Stimulation of fat storage by prostacyclin and selective agonists of prostanoid IP receptor during the maturation phase of cultured adipocytes

Ferdous Khan · Pinky Karim Syeda · Michael Nii N. Nartey ·
Mohammad Shahidur Rahman · Mohammad Safiqul Islam · Kohji Nishimura ·
Mitsuo Jisaka · Fumiaki Shono · Kazushige Yokota

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Abstract We have previously shown that cultured adipocytes have the ability to biosynthesize prostaglandin (PG) I_2 called alternatively as prostacyclin during the maturation phase by the positive regulation of gene expression of PGI synthase and the prostanoid IP receptor. To clarify how prostacyclin regulates adipogenesis, we investigated the effects of prostacyclin and the specific agonists or antagonists for the IP receptor on the storage of fats during the maturation phase of cultured adipocytes. Exogenous PGI₂ and the related selective agonists for the IP receptor including MRE-269 and treprostinil rescued the storage of fats attenuated by aspirin, a cyclooxygenase inhibitor. On the other hand, selective antagonists for IP such as

CAY10441 and CAY10449 were effective to suppress the accumulation of fats as GW9662, a specific antagonist for peroxisome proliferator-activated receptor (PPAR) γ . Thus, pro-adipogenic action of prostacyclin can be explained by the action mediated through the IP receptor expressed at the maturation stage of adipocytes. Cultured adipocytes incubated with each of PGI₂ and MRE-269 together with troglitazone, an activator for PPAR γ , exhibited additively higher stimulation of fats storage than with either compound alone. The combined effect of MRE-269 and troglitazone was almost abolished by co-incubation with GW9662, but not with CAY10441. Increasing concentrations of troglitazone were found to reverse the inhibitory effect of CAY10441 in a dose-dependent manner while those of MRE-269 failed to rescue adipogenesis suppressed by GW9662, indicating the critical role of the PPAR γ activation as a downstream factor for the stimulated adipogenesis through the IP receptor. Treatment of cultured adipocytes with cell permeable stable cAMP analogues or forskolin as a cAMP elevating agent partly restored the inhibitory effect of aspirin. However, excess levels of cAMP stimulated by forskolin attenuated adipogenesis. Supplementation with H-89, a cell permeable inhibitor for protein kinase A (PKA), had no effect on the promoting action of PGI₂ or MRE-269 along with aspirin on the storage of fats, suggesting that the promotion of adipogenesis mediated by the IP receptor does not require the PKA activity.

F. Khan · P. K. Syeda · M. N. N. Nartey ·
M. S. Rahman · M. S. Islam · M. Jisaka · K. Yokota (✉)
Department of Life Science and Biotechnology, Shimane
University, 1060 Nishikawatsu-cho, Matsue,
Shimane 690-8504, Japan
e-mail: yokotaka@life.shimane-u.ac.jp

K. Nishimura
Department of Molecular and Functional Genomics,
Center for Integrated Research in Science, Shimane
University, 1060 Nishikawatsu-cho, Matsue,
Shimane 690-8504, Japan

F. Shono
Department of Clinical Pharmacy, Faculty of
Pharmaceutical Sciences, Tokushima Bunri University,
180 Yamashiro-cho, Tokushima-shi,
Tokushima 7700-8514, Japan

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Abbreviations

PG	Prostaglandin
PPAR	Peroxisome proliferator-activated receptor
PKA	Protein kinase A
C/EBP	CCAAT enhancer-binding protein
15d-PGJ ₂	15-Deoxy- $\Delta^{12,14}$ -PGJ ₂
IBMX	3-Isobutyl-1-methylxanthine
COX	Cyclooxygenase
DMEM-	Dulbecco's modified Eagle
HEPES	medium with 25 mM HEPES
FBS	Fetal bovine serum
8-CPT-cAMP	8-(4-Chlorophenyl)thio-cAMP
8-CPT-2'-O-Me-cAMP	8-(4-Chlorophenyl)thio-2'-O-methyl-cAMP
GM	Growth medium
DM	Differentiation medium
MM	Maturation medium
PBS (–)	Phosphate-buffered saline without Ca ²⁺ and Mg ²⁺

Introduction

Adipocytes are involved in the control of energy homeostasis by regulating the storage of fats and mobilization of free fatty acids in adipose tissue depending on the nutritional and hormonal conditions. As well, adipocytes serve as an endocrine organ to secrete a variety of bioactive molecules called adipocytokines (Galic et al. 2010). Adipogenesis requires the differentiation of preadipocytes into mature adipocytes by transcriptional programs to regulate specific expression of adipogenic genes. Recent progress has revealed that different nuclear factors play a pivotal role in triggering the adipogenic process. The most active studies have been conducted on the families of transcription factors, including the CCAAT enhancer-binding proteins (C/EBPs) and peroxisome proliferator-activated receptors (PPARs) (Gregoire et al. 1998). Of these, PPAR γ , a member of the nuclear hormone superfamily, is a predominant form of adipocytes and is considered as a master regulator of adipogenesis (Chawla et al. 1994). Since PPAR γ is a ligand-activated transcription factor,

the activation of this factor should require the active ligands. Various lipophilic molecules such as certain polyunsaturated fatty acids and their metabolites like eicosanoids can be effective to activate PPAR γ as natural ligands. Since some of J₂ series of prostaglandins (PGs) have been shown to be a potent activator of PPAR γ (Forman et al. 1995; Kliewer et al. 1995), much attention has been paid to the role of PGs in the differentiation and maturation processes of adipocytes. Indeed, NIH 3T3-L1 cells ectopically expressing PPAR γ can be converted to adipocytes by PPAR activators including 15-deoxy- $\Delta^{12,14}$ -PGJ₂ (15d-PGJ₂) (Forman et al. 1995). However, several types of PGs with opposite effects on adipogenesis are produced at different stages of adipocytes.

Several preadipogenic cell lines have been utilized for understanding many different processes involved in the regulation of adipogenesis. The most notable one is mouse preadipogenic cell line, 3T3-L1 cells (Green and Kehinde 1974, 1975), which can be induced to differentiate into mature adipocytes under the controlled culture conditions involving the growth, differentiation, and maturation phases. For the initiation of the differentiation program of this cell line, the growth-arrested cells were generally stimulated with a hormone mixture of insulin, dexamethasone, and 3-isobutyl-1-methylxanthine (IBMX) in the differentiation medium, which leads to the programmed expression of C/EBPs and PPAR γ . Following the induction of differentiation program, mature adipocytes with accumulated fats are generated by the promotion of adipogenesis during the maturation phase (Ham et al. 2001; Petersen et al. 2003). We have been making use of cultured 3T3-L1 cells for studying the regulation of the arachidonate cyclooxygenase (COX) pathway at different life stages of adipocytes (Xu et al. 2006; Mazid et al. 2006; Rahman et al. 2014). For example, PGE₂ and PGF_{2 α} are known as anti-adipogenic prostanoids in most preadipogenic cells. A previous study has reported that PGE₂ added to both the differentiation and maturation media suppresses the adipocyte differentiation of 3T3-L1 cells through the prostanoid EP4 receptor coupled with the elevation of cAMP (Tsuboi et al. 2004). We have shown that the preadipocytes during the growth phase have the highest ability to stimulate the biosynthesis of PGE₂ and PGF_{2 α} in response to cell stimuli (Xu et al. 2006). On the other hand, our previous studies have reported the up-regulation of the endogenous synthesis of pro-adipogenic prostanoids, such as PGJ₂ derivatives including 15d-PGJ₂ (Mazid et al. 2006) and Δ^{12} -PGJ₂ (Hossain

et al. 2011), by the non-enzymatic dehydration of PGD₂ in cultured 3T3-L1 cells during the maturation phase. We also confirmed that each of 15d-PGJ₂ and Δ^{12} -PGJ₂ added to the maturation medium was able to rescue the inhibitory effect of COX inhibitors on adipogenesis (Mazid et al. 2006; Hossain et al. 2011). These findings indicate the autocrine role for those PGJ₂ derivatives in the promotion of adipogenesis during the maturation phase.

Earlier studies described that carbaprostacyclin, a stable analogue of PGI₂ also called prostacyclin, exerted pro-adipogenic effects on Ob1771 mouse-pre-adipose cells (Négrel et al. 1989; Catalioto et al. 1991). Alternatively, growing 3T3-L1 cells have been reported to produce PGI₂ upon acute stimulation with calcium ionophore A23187 (Hyman et al. 1982). More recently we have provided the evidence that cultured 3T3-L1 adipocytes during the maturation phase are more capable of generating endogenous PGI₂ as determined by the amount of 6-keto-PGF_{1 α} as the stable hydrolysis product of PGI₂ by its specific immunological assay, which is accompanied by the coordinated gene expression of PGI synthase and the prostanoid IP receptor for PGI₂ (Rahman et al. 2014). These findings led us to suggest the pro-adipogenic action of endogenous PGI₂ as an autocrine factor in mature adipocytes. However, the action of natural PGI₂ appears to be uncertain due to the short half-life in biological fluids. By the extension of our recent results, we aim to obtain more insight into the role for prostacyclin and the related compounds in the up-regulation of adipogenesis in cultured adipocytes during the maturation phase. In this study we attempt to determine the specific action of the parent prostacyclin and the related agonists or antagonists for the specific IP receptor on adipogenesis in combination with the agents that influence the activation of PPAR γ and the elevation of cAMP. We discuss the mode of how prostacyclin affects adipogenesis through the IP receptor during the maturation phase of adipocytes.

Materials and methods

Materials

Dulbecco's modified Eagle medium with 25 mM HEPES (DMEM-HEPES), penicillin G potassium salt, streptomycin sulfate, dexamethasone, and recombinant human insulin were supplied by Sigma (St.

Louis, MO, USA). L-Ascorbic acid phosphate magnesium salt n-hydrate, 3-isobutyl-1-methylxanthine (IBMX), and Triglyceride E-Test Kit were provided by Wako (Osaka, Japan). Fetal bovine serum (FBS) was purchased from MP Biomedicals (Solon, OH, USA). PGI₂ (sodium salt), indomethacin, aspirin, troglitazone, GW9662, carbaprostacyclin, MRE-269, treprostinil, CAY10441, CAY10449, forskolin, 8-bromo-cAMP, 8-(4-chlorophenyl)thio-cAMP (8-CPT-cAMP), and H-89 were products of Cayman Chemical (Ann Arbor, MI, USA). Dibutylryl-cAMP was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). 8-(4-Chlorophenyl)thio-2'-O-methyl-cAMP (8-CPT-2'-O-Me-cAMP) was obtained from Tocris Bioscience (Bristol, UK). Petri dishes of Iwaki brand for tissue culture were supplied by Asahi Glass (Tokyo, Japan). All other chemicals used are of reagent or tissue culture grade.

Cell culture of 3T3-L1 cells and adipogenesis during the maturation phase

The preadipogenic mouse 3T3-L1 cells (JCRB9014) were employed for the differentiation and maturation of adipocytes under the established cultured conditions as described earlier (Ham et al. 2001; Tsuboi et al. 2004; Lu et al. 2004; Xu et al. 2006; Rahman et al. 2014). 3T3-L1 cells were plated at 5×10^4 cells/ml in the growth medium (GM) containing DME-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. The confluent cells were replaced with the differentiation medium (DM) supplemented with 1 μ M dexamethasone, 0.5 mM IBMX, and 10 μ g/ml insulin and treated for 45 h. After the triggering of the differentiation process, the culture medium was changed to the maturation medium (MM) with 5 μ g/ml insulin and refed with the fresh MM every 2 days for a total of 10 days to promote adipogenesis for fat storage.

For the evaluation of the effect of a variety of compounds on the storage of fats in cultured adipocytes, the cultured cells after the differentiation phase were exposed to the compounds to be tested in MM during the maturation phase by replacing with the fresh MM with the same compounds every 2 days. The compounds were dissolved in ethanol as a vehicle and added to MM to allow the volume of ethanol to be 0.2 %.

Determination of cellular levels of triacylglycerol and protein

For the determination of cellular levels of fat storage and cellular proteins, cultured adipocytes were harvested as cell suspension after the incubation at 37 °C with 0.05 % trypsin and 0.53 mM EDTA in phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS (–)) for 5 min. The resulting cell suspensions were divided into two parts after washing with PBS (–). The divided ones were individually applied to the determination of the amounts of cellular triacylglycerols and proteins after homogenizing in 25 mM Tris–HCl buffer (pH 7.4) containing 1 mM EDTA, and 1 N NaOH, respectively. The accumulation of triacylglycerol in adipocytes during the maturation phase was quantified using the Triglyceride E-Test Kit by assaying the released amount of glycerol through the action of microbial lipoprotein lipase and the related enzymes according to the procedures of the manufacturer as described previously (Xu et al. 2006; Chu et al. 2009; Rahman et al. 2014). Cellular proteins in cultured adipocytes were determined using fatty acid-free bovine serum albumin as a standard by the method of Lowry et al. (1951) after precipitated with cold 6 % trichloroacetic acid to remove the interfering substances for the assays (Markwell et al. 1981).

Microscopic and macroscopic observation of cultured adipocytes

The status of cultured adipocytes was observed by phase-contrast microscopy using a Nikon inverted microscope system, Eclipse TE300 (Nikon, Tokyo, Japan), and the micrographs were recorded by Nikon digital camera D-5200 attached to the inverted microscope system. Oil droplets stored in adipocytes were stained with Oil Red O for the observation by differential-interference microscopy and macroscopic views of cultured cells in Petri dishes as described previously (Kuri-Harcuch and Green 1978).

Others

The data on the storage of fats were normalized to protein content and are expressed as the mean $\pm$ SEM of three independent experiments. Statistically significant differences between mean values were evaluated

by the Student's *t* test. Differences were considered to be significant when $p < 0.05$.

Results

Effect of prostacyclin and selective agonists or antagonist for IP receptor on the storage of fats attenuated by aspirin during the maturation phase of adipocytes

Our previous studies have shown that cultured adipocytes during the maturation phase are able to increasingly biosynthesize pro-adipogenic prostanooids, such as PGD_2 , PGJ_2 derivatives, and PGI_2 (Mazid et al. 2006; Hossain et al. 2011; Rahman et al. 2014). The treatment of cultured adipocytes with 500 μM aspirin, a well-known inhibitor for COX enzymes, during the maturation phase resulted in a significant reduction in the generation of the accumulation of triacylglycerols (Fig. 1a). The inhibitory effect of aspirin was rescued by the addition of exogenous PGI_2 (sodium salt) at each concentration of 50 and 100 nM. The microscopic and macroscopic views of cultured adipocytes by Oil Red O staining confirmed the increased number of oil droplets by the supplementation with increasing concentrations of PGI_2 in the presence of aspirin (Fig. 1b). We then investigated effects of selective agonists for the IP receptor or $\text{PPAR}\gamma$ on the storage of fats attenuated by the addition of aspirin. The supplementation with selective agonists for the IP receptor including carbaprostacyclin (Whittle et al. 1980), MRE-269 (Kuвано et al. 2007), and treprostinil (Olschewski et al. 2004) at different concentrations of 0.1 and 0.5 μM were found to be effective in appreciably stimulating adipogenesis in cultured adipocytes during the maturation phase as can be seen with 0.1 and 1 μM troglitazone (Willson et al. 2000), an activator of $\text{PPAR}\gamma$ (Fig. 2a). The promoting effect of the selective agonists for the IP receptor along with aspirin on the accumulation of oil droplets was also evident by the observation of cultured adipocytes stained with Oil Red O (Fig. 2b).

To determine additionally the involvement of the IP receptor in the up-regulation of adipogenesis, cultured adipocytes were incubated with selective antagonists for the IP receptor during the maturation phase. The IP antagonists CAY10441 (Clark et al. 2004) and

Fig. 1 Effect of sodium salt of PGI₂ together with aspirin on the storage of fats in cultured adipocytes during the maturation phase. 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 100 % confluence. After the resulting cells were treated with 2 ml of DM for 45 h to induce the differentiation phase, the cells were furthermore cultured for a total of 10 days of the maturation phase by replacing every 2 days with 2 ml of fresh MM with 0.2 % ethanol as a vehicle, 500 μ M aspirin, or 50 nM or 100 nM PGI₂ together with aspirin. The mature adipocytes at the terminal differentiation were harvested and then subjected to

the determination of the amounts of cellular triacylglycerols (a). Data represent the mean $\pm$ SEM of three independent experiments. * $p < 0.05$ compared with the cells treated with vehicle. # $p < 0.05$ compared with the cells treated with aspirin only. Moreover, the microscopic views of cultured adipocytes were observed by phase-contrast microscopy (upper panels) (b). Alternatively, the cultured adipocytes were stained with Oil Red O and then subjected to the observation of microscopic views by differential-interference microscopy (middle panels) or macroscopic views (lower panels) (b). Pictures are shown from a representative one done in three experiments. Scale bar 50 μ m

Fig. 2 Effect of selective agonists for the IP receptor or PPAR γ together with aspirin on the storage of fats during the maturation phase. 3T3-L1 cells were cultured, differentiated, and matured to adipocytes as described in Fig. 1. During the maturation phase, cultured cells were treated for a total of 10 days with vehicle, 1 μ M indomethacin, 500 μ M aspirin, or different concentrations of either troglitazone, carbaprostacyclin, MRE-269, or treprostinil together with 500 μ M aspirin. The resulting cultured adipocytes were harvested for the determination of the amounts of cellular triacylglycerols (a). Data represent the

mean $\pm$ SEM of three independent experiments. * $p < 0.05$ compared with the cells treated with vehicle. # $p < 0.05$ compared with the cells treated with aspirin only. Moreover, the microscopic views of cultured adipocytes were observed by phase-contrast microscopy (upper panels) (b). Alternatively, the cultured adipocytes were stained with Oil Red O and then subjected to the observation of microscopic views by differential-interference microscopy (middle panels) or macroscopic views (lower panels) (b). Pictures are shown from a representative one done in three experiments. Scale bar 50 μ m

CAY10449 (Clark et al. 2004) at concentrations of 0.05 and 0.1 μ M significantly suppressed the storage of fats as 0.1 and 1 μ M GW9662 (Bendixen et al.

2001), a selective antagonist for PPAR γ , did under the same culture conditions (Fig. 3a). The observation of cultured adipocytes after Oil Red O staining also

revealed the efficacy of the IP antagonists in the attenuation of adipogenesis after 10 days of the maturation phase (Fig. 3b). These results indicate that the pro-adipogenic action of PGI₂ can be explained by the action mediated through the IP receptor in cultured adipocytes.

Combined effect of a selective agonist for IP receptor and an activator for PPAR γ on adipogenesis along with aspirin during the maturation phase of adipocytes

To obtain the information on the combined effect of a selective agonist for IP receptor and an activator for PPAR γ , cultured cells are exposed to a mixture of troglitazone and PGI₂ or that of troglitazone and MRE-269 in the presence of aspirin during the maturation phase. The co-incubation with 1 μ M troglitazone and 100 nM PGI₂ or 1 μ M troglitazone and 0.5 μ M MRE-269 resulted in significantly higher stimulation of fat storage than that with troglitazone, PGI₂, or MRE-269 alone (Fig. 4). The increased levels of stored fats exceeded the control ones without aspirin after 10 days of the maturation phase. The findings suggest that the activation of the IP receptor and PPAR γ exert an additive effect on the promotion

of adipogenesis in cultured adipocytes during the maturation phase.

We next examined the influence of GW9662, an antagonist for PPAR γ , or CAY10441, an antagonist for the IP receptor, on the additive action of troglitazone and MRE-269 to stimulate the accumulation of fats. The supplementation with GW9662 was found to abolish completely the stimulation of adipogenesis caused by a mixture of troglitazone and MRE-269, whereas CAY10441 suppressed partly the storage of fats enhanced by the mixture (Fig. 5a). Moreover, it was also apparent that there was the difference in the inhibition of adipogenesis between GW9662 and CAY10441 by the microscopic and macroscopic views of cultured adipocytes after Oil Red staining (Fig. 5b).

To determine the role of PPAR γ in the stimulation of adipogenesis by a selective agonist for the IP receptor, cultured adipocytes were treated with increasing concentrations of MRE-269 or troglitazone in the presence of 1 μ M GW9662 or 0.1 μ M CAY10441, respectively. The treatment of cultured adipocytes with increasing concentrations of MRE-269 did not rescue the inhibitory effect of GW9662 on the storage of fats during the maturation phase even if higher concentrations were used (Fig. 6a). On the

Fig. 3 Effect of selective antagonists for the IP receptor or PPAR γ on the storage of fats during the maturation phase. 3T3-L1 cells were cultured, differentiated, and matured to adipocytes as described in Fig. 1. During the maturation phase, cultured cells were treated for a total of 10 days with vehicle or different concentrations of GW9662, CAY10449, and CAY10441. The resulting cultured adipocytes were harvested for the determination of the amounts of cellular triacylglycerols (**a**). Data represent the mean $\pm$ SEM of three independent experiments.

* $p < 0.05$ compared with the cells treated with vehicle. Moreover, the microscopic views of cultured adipocytes were observed by phase-contrast microscopy (*upper panels*) (**b**). Alternatively, the cultured adipocytes were stained with Oil Red O and then subjected to the observation of microscopic views by differential-interference microscopy (*middle panels*) or macroscopic views (*lower panels*) (**b**). Pictures are shown from a representative one done in three experiments. Scale bar 50 μ m

Fig. 4 Combined effect of selective agonists for the IP receptor and PPAR γ on the storage of fats during the maturation phase. 3T3-L1 cells were cultured, differentiated, and matured to adipocytes as described in Fig. 1. During the maturation phase, cultured cells were treated for a total of 10 days with vehicle, 500 μ M aspirin alone, or either of 1 μ M troglitazone, 0.5 μ M MRE-269, 100 nM PGI₂, a mixture of troglitazone and MRE-269, or a mixture of troglitazone and PGI₂ in the presence of 500 μ M aspirin. The resulting cultured adipocytes were harvested for the determination of the amounts of cellular triacylglycerols. Data represent the mean $\pm$ SEM of three independent experiments. * p < 0.05 compared with the cells treated with vehicle. # p < 0.05 compared with the cells treated with aspirin only

other hand, increasing concentrations of troglitazone were effective in a dose-dependent manner to reverse the storage of fats suppressed by CAY10441 under the same conditions (Fig. 6b). The results indicate that the activation of PPAR γ is essential as a downstream factor for the promotion of adipogenesis by the action of selective agonists for the IP receptor.

Action of cAMP analogues, forskolin, and a protein kinase A (PKA) inhibitor on the storage of fats during the maturation phase of adipocytes

The prostanoid IP receptor is generally coupled to Gs protein to increase intracellular cAMP (Narumiya et al. 1999; Wise 2003). Hence, to know the involvement of cAMP as a second messenger in the signal transduction of the action of PGI₂ and the related agonists, cultured adipocytes were exposed to cell-permeable stable cAMP analogues at 100 μ M during the maturation phase (Fig. 7a). The compounds, such

as dibutyryl-cAMP (Steinberg and Agard 1981), 8-bromo-cAMP (Sandberg et al. 1991), and 8-CPT-cAMP (Sandberg et al. 1991), were found to partly stimulate adipogenesis attenuated by aspirin. In addition, 8-CPT-2'-O-Me-cAMP, a specific, cell permeable activator of the Epac cAMP receptor (Christensen et al. 2003), also exhibited a promoting activity for the storage of fats although the level did not reach the control one without aspirin. Alternatively, we investigated the effects of increasing concentrations of forskolin (Insel and Ostrom 2003) used as an activator of adenylyl cyclase to raise intracellular level of cAMP on the accumulation of fats in the presence or absence of aspirin (Fig. 7b). The inhibitory effect of aspirin was rescued by increasing concentrations of forskolin to a higher extent. By contrast, the concentrations of forskolin at higher than 10 μ M without aspirin suppressed adipogenesis after 10 days of the maturation phase. These findings suggest that the elevation of intracellular levels of cAMP exert opposite effects on adipogenesis during the maturation phase depending on the extent of accumulated fats in mature adipocytes.

Next, to determine the involvement of PKA activity in the stimulatory effects of PGI₂ and MRE-269 on adipogenesis in the presence of aspirin, we examined the influence of 10 μ M H-89 known as a potent, cell permeable inhibitor of PKA on adipogenesis. As shown in Fig. 8a, H-89 had almost no effect on the accumulation of fats in cultured adipocytes during the maturation phase, which was contrary to the significant inhibition of adipogenesis by GW9662, an antagonist of PPAR γ . The observation indicates that the up-regulation of adipogenesis by PGI₂ and the related IP agonists did not depend on a PKA-sensitive pathway. On the other hand, we recognized significant inhibitory effects on adipogenesis when cultured adipocytes were treated with the compounds that can lead to the activation of PKA activity, including forskolin, dibutyryl-cAMP, 8-bromo-cAMP, and 8-CPT-cAMP, in the absence of aspirin (Fig. 8b). However, 8-CPT-2'-O-Me-cAMP serving as a selective activator of Epac, but not as PKA activator, did not show an inhibitory effect on adipogenesis. H-89 acting as a specific PKA inhibitor (Davies et al. 2000) was able to reverse the suppression of adipogenesis caused by the agents that could lead to the activation of PKA. These results support the idea that inhibitory effects of the cAMP analogues and forskolin on

Fig. 5 Inhibitory effect of a selective antagonist for either the IP receptor or PPAR γ on the storage of fats enhanced by a combination of troglitazone and MRE-269 in the presence of aspirin. 3T3-L1 cells were cultured, differentiated, and matured to adipocytes as described in Fig. 1. During the maturation phase, cultured cells were treated for a total of 10 days with vehicle, 500 μ M aspirin alone, or a mixture of 1 μ M troglitazone, 0.1 μ M MRE-269, and 500 μ M aspirin together with either vehicle, 1 μ M GW9662, or 0.1 μ M CAY10441. The resulting cultured adipocytes were harvested for the determination of the amounts of cellular triacylglycerols (**a**). Data

represent the mean $\pm$ SEM of three independent experiments. * $p < 0.05$ compared with the cells treated with aspirin only. # $p < 0.05$ compared with the cells treated with a mixture of 1 μ M troglitazone, 0.1 μ M MRE-269, and 500 μ M aspirin. Moreover, the microscopic views of cultured adipocytes were observed by phase-contrast microscopy (*upper panels*) (**b**). Similarly, the cultured adipocytes were stained with Oil Red O and then subjected to the observation of microscopic views by differential-interference microscopy (*middle panels*) or macroscopic views (*lower panels*) (**b**). Pictures are shown from a representative one done in three experiments. Scale bar 50 μ m

adipogenesis during the maturation phase is more likely to be mediated by the activation of PKA although the promotion of adipogenesis by the activation of the IP receptor does not involve the PKA-dependent pathway.

Discussion

Preadipogenic mouse 3T3-L1 cells have been utilized widely as a useful model for adipogenesis from undifferentiated preadipocytes to mature adipocytes displaying the growth, differentiation, and maturation phases (Green and Kehinde 1974, 1975; Hyman et al. 1982). Usually, the differentiation of confluent cultured 3T3-L1 preadipocytes is initiated by exposure to a mixture of hormonal inducers including insulin, dexamethasone, and IBMX to induce a sequential activation of transcription factors, which is followed by the continued cell culture in the maturation medium with insulin for the promotion of adipogenesis (Ham et al. 2001; Petersen et al. 2003). More recently, we have reported that the cultured adipocytes during the

maturation phase can biosynthesize PGI $_2$ as determined by the amount of stable 6-keto-PGF $_{1\alpha}$ reflecting the generation of endogenous PGI $_2$, an unstable COX metabolite, at much higher levels than the preadipocytes at growth and differentiation phases, which is associated with up-regulation of the gene expression of PGIS and the IP receptor (Rahman et al. 2014). These results led us to investigate the role of endogenous prostacyclin as pro-adipogenic prostanooids in an autocrine manner. Hence, the present study focused on the specific action of prostacyclin and its specific agonists or antagonist for the IP receptor on cultured adipocytes during the maturation phase. Our previous studies have also revealed that cultured 3T3-L1 adipocytes are also capable of generating other pro-adipogenic prostanooids such as PGD $_2$ and PGJ $_2$ derivatives during the maturation phase, which are involved in the stimulation of adipogenesis (Mazid et al. 2006; Hossain et al. 2011). As expected, we found that the treatment of cultured cells with each of aspirin and indomethacin, well-known COX inhibitors, resulted in a significant suppression of adipogenesis during the maturation phase. The current study

Fig. 6 Effect of a selective agonist for either the IP receptor or PPAR γ on the storage of fats suppressed by a selective antagonist for either of them. 3T3-L1 cells were cultured, differentiated, and matured to adipocytes as described in Fig. 1. During the maturation phase, cultured cells were treated for a total of 10 days with increasing concentrations of MRE-269 in the presence or absence of 1 μ M GW9662 (**a**). Similarly, cultured cells during the maturation phase were exposed to increasing concentrations of troglitazone in the presence or absence of 0.1 μ M CAY10441 (**b**). The resulting cultured adipocytes were harvested for the determination of the amounts of cellular triacylglycerols. Data represent the mean $\pm$ SEM of three independent experiments. * p < 0.05 compared with the cells treated by vehicle

showed that natural PGI₂ added to the maturation medium every 2 days for a total of 10 days were able to rescue the inhibitory effect of aspirin on the storage of fats even although PGI₂ in biological fluids is generally known to be unstable with the half-life of a few minutes. The finding indicates that natural prostacyclin can exert its pro-adipogenic effect in short time prior to its spontaneous degradation in the maturation medium. Thus, it is conceivable that endogenous PGI₂ generated by cultured adipocytes contributes to the positive regulation of adipogenesis after the maturation phase in an autocrine manner. This study found the effectiveness of exogenous PGI₂ at the concentration of 50 nM to rescue the inhibitory

Fig. 7 Effect of cAMP analogues and forskolin on the storage of fats during the maturation phase. 3T3-L1 cells were cultured, differentiated, and matured to adipocytes as described in Fig. 1. **a** During the maturation phase, cultured cells were treated for a total of 10 days with either of cAMP analogues at 100 μ M in the presence of 500 μ M aspirin. The resulting cultured adipocytes were harvested for the determination of the amounts of cellular triacylglycerols. Data represent the mean $\pm$ SEM of three independent experiments. * p < 0.05 compared with the cells treated with vehicle. # p < 0.05 compared with the cells treated with aspirin only. **b** Similarly, cultured cells during the maturation phase were exposed to increasing concentrations of forskolin in the presence or absence of 500 μ M aspirin. The resulting cultured adipocytes were harvested for the determination of the amounts of cellular triacylglycerols and data are shown as described above. * p < 0.05 compared with the cells treated with aspirin only. # p < 0.05 compared with the cells treated with vehicle

effect of aspirin. This dose is almost near to the level of endogenous levels of PGI₂ as determined by the level of 6-keto-PGF_{1 α} previously (Rahman et al. 2014).

The following lines of evidence support that the pro-adipogenic action of prostacyclin is mediated through the binding to the IP receptor in cultured 3T3-L1 adipocytes. At first, all of the specific agonists for the IP receptor including carbaprostacyclin, MRE-269, and treprostinil were found to be effective in

Fig. 8 Effect of H-89, a specific inhibitor for PKA, on the storage of fats during the maturation phase. 3T3-L1 cells were cultured, differentiated, and matured to adipocytes as described in Fig. 1. **a** During the maturation phase, cultured cells were treated for a total of 10 days with either of 10 μ M H-89 or 1 μ M GW9662 together with 100 nM PGI₂ or 0.5 μ M MRE-269 in the presence of 500 μ M aspirin. The resulting cultured adipocytes were harvested for the determination of the amounts of cellular triacylglycerols. Data represent the mean $\pm$ SEM of three independent experiments. * p < 0.05 compared with the cells treated with a mixture of 100 nM PGI₂ and 500 μ M aspirin. # p < 0.05 compared with the cells treated with a mixture of 0.5 μ M MRE-269 and 500 μ M aspirin. **b** Similarly, cultured cells during the maturation phase were exposed to vehicle, 20 μ M forskolin, or either of cAMP analogues at 100 μ M in the presence or absence of 10 μ M H-89. The resulting cultured adipocytes were harvested for the determination of the amounts of cellular triacylglycerols and data are shown as described above. * p < 0.05 compared with the cells treated without H-89 in the presence of vehicle or either of forskolin and cAMP analogues

rescuing the inhibitory effect of aspirin on adipogenesis during the maturation phase in this study. Next, we also confirmed that specific antagonists for the IP receptor, such as CAY10441 and CAY10449, suppressed the storage of fats in cultured adipocytes after 10 days of the maturation phase. Moreover, a previous study of us has shown that the gene expression of the IP receptor is up-regulated more extensively after 6–10 days of the maturation phase as compared with that during the growth and differentiation phases (Rahman et al. 2014). The higher expression levels of mRNA for the IP receptor was also recognized after several days of the induced adipocyte differentiation from cultured 3T3-L1 preadipocytes in an earlier report (Tsuboi et al. 2004). These observations are consistent with the idea that endogenous prostacyclin can exert a promoting effect on adipogenesis through the IP receptor during the maturation phase of cultured adipocytes. Interestingly, a previous study described that wild-type mother mice fed a high-fat diet rich in linoleic acid during the pregnancy-lactation period resulted in the promotion of adipose tissue development in their newborn mice at 8 weeks of age while the IP-deficient mice fed the same diet failed to do it (Massiera et al. 2003). The finding indicates the contribution of the prostacyclin signaling through the IP receptor to adipose tissue development in animals in vivo.

In this study, the inhibitory effect of aspirin on adipogenesis during the maturation phase of cultured 3T3-L1 adipocytes was reversed by troglitazone, a specific activator for PPAR γ , as well as by PGI₂ or MRE-269, a selective agonist for the IP receptor. Here, we found that a mixture of troglitazone and PGI₂ had more potent effect on the storage of fats than either troglitazone or PGI₂ alone. Similarly, the co-incubation of cultured adipocytes with troglitazone and MRE-269 was more effective to promote adipogenesis than each of them. These observations suggest that the simultaneous activation of the IP receptor and PPAR γ resulted in the additive effect on the stimulation of adipogenesis in cultured adipocytes during the maturation phase. Moreover, the stimulatory effect of a mixture of troglitazone and MRE-269 on adipogenesis along with aspirin was blocked completely by GW9662, a specific antagonist for PPAR γ . On the

other hand, CAY10441, a selective antagonist for the IP receptor, attenuated the storage of fats to a lower extent than that of PPAR γ antagonist. The findings imply the crucial role of the activation of PPAR γ in the pro-adipogenic effects of PGI $_2$ and MRE-269 through the IP receptor. This idea is supported additionally by our data, as follows. The suppression of adipogenesis by GW2669 was not rescued by increasing concentrations of MRE-269. By contrast, troglitazone was able to stimulate the adipogenesis attenuated by CAY10441 in a doses-dependent fashion. These results indicate that the pro-adipogenic effects of PGI $_2$ and the related agonists for the IP receptor require the activation of the nuclear hormone receptor PPAR γ as a downstream signaling factor for the stimulation of adipogenesis during the maturation phase (Fig. 9).

PPAR γ is predominantly expressed after the maturation phase of cultured 3T3-L1 adipocytes (Lu et al. 2004; Xu et al. 2006; Mazid et al. 2006). Since PPAR γ is a master regulator of adipogenesis, it is possible to consider that prostacyclin and the selective IP agonists may exert their pro-adipogenic actions through the direct binding to PPAR γ to activate the related signaling leading to the stimulation of adipogenesis. Previous studies have described that some of stable prostacyclin analogues including carbaprostacyclin and iloprost act as active ligands for PPAR α and PPAR δ (Forman et al. 1997). A novel pathway of prostacyclin signaling through PPAR δ is thought to be operative in certain systems (Lim and Dey 2002). An

earlier study has reported that PPAR δ is expressed in cultured 3T3-L1 preadipocytes, but the activation of PPAR δ only modestly promote terminal differentiation, indicating that the activation of PPAR δ is not a decisive factor in terminal differentiation of adipocytes (Hansen et al. 2001). On the other hand, natural PGI $_2$ and the prostacyclin analogues have been shown to be inactive ligands in the activation of PPAR γ , a key regulator of terminal adipocyte differentiation (Forman et al. 1997). Considering these findings, it is not conceivable that the stimulation of adipogenesis by prostacyclin and the related analogues in our study was mediated by the activation of PPAR γ through the direct interaction with their ligands. Instead, the upstream signaling through the IP receptor is more likely to be responsible for the promotion of adipogenesis mediated by the activation of PPAR γ in cultured 3T3-L1 adipocytes. Alternatively, PGI $_2$ may regulate the gene expression of PPAR γ through the IP receptor. Further detailed studies remain to be done. Interestingly, a recent study using a cell-based reporter gene assay in HEK-293 cells stably expressing the IP receptor have provided the evidence that activation of PPAR γ by prostacyclin analogues contributing to anti-growth effects is dependent on the presence of the IP receptor although the mechanism of activation is unknown (Falcetti et al. 2007).

Prostacyclin and its stable analogues are known to exert their biological effects by binding to the cell-surface membrane IP receptor, which couples to the Gs protein to activate adenylyl cyclase and elevate

Fig. 9 Proposed mechanism for the action of prostacyclin and its selective agonists on the stimulated adipogenesis through the IP receptor and PPAR γ in cultured adipocytes during the maturation phase

cAMP as an intracellular second messenger (Narumiya et al. 1999; Wise 2003). The present study revealed that the inhibitory effect of aspirin on adipogenesis during the maturation phase was partly reversed by the supplementation of cell-permeable stable cAMP analogues including dibutyl-cAMP, 8-bromo-cAMP, and 8-CPT-cAMP. As well, we showed that forskolin, an activator of adenylyl cyclase to increase intracellular level of cAMP, appreciably enhanced the storage of fats during the maturation phase. These findings raise the possibility that the promotion of adipogenesis by prostacyclin and its selective agonists might be partly mediated by the activation of cAMP-dependent PKA. However, the PKA inhibitor H-89 (Davies et al. 2000) had no inhibitory effects on the storage of fats stimulated by PGI₂ and MRE-269 in the presence of aspirin. This observation indicates that elevated cAMP levels through the IP receptor promote adipogenesis in a PKA-independent manner. Instead, we also confirmed the effectiveness of 8-CPT-2'-O-Me-cAMP, a specific, cell permeable specific activator of the Epac cAMP receptor (Christensen et al. 2003), in partly rescuing adipogenesis attenuated by aspirin to the same extent as other stable cAMP analogues. The activation of Epac I is considered to be linked with the signaling with Akt/PKB necessary for terminal adipocyte differentiation as a part of the signaling through the IP receptor (Mei et al. 2002). On the other hand, we noticed the suppression of normal adipogenesis by stable cAMP analogues and forskolin in the absence of aspirin. The inhibition of adipogenesis by these compounds was reversed completely by co-incubation with H-89, indicating that the inhibitory effects of stable cAMP and forskolin are mediated by the activation of PKA. Nevertheless, more studies remain to be done to unravel the detailed cellular mechanism for the promotion of adipogenesis by prostacyclin and its selective agonists during the maturation phase.

In conclusion, we demonstrated that natural prostacyclin and its selective agonists for the IP receptor stimulated adipogenesis attenuated by aspirin during the maturation phase. The pro-adipogenic effects of these compounds were blocked by a specific antagonist for PPAR γ although prostacyclin and the related agonists are not known as the direct activator of PPAR γ . These findings indicate that the action of prostacyclin through the IP receptor is linked with the

activation of PPAR γ as a downstream factor. The up-regulation of adipogenesis by prostacyclin appears to be partly dependent on the elevated levels of cAMP, but is not dependent on the PKA activity.

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Compliance with ethical standards

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

References

- Bendixen AC, Shevde NK, Dienger KM, Willson TM, Funk CD, Pike JW (2001) IL-4 inhibits osteoclast formation through a direct action on osteoclast precursors via peroxisome proliferator-activated receptor γ 1. *Proc Natl Acad Sci USA* 98:2443–2448
- Catalioto RM, Gaillard D, Maclouf J, Ailhaud G, Négrel R (1991) Autocrine control of adipose cell differentiation by prostacyclin and PGF_{2 α} . *Biochim Biophys Acta* 1091:364–369
- Chawla A, Schwartz EJ, Dimaculangan DD, Lazar MA (1994) Peroxisome proliferator-activated receptor (PPAR) γ : adipose-predominant expression and induction early in adipocyte differentiation. *Endocrinology* 135:798–800
- Christensen AE, Selheim F, de Rooij J, Dremier S, Schwede F, Dao KK, Martinez A, Maenhaut C, Bos JL, Genieser HG, Døskeland SO (2003) cAMP analog mapping of Epac1 and cAMP kinase. Discriminating analogs demonstrate that Epac and cAMP kinase act synergistically to promote PC-12 neurite extension. *J Biol Chem* 278:35394–35402
- Chu X, Xu L, Nishimura K, Jisaka M, Nagaya T, Shono F, Yokota K (2009) Suppression of adipogenesis program in cultured preadipocytes transfected stably with cyclooxygenase isoforms. *Biochim Biophys Acta* 1791:273–280
- Clark RD, Jahangir A, Severance D, Salazar R, Chang T, Chang D, Jett MF, Smith S, Bley K (2004) Discovery and SAR development of 2-(phenylamino) imidazolines as prostacyclin receptor antagonists. *Bioorg Med Chem Lett* 14:1053–1056
- Davies SP, Reddy H, Caivano M, Cohen P (2000) Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem J* 351:95–105
- Falcetti E, Flavell DM, Staels B, Tinker A, Haworth SG, Clapp LH (2007) IP receptor-dependent activation of PPAR γ by stable prostacyclin analogues. *Biochem Biophys Res Commun* 360:821–827
- Forman BM, Tontonoz P, Chen J, Brun RP, Spiegelman BM, Evans RM (1995) 15-Deoxy- $\Delta^{12,14}$ -PGJ₂ is a ligand for the adipocyte determination factor PPAR γ . *Cell* 83:803–812
- Forman BM, Chen J, Evans RM (1997) Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors α and δ . *Proc Natl Acad Sci USA* 94:4312–4317

- Galic S, Oakhill JS, Steinberg GR (2010) Adipose tissue as an endocrine organ. *Mol Cell Endocrinol* 316:129–139
- Green H, Kehinde O (1974) Sublines of mouse 3T3 cells that accumulate lipid. *Cell* 1:113–116
- Green H, Kehinde O (1975) An established preadipose cell line and its differentiation in culture. II. Factors affecting the adipose conversion. *Cell* 5:19–27
- Gregoire FM, Smas CM, Sul HS (1998) Understanding adipocyte differentiation. *Physiol Rev* 78:783–809
- Ham JK, Park BH, Farmer SR (2001) A role for C/EBP β in regulating peroxisome proliferator-activated receptor γ activity during adipogenesis in 3T3-L1 preadipocytes. *J Biol Chem* 276:18464–18471
- Hansen JB, Zhang H, Rasmussen TH, Petersen RK, Flindt EN, Kristiansen K (2001) Peroxisome proliferator-activated receptor δ (PPAR δ)-mediated regulation of preadipocyte proliferation and gene expression is dependent on cAMP signaling. *J Biol Chem* 276:3175–3182
- Hossain MS, Chowdhury AA, Rahman MS, Nishimura K, Jisaka M, Nagaya T, Shono F, Yokota K (2011) Development of enzyme-linked immunosorbent assay for Δ^{12} -prostaglandin J_2 and its application to the measurement of the endogenous product generated by cultured adipocytes during the maturation phase. *Prostaglandins Other Lipid Mediat* 94:73–80
- Hyman BT, Stoll LL, Spector AA (1982) Prostaglandin production by 3T3-L1 cells in culture. *Biochim Biophys Acta* 713:375–385
- Insel PA, Ostrom RS (2003) Forskolin as a tool for examining adenylyl cyclase expression, regulation, and G protein signaling. *Cell Mol Neurobiol* 23:305–314
- Kliwer SA, Lenhard JM, Wilson TM, Patel I, Morris DC, Lehmann JM (1995) A prostaglandin J_2 metabolite binds peroxisome proliferator-activated receptor γ and promotes adipocyte differentiation. *Cell* 83:813–819
- Kuri-Harcuch W, Green H (1978) Adipose conversion of 3T3 cells depends on a serum factor. *Proc Natl Acad Sci USA* 75:6107–6109
- Kuwano K, Hashino A, Asaki T, Hamamoto T, Yamada T, Okubo K, Kuwabara K (2007) 2-[4-[(5,6-Diphenylpyrazin-2-yl)(isopropyl)amino]butoxy]-N-(methylsulfonyl)acetamide (NS-304), an orally available and long-acting prostacyclin receptor agonist prodrug. *J Pharmacol Exp Ther* 322:1181–1188
- Lim H, Dey SK (2002) Minireview: a novel pathway of prostacyclin signaling-hanging out with nuclear receptors. *Endocrinology* 143:3207–3210
- Lowry OH, Rosebrough NJ, Farr AL, Randall RL (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* 193:265–275
- Lu S, Nishimura K, Hossain MA, Jisaka M, Nagaya T, Yokota K (2004) Regulation and role of arachidonate cascade during changes in life cycle of adipocytes. *Appl Biochem Biotechnol* 118:133–153
- Markwell MA, Hass SM, Toleret NE, Bieber LI (1981) Protein determination in membrane and lipoprotein samples: manual and automated procedures. *Methods Enzymol* 72:296–303
- Massiera F, Saint-Marc P, Seydoux J, Murata T, Kobayashi T, Narumiya S, Guesnet P, Amri EZ, Négrel R, Ailhaud G (2003) Arachidonic acid and prostacyclin signaling promote adipose tissue development: a human health concern? *J Lipid Res* 44:271–279
- Mazid MA, Chowdhury AA, Nagao K, Nishimura K, Jisaka M, Nagaya T, Yokota K (2006) Endogenous 15-deoxy- $\Delta^{12,14}$ -PGJ $_2$ synthesized by adipocytes during maturation phase contributes to upregulation of fat storage. *FEBS Lett* 580:6885–6890
- Mei FC, Qiao J, Tsygankova QM, Meinkoth JL, Quilliam LA, Chen X (2002) Differential signaling of cyclic AMP: opposing effects of exchange protein directly activated by cyclic AMP and cAMP-dependent protein kinase on protein kinase B activation. *J Biol Chem* 277:11497–11504
- Narumiya S, Sugimoto Y, Ushikubi F (1999) Prostanoid receptors: structures, properties, and functions. *Physiol Rev* 79:1193–1226
- Négrel R, Gaillard D, Ailhaud G (1989) Prostacyclin as a potent effector of adipose-cell differentiation. *Biochem J* 257:399–405
- Olschewski H, Rose F, Schermuly R, Ghofrani HA, Enke B, Olschewski A, Seeger W (2004) Prostacyclin and its analogues in the treatment of pulmonary hypertension. *Pharmacol Ther* 102:139–153
- Petersen RK, Jørgensen C, Rustan AC, Frøylund L, Muller-Decker K, Furstenberger G, Berge RK, Kristiansen K, Madsen L (2003) Arachidonic acid-dependent inhibition of adipocyte differentiation requires PKA activity and is associated with sustained expression of cyclooxygenases. *J Lipid Res* 44:2320–2330
- Rahman MS, Khan F, Syeda PK, Nishimura K, Jisaka M, Nagaya T, Shono F, Yokota K (2014) Endogenous synthesis of prostacyclin was positively regulated during the maturation phase of cultured adipocytes. *Cytotechnology* 66:635–646
- Sandberg M, Butt E, Nolte C, Fischer L, Halbrügge M, Beltman J, Jahnsen T, Genieser HG, Jastorff B, Walter U (1991) Characterization of Sp-5,6-dichloro-1- β -D-ribofuranosylbenzimidazole-3',5'-monophosphorothioate (Sp-5,6-DCI-cBiMPS) as a potent and specific activator of cyclic-AMP-dependent protein kinase in cell extracts and intact cells. *Biochem J* 279:521–527
- Steinberg RA, Agard DA (1981) Turnover of regulatory subunit of cyclic AMP-dependent protein kinase in S49 mouse lymphoma cells. Regulation by catalytic subunit and analogs of cyclic AMP. *J Biol Chem* 256:10731–10734
- Tsuboi H, Sugimoto Y, Kainoh T, Ichikawa A (2004) Prostanoid EP4 receptor is involved in suppression of 3T3-L1 adipocyte differentiation. *Biochem Biophys Res Commun* 322:1066–1072
- Whittle BJ, Moncada S, Whiting F, Vane JR (1980) Carbacyclin—a potent stable prostacyclin analogue for the inhibition of platelet aggregation. *Prostaglandins* 19:605–627
- Willson TM, Brown PJ, Sternbach DD, Henke BR (2000) The PPARs: from orphan receptors to drug discovery. *J Med Chem* 43:528–550
- Wise H (2003) Multiple signalling options for prostacyclin. *Acta Pharmacol Sin* 24:625–630
- Xu L, Nishimura K, Jisaka M, Nagaya T, Yokota K (2006) Gene expression of arachidonate cyclooxygenase pathway leading to the delayed synthesis of prostaglandins E_2 and $F_{2\alpha}$ in response to phorbol 12-myristate 13-acetate and action of these prostanoids during life cycle of adipocytes. *Biochim Biophys Acta* 1761:434–444