation medium to be used for cultured adipocytes was fortified with increasing amounts of authentic 6-keto-PGF_{1α} in a range of 0–200 pg/ml. The resulting samples were diluted and applied to the quantification of 6-keto-PGF_{1α} by ELISA

Fig. 5 Endogenous synthesis of 6-keto-PGF_{1α} by cultured adipocytes at different life stages of adipocytes. 3T3-L1 cells were plated at 5×10^4 cells/ml in a 35-mm Petri dish containing 2 ml of GM, and grown to confluence. The confluent cells were exposed to DM for the induction of the differentiation phase, and then matured to terminal differentiation up to the 14th day of the maturation phase by changing MM every 2 days. At the indicated time, the culture medium was collected and subjected to the quantification of 6-keto-PGF_{1α} by ELISA. Data represent the mean $\pm$ SEM of three independent experiments

the stimulated accumulation of fats in adipocytes, which was more evident after 6 days of the maturation phase.

Gene expression of PGIS and IP receptor during the maturation phase

Cultured 3T3 cells have been shown to express the arachidonate COX pathway involving two types of COX isoforms, constitutive COX-1 and inducible

Fig. 6 Accumulation of fats by cultured adipocytes during the maturation phase. 3T3-L1 cells were plated and grown to the confluence as described in Fig. 5. The confluent cells were exposed to DM for the induction of the differentiation phase, and then matured to terminal differentiation up to the 14th day of the maturation phase by changing MM every 2 days. At the indicated time, the cultured cells were harvested to determine the accumulation of triacylglycerols (a). Data represent the

mean $\pm$ SEM of three independent experiments. Alternatively, the unstained cultured cells were viewed by phase-contrast microscopy (*upper panels*), or the cultured cells after staining with Oil Red O were observed by differential-interference microscopy (*middle panels*) as well as macroscopic views (*lower panels*) (b). Data are shown from a representative one done in replicate experiments. Scale bar 50 μ m

COX-2, which are involved in the biosynthesis of different prostanoids with opposite effects on adipogenesis (Mazid et al. 2006; Xu et al. 2006). For the endogenous synthesis of PGI₂, the arachidonate COX pathway requires PGIS, the biosynthetic enzyme using PGH₂ as the substrate, which is the product of COX isoforms. In addition, PGI₂ exerts its biological activity through the specific PGI₂ receptor, the IP receptor (Narumiya et al. 1999; Wise 2003). Here, we investigated the gene expression profiles of PGIS and IP receptor during the maturation phase of adipocytes (Fig. 7). The analysis revealed that the transcript levels of both PGIS and IP receptor increased gradually and reached the highest levels after 6 days of the

maturation phase although the gene expression of them was detectable at earlier stages including growth and differentiation phases. However, their mRNA levels at the differentiation phase were found to be much lower than in other phases, presumably due to the presence of dexamethasone or IBMX in DM. These results suggest that the enhanced gene expression of PGIS was responsible for the stimulation of the PGI₂ synthesis as determined by the production of 6-keto-PGF_{1 α} during the maturation phase as described in Fig. 5. As well, a co-expression of IP receptor in a similar way could effectively contribute to the action of PGI₂ synthesized endogenously by adipocytes during the process of adipogenesis.

Fig. 7 Gene expression of PGIS and IP receptor at different life stages of adipocytes. 3T3-L1 cells were plated at 5×10^4 cells/ml in a 60-mm Petri dish containing 4 ml of GM, and grown to the confluence. The resulting cultured cells were differentiated and matured to terminal differentiation up to the 10th day of the maturation phase by changing MM every 2 days. At the

indicated time, total RNA was extracted from the cultured cells at different life stages of adipocytes and subjected to the analysis of mRNA levels of PGIS, IP receptors and β -actin (reference). Data are shown from a representative one done in replicate experiments

Discussion

A role of prostacyclin PGI_2 as a potent inhibitor of platelet aggregation and a powerful vasodilator is well established in the blood vascular system (Kelton and Blajchman 1980). In adipose tissue, earlier studies have described that carbaprostacyclin, a stable analogue of prostacyclin, promotes terminal differentiation of cultured mouse OB1771 preadipose cells and 3T3-F442A cells under serum-free culture conditions (Negrel et al. 1989; Catalioto et al. 1991). Nevertheless, until now the endogenous synthesis of PGI_2 has not been determined quantitatively at different life stages of adipocytes from the growth, differentiation, and maturation phases. PGI_2 is a well-known unstable product generated by the arachidonate COX pathway and converted rapidly to 6-keto- $\text{PGF}_{1\alpha}$ by spontaneous hydrolysis (Stehle 1982). Hence, to monitor the endogenous synthesis of PGI_2 , the target of the measurement should be a stable 6-keto- $\text{PGF}_{1\alpha}$ although this compound is biologically inactive. In order to apply the quantification of 6-keto- $\text{PGF}_{1\alpha}$ as a measure of the endogenous levels of PGI_2 in different culture media used for adipogenesis of cultured 3T3-L1 cells, we currently attempted to develop a sensitive solid-phase ELISA specific for 6-keto- $\text{PGF}_{1\alpha}$ in biological fluids. This immunological assay using the immobilized antigen is a convenient and useful method that can be used for the measurement of many samples at the same time with low-cost instruments. Our solid-phase ELISA was found to be sensitive

enough to quantify the amount of over 1 pg in an assay that corresponds to the concentration of 20 pg/ml in a biological fluid in our system. In this study, we also demonstrated that our immunological assay was specific for 6-keto- $\text{PGF}_{1\alpha}$ due to a very low cross-reaction of the antibody with other prostanoids and the related compounds, with values of less than 0.1 %. Thus, the sensitivity and specificity of our assay were almost comparable with other ELISAs for each of 15d- PGJ_2 and Δ^{12} - PGJ_2 using its specific antiserum as reported recently (Mazid et al. 2006; Hossain et al. 2011). As long as cultured 3T3-L1 cells are used for the generation of PGI_2 , the end product is more likely to be 6-keto- $\text{PGF}_{1\alpha}$ without further metabolism to other products, since there are no further oxidation systems in adipocytes and the related precursor cells, which is different from blood circulation system in vivo.

To monitor the endogenous production of 6-keto- $\text{PGF}_{1\alpha}$ derived from PGI_2 at different life stages of adipocytes, we employed three types of culture media including the GM, DM, and MM corresponding to the growth, differentiation, and maturation phases, respectively. Hence, it is crucial to demonstrate that non-specific interference can be excluded in individual assays with different culture media. We tried to verify the accuracy of our ELISA for the quantification of 6-keto- $\text{PGF}_{1\alpha}$ by generating the calibration curve using an ELISA buffer mixed equally with the desired culture medium. The resulting analysis provided evidence that the measured values of 6-keto- $\text{PGF}_{1\alpha}$

in the culture medium were linearly correlated with those fortified with increasing amounts of the authentic compound in the respective one. The findings indicated that we successfully confirmed the accuracy of our ELISA for the determination of the levels of 6-keto-PGF_{1 α} in the individual culture medium used for life stages leading to adipogenesis in cultured adipocytes. The combined results led us to know that non-specific interference was not detectable in our immunological assays for 6-keto-PGF_{1 α} due to the presence of various components in different culture media.

After confirming the validity of our developed solid-phase ELISA, we applied our developed solid-phase ELISA to the determination of endogenous synthesis of 6-keto-PGF_{1 α} at different life stages of cultured 3T3-L1 cells leading to adipocytes from undifferentiated preadipocytes. In this study, we showed that preadipocytes at the growth phase produced 6-keto-PGF_{1 α} reflecting the endogenous synthesis of PGI₂. However, when the cultured cells were induced to undergo the differentiation phase, the synthesis of 6-keto-PGF_{1 α} was suppressed substantially. This suppression is considered to be due to the presence of dexamethasone in DM, a well-known anti-inflammatory synthetic compound acting through the glucocorticoid receptor (Goppelt-Strube et al. 1989). Once DM was removed and cells were refed with MM every 2 days, adipocytes were found to recover the ability to synthesize 6-keto-PGF_{1 α} after the maturation phase of adipocytes, exhibiting the highest level during the periods of 4–6 days. Then, the capability of cultured adipocytes to generate 6-keto-PGF_{1 α} was gradually attenuated at later maturation phase while the stimulation of fat storage was more evident in later maturation phase. This up-regulation of the endogenous synthesis of 6-keto-PGF_{1 α} was consistent with our current data on the gradual increase in gene expression levels of PGIS responsible for the synthesis of PGI₂ from PGH₂ with the highest level after 6 days of the maturation phase. We have already reported the gene expression of biosynthetic enzymes in the arachidonate COX pathway necessary for the formation of PGH₂ from arachidonic acid in cultured 3T3-L1 adipocytes (Mazid et al. 2006). These include cytosolic phospholipase A₂ α for the release of free arachidonic acid from membrane phospholipids and COX isoforms for the oxygenation of free arachidonic acid to generate PGH₂, such as the constitutive COX-1 and the inducible COX-2. Thus, both COX isoforms

appear to be involved in the generation of PGI₂ during the maturation phase.

Previous studies have described the synthesis of PGI₂ by cultured 3T3-L1 cells as determined by the amount of 6-keto-PGF_{1 α} . For example, an earlier study has described that rapidly growing cultured 3T3-L1 preadipocytes can produce PGI₂ as an acute response to the stimulation with the calcium ionophore A23187 or incubation with exogenous arachidonic acid (Hyman et al. 1982). According to this study, the capability to form PGI₂ was lower in cultured adipocytes than preadipocytes. Alternatively, Xie et al. (2006) have documented the reduced levels of 6-keto-PGF_{1 α} in 3T3-L1 adipocytes compared with preadipocytes. However, the developmental stages of cultured cells and the culture conditions used were not specified in that study. In contrast, Fajas et al. (2003) have reported gradual increase in the generation of 6-keto-PGF_{1 α} after cultured 3T3-L1 cells had been induced to the differentiation phase for 2 days in the presence of IBMX, dexamethasone, and insulin. The reason for the discrepancy might be explained by differences in their culture conditions, sampling periods, or assay methods. Neither of the above studies determined the endogenous synthesis of 6-keto-PGF_{1 α} at different life stages continuously from undifferentiated preadipocytes to mature adipocytes. On the other hand, we found that the ability of cultured cells to generate 6-keto-PGF_{1 α} was reduced when the cells were shifted from the growth phase to the differentiation phase. Instead, the up-regulation of the endogenous synthesis of 6-keto-PGF_{1 α} was observed markedly during the maturation phase, which was accompanied by the enhanced gene expression of PGIS. As a separate cell culture system, Ob1771 pre-adipose cells have been used for the synthesis of prostanoids including PGI₂ although the changes in the capability of cells to form PGI₂ were not monitored at the different stages of adipogenesis (Catalioto et al. 1991). In this case, cultured cells were maintained in serum-free hormone-supplemented medium. Therefore, the culture conditions are largely different from our culture conditions of 3T3-L1 cells.

We found the highest capability of cultured adipocytes to endogenously synthesize 6-keto-PGF_{1 α} reflecting PGI₂ around the periods of 4–6 days during the maturation phase. Following this endogenous synthesis of PGI₂, the accumulation of fats occurred more increasingly at a later maturation phase. Moreover,

the gene expression of the cell surface IP receptor was closely linked with the generation of PGI₂ and the transcript levels of PGIS. Considering these findings, endogenously synthesized PGI₂ appears to potentially contribute to the positive regulation of adipogenesis in an autocrine manner. In support of this idea, we have recently reported that well-known COX inhibitors including aspirin and indomethacin attenuated fat storage during the maturation phase. However, we have recently shown that other pro-adipogenic prostanoids including J₂ series PGs were generated during the maturation phase (Mazid et al. 2006; Hossain et al. 2011). Hence, the up-regulation of adipogenesis is not explained solely by the action of PGI₂. Furthermore, since biologically active PGI₂ is an unstable compound in biological fluids, the effectiveness of intact PGI₂ remains yet to be determined during the maturation phase of adipocytes. Nonetheless, it is conceivable that the endogenous PGI₂ could partly be efficacious to up-regulate adipogenesis through its IP receptor. Just recently, we have noticed that specific IP agonists can partly rescue the inhibition of adipogenesis in the presence of indomethacin or aspirin. As well, specific antagonists for the IP receptor were able to attenuate fat storage during the maturation phase under our experimental conditions (data not shown). Although the cell line and the culture conditions are different from the ones used in the present study, earlier studies have reported that carbaprostacyclin, a stable analogue of prostacyclin, stimulated the terminal differentiation of Ob1771 pre-adipose cells in serum-free hormone-supplemented medium, indicating the pro-adipogenic action through the IP receptor (Négrel et al. 1989; Catalioto et al. 1991). In these studies, they did not show any results using a natural PGI₂ itself presumably due to the instability of this prostanoids. Interestingly, a recent study using a cell-based reporter gene assay in HEK-293 cells stably expressing the IP receptor has reported that PPAR γ , a master regulator of adipogenesis, is activated through the IP receptor via a cAMP-independent mechanism by stable prostacyclin analogues (Falcetti et al. 2007). Another study has described that fat mass gain is suppressed in IP-deficient mice compared with wild-type mice when mother mice were fed high-fat diet rich in linoleic acid, indicating the contribution of the signaling through the IP receptor to adipose tissue development (Massiera et al. 2003). Thus, the modulation of adipogenesis by PGI₂ analogues through the IP receptor is more likely to

occur in cultured cells or adipose tissues. In addition, the present study supports the potential idea that the increased synthesis of endogenous prostacyclin by adipocytes during the maturation phase can stimulate adipogenesis in an autocrine manner by interacting with its specific IP receptor, the expression of which is regulated coordinately with PGIS. The resulting signaling process would involve the indirect activation of PPAR γ , a master regulator of adipogenesis. However, much work remains to be done regarding the role of naturally occurring PGI₂ itself in adipogenesis in terms of an autocrine or paracrine control.

In conclusion, we have developed a sensitive solid-phase ELISA specific for 6-keto-PGF_{1 α} , a stable hydrolysis product of an unstable parent PGI₂ called also prostacyclin. Our ELISA was validated for the application to the quantification of 6-keto-PGF_{1 α} reflecting the biosynthesis of PGI₂ at different life stages of adipogenesis. We revealed that biosynthesis of PGI₂ was up-regulated at the highest levels around 4–6 days the maturation phase of adipocytes, which was accompanied by gene expression of PGIS and the IP receptor. Following these responses, the accumulation of fats occurred increasingly at later maturation phase. These findings provided the useful information on the generation of endogenous PGI₂ and raised the potential role of this prostanoid as one of the pro-adipogenic factors in an autocrine manner in cultured adipocytes. Thus, the biosynthesis of prostacyclin can be used as a biomarker of adipocyte differentiation.

Acknowledgments This work was supported by JSPS KAKENHI Grant Numbers 25450128 and 25-03091.

Conflict of interest The authors declare that there are no conflicts of interest.

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