Specific action of prostacyclin on adipogenesis at different stages of cultured adipocytes

[培養脂肪細胞の異なるステージでの脂肪細胞形成に対するプロスタサイクリンの特異的作用]

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Abbreviations

AA	arachidonic acid
20:4 (n-6)	arachidonic acid
aP-2	adipocyte protein 2
8-CPT-2'-*O*-Me-cAMP	8-(4-chlorophenyl)thio-2'-*O*-methyl-cAMP
8-CPT-cAMP	8-(4-chlorophenyl)thio-cAMP
C/EBP	CCAAT enhancer-binding protein
COX	cyclooxygenase
CRTH2	chemoattractant receptor-homologous molecule expressed on Th2 cells
Dex	dexamethasone
DM	differentiation medium
22:6 (n-3)	4, 7, 10, 13, 16, 19-docosahexaenoic acid
DMEM-HEPES	Dulbecco's modified Eagle medium with 25 mM HEPES
20:5 (n-3)	5, 8, 11, 14, 17-eicosapentaenoic acid
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
GLUT	glucose transporter
GM	growth medium
IBMX	3-isobutyl-1-methylxanthine
Indo	indomethacin
18:2 (n-6)	linoleic acid
18:3 (n-3)	α-linolenic acid
LPL	lipoprotein lipase
MM	maturation medium
PBS (-)	phosphate-buffered saline without Ca^{2+} and Mg^{2+}
PCR	polymerase chain reaction
PG	prostaglandin
L-PGDS	lipocalin-type PGD synthase
cPGES	cytosolic PGE synthase
mPGES	membrane-bound PGE synthase
PGFS	PGF synthase
PGIS	PGI synthase

15d-PGJ_2	15-deoxy-$\Delta^{12,14}$-PGJ_2
PKA	protein kinase A
PPAR	peroxisome proliferator-activated receptor
RT	reverse transcriptase

Chapter 1

Introduction

1.1 Different life stages of adipocytes

Excess accumulation of fat is termed as obesity, which is characterized by the increase in the number and/or size of adipocytes (Flier 1995). The life cycle of adipocytes includes three distinct phases: the cell growth of preadipocytes, triggering of differentiation by appropriate hormonal and biochemical stimulation and the terminal differentiation. The differentiation of adipocyte is associated with the conversion of cells from fibroblastic to spherical shape (Rosen and MacDougald 2006). These morphological modifications are accomplished by changes in the level of extracellular matrix component and the level of cytoskeletal components (Gregoire et al. 1998). Recently, progression has been made in understanding the process of adipocytes differentiation and the cellular and molecular basis of adipose tissue growth. However, complete understanding of the cellular and molecular mechanism of adipose tissue growth in physiological and pathophysiological states is important to develop newer therapeutic strategies for the prevention and treatment of obesity. The most frequently employed cell lines to understand adipogenesis are 3T3-F442A and 3T3-L1. These were clonally isolated from Swiss 3T3 cells derived from disaggregated 17 to 19-days mouse embryos (Green and Kehinde 1975). 3T3-L1 preadipocyes spontaneously differentiate over a period of several weeks into fat-cell clusters when maintained in culture with fetal calf serum (Fig. 1-1).

During the growth phase, cells of preadipocyte lines as well as primary preadipocytes are morphologically similar to fibroblasts. At confluence, induction of differentiation by appropriate combination of hormonal and biochemical stimulators leads to dramatic change in cell shape (Fan et al. 1983). For example, for triggering the differentiation of murine 3T3-L1 preadipocytes in vitro, an adequate combination of glucocorticoid, insulin and cyclic AMP (cAMP) elevating agent in presence of fetal bovine serum in Dulbecco's modified Eagle's medium (DMEM) is necessary (Student et al. 1980; Rubin et al. 1978). The absence of any one of these abovementioned inducers in DMEM results in severe impediment of differentiation and eventually decreased lipid accumulation in 3T3-L1 cells (Madsen et al. 2005; Petersen et al. 2003). The preadipocyte converts to a spherical shape, accumulates lipid droplets, and progressively acquires the morphological and biochemical

characteristics of the mature adipocytes (Gregoire et al. 1998). During the terminal phase of differentiation, activation of the transcriptional cascade leads to increased activity, proteins, and mRNA levels for enzymes involved in triacylglycerol synthesis and degradation (Gregoire 2001).

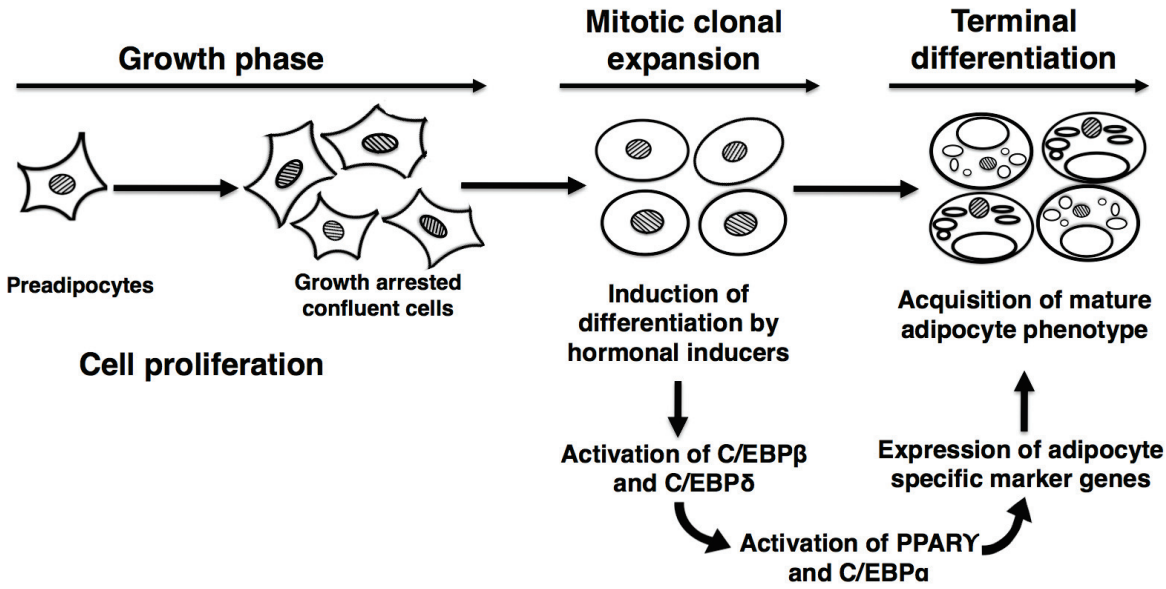

Fig. 1-1. Schematic presentation of life cycle of mouse embryogenic 3T3-L1 preadipocytes and the regulation by mouse embryonic 3T3-L1 cells and the regulation by C/EBPs and PPARγ

1.2 Transcriptional regulation of adipogenesis

During adipogenesis fibroblast-like preadipocytes differentiate into lipid-laden, insulin sensitive adipocyte via a complex processes including coordinated changes in hormone sensitivity and gene expression (Lefterova and Lazar 2009; Fujimori et al. 2012). The treatment of confluent preadipocytes by the optimum combination of insulin, glucocorticoid and cAMP elevating agent during the clonal expansion phase can optimally trigger several signal transduction events, which eventually culminates into the enhanced expression of adipocyte-specific marker genes, and hence formation of mature adipose cells. Genes differentially regulated during adipogenesis has been categorized into early, intermediate and late mRNA/protein markers (Rangwala and Lazzar, 2000; Morrison and Farmer, 2000; Rosen and Spiegelman, 2000; Boone et al. 2000; Ntambi and Young 2000). This process involves the orchestrated activation of several signal transduction events in which numerous signaling molecules play very important roles through cell membrane receptors and nuclear receptors (Ailhaud 1999). Based on two-dimensional electrophoresis of cell extract before, during, and after adipose conversion, researchers have estimated that

at least 300 proteins are differentially expressed from the commencement of hormonal and biochemical induction up to the commitment of mature adipocyte phenotype (Sadowski et al. 1992).

Among numerous signaling molecules CCAAT/enhancer binding protein β and δ (C/EBPβ and C/EBPδ) have been reported to be the first transcription factors induced after exposure of preadipocytes to differentiation cocktail containing dexamethasone, insulin and 3-isobutyl-1-methylxanthine (IBMX) in presence of fetal bovine serum (FBS) (Aubert et al. 2000; Cornelius et al. 1994; Ntambi and Young 2000; Otto and Lane 2005). Among these inducers IBMX increases cAMP, which in turn control the expression of numerous genes, including C/EBPβ, through protein kinase A (PKA) mediated phosphorylation of cAMP responsive element binding protein (CREB) (Reusch et al. 2000; Zhang et al. 2004). The induction of C/EBPβ and C/EBPδ has been reported to mediate the expression of two master regulators (Lefternova and Lazar 2009) of adipogenesis: C/EBPα (Lane et al. 1999) and peroxisome proliferator-activated receptor gamma (PPARγ) (Clarke et al. 1997; Wu et al. 1995; Ham et al. 2001). Although the expression of C/EBPα is not strictly adipocyte-specific, PPARγ is the most adipocyte-specific among the three PPAR isoforms (Gregorie et al. 1998). After activation PPARγ and C/EBPα can cross-regulate the expression and activity of each other (Shao and Lazar 1997). Moreover combined expression of these two factors is reported to possess synergistic effect on the differentiation of 3T3-L1 adipocytes by up-regulating the expression of several adipocyte-specific genes encoding proteins and enzymes involved in the accumulation of lipid (Tontonz et al. 1994a; Gregorie et al. 1998). For example, PPARγ and C/EBPα alone or in combination have been reported to co-ordinate the activation of genes encoding adipocyte-specific fatty acid binding protein (aP2) (Tontonoz et al 1994b; Herrera et al. 1989), GLUT4, adiponectin, (Long and Pekala 1996) and leptin (Hollenberg et al. 1997).

1.3 Role of polyunsaturated fatty acid (PUFA) and cyclooxygenase cascade in adipogenesis

Arachidonic acid, an omega-6 (n-6) polyunsaturated fatty acid, is released from the membrane phospholipids in response to a variety of stimuli. After release with the help of distinct enzymatic pathways such as cyclooxygenase and lipoxygenase pathways arachidonic acid is converted into bioactive lipid mediators, which are also known as

eicosanoids (Needleman et al. 1986; Yamamoto 1992). The rate-limiting step in the biosynthesis of prostanoids is the conversion of arachidonic acid into prostaglandin H_2, which is catalyzed by the prostaglandin endoperoxide H synthases-1 and 2 (PGHS-1 and PGHS-2) also known as cyclooxygenases-1 and 2 (COX-1 and COX-2) (Smith et al. 2000). The constitutive COX-1 is mainly utilized in the immediate PG biosynthesis, which occurs within several minutes after stimulation with Ca^{2+} mobilizers, where as the inducible COX-2 is an absolute requirement for delayed PG biosynthesis, which last for several hours after triggering by proinflamatory stimuli (Rahman et al. 2013). PGH_2 is subsequently used as substrate by several terminal prostaglandin synthases for the biosynthesis of corresponding prostaglandins. Prostaglandins produced by arachidonate cyclooxygenase pathway possess a diverse range of functions depending on the PG types and cell target. Among those effects, prostaglandins and their metabolites play either pro-adipogenic or anti-adipogenic role in controlling the differentiation of preadipocytes to mature adipocytes. For example, PGE_2 and $PGF_{2\alpha}$ are reported as anti-adipogenic (Serrero et al. 1992a and b) on the other hand, PGI_2, PGD_2, 15-deoxy $\Delta^{12,14}$-PGJ_2 have been reported as pro-adipogenic (Forman et al. 1995a and b; Forman et al. 1997; Kliewer et al. 1995; Massiera et al. 2003) prostaglandins. Polyunsaturated fatty acids (PUFA) of n-3 series, for example eicosapentaenoic acid (EPA), docosahexaenoic acid decreased adipose tissue mass and suppress the development of obesity in rodents whereas n-6 PUFAs (arachidonic acid or linoleic acid) exerted either anti- or pro-adipogenic effects depending on the experimental conditions when used for triggering adipogenesis in growth arrested post-confluent preadipocytes, (Madsen et al. 2005).

1.4 Role of prostacyclin in adipogenesis

Prostacyclin also known as prostaglandin (PG) I_2 is an unstable metabolite, which is converted rapidly to 6-keto-$PGF_{1\alpha}$ by spontaneous hydrolysis (Kelton and Blajchman 1980). Earlier studies have suggested that prostacyclin analogues can act as a potent inducer of pre-adipocyte differentiation. For example, carbaprostacyclin, a stable analogue of prostacyclin stimulates terminal differentiation of Ob1771 mouse pre-adipose cells and 3T3-F442A cells in serum-free hormone-supplemented medium (Negrel et al. 1989; Catalioto et al. 1991). A recent study using a cell-based reporter gene assay, in HEK-293 cells stably expressing the IP receptor, has reported that PPARγ, 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-knockout mice compared with wild-type mice when mother mice were fed high-fat diet supplemented with linoleic acid, suggesting the contribution of the signaling through the IP receptor to adipose tissue development (Massiera et al. 2003). In addition, our previous 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 (Rahman et al. 2014). But interestingly, Hopkins and Gorman reported that the repeated addition of exogenous PGI_2 to cultured 3T3-L1 cells inhibited insulin- and indomethacin-mediated adipocyte differentiation (Hopkins and Gorman 1981).

Prostacyclin has been reported to be the primary product of COX-2 in many systems (McAdam et al. 1999) and preferentially synthesized by preadipocytes (Hyman et al. 1982). In our earlier study, we reported that the biosynthesis of prostacyclin was gradually suppressed after the induction of differentiation by dexamethasone, insulin and IBMX; but this suppression was rescued during the maturation phase, when the terminally differentiating 3T3-L1 cells were exposed to DMEM containing 10% FBS and 5 μg/ml insulin (Rahman et al. 2014). This suppressed biosynthesis of PGI_2, during the induction of differentiation, might be attributed to the presence of dexamethasone, an anti-inflammatory synthetic compound acting through the glucocorticoid receptor (Goppelt-Struebe et al. 1989). Recently Lasa et al reported that dexamethasone can destabilize COX-2 mRNA by inhibiting mitogen-activated protein kinase (MAPK) p38 (Lasa et al. 2001).

1.5 Intertwined relationship of PUFAs, prostaglndins and cAMP in the life cycle of adipocytes

Besides prostaglandins another potent effector of adipogenesis is the second messenger molecule, cyclic AMP (cAMP) an important intracellular signaling molecule, generally control several signal transduction events via the activation of protein kinase A (PKA) (Kato et al. 2007). Arachidonic acid through the synthesis of prostacyclin (Négrel 1999) and PGE_2 (Tsuboi et al. 2004) is able to increase the cAMP level (Hans-Erik 1980). Conversely, Griesmacher and colleagues reported that the endothelial cells of human umbilical vein is able to release prostacyclin that is dependent on intracellular Ca^{2+} ion

mobilization but independent of intracellular cAMP level (Griesmacher et al. 1992). Moreover, Williams and co-workers reported that, forskolin is able to stimulate prostacyclin synthesis in rabbit heart that was independent of cAMP but dependent on intracellular and extracellular calcium (Williams and Malik 1990). However, forskolin, a potent receptor-independent adenylyl cyclase activator, rapidly elevated cAMP synthesis and under this condition cultured mouse peritoneal macrophages were able to suppress the synthesis of prostacyclin measured as 6-keto-$PGF_{1\alpha}$ (Chang et al. 1984). A widely used component of adipogenic cocktail, 3-isobutyl-1-methylxanthine (IBMX) through the inhibition of phosphodiesterases (PDE) elevates the level of cAMP (Fawcett et al. 2000). Though baseline cAMP is necessary for triggering the differentiation of 3T3-L1 cells, elevation of cAMP above a critical level is reported to activate anti-adipogenic cellular signaling processes, which results in reduced fat accumulation (Li et al. 2008; Petersen et al. 2003).

The pro- or anti-adipogenic effect of n-6 polyunsaturated fatty acids (PUFAs) is attributed to the balance between carbohydrate and protein in diet of C57BL/6JBomTac mice. A high protein/carbohydrate ratio in diet is translated into a high glucagon/insulin ratio in blood, which enhances cAMP-dependent signaling. In this condition of augmented cAMP-dependent signaling, n-6 PUFAs are able to stimulate the production of anti-adipogenic prostaglandins. This anti-adipogenic action can be restored by lowering the cAMP level either by the exclusion of n-6 PUFA or IBMX or by suppressing anti-adipogenic prostanoid synthesis by inclusion of cyclooxygenase inhibitor (Madsen et al. 2008). These results also support the notion that augmented synthesis of PGE_2 and $PGF_{2\alpha}$ can thwart the conversion preadipocytes to mature adipocytes (Reginato et al. 1998). Alternatively, Casimir and co-workers observed reduced production of endogenous $PGF_{2\alpha}$ in differentiating cells compared to un-stimulated preadipocytes and hence they concluded that $PGF_{2\alpha}$ production by preadipocytes is one of the several prerequisites for the maintenance of the undifferentiated state (Casimir et al. 1996). Moreover, during the early phase of adipogenesis, enhancement of COX-2 activity, through $PGF_{2\alpha}$-activated FP receptor/extracellular-signal-regulated kinase (ERK)/cyclic AMP response element binding protein (CREB) cascade, preadipocytes can enhance the production of $PGF_{2\alpha}$ and PGE_2, and thus can form positive feed back loop of inhibitory action (Ueno and Fujimori 2011).

1.6 Mitotic clonal expansion and cAMP-PKA-dependent process

Growth-arrested confluent preadipocytes, if exposed to appropriate mitogenic and adipogenic signals, re-enter the cell cycle (Qiu et al. 2001) and undergo at least one round of DNA replication followed by the doubling of cell numbers (Gregorie et al. 1998), which is termed as mitotic clonal expansion (Lefterova and Lazar, 2009). Cyclic AMP (cAMP) is an important intracellular signaling molecule, generally control several signal transduction events via the activation of cAMP-dependent protein kinase A (PKA) (Kato et al. 2007). The activation of PKA is necessary for mitotic clonal expansion (MCE) (Martini et al. 2009), and for cAMP responsive element binding protein (CREB)-dependent stimulation of CCAAT/enhancer binding protein β and δ (C/EBPβ and C/EBPδ) gene expression (Belmonte et al. 2001). Both MCE and C/EBPβ and C/EBPδ gene expression are very important events during first 2 days after induction of differentiation (Gregorie et al. 1998). In the hierarchy of molecular and cellular events the expression of C/EBPβ and C/EBPδ is followed by the expression of PPARγ and C/EBPα: the master regulators (Lefternova and Lazar 2009) of adipocyte conversion (Gregorie et al. 1998; Cornelius et al. 1994; Ntambi and Young 2000; Clarke et al. 1997; Wu et al. 1995). C/EBPα through its antimitotic activity (Umek et al. 1991) and PPARγ through its ability to induce cell cycle withdrawal are strongly implicated in the growth arrest (Altiok et al. 1997). MCE is followed by the expression of adipogenic genes leading to adipocyte differentiation (Bernlohr et al. 1985; Conrnelius et al. 1994; MacDougald and Lane 1995). Numerous studies support the necessity of MCE in the differentiation of preadipocytes to mature adipocytes. For example, tumor necrosis factor alpha (TNFα) inhibits 3T3-L1 cell differentiation by blocking the MCE through the perturbation of p130 and p107 protein levels: two members of retinoblastoma protein family, which are involved in the regulation of cell cycle events (Lyle et al. 1998). Consistent with this, Patel and Lane reported that inhibition of calpain activity by N-acetyl-Leu-Leu-norleucinal (ALLN), during the early stages, blocks both MCE and differentiation of 3T3-L1 cells (Patel and Lane 2000). However, controversial evidences made the role and necessity of MCE, and its mediator PKA, obscure in the differentiation of 3T3-L1 preadipocytes. For example, cAMP-PKA-dependent signaling is necessary for MCE but cAMP-PKA-independent signaling is necessary for the differentiation of 3T3-L1 preadipocytes (Martini et al. 2009).

Although PKA was initially thought to be the only protein activated by cAMP;

PKA-independent cAMP-mediated cellular signaling processes have been reported (Chin and Abayasekara 2004; Holz et al. 2006; Kanda and Wantanabe 2007; Yin et al. 2006), which are also involved in controlling adipogenesis. For example, cAMP can activate Epac (exchange protein directly activated by cAMP); synergistic action between Epac and PKA is necessary to promote adipogenesis of 3T3-L1 cells (Petersen et al. 2008). Additionally, activated PKA can activate protein kinase B (PKB also known as Akt), through a PI3-kinase-independent pathway (Filippa et al. 1999). Cyclic AMP mediated activation of PKA and PKB is necessary for the transcriptional activation of PPARγ (Kim et al. 2010). Arachidonic acid through the synthesis of prostacyclin (Négrel 1999) and 3-isobutyl-1-methylxanthine (IBMX) through the inhibition of phosphodiesterases (PDE) (Fawcett et al. 2000) are able to elevate the level of cAMP; therefore co-administration of these two agents in the adipogenic cocktail may result in excessive elevation of cAMP level and activation PKA-dependent processes. All these evidences consolidate the notion that mitotic clonal expansion, a milestone in the highway of preadipocyte differentiation, is not an unavoidable step in the process of adipogenesis.

1.7 Prostaglandins can promote and block adipogenesis through cell surface and nuclear receptors

Certain cell surface receptors (Tsuboi et al. 2004) and nuclear receptors (Yu et al. 1995) are involved in controlling the series of signal transduction events leading to the commitment of preadipose cells to mature adipocytes (Ailhaud 1999). Extensive studies over last few decades have reveled that prostaglandins, which are naturally produced in different life stages of adipocytes, play a variety of roles in adipogenesis through the cell-surface receptors and nuclear receptors (Clarke et al. 1997). For example, prostacyclin produced near the cell membrane can act as an autocrine or a paracrine effector molecule by activating the G-protein coupled seven transmembrane IP-receptor of the same cell or neighboring cells respectively, to activate adenylyl cyclase and increase cAMP (Narumiya et al. 1999; Wise 2003). On the other hand, prostacyclin produced near the nucleus if transported into the nucleus may act as a ligand for nuclear receptors: peroxisome proliferator-activated receptors (PPARs), the nuclear receptor superfamily of ligand-activated transcription factors, both in presence and in absence of functional IP receptor and can influence gene expression and thus may be considered as an intracrine mediator of cell signaling process (Lim and Dey 2002). For example, prostacyclin

analogues are able to induce the activation of PPARα and PPARδ in monkey kidney CV-1 and human hepatoma HepC2 cells not expressing the IP receptor (Hertz et al. 1996). In a recent study, Falcetti and co-workers reported that human embryonic kidney (HEK-293) cells stably expressing the IP receptor are able to activate PPARγ through the cell surface IP receptor via a cAMP-independent mechanism by stable prostacyclin analogues (Falcetti et al. 2007). In our earlier study we reported that cell surface IP-receptor, which is expressed in preadipocytes, is temporarily suppressed after hormonal induction of differentiation and up-regulated during the terminal differentiation and eventually down-regulated in the matured adipocytes. This peaks and valleys in the expression of IP receptor was also reflected as synchronized augmentation and diminution of the expression of prostacyclin synthase (PGIS) and hence prostacyclin itself. This enhancement of IP receptor mediated activity was also confirmed by the ability of IP agonists to partially rescue cyclooxygenase inhibitor suppressed adipogenesis during the maturation phase of 3T3-L1 cells (Rahman et al. 2014).

Prostaglandins are also able to interact with PPARγ, the master regulator of adipogenesis and thus can promote or block adipogenesis (Reginato et al. 1998). For example, derivatives of prostaglandin D_2 (PGD_2) including 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) and Δ^{12}-prostaglandin J_2 (Δ^{12}-PGJ_2) are ligands of PPARγ (Forman et al. 1995b; Mazid et al. 2006). In our earlier studies, we have also reported that the biosynthesis of 15d-PGJ_2 (Mazid et al. 2006) and Δ^{12}-PGJ_2 (Syeda et al. 2012a), natural ligands of PPARγ, are increased during the maturation phase of adipogenesis when the gene expression of PPARγ also reach to its maximum level. Conversely, anti-adipogenic prostaglandin PGE_2, which is synthesized mainly by undifferentiated preadipocytes, through EP4 receptor, is able to increase intracellular cAMP level (Fujimori, 2012), which inhibits PPARγ activity and eventually down-regulates the early stages of 3T3-L1 preadipocytes differentiation (Tsuboi et al. 2004). Another antiadipogenic prostaglandin, $PGF_{2\alpha}$, blocks adipogenesis through the activation of mitogen-activated protein kinase, resulting in inhibitory phosphorylation of PPARγ. Both mitogen-activated protein kinase activation and PPARγ phosphorylation are required for the anti-adipogenic effects of $PGF_{2\alpha}$. Thus, PG signals, generated through cell surface receptor, regulate the activities of a nuclear hormone receptor that can also be directly activated or inhibited by other PGs. The balance among PGI_2, PGE_2, $PGF_{2\alpha}$, 15d-PGJ_2 and Δ^{12}-PGJ_2, and their signaling may be

central to the development of obesity and diabetes (Reginato et al. 1998).

1.8 PPARγ the master regulator of adipogenesis

The three peroxisome proliferator-activated receptor (PPAR) isotypes, PPARα, PPARβ (also known as PPARδ), and PPARγ form a subfamily of nuclear receptors mainly involved in lipid and glucose homeostasis, regulation of food intake and body weight, control of inflammation and wound healing (Chawla et al. 1994; Farmer 2005). Among these three, PPARγ is preferentially expressed expressed in adipose tissue (Tontonz et al. 1994b). Ectopic expression of PPARγ in non-adipogenic mouse fibroblasts, which is known as gain of function study, revealed that PPARγ alone can initiate the entire adipogenic program with the accomplishment of mature adipocyte phenotype (Tontonz et al. 1994a). Conversely, loss of function studies reconfirms the necessity of PPARγ in fat cell formation (Farmer 2006; Kim et al. 2010). Transfection of dominant-negative mutant of PPARγ into mature 3T3-L1 adipocytes causes loss of lipid accumulation and decreased expression of adipocyte markers, suggesting the requirement of PPARγ for the maintenance of differentiated state (Tamori et al. 2002). PPARγ null fibroblasts and embryonic stem cells as well as dominant negative PPARγ mutant were failed to differentiate in vitro (Kubota et al. 1999; Rosen et al. 1999; Barroso et al. 1999). Numerous reports gathered in last few years consolidated the notion that no transcriptional regulator has been discovered that promotes adipocyte differentiation in the absence of PPARγ; hence all pro-adipogenic factors somehow exert their effect through stimulating PPARγ gene expression or activity (Christodoulides and Vidal 2010). Conversely, many of the anti-adipogenic factors exert their activity by suppressing the gene expression of PPARγ or by deactivating PPARγ by post-translational modification (Reginato et al. 1998; Fujimori 2012; Anghel 2007). Thus numerous studies uphold the notion that PPARγ activity is absolutely necessary for adipogenesis, and no factor has been discovered until now which can promote adipogenesis in the absence of PPARγ activity (Christodoulides and Vidal 2010).

1.9 Aim of the present studies

By considering the intertwined role of cAMP, PUFAs and their products prostaglandins and the receptors of prostaglandins in adipogenesis, in this study, we attempted to determine the effect of the pretreatment of cultured 3T3-L1 preadipocytes with AA during the differentiation phase without IBMX. Our aim of this study was to investigate the impact

of this pre-treatment upon adipogenesis of cultured adipocytes after the maturation phase, and to seek the cellular mechanisms underlying the effect of AA. Moreover, archidonic acid can be converted to prostacyclin and the prostacyclin can elevate cAMP level as well as can interact with not only cell surface IP receptor but also nuclear receptor PPARγ. The gene expression of these two receptors reaches to the maximum level during the maturation phase of 3T3-L1 cells. Hence in the subsequent part of our study, we attempt to determine the specific action of the parent prostacylin and the related agonists or antagonists for the specific IP receptor on adipogenesis in combination with the agents that influence the activation of PPARγ and the elevation of cAMP. We discuss the mode of how prostacylin affects adipogenesis through the IP receptor during the maturation phase of adipocytes.

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Chapter 2

Pretreatment of cultured preadipocytes with arachidonic acid during the differentiation phase without a cAMP-elevating agent enhances fat storage after the maturation phase

1. Introduction

Adipose tissue functions as the storage site of triacyglycerols, which can be used for the mobilization of free fatty acids depending on nutritional status and hormonal response. The body fat mass is controlled by adipogenesis involving the differentiation of preadipocytes into mature adipocytes. Excess uptake of fuel molecules and lower energy expenditure are well known to generate obesity with an increase in the number or size of white adipocytes. In addition, adipocytes serve as an endocrine organ to secrete various bioactive molecules called adipokines, such as leptin, adiponectin, and resistin, to affect other tissues in vivo (Camfield et al. 1955; Kadowaki and Yamaguchi 2005; Sethi and Hotamisligul 1999). Hypertrophic adipocytes from obese tissue show alterations in the profiles of adipokines to secrete pro-inflammatory factors including tumor necrosis factor-α, interleukin-6, and monocyte chemoattractant protein-1, which are associated with adipocyte inflammation and insulin resistance (Permana et al. 2006). Under these conditions, free fatty acids are released to impair the oxidation of glucose though the inhibition of glucose uptake in adipose tissue and other organs, contributing to the onset of insulin resistance (Boden et al. 1994). However, certain types of fatty acids and their metabolites have been shown to act as endogenous ligands for peroxisome proliferator-activated receptor (PPAR) γ, which serves as a master regulator of adipogenesis and a positive regulator of insulin sensitivity (Yu et al. 1995). Different classes of free fatty acids, such as saturated, monounsaturated, n-6 and n-3 polyunsaturated fatty acids, are considered to affect the adipogenic process in different manners. Nevertheless, the cellular mechanisms underlying these opposite effects have not been understood fully.

Arachidonic acid (AA), a member of n-6 polyunsaturated fatty acids, can be converted to several prostanoids with pro-adipogenic or anti-adipogenic effects through the arachidonate cyclooxygenase (COX) pathway with two types of COX isoforms, the rate-limiting enzymes of this pathway (Smith et al. 2000). A previous animal study described that heterozygous mice deficient for the COX-2 gene exhibit more increased fat

mass as compared with wild-type mice, suggesting the functional coupling of COX-2 with the generation of anti-adipogenic prostanoids (Fain et al. 2001). Preadipogenic mouse 3T3-L1 cells have been utilized as a useful model cell culture system for studying different life stages of adipogenesis under the defined cultured conditions including growth, differentiation, and maturation phases (Green and Kehinde 1974; Green and Kehinde 1975). Recent studies have established that prostaglandin (PG) E_2 and $PGF_{2\alpha}$ are synthesized preferentially in cultured 3T3-L1 preadipocytes and serve as anti-adipogenic prostanoids (Xu et al. 2006). PGE_2 has been shown to inhibit the differentiation of cultured 3T3-L1 cells through its specific EP4 receptor, one of the receptor subtypes for PGE2 (Tsuboi et al. 2004). Alternatively, $PGF_{2\alpha}$ can also inhibit the differentiation of 3T3-L1 preadipocytes into the adipocytes by interacting with its FP receptor (Reginato et al. 1998). On the other hand, previous studies have reported the selective expression of lipocalin-type PGD synthase (L-PGDS) necessary for the biosynthesis of PGD_2 after the maturation phase of cultured 3T3-L1 cells (Jowsey et al. 2003; Xie et al. 2006). PGD_2 readily undergo the non-enzymatic dehydration to give biologically active PGJ_2 derivatives including 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2) and Δ^{12}-PGJ_2 (Fitzpatrick and Wynalda 1983; Shibata et al. 2002). Of these, 15d-PGJ_2 is the most potent natural activator for the nuclear hormone receptor PPARγ (Forman et al. 1995; Kliewer et al 1995). We have also shown that cultured adipocytes after the maturation phase have the ability to increasingly produce endogenous PGs of J_2 series and contribute to the up-regulation of adipogenesis (Mazid et al. 2006). Therefore, PGD_2 and the related PGJ_2 derivatives can be regarded as pro-adipogenic prostanoids. More recently, we have reported that endogenous synthesis of prostacyclin, PGI_2, is also positively regulated after the maturation phase of cultured 3T3-L1 adipocytes (Rahman et al. 2014).

Cultured 3T3-L1 preadipocytes have been usually exposed to the differentiation medium supplemented with 3-isobutyl-1-methylxanthine (IBMX), insulin, and dexamethasone to induce the program to drive the resting cells into adipocytes (Xu et al. 2006; Tsuboi et al. 2004; Mazid et al. 2006; Rahman et al. 2014; Casimir et al. 1996; Kamon et al. 2001; Petersen et al. 2003). The addition of exogenous AA to the differentiation medium has been shown to suppress the differentiation of cultured 3T3-L1 preadipocytes (Casimir et al. 1996; Kamon et al. 2001; Petersen et al. 2003). On the other hand, earlier reports described that exogenous AA in the culture medium without IBMX was effective to induce the differentiation of Ob1771 preadipose cells (Gaillard et al 1989;

Catalioto et al. 1991) and 3T3-F442A cells (Gaillard et al 1989). Hence, we hypothesized that the opposite effects of exogenous AA on adipogenesis in different cell lines could be explained by presence or absence of IBMX, a cAMP elevating agent. In this study, we attempted to determine the effect of the pretreatment of cultured 3T3-L1 preadipocytes with AA during the differentiation phase without IBMX on adipogenesis of cultured adipocytes after the maturation phase, and to seek the cellular mechanisms underlying the effect of AA.

2. Materials and methods

2.1 Materials

Dulbecco's modified Eagle's medium with HEPES (DMEM-HEPES), penicillin G potassium salt, streptomycin sulfate, dexamethasone, recombinant human insulin, fatty acid-free bovine serum albumin, and ExtraAvidin-peroxidase conjugate were obtained from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS) was the product of MP Biochemicals (Solon, OH, 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). Biotin-conjugated rabbit anti-mouse IgG antibody was supplied by Jackson ImmunoResearch Laboratories (West Grove, PA, USA). AA, aspirin, indomethacin, H-89, authentic fatty acids, PGs, MRE-269, CAY10441, and cAMP EIA kit were purchased from Cayman Chemical (Ann Arbor, MI, USA). M-MLV reverse transcriptase (RT) (Ribonuclease H minus, point mutant) and polymerase chain reaction (PCR) MasterMix were obtained from Promega (Madison, WI, USA). Oligonucleotides used for the PCR amplification were provided by Sigma Genosys Japan (Ishikari, Japan). 96-Well microplates for enzyme-linked immunosorbent assay (ELISA) were supplied by BD Falcon (Durham, NC, USA), and other Petri dishes for cull culture were from Asahi Glass (Tokyo, Japan). Antibodies specific for PGE_2, $PGF_{2\alpha}$, and 6-keto-$PGF_{1\alpha}$ were prepared in our laboratory and used for the development of ELISA for each of them as described earlier (Xu et al. 2006; Rahman et al. 2014; Shono et al. 1988; Yokota et al. 1996). All other chemicals used here are of reagent or tissue culture grade.

2.2 Cell culture of 3T3-L1 cells and differentiation to adipocytes

The mouse 3T3-L1 preadipogenic cell line (JCRB9014) was obtained from JCRB Cell Bank (Osaka, Japan). The cells were plated at $5x10^4$ cells/ml in the growth medium (GM) containing DMEM-HEPES, 10% FBS, 100 units/ml penicillin G, 100 μg/ml streptomycin sulfate, and 200 μM ascorbic acid, and grown until confluence at 37 °C under 7% CO2. Under the standard culture conditions, the confluent monolayer cells were exposed to the differentiation medium (DM) supplemented with 1 μM dexamethasone, 0.5 mM IBMX, and 10 μg/ml insulin for 2 days to enter the differentiation phase as described earlier (Xu et al. 2006; Rahman et al. 2014; Lu et al. 2004). To promote the storage of fats in adipocytes during the maturation phase, the treated cells were cultured furthermore in the maturation medium (MM) with 5 μg/ml insulin for a total of 10 days by replacing with the fresh MM every 2 days.

2.3 Adipogenesis treatments

To assess the influence of the pretreatment of cultured preadipocytes with AA or other compounds during the differentiation phase without IBMX on the storage of fats after the maturation phase, the confluent monolayer cells were mainly exposed to DM supplemented with 1 μM dexamethasone and 10 μg/ml insulin without IBMX for 48 h during the differentiation phase in the presence or absence of 50 μM AA or either of other compounds including various polyunsaturated fatty acids at 50 μM, prostanoids and MRE-269 at 1 μM, 100 nM PGI_2, 500 μM aspirin, 1 μM indomethacin, 0.1 μM or 1 μM CAY10441, and 20 μM H-89. The compounds to be tested were dissolved in ethanol as a vehicle and added to DM to adjust the volume of ethanol to 0.2%. After this pretreatment, the cells were cultured furthermore in the standard MM as above up to 10 days of the maturation phase to determine the accumulation of triacylglycerols. Moreover, gene expression levels of adipocyte marker proteins were evaluated by harvesting the cells at different days after the maturation phase. Cell number of cultured cells attached to the surface of Petri dishes was counted by suspending the cells after the incubation with 0.05% trypsin and 0.53 mM EDTA in phosphate-buffered saline for 5 min.

2.4 Prostanoid quantification by ELISA

PGE_2, $PGF_{2\alpha}$, and 6-keto-$PGF_{1\alpha}$ levels were measured in DM of preadipocytes exposed to vehicle or 50 μM AA for 48 h during the differentiation phase. Briefly, for the

quantification of PGE_2 by its specific ELISA, the conjugate of PGE_2 and fatty acid-free bovine serum albumin was used as an immobilized antigen in 96-well microplates for ELISA. The immobilized antigen in each 96-well was incubated competitively with a diluted mouse monoclonal antibody specific for PGE_2 in a standard or a sample to be tested. The resulting immunocomplex was detected spectrophotometrically by monitoring the peroxidase activity using 0-phenylenediamine as a substrate after binding to biotin-conjugated rabbit anti-mouse IgG antibody and the subsequent ExtrAvidin-peroxidase conjugate as described previously (Yokota et al. 1996). Alternatively, to determine the amounts of $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ reflecting the biosynthesis of PGI_2, a polyclonal mouse antiserum specific for each of them was prepared and used for the development of the respective solid-phase ELISA using the corresponding immobilized antigen as reported earlier (Rahman et al. 2014). For the measurement of the levels of each prostanoid biosynthesized during the differentiation phase, the fresh culture medium of DM without IBMX was used for the generation of a standard curve for each prostanoid species.

2.5 Gene expression analysis

Total RNA was extracted from cultured 3T3-L1 cells during the differentiation phase or the cultured adipocytes after the maturation phase by the method of acid guanidium thiocyanate/phenol/chloroform mixture (Chomczynski and Sacchi 1987). For the specific detection of mRNA levels of target genes, total RNA (1 μg) from each dish was subjected to the amplification of desired DNA fragments by RT-PCR using M-MLV reverse-transcriptase (Ribonuclease H minus, point mutant) and 1 x PCR MasterMix as described previously (Xu et al. 2006; Lu et al. 2004). In brief, for the synthesis of single stranded cDNA by the RT reaction, oligo-(dT)15 and a random 9 mer (Promega) were used as primers. The cDNA fragments for the target genes from mouse were amplified by PCR in a semi-quantitative manner using a combination of 5'- and 3'-primers specific for each of PPARγ, C/EBPα, LPL, GLUT4, adiponectin, leptin, aP2, and β-actin as reported earlier (Xu et al. 2006; Mazid et al. 2006; Chu et al. 2009). The amplified DNA fragments were separated by 1.5% agarose gel electrophoresis and detected by staining with ethidium bromide. For the confirmation of the target genes, the nucleotide sequences of those genes were determined by an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) after the sequencing reaction with BigDye Terminator v.1.1 Cycle Sequence Kit (Applied Biosystems) according to our previous methods (Xu et al. 2006; Chu et al. 2009).

For the determination of the transcript levels of COX isoforms, other biosynthetic enzymes, receptors, and adipogenic markers, quantitative real-time PCR was conducted with the kit of SYBR Premix Ex Taq (Tli RNaseH Plus) (Takara Bio Inc., Ohtsu, Japan) and analyzed by a Thermal Cycler Dice Real Time System Single (Takara Bio Inc.) according to the manufacturer's recommended procedures. Oligonucleotides used here were mouse sequences corresponding to-

5'-ATCGGCCTGGCCTTCTAAAC-3' as 5'-primer and

5'- CTGCCGAAGGTCCACCATTT-3' as 3'-primer for PPARγ,

5'- GCCAAGAAGTCGGTGGACA-3' as 5'-primer and

5'-GTCTCCACGTTGCGTTGTTT-3' as 3'-primer for C/EBPα,

5'-GGATTCCATCCCACAAGGCA-3' as 5'-primer and

5'-CCAACACGGCCAAGACATTG-3' as 3'-primer for GLUT4,

5'-TTTCACACACGCAGTCGGTA-3' as 5'-primer and

5'-CACATTTTGGGAAGGCAGGC-3' as 3'-primer for leptin,

5'-ACCATCTCTATCACTGGCATC-3' as 5'-primer and

5'-TATTCATTGAAGGGCTGTAGG-3' as 3'-primer for COX-1,

5'-GTTTGTTGAGTCATTCACCAG-3' as 5'-primer and

5'-CAGAATTGAAAGCCCTCTACA-3' as 3'-primer for COX-2,

5'-TTTCTGCTCTGCAGCACACT-3' as 5'-primer and

5'-GGGTCCCAGGAATGAGTACA-3' as 3'-primer for mPGES-1,

5'-TTGGAAAAACTGGGAGGATG-3' as 5'-primer and

5'-AAAATCCAGGCGATGACAAC-3' as 3'-primer for cPGES,

5'-CAAGCCTGAAGATCCGTCTC-3' as 5'-primer and

5'-CACCCTCCAGTTCCTGTTGT-3' as 3'-primer for PGF synthase (PGFS),

5'-TGCCAGCTTCCTTACCAGGAT-3' as 5'-primer and

5'-TTCTCCTCACTGGGGTTGAAA-3' as 3'-primer for PGI synthase (PGIS),

5'-GATGTTCATCTTCGGGGTGGT-3' as 5'-primer and

5'-GTGCTATAGTCACACAGTGCC-3' as 3'-primer for EP4,

5'-CATCTTCATGACAGTGGGGAT-3' as 5'-primer and

5'-GACTGATCAAAGCGGATCCAG-3' as 3'-primer for FP,

5'-CCTGCTGGAATATCACCTACG-3' as 5'-primer and

5'-GCATAGGCCACAAACACTGCA-3' as 3'-primer for IP,

5'-GAGCAAGAGAGGTATCCTGAC-3' as 5'-primer and

5’-GTGTTGAAGGTCTCAAACATG-3’ as 3’-primer for β-actin
The reaction was carried out at 95^0C for 30 s and followed by 40 cycles of amplification at 95^0C for 5s and 60^0C for 30 s. Following further reaction at 95^0C for 15 s and 60^0C for 30 s, the transcript levels of each target gene were determined by the normalization to those of β-actin as a control.

2.6 Triacylglycerol content and Oil Red O staining

The storage of fats was determined as the accumulation of triacylgycerols in adipocytes after the maturation phase using the Triglyceride E-Test Kit Chu et al. 2009; Chu et al. 2010). Data of triacylglycerol content were expressed as mg triacylglycerol per mg protein. The storage of oil droplets in adipocytes was stained with Oil Red O for the observation of cultured cells by differential-interference microscopy and macroscopic views of cultured dishes as described earlier (Kuri-Harcuch and Geen 1978).

2.7 Intracellular cAMP measurement

For the measurement of intracellular cAMP in cultured 3T3-L1 cells, the cells were plated at $5x10^4$ cells/ml and grown to confluence. The confluent cells were treated with vehicle, 50 μM AA, 100 nM PGI_2, 1 μM MRE-269, or a mixture of 50 μM AA and 0.1 μM CAY10441 for 30 min in DM without IBMX. After removing DM, cAMP was extracted from the cells with 0.1 M HCl and measured using a cAMP EIA kit according to manufacturer’ s instructions.

2.8 Other procedures

Cellular proteins were quantified by the method of Lowry et al. (Lowry et al. 1951) using bovine serum albumin as a standard after the proteins to be assayed were precipitated with cold trichloroacetic acid to remove the interfering substances (Markwell et al. 1981). Statistical significance was evaluated by the Student’s t test, and the difference was considered to be significant when $P<0.05$.

3. Results

3.1 Pretreatment of cultured preadipocytes with AA during the differentiation phase without IBMX stimulate the storage of fats after the maturation phase

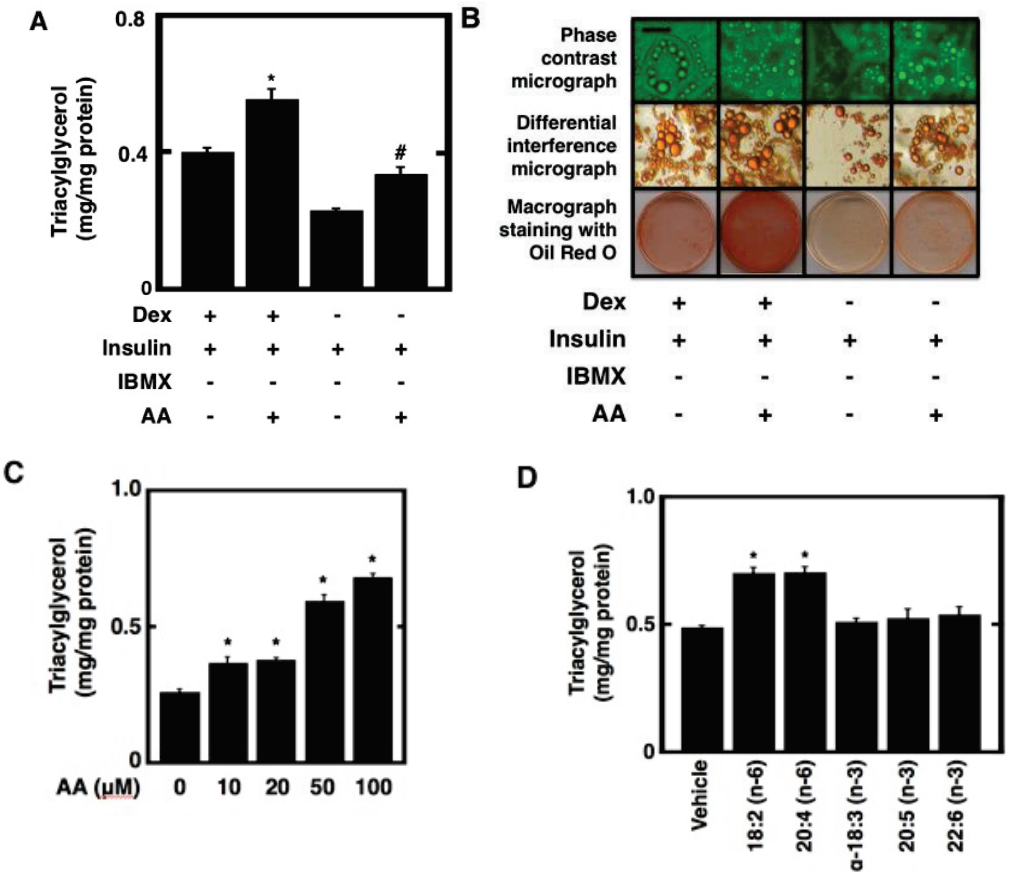

Fig. 2-1. Effect of the pretreatment of cultured preadipocytes with AA or either of n-6 and n-3 polyunsaturated fatty acids on the storage of fats after the maturation phase. 3T3-L1 cells were plated at 5 x 10^4 cells/ml in a 35-mm Petri dish containing 2 ml of GM and grown until 100% confluence. The resulting confluent cells were pretreated for 48 h during the differentiation phase with 2 ml of DM without IBMX in the presence or absence of 50 μM AA. 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. The mature adipocytes at the terminal differentiation were harvested and then subjected to the determination of the amounts of cellular triacylglycerols per mg protein (A). Data represent the mean ± S.E.M. of three independent experiments. *$p<0.05$ compared with the cells treated with vehicle in DM without IBMX. $^{\#}p<0.05$ compared with the cells treated with vehicle in DM without IBMX and dexamethasone. 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

representative of one of three experiments. Scale bar, 50 μm. Label: Dex, dexamethasone. Separately, the confluent preadipocytes were pretreated with increasing concentrations of AA (C) or either of n-6 and n-3 polyunsaturated fatty acids at 50 μM (D) for 48 h in DM without IBMX during the differentiation phase. The cells were furthermore cultured for a total of 10 days of the maturation phase as described above. The mature adipocytes were harvested for the determination of the amounts of cellular triacylglycerols per mg protein. Data represent the mean ± S.E.M. of three independent experiments. *$p<0.05$ compared with the control cells treated with vehicle. Labels: 18:2 (n-6), linoleic acid; 20:4 (n-6), arachidonic acid; 18:3 (n-3), α-linolenic acid; 20:5 (n-3), 5, 8, 11, 14, 17-eicosapentaenoic acid; 22:6 (n-3), 4, 7, 10, 13, 16, 19-docosahexaenoic acid.

Under the standard culture conditions of the differentiation phase of cultured 3T3-L1 cells, the confluent preadipocytes have been pretreated for 2 days with DM with dexamethasone, insulin, and IBMX as hormone stimulation prior to the further culture for adipogenesis after the maturation phase (Xu et al. 2006; Rahman et al. 2014; Lu et al. 2004; Bernlohr et al. 1985; Ham et al. 2001). IBMX is well known as a cAMP-elevating agent by acting as an inhibitor of cAMP phosphodiesterase (Beavo et al. 1970). When the confluent preadipocytes were pretreated with AA in the standard DM with IBMX during the differentiation phase, we also recognized the AA-dependent suppression of fat storage after the maturation phase (Fig. 2-S1) as described earlier (Casimir et al. 1996; Kamon et al. 2001; Petersen et al. 2003). On the other hand, the pretreatment of cultured preadipocytes with 50 μM AA during the differentiation phase without IBMX significantly enhanced the storage of fats regardless of the presence or absence of dexamethasone (Fig. 2-1A) without affecting the contents of cellular proteins. The increased number and size of oil droplets were confirmed by microscopic and macroscopic observations of cultured adipocytes treated with AA (Fig. 2-1B). Moreover, AA stimulated the storage of fats in a dose-dependent manner up to 100 μM (Fig. 2-1C). To determine the specificity for the stimulatory effect of AA on the storage of fats, we pretreated with either of different n-6 and n-3 polyunsaturated fatty acids during the differentiation phase without IBMX and determined the accumulation of triacylglycerols after the maturation phase (Fig. 2-1D).

The analysis revealed that n-6 polyunsaturated fatty acids including linoleic acid and AA enhanced the storage of fats to a significant extent. By contrast, n-3 polyunsaturated fatty acids, such as α-linolenic acid, 5, 8, 11, 14, 17-eicosapentaenoic acid, and 4, 7, 10, 13, 16, 19-docosahexaenoic acid, failed to stimulate the storage of fats. We then investigated whether the stimulated fat storage by AA could be due to a positive regulation of

adipogenesis. For this, cultured preadipocytes were pretreated similarly with or without AA and then cultured after the maturation phase up to 8 days.

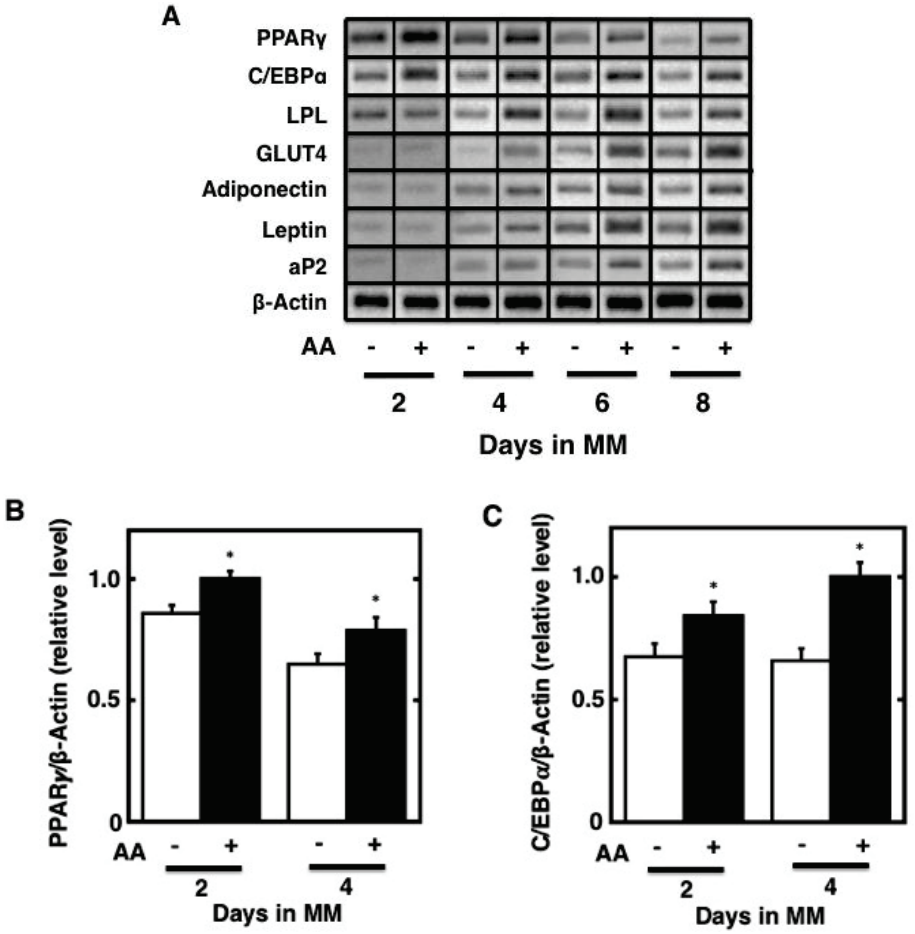

Fig. 2-2. Gene expression of adipocyte-specific markers during the maturation phase after the pretreatment of cultured preadipocytes with AA. 3T3-L1 cells were plated at 5 x 10^4 cells/ml in a 60-mm Petri dish containing 4 ml of GM and grown until confluence. The resulting confluent cells were pretreated with or without 50 μM AA for 48 h in DM without IBMX during the differentiation phase. The cells were furthermore cultured up to 8 days of the maturation phase by replacing every 2 days with fresh MM. At the indicated days, the mature adipocytes were harvested for the extraction of total RNA. The resulting RNA was used for the RT-PCR analysis of mRNA levels of PPARγ, C/EBPα, LPL, GLUT4, adiponectin, leptin, aP2, and β-actin (control) (A). Moreover, quantitative real-time PCR analysis of the transcript levels of GLUT4 (B) and leptin (C) was performed against those levels of β-actin. Data represent the mean ± S.E.M. of three independent experiments. *$p<0.05$ compared with the control cells treated with vehicle.

The gene expression analysis of adipocyte-specific markers provided the clear evidence for the increases in the transcript levels of PPARγ and CCAAT/enhancer-binding protein (C/EBP) α, master regulators of adipogenesis, and other downstream

adipocyte-specific genes, such as lipoprotein lipase (LPL), glucose transporter (GLUT)-4, adiponectin, leptin, and adipocyte protein 2 (aP-2) (Fig. 2-2A). The quantitative analysis of mRNA levels also confirmed the significant increases in the expression of PPARγ (Fig. 2-2B), C/EBPα (Fig. 2-2C), GLUT4 (Fig. 2-2D), and leptin (Fig. 2-2E). These findings support the positive regulation of adipogenesis for the storage of fats after the maturation phase due to the pretreatment of cultured preadipocytes with AA in the absence of IBMX.

3.2 Effect of the pretreatment of cultured preadipocytes with AA together with either of COX inhibitors, a protein kinase A (PKA) inhibitor, and a IP receptor antagonist, or with prostanoids on the storage of fats

To investigate whether the stimulatory effect of the pretreatment of cultured preadipocytes with AA during the differentiation phase without IBMX on the storage of fats after the maturation phase was caused by endogenous prostanoids, the cultured cells were co-incubated with aspirin or indomethacin, a specific inhibitor for COX, together with or without AA. The pretreatment with each of aspirin and indomethacin in the presence or absence of AA was found to be effective to inhibit significantly the accumulation of triacylglycerols after the maturation phase (Fig. 2-3A). Similarly, the microscopic views of cultured adipocytes after staining with Oil Red O supported the inhibitory effects of aspirin and indomethacin on the storage of fats in mature adipocytes when the cultured cells was pretreated with either of COX inhibitors in DM without IBMX (Fig. 2-3B). Next, we examined the effect of the pretreatment of cultured preadipocytes with each of different exogenous prostanoids and MRE-269, a specific IP agonist for PGI_2 receptor (Kuwano et al. 2007), during the differentiation phase without IBMX on the accumulation of fats after the maturation phase (Fig. 2-3C). The pretreatment with 1 μM MRE-269 was the most effective to simulate appreciably the storage of fats. In addition, the stimulatory effect of AA was significantly blocked by CAY104441, a selective IP antagonist (Clark et al. 2004) (Fig. 2-3D). Moreover, we confirmed that the effects of PGI_2 and MRE-269 were inhibited by CAY10441 (Fig. 2-S2). These findings suggest a predominant role for PGI_2 in the stimulation of adipogenesis program during the differentiation phase without IBMX. The supplementation of cultured preadipocytes with either of PGE_2 and $PGF_{2\alpha}$ in DM without IBMX caused the suppression of adipogenesis after the maturation phase. On the other hand, no stimulatory effects were observed by the pretreatment with either of PGD_2, Δ^{12}-PGJ_2, and 15d-PGJ_2.

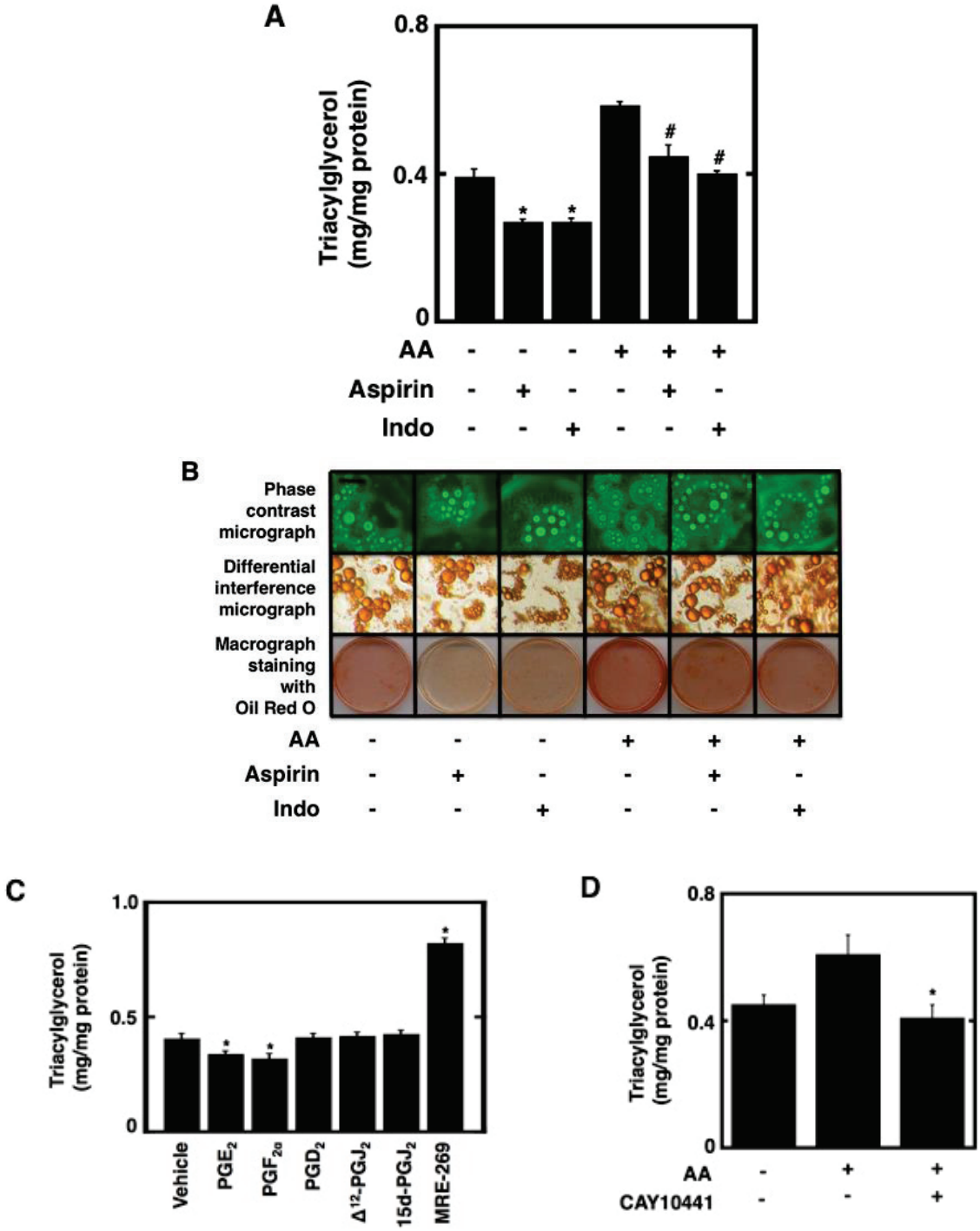

Fig. 2-3. Effect of the pretreatment of cultured preadipocytes with either of COX inhibitors together with AA, with either of prostanoids and a selective IP agonist, or with a selective IP antagonist together with AA on the storage of fats after the maturation phase. 3T3-L1 cells were plated and grown until confluence as described in Fig. 2-1. The resulting confluent cells were pretreated with either of 500 μM aspirin or 1 μM indomethacin in the presence or absence of 50 μM AA for 48 h in DM without IBMX during the differentiation phase. The cells were furthermore cultured for a total of 10 days of the maturation phase by

replacing every 2 days with fresh MM. The mature adipocytes were harvested for the determination of the amounts of cellular triacylglycerols per mg protein (A). Data represent the mean ± S.E.M. of three independent experiments. *$p<0.05$ compared with the control cells treated with vehicle. $^{\#}p<0.05$ compared with the cells treated with AA. 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 representative of one of three experiments. Scale bar, 50 μm. Label: Indo, indomethacin. Separately, the confluent preadipocytes were pretreated with either of prostanoids and MRE-269, a selective IP agonist, at 1 μM (C), or with 50 μM AA in the presence or absence of 0.1 μM CAY10441, a selective IP antagonist (D) for 48 h in DM without IBMX during the differentiation phase. The cells were furthermore cultured for a total of 10 days of the maturation phase as described above. The mature adipocytes were harvested for the determination of the amounts of cellular triacylglycerols per mg protein. Data represent the mean ± S.E.M. of three independent experiments. *$p<0.05$ compared with the cells treated with vehicle (C) or with AA (D).

PGI_2 has been shown to act through its specific receptor, the IP receptor, which is coupled with the generation of cAMP as a second messenger (Narumiya et al. 1999). The present study also revealed that all the pretreatments with AA, PGI_2, and MRE-269 were found to transiently increase the cAMP levels (Fig. 2-S3). Furthermore, the effect of AA to stimulate cAMP formation was appreciably abolished by CAY10441. This finding suggests the mediation of cAMP-dependent PKA in the action of AA during the differentiation phase. To determine the involvement of PKA in the stimulatory effect of the pretreatment with AA on adipogenesis, cultured preadipocytes were exposed to H-89, a specific PKA inhibitor (Engh et al. 1996), in the presence or absence of AA during the differentiation phase without IBMX. We found that H-89 suppressed the cell growth stimulated with AA during the differentiation phase without IBMX, indicating the interference of mitotic clonal expansion (Fig. 2-4A). However, the pretreatment of cultured preadipocytes with H-89 had no effect on the increase in the accumulation of fats caused by AA after the maturation phase (Fig. 2-4B).

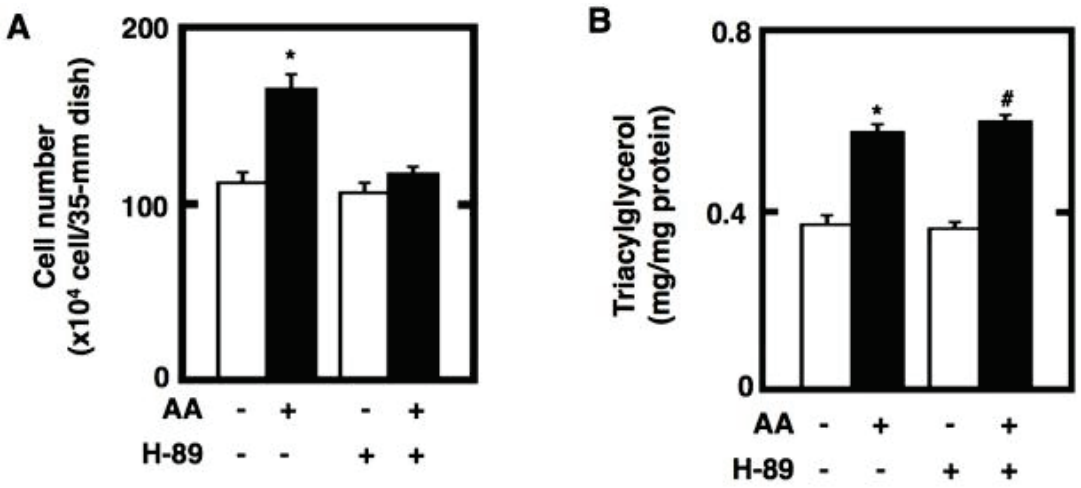

Fig. 2-4. Effect of the pretreatment of cultured preadipocytes with H-89, a specific inhibitor for PKA, on the storage of fats and cell growth after the maturation phase. 3T3-L1 cells were plated and grown until confluence as described in Fig. 2-1. The resulting confluent cells were pretreated with or without 50 μM AA in the presence or absence of 20 μM H-89 for 48 h in DM without IBMX during the differentiation phase. After the differentiation phase, cell number was measured (A). The pretreated cells were furthermore cultured for a total of 10 days of the maturation phase by replacing every 2 days with fresh MM. The mature adipocytes were harvested for the determination of the amounts of cellular triacylglycerols per mg protein (B). *$p<0.05$ compared with the control cells treated with vehicle. #$p<0.05$ compared with the cells treated with H-89.

3.3 Gene expression of isoformic enzymes in the COX pathway in cultured preadipocytes treated with AA

To evaluate the regulation of the arachidonate COX pathway in cultured preadipocytes supplemented with AA during the differentiation phase without IBMX, the transcript levels of COX isoforms were determined by quantitative real-time PCR. The mRNA levels of COX-1 (Fig. 2-5A) and COX-2 (Fig. 2-5B) tend to increase during the differentiation phase in the absence of AA. On the other hand, the supplementation with AA resulted in the substantial reduction in the transcript levels of COX-2 after 24 h of the differentiation phase while those of COX-1 reached to the steady levels.

We also investigated the gene expression of isoformic biosynthetic enzymes for PGE_2, $PGF_{2\alpha}$, and PGI_2 in cultured preadipocytes. The quantitative analysis revealed that the transcript levels of mPGES-1 increased after 24 h and 48 h of the differentiation phase in the absence of AA, whereas the treatment of the cells with AA induced a transient

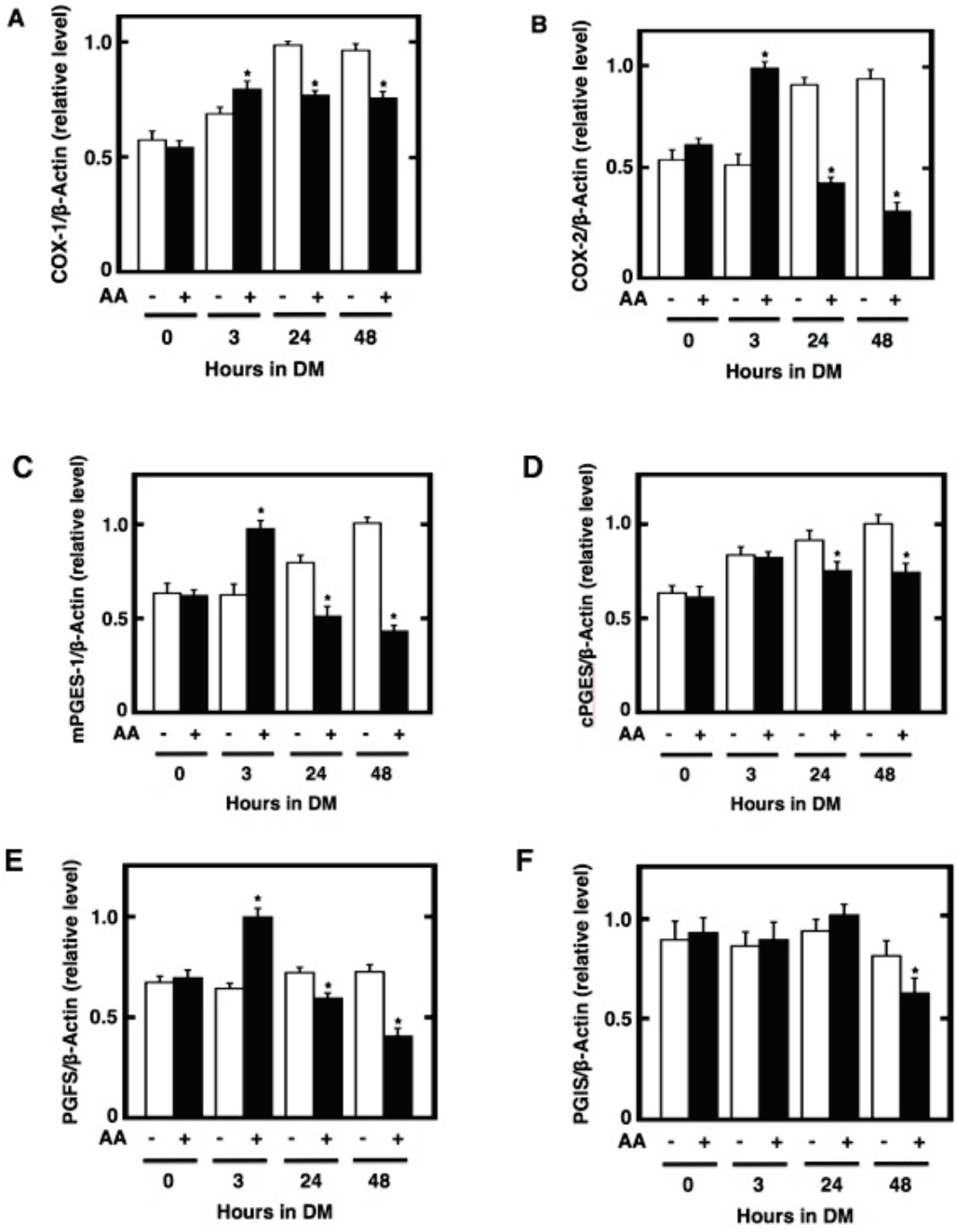

Fig. 2-5. Gene expression of COX isoforms and other isoformic enzymes in the arachidonate COX pathway in cultured preadipocytes treated with AA. 3T3-L1 cells were plated and grown until confluence as described in Fig. 2-2. The resulting confluent cells were treated with or without 50 μM AA for 48 h in DM without IBMX during the differentiation phase. At the indicated time, the treated cells were harvested for the extraction of total RNA. The resulting RNA was used for the quantitative real-time PCR analysis of mRNA levels of COX-1 (A), COX-2 (B), mPGES-1 (C), cPGES (D), PGFS (E), and PGIS (F) against those levels of β-actin (control). Data represent the mean ± S.E.M. of three independent experiments. *$p<0.05$ compared with the control cells treated with vehicle.

increase in the expression level around 3 h and then brought about the appreciable declines in those expression levels after 24 h (Fig. 2-5C). In contrast, the gene expression levels of cPGES remained steady in the presence of AA although the transcript levels are lower than those without AA (Fig. 2-5D). The mRNA levels of PGFS remained unchanged without supplementation of AA, whereas the treatment with AA induced the highest level after 3 h and resulted in a decrease in the level after 24 h (Fig. 2-5E). The gene expression levels of PGIS were found to be almost constant besides the supplementation with AA for 48 h (Fig. 2-5F). This finding indicates the constitutive expression of the arachidonate COX pathway for the generation of PGI_2. By contract, the gene expression level of L-PGDS was not appreciably detectable under the same conditions (data not shown).

3.4 Biosynthesis of prostanoids by cultured preadipocytes treated with AA

We subsequently determined the ability of cultured preadipocytes to synthesize endogenous prostanoids during the differentiation phase without IBMX. The amounts of endogenous prostanoids were determined by ELISA specific for each of prostanoids. The biosynthesis of PGE_2 was significantly suppressed in the presence of AA when compared with the treatment without AA (Fig. 2-6A). Similarly, the production of $PGF_{2\alpha}$ was found to be appreciably lower in cultured preadipocytes supplemented with AA (Fig. 2-6B). On the other hand, the addition of AA to DM without IBMX enhanced significantly the generation of 6-keto-$PGF_{1\alpha}$, the stable product derived from unstable PGI_2, by its spontaneous hydrolysis (Stehle 1982) (Fig. 2-6C). These results provided the evidence that endogenous synthesis of PGE_2 and $PGF_{2\alpha}$ serving as anti-adipogenic factors was down-regulated while the generation of pro-adipogenic PGI_2 was up-regulated by the supplementation of AA at the end of the differentiation phase without IBMX, a cAMP-elevating agent.

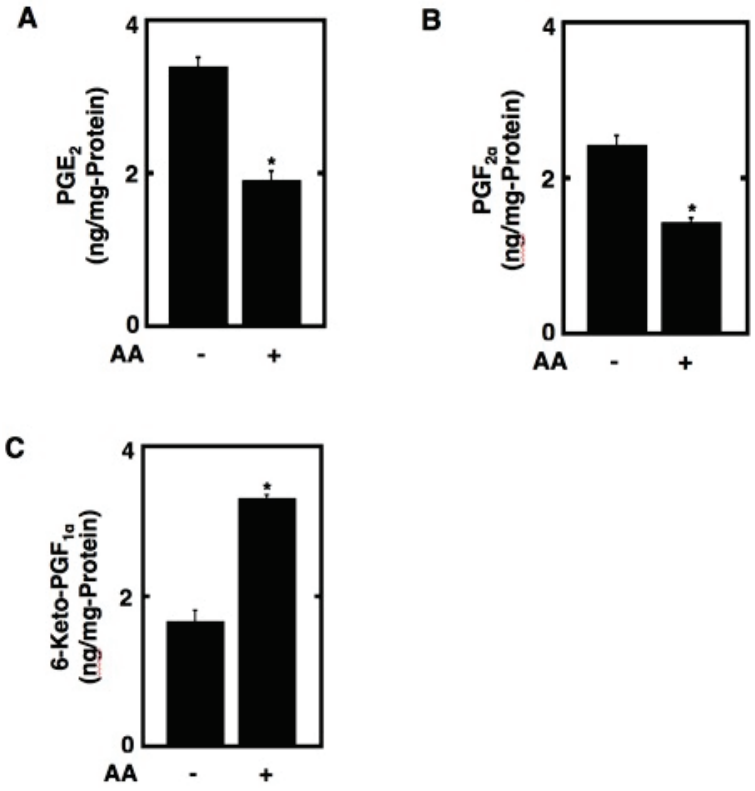

Fig. 2-6. Biosynthesis of prostanoids by cultured preadipocytes treated with AA. 3T3-L1 cells were plated and grown until confluence as described in Fig. 2-1. The resulting confluent cells were treated with or without 50 μM AA for 48 h in DM without IBMX during the differentiation phase. The culture medium was collected for the determination of the levels of PGE_2 (A), $PGF_{2\alpha}$ (B), and 6-keto-$PGF_{1\alpha}$ (C) by ELISA using specific antibodies for each of them. Data represent the mean ± S.E.M. of three independent experiments. *$p<0.05$ compared with the control cells treated with vehicle.

3.5 Gene expression of prostanoid receptors in cultured preadipocytes treated with AA

Moreover, the gene expression levels of prostanoid receptors were evaluated during the differentiation phase without IBMX in response to the addition of AA. A previous study has shown that EP4, a subtype of PGE_2 receptors, is expressed predominantly during the differentiation phase of cultured 3T3-L1 cells (Tsuboi et al. 2004). Here, we determined the transcript levels of EP4, FP, and IP corresponding to the specific receptors for PGE_2, $PGF_{2\alpha}$, and PGI_2. Quantitative real-time PCR analysis revealed that the transcript levels of EP4 (Fig. 2-7A) and FP (Fig. 2-7B) tended to increase in the absence of AA while those mRNA levels declined after 24 h of the supplementation with AA. In sharp contrast, the gene expression levels of IP remained relatively constant regardless of the presence and absence of AA throughout the differentiation phase with IBMX, suggesting the continued action of

IP (Fig. 2-7C). PGD_2 is known to exert its effect through two types of cell-surface receptor subtypes, DP1 (Boie et al. 1995) and chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) alternatively called DP2 (Hirai et al. 2001; Spik et al. 2005). Although the transcript levels of these two receptors became slightly detectable after 48 h of the differentiation phase without IBMX, the expression levels were much lower as compared with those of EP4, FP, and IP (data not shown).

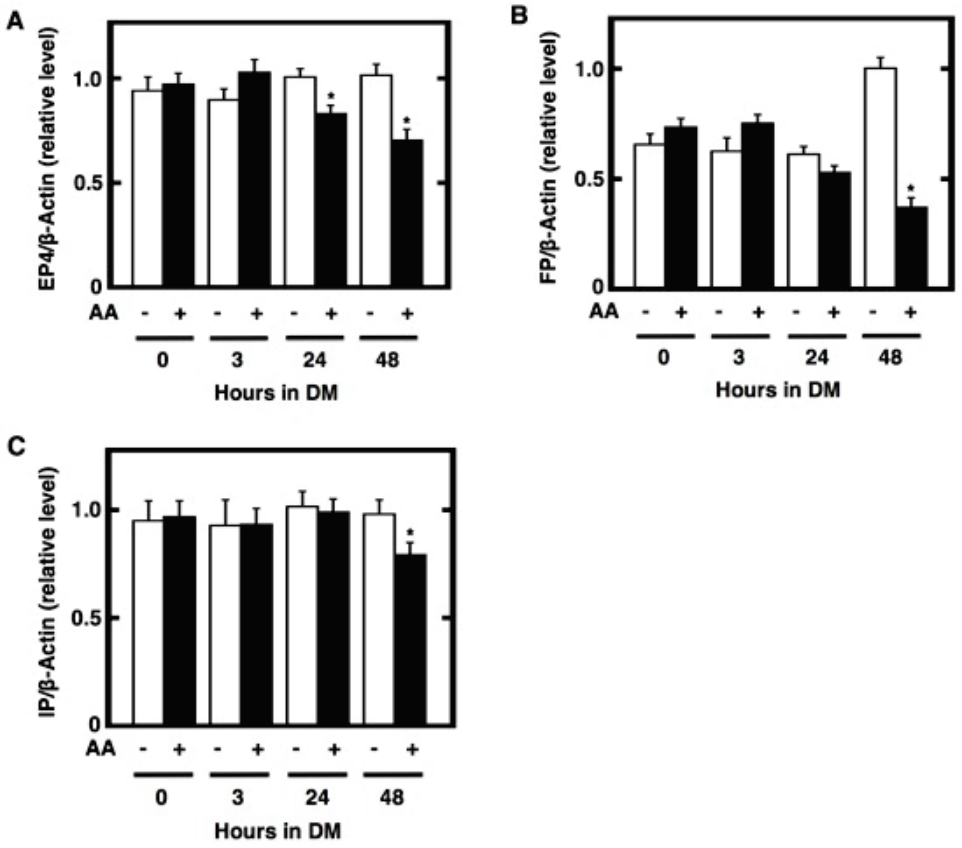

Fig. 2-7. Gene expression of prostanoid receptors in cultured preadipocytes treated with AA. 3T3-L1 cells were plated and grown until confluence as described in Fig. 2-2. The resulting confluent cells were treated with or without 50 μM AA for 48 h in DM without IBMX during the differentiation phase. At the indicated time, the treated cells were harvested for the extraction of total RNA. The resulting RNA was used for the quantitative real-time PCR analysis of the transcript levels of EP4 (A), FP (B), and IP (C) against those levels of β-actin (control). Data represent the mean ± S.E.M. of three independent experiments. *$p<0.05$ compared with the control cells treated with vehicle.

4. Discussion

Usually growth-arrested cultured 3T3-L1 preadipocytes are exposed to a hormone cocktail containing insulin, dexamethasone, and IBMX to trigger the differentiation stage to irreversibly program the fibroblasts to differentiate into adipocytes (Casimir et al. 1996; Bernlohr et al. 1985; Hamm et al. 2001). After the triggering of the differentiation phase, adipocytes accumulate triacylglycerols by stimulating adipogenesis after the maturation phase. In this study, we confirmed that the pretreatment of cultured 3T3-L1 cells with the standard DM without IBMX resulted in the appreciable attenuation of adipogenesis in mature adipocytes. This has been explained by the suppression of C/EBPβ activity affecting the subsequent expression of PPARγ due to the lack of IBMX, a cAMP-elevating agent, as described before (Hamm et al. 2001). In sharp contrast, our studies provided the evidence to show the efficacy of the pretreatment with AA in DM without IBMX to stimulate the storage of fats in adipocytes. Here, cultured preadipocytes were only exposed to AA in the standard DM without IBMX for 48 h and then replaced with MM after the DM was removed. These results indicate that the continuous presence of AA is not required to drive the conversion of preadipocytes to adipocytes. Moreover, we showed that the increase in the accumulation of fats was caused by the typical stimulation of adipogenesis as evident with precedent gene expression of C/EBPα and PPARγ serving as master regulators of later expression of adipocyte-specific genes including LPL, GLUT-4, adiponectin, leptin, and aP2, leading to the differentiated adipocyte phenotype.

The present study showed that the pretreatment with AA during the differentiation phase without IBMX stimulated the storage of fats in mature adipocytes. The effect of AA was found to be dose-dependent and the stimulation was observed similarly with linoleic acid, another n-6 family. By contrast, n-3 polyunsaturated fatty acids were ineffective. These findings suggest that certain AA metabolites would be responsible for the stimulatory effect on adipogenesis. Indeed, the stimulatory effect of the pretreatment with AA was appreciably reversed by the co-incubation with each of COX inhibitors such as aspirin and indomethacin. Furthermore, we recognized the substantial stimulatory effect of MRE-269, a selective agonist for PGI_2 (Kuwano et al. 2007), on the storage of fats among prostanoids, suggesting the predominant role for endogenous PGI_2 generated from exogenous AA. This idea was supported by the result that the stimulatory effect of the pretreatment with AA was abolished by CAY10441, a selective IP antagonist (Clark et al. 2004). As well, we confirmed that the stimulatory effects of exogenous PGI_2 and MRE-269

on adipogenesis were suppressed by CAY10441. Since PGI_2 is known to generate intracellular cAMP by acting on its specific IP receptor (Narumiya et al. 1999), we measured cAMP formation by AA, PGI_2, and MRE-269. Indeed, all the compounds were found to be effective to increase the generation of cAMP. Moreover, the stimulatory effect of AA on cAMP formation was blocked by CAY10441, additionally supporting the mediation of the IP receptor in the action of the endogenous PGI_2 produced from AA. The increased levels of intracellular cAMP by AA and other IP agonists did not inhibit adipogenesis presumably due to the much lower production of cAMP than those by IBMX although the presence of both IBMX and exogenous AA has been reported to inhibit terminal differentiation of 3T3-L1 adipocytes (Casimir et al. 1996; Kamon et al. 2001; Perersen et al. 2003). We also noticed that H-89, a specific PKA inhibitor (Engh et al. 1996), had no effect on the stimulation of adipogenesis by the pretreatment with AA. Taking these into account, the stimulation of adipogenesis by AA was caused by the specific action of endogenous PGI_2 through the IP receptor via cAMP-independent mechanism. Regarding the stimulatory effect of a PGI_2 analogue, earlier studies described the promoting effect of carbaprostacyclin, a stable analogue of PGI_2, on the terminal differentiation of other cell lines of cultured Ob1771 cells (Gaillard et al. 1989; Catalioto et al. 1991; Negrel et al. 1989) and 3T3-F442A cells (Gaillard et al. 1989; Auburt et al. 1996) although the culture conditions and cell properties are different.

Cultured 3T3-L1 preadipocytes have been shown to have the arachidonate COX pathway to generate endogenous prostanoids including PGE_2, $PGF_{2\alpha}$, and PGI_2 during the growth and differentiation phases under the standard culture conditions (Xu et al. 2006; Casimir et al. 1996; Hyman et al. 1982; Fajas et al 2003). The present study evaluated the effect of the pretreatment with AA on the gene expression of isoformic biosynthetic enzymes involved in the endogenous synthesis of the related prostanoids during the differentiation phase in DM deficient in IBMX. The gene expression levels of COX-2, mPGES-1, and PGFS similarly tended to increase after 24 h of the differentiation phase without IBMX and AA. In sharp contrast, we observed the transiently enhanced levels of their transcription and the subsequent significant reduction in the mRNA levels when the preadipocytes were exposed to AA. These results reconfirmed the inducible nature of those enzymes in our experimental system as described earlier in other cells (Smith et al. 2000). It remains still unknown about the detailed cellular mechanism underlying the difference between the presence and

absence of AA in DM without IBMX. On the other hand, the addition of AA resulted in the maintenance of steady expression levels of COX-1, cPGES, and PGIS, implying the relatively constitutive expression of those enzymes. In agreement with these results, we observed the appreciable suppression of the endogenous synthesis of PGE_2 and $PGF_{2\alpha}$ by the supplementation with AA, indicating the functional coupling of inducible COX-2 with mPGES-1 and PGFS. Contrary to these results, the endogenous production of 6-keto-$PGF_{1\alpha}$ derived from the unstable PGI_2 was elevated significantly by the treatment with AA. This observation could be explained by the biosynthesis of PGI_2 through the functional coupling of the constitutive COX-1 and PGIS. The combined results indicate that the pretreatment of cultured preadipocytes with AA in DM without IBMX caused the preferential reduction in the biosynthesis of PGE_2 and $PGF_{2\alpha}$ serving as anti-adipogenic factors and the stimulated biosynthesis of pro-adipogenic PGI_2 during the differentiation phase, eventually contributing to promotion of terminal adipogenesis.

The action of PGE_2 and $PGF_{2\alpha}$ was earlier demonstrated to occur through their specific EP4 (Tsuboi et al. 2004) and FP (Casimir et al. 1996) receptors, respectively, in cultured 3T3-L1 preadipocytes. On the other hand, until now, alternations in the gene expression levels of their prostanoid receptors have not been examined during the differentiation phase without IBMX by the addition of AA. Currently, we also detected the gene expression of the EP4, FP, and IP receptors for PGE_2, $PGF_{2\alpha}$, and PGI_2, respectively, in cultured preadipocytes treated with AA during the differentiation phase in DM deficient in IBMX. The results from our present study indicated that the supplementation of cultured preadipocytes with AA suppressed gradually the transcript levels of EP4 and FP after 24 h while their expression levels remained higher in the absence of AA. By contrast, we found the stable expression of IP during the differentiation medium regardless of the presence or absence of AA. These findings also support a specific role for endogenous PGI_2 formed from exogenous AA in programming 3T3-L1 preadipocytes to differentiate into adipocytes. The contribution of the IP receptor to adipose tissue development was earlier described in IP-deficient mice in which fat mass gain is suppressed as compared with wild-type mice (Massiera et al. 2003). However, the specific role of IP remains to be clarified at different stages of adipogenesis.

Taken together, the present study demonstrated that the pretreatment of cultured preadipocytes with AA during the differentiation phase without IBMX, a AMP-elevating

agent, stimulated the adipogenesis program to generate mature adipocytes. The stimulatory effect of AA was caused by decreased synthesis of anti-adipogenic prostanoids including PGE_2 and $PGF_{2\alpha}$ and enhanced generation of pro-adipogenic PGI_2. Moreover, relatively stable expression of the IP receptor and reduced expression of EP4 and FP by AA also could contribute to the positive regulation of differentiation program leading to terminal adipogenesis. Our findings identify a predominant role of endogenous PGI_2 in stimulatory effect of AA added to the differentiation medium deficient in IBMX.

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Supplementary Figures

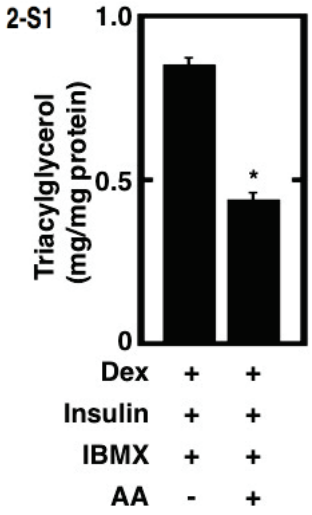
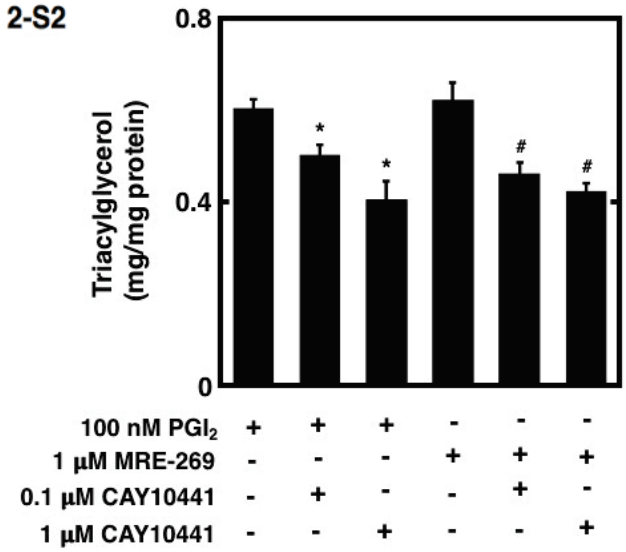
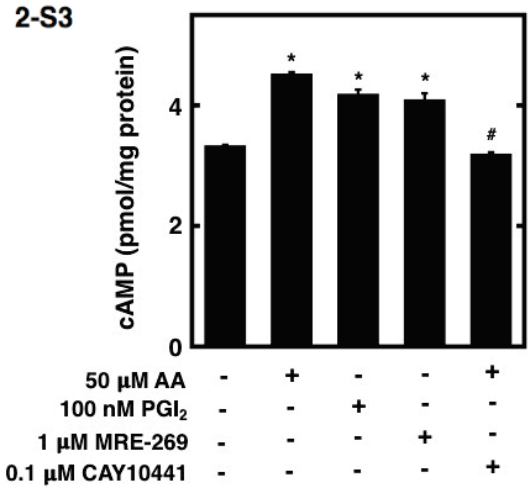


Fig. 2-S1. Addition of AA with the conventional adipogenic cocktail of dexamethasone, insulin and IBMX caused lowering of fact accumulation. 3T3-L1 cells were plated at 5 x 10^4 cells/ml in a 35-mm Petri dish containing 2 ml of GM and grown until 100% confluence. The resulting confluent cells were pretreated for 48 h during the differentiation phase with 2 ml of DM without IBMX in the presence or absence of 50 μM AA. 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. The mature adipocytes at the terminal differentiation were harvested and then

subjected to the determination of the amounts of cellular triacylglycerols per mg protein. Data represent the mean ± S.E.M. of three independent experiments. *p<0.05 compared with the cells treated with vehicle in DM without AA.



2-S2. Effect of the pretreatment of cultured preadipocytes with either of PGI_2 or MRE-269 together with CAY10441 on the storage of fats after the maturation phase. 3T3-L1 cells were plated at $5x10^4$ cells/ml in a 35-mm Petri dish containing 2 ml of GM and grown until 100% confluence. The confluent cells were pretreated for 48 h during the differentiation phase with 2 ml of DM containing either of 100 nM PGI_2 or 1 μM MRE-269 together with CAY10441 (0.1 μM or 1 μM). The cells were furthermore cultured in MM for 10 days by replacing every 2 days with 2 ml of fresh MM. The mature adipocytes at the terminal differentiation were harvested and then subjected to the determination of the amounts of cellular triacylglycerols per mg protein. Data represent the mean ± S.E.M. of three independent experiments. *p<0.05 compared with the cells treated with vehicle in DM with PGI_2. $^{\#}$p<0.05 compared with the cells treated with MRE-269.



2-S3. Effect of treatmenting cultured preadipocytes with either of PGI_2 or MRE-269 together with CAY10441 on the biosynthesis of cAMP. 3T3-L1 cells were plated at $5x10^4$ cells/ml in a 35-mm Petri dish containing 2 ml of GM and grown until 100% confluence. The confluent cells were treated with either of vehicle, 50 μM AA, 100 nM PGI_2, 1 μM MRE-269, or a mixture of 50 μM AA and 0.1 μM CAY10441 for 30 min in DM without IBMX as described in section 2.7 for the determination of cAMP. Data represent the mean ± S.E.M. of three independent experiments. *$p<0.05$ compared with the cells treated with vehicle in DM. $^{\#}p<0.05$ compared with the cells treated with AA in DM.

Chapter 3

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

1. 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 preadipoctyes 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, PPARγ, 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 PPARγ 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 PPARγ as natural ligands. Since some of J_2 series of prostaglandins (PGs) have been shown to be a potent activator of PPARγ (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 PPARγ can be converted to adipocytes by PPAR activators including 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2) (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 Kinde 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 PPARγ. 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, PGE_2 and $PGF_{2\alpha}$ are known as anti-adipogenic prostanoids in most preadipogenic cells. A previous study has reported that PGE_2 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 PGE_2 and $PGF_{2\alpha}$ 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 PGJ_2 derivatives including 15d-PGJ_2 (Mazid et al. 2006) and Δ^{12}-PGJ_2 (Hossain et al. 2011), by the non-enzymatic dehydration of PGD_2 in cultured 3T3-L1 cells during the maturation phase. We also confirmed that each of 15d-PGJ_2 and Δ^{12}-PGJ_2 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 PGJ_2 derivatives in the promotion of adipogenesis during the maturation phase.

Earlier studies described that carbaprostacyclin, a stable analogue of PGI_2 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 PGI_2 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 PGI_2 as determined by the amount of 6-keto-$PGF_{1\alpha}$ as the stable hydrolysis product of PGI_2 by its specific immunological assay, which is accompanied by the coordinated gene expression of PGI synthase and the prostanoid IP receptor for PGI_2 (Rahman et al. 2014). These findings lead us to suggest the pro-adipogenic action of endogenous PGI_2 as an autocrine factor in mature adipocytes. However, the action of natural PGI_2 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 prostacylin and the related agonists or antagonists for the specific IP receptor on adipogenesis in combination with the agents that influence the activation of PPARγ and the elevation of cAMP. We discuss the mode of how prostacylin affects adipogenesis through the IP receptor during the maturation phase of adipocytes.

2. Materials and methods

2.1 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). PGI_2 (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). Dibutyryl-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.

2.2 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 (Hamm 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 $5x10^4$ cells/ml in the growth medium (GM) containing DME-HEPES, 10% FBS, 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, and 200 μM ascorbic acid, and then cultured at 37 °C under 7% CO_2. The confluent

cells were replaced with the differentiation medium (DM) supplemented with 1 μM dexamethasone, 0.5 mM IBMX, and 10 μg/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 μg/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%.

2.3 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. (Lowry et al. 1951) after precipitated with cold 6% trichloroacetic acid to remove the interfering substances for the assays (Markwell et al. 1981).

2.4 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).

2.5 Others

The data on the storage of fats were normalized to protein content and are expressed as the mea ± S.E.M. 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 $P<0.05$.

3. Results

3.1 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 prostanoids, 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. 3-1A). The inhibitory effect of aspirin was rescued by the addition of exogenous PGI_2 (sodium salt) at each concentration of 50 nM 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. 3-1B). We then investigated effects of selective agonists for the IP receptor or PPARγ 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 (Kuwano et al. 2007), and treprostinil (Olschewski et al. 2004) at different concentrations of 0.1 μM 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 μM and 1 μM troglitazone (Willson et al. 2000), an activator of PPARγ (Fig. 3-2A). 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. 3-2B).

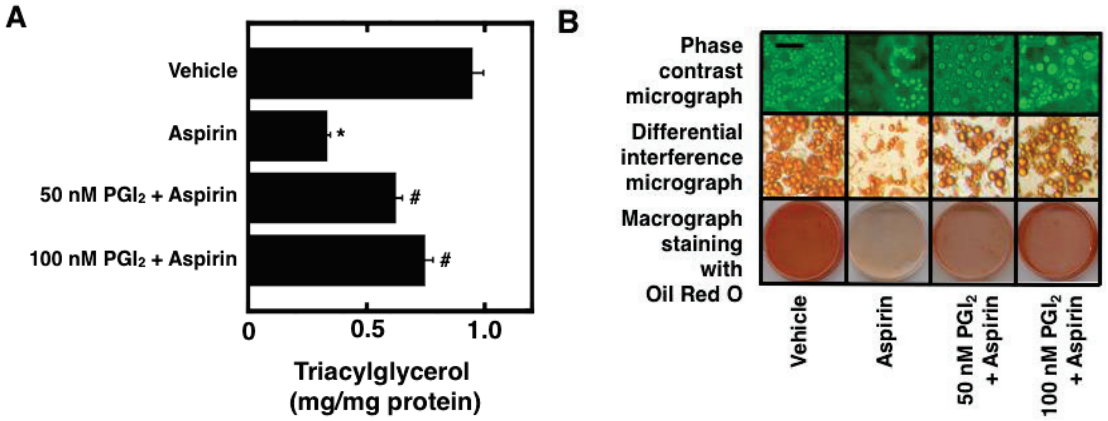


Fig. 3-1. Effect of sodium salt of PGI_2 together with aspirin on the storage of fats in cultured adipocytes during the maturation phase. 3T3-L1 cells were plated at 5 x 10^4 cells/ml in a 35-mm Petri dish containing 2 ml of GM and grown to 100% confluence. After the resulting cells were treated with 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 μM aspirin, or 50 nM or 100 nM PGI_2 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 ± S.E.M. 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 μm.

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 CAY10449 (Clark et al. 2004) at concentrations of 0.05 μM and 0.1 μM significantly suppressed the storage of fats as 0.1 μM and 1 μM GW9662 (Bendixen et al. 2001), a selective antagonist for PPARγ, did under the same culture conditions (Fig. 3-3A). 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. 3-3B). These results indicate that the pro-adipogenic action of PGI_2 can be explained by the action mediated through the IP receptor in cultured adipocytes.

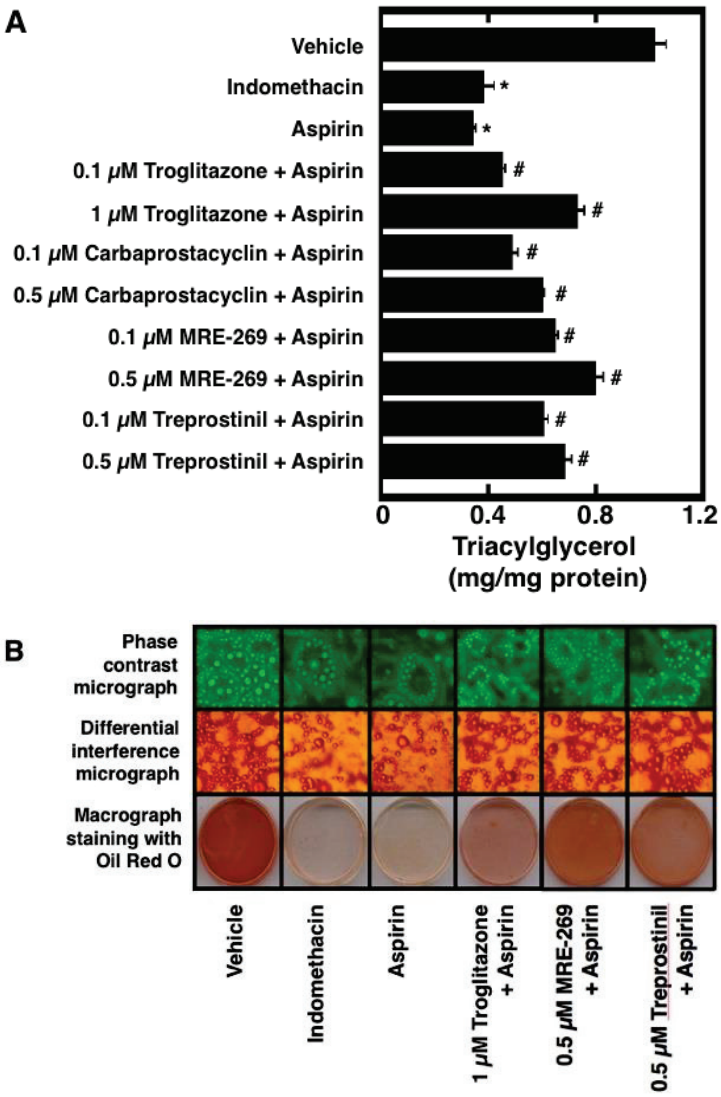


Fig. 3-2. Effect of selective agonists for the IP receptor or PPARγ together with aspirin on the storage of fats during the maturation phase. 3T3-L1 cells were cultured, differentiated, and matured to adipocytes as described in Fig. 3-1. During the maturation phase, cultured cells were treated for a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

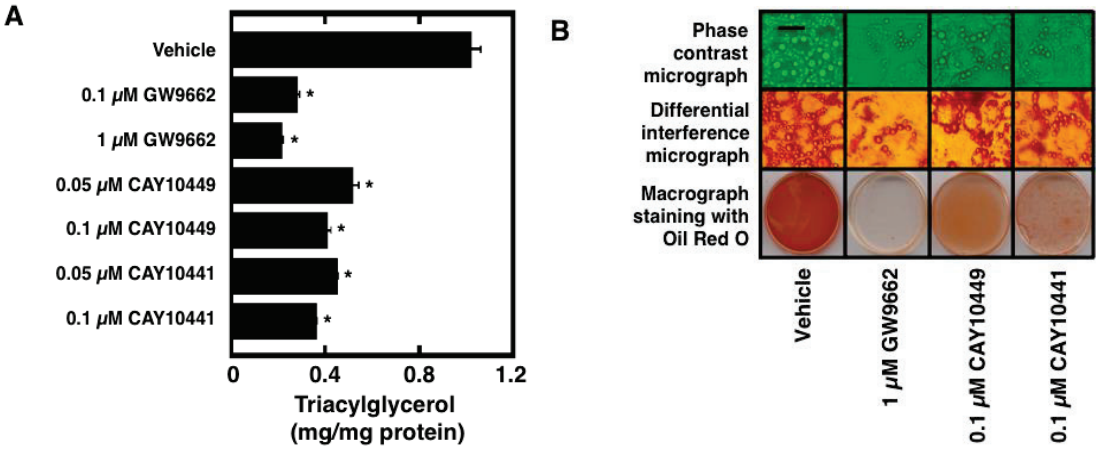


Fig. 3-3. Effect of selective antagonists for the IP receptor or PPARγ on the storage of fats during the maturation phase. 3T3-L1 cells were cultured, differentiated, and matured to adipocytes as described in Fig. 3-1. During the maturation phase, cultured cells were treated for a total of 10 days with 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 ± S.E.M. 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 μm.

3.2 Combined effect of a selective agonist for IP receptor and an activator for PPARγ 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 PPARγ, cultured cells are exposed to a mixture of troglitazone and PGI_2 or that of troglitazone and MRE-269 in the presence of aspirin during the maturation phase. The co-incubation with 1 μM troglitazone and 100 nM PGI_2 or 1 μM troglitazone and 0.5 μM MRE-269 resulted in significantly higher stimulation of fat storage than that with troglitazone, PGI_2, or MRE-269 alone (Fig. 3-4). 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 PPARγ exert an additive effect on the promotion of adipogenesis in cultured adipocytes during the maturation phase.

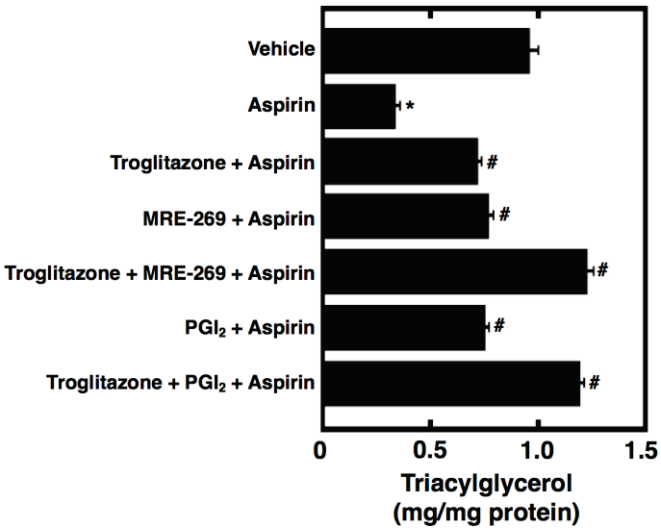


Fig. 3-4. Combined effect of selective agonists for the IP receptor and PPARγ on the storage of fats during the maturation phase. 3T3-L1 cells were cultured, differentiated, and matured to adipocytes as described in Fig. 3-1. During the maturation phase, cultured cells were treated for a total of 10 days with vehicle, 500 μM aspirin alone, or either of 1 μM troglitazone, 0.5 μM MRE-269, 100 nM PGI_2, a mixture of troglitazone and MRE-269, or a mixture of troglitazone and PGI_2 in the presence of 500 μM aspirin. The resulting cultured adipocytes were harvested for the determination of the amounts of cellular triacylglycerols. Data represent the mean ± S.E.M. 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.

We next examined the influence of GW9662, an antagonist for PPARγ, 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 CAY10441suppressed partly the storage of fats enhanced by the mixture (Fig. 3-5A). 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. 3-5B).

To determine the role of PPARγ 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 μM GW9662 or 0.1 μM 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

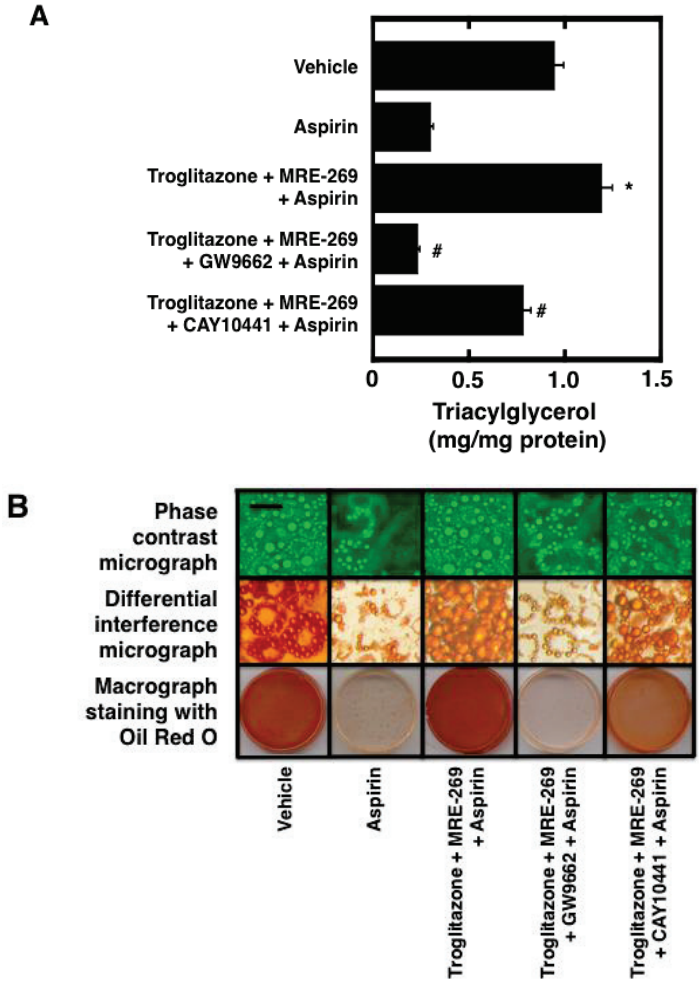


Fig. 3-5. Inhibitory effect of a selective antagonist for either the IP receptor or PPARγ on the storage of fats enhanced by a combination of troglitazone and MRE-269 in the presence of aspirin. 3T3-L1 cells were cultured, differentiated, and matured to adipocytes as described in Fig. 3-1. During the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

maturation phase even if higher concentrations were used (Fig. 3-6A). On the 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. 3-6B). The results indicate that the activation of PPARγ is essential as a downstream factor for the promotion of adipogenesis by the action of selective agonists for the IP receptor.

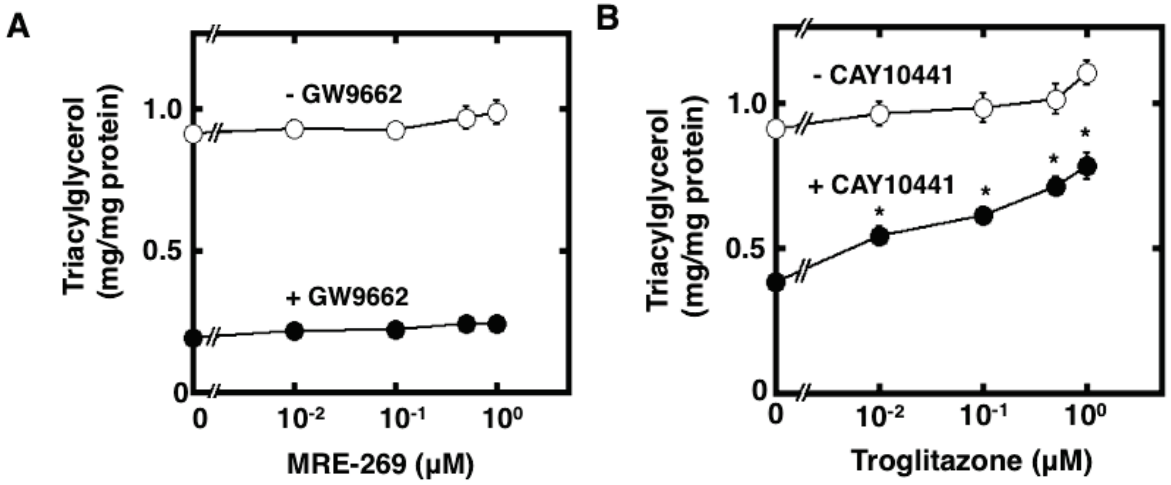


Fig. 3-6. Effect of a selective agonist for either the IP receptor or PPARγ on the storage of fats suppressed by a selective antagonist for either of them. 3T3-L1 cells were cultured, differentiated, and matured to adipocytes as described in Fig. 3-1. During the maturation phase, cultured cells were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

3.3 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 et al. 2003). Hence, to know the involvement of cAMP as a second messenger in the signal transduction of the action of PGI_2 and the related agonists, cultured adipocytes were exposed to cell-permeable stable cAMP analogues at 100 μM during the maturation phase (Fig. 3-7A). The compounds, such as dibutyryl-cAMP (Steinberg et al. 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 et al. 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. 3-7B). 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 μM 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.

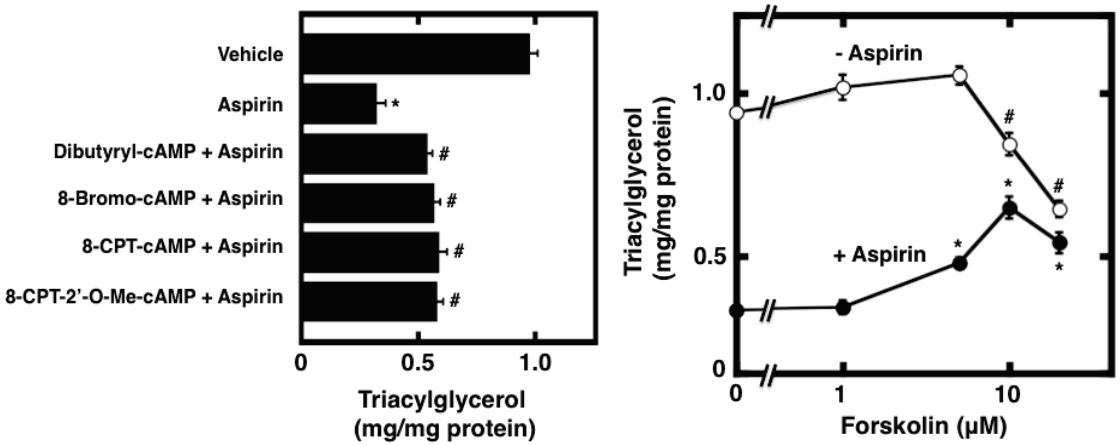


Fig. 3-7. Effect of cAMP analogues and forskolin on the storage of fats during the maturation phase. 3T3-L1 cells were cultured, differentiated, and matured to adipocytes as described in Fig. 3-1. (A) During the maturation phase, cultured cells were treated for a total of 10 days with either of cAMP analogues at 100 μM in the presence of 500 μM aspirin. The resulting cultured adipocytes were harvested for the determination of the amounts of cellular triacylglycerols. Data represent the mean ± S.E.M. 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 μM 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.

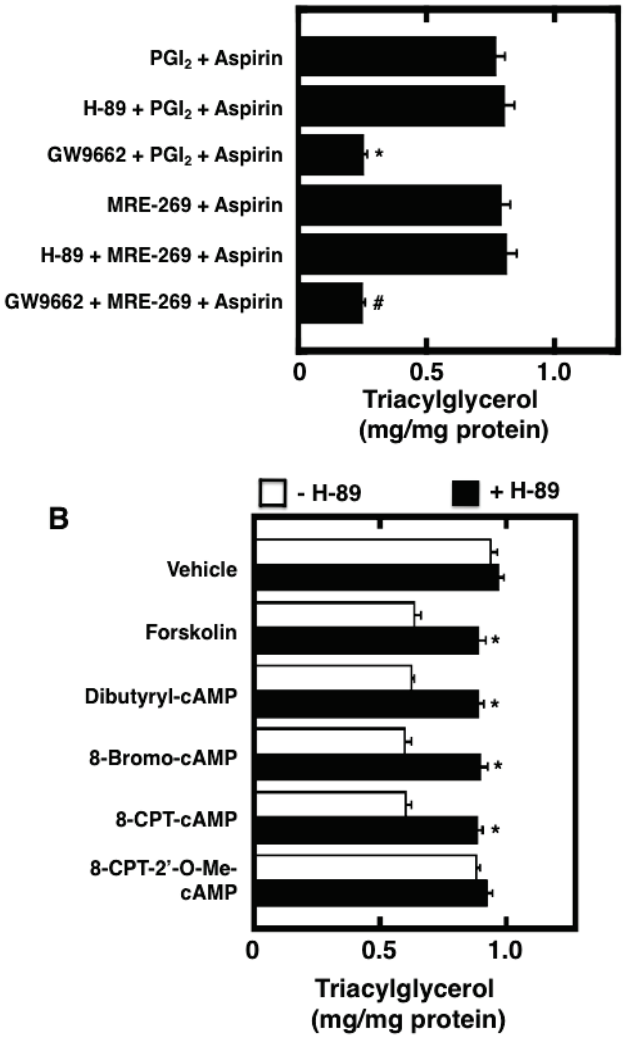


Fig. 3-8. Effect of H-89, a specific inhibitor for PKA, on the storage of fats during the maturation phase. 3T3-L1 cells were cultured, differentiated, and matured to adipocytes as described in Fig. 3-1. (A) During the maturation phase, cultured cells were treated for a total of 10 days with either of 10 μM H-89 or 1 μM GW9662 together with 100 nM PGI_2 or 0.5 μM MRE-269 in the presence of 500 μM aspirin. The resulting cultured adipocytes were harvested for the determination of the amounts of cellular triacylglycerols. Data represent the mean ± S.E.M. of three independent experiments. *$p<0.05$ compared with the cells treated with a mixture of 100 nM PGI_2 and 500 μM aspirin. $^{\#}p<0.05$ compared with the cells treated with a mixture of 0.5 μM MRE-269 and 500 μM aspirin. (B) Similarly, cultured cells during the maturation phase were exposed to vehicle, 20 μM forskolin, or either of cAMP analogues at 100 μM in the presence or absence of 10 μM 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.

Next, to determine the involvement of PKA activity in the stimulatory effects of PGI_2 and MRE-269 on adipogenesis in the presence of aspirin, we examined the influence of 10 μM 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 PPARγ. The observation indicates that the up-regulation of adipogenesis by PGI_2 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, dibutyrl-cAMP, 8-bromo-cAMP, and 8-CPT-cAMP, in the absence of aspirin (Fig. 3-8B). 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 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.

4. 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 Kinde 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 prostanoids 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 prostanoids such as PGD_2 and PGJ_2 derivatives during the maturation phase, which are involved in the stimulation of adipogensis (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 showed that natural PGI_2 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 PGI_2 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 PGI_2 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 PGI_2 at the concentration of 50 nM to rescue the inhibitory effect of aspirin. This dose is almost near to the level of endogenous levels of PGI_2 as determined by the level of 6-keto-$PGF_{1\alpha}$ 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 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 PPARγ, as well as by PGI_2 or MRE-269, a selective agonist for the IP receptor. Here, we found that a mixture of troglitazone and PGI_2 had more potent effect on the storage of fats than either troglitazone or PGI_2 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 PPARγ 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 PPARγ. On the other hand, CAY10441, a selective antagonist for the IP receptor, attenuated the storage of fats to a lower extent than that of PPARγ antagonist. The findings imply the crucial role of the activation of PPARγ in the pro-adipogenic effects of PGI_2 and MRE-269 through the IP receptor. This idea is supported additionally by the following our data. 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 PPARγ as a downstream signaling factor for the stimulation of adipogenesis during the maturation phase (Fig. 3-9).

PPARγ 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 PPARγ 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 PPARγ 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 PPARα and PPARδ (Forman et al. 1997). A novel pathway of prostacyclin signaling through PPARδ is thought to be operative in certain systems (Lim and Dey 2002). An earlier study has reported that PPARδ is expressed in cultured 3T3-L1 preadipocytes, but the activation of PPARδ only modestly promote terminal differentiation, indicating that the activation of PPARδ 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 PPARγ, 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 PPARγ 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 PPARγ in cultured 3T3-L1 adipocytes. Alternatively, PGI_2 may regulate the gene expression of PPARγ 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 PPARγ 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).

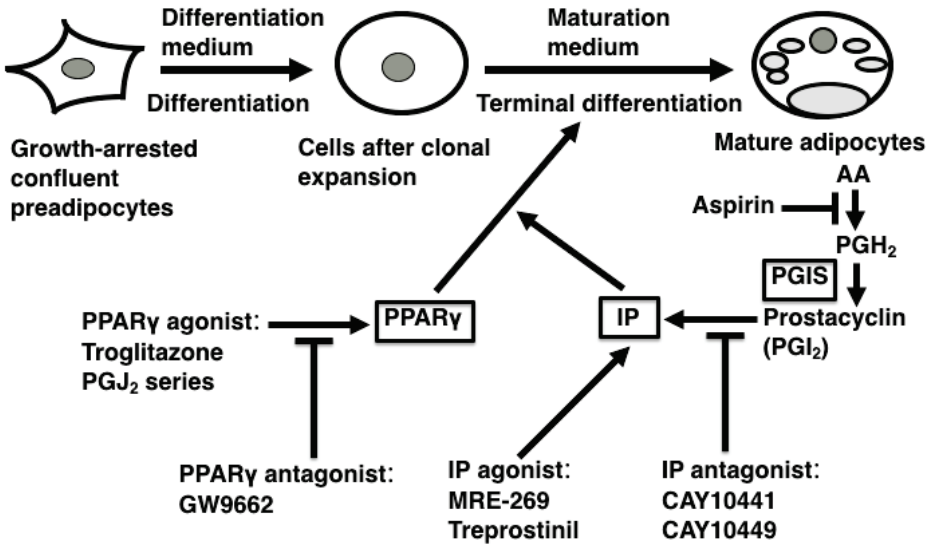


Fig. 3-9. Proposed mechanism for the action of prostacylin and its selective agonists on the stimulated adipogenesis through the IP receptor and PPARγ in cultured adipocytes during the maturation phase.

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 cAMP as an intracellular second messenger (Narumiya et al. 1999; Wise et al. 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 PGI_2 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 adipogeneis 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 PPARγ although prostacyclin and the related agonists are not known as the direct activator of PPARγ. These findings indicate that the action of prostacyclin through the IP receptor is linked with the activation of PPARγ 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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Chapter 4

Conclusion

Summary

The present study attempted to clarify the specific action of prostacyclin, alternatively termed prostaglandin (PG) I_2, on adipogenesis at different stages of cultured adipocytes from two points of view. Initially, I studied the influence of pretreatment of cultured preadipocytes with arachidonic acid during the differentiation phase without a cAMP-elevating agent on adipogenesis after the maturation phase. Next research was conducted to unravel the effects of prostacyclin and selective agonists of prostanoid IP receptor on the storage of fats during the maturation phase of cultured adipocytes.

Arachidonic acid (AA) and the related prostanoids exert complex effects on the adipocyte differentiation depending on the culture conditions and life stages. Here, I investigated the effect of the pretreatment of cultured 3T3-L1 preadipocytes with exogenous AA during the differentiation phase without 3-isobutyl-1-methylxanthine (IBMX), a cAMP-elevating agent, on the storage of fats after the maturation phase. This pretreatment with AA stimulated appreciably adipogenesis after the maturation phase as evident with the up-regulated gene expression of adipogenic markers. The stimulatory effect of the pretreatment with AA was attenuated by the co-incubation with each of cyclooxygenase (COX) inhibitors. Among exogenous prostanoids and related compounds, the pretreatment with MRE-269, a selective agonist of the IP receptor for PGI_2, strikingly stimulated the storage of fats in adipocytes. The gene expression analysis of arachidonate COX pathway revealed that the transcript levels of inducible COX-2, membrane-bound PGE synthase-1, and PGF synthase declined more greatly in cultured preadipocytes treated with AA. By contrast, the expression levels of COX-1, cytosolic PGE synthase, and PGI synthase remained constitutive. The treatment of cultured preadipocytes with AA resulted in the decreased synthesis of PGE_2 and $PGF_{2\alpha}$ serving as anti-adipogenic PGs although the biosynthesis of pro-adipogenic PGI_2 was up-regulated during the differentiation phase. Moreover, the gene expression levels of EP4 and FP, the respective prostanoid receptors for PGE_2 and $PGF_{2\alpha}$, were gradually suppressed by the supplementation with AA, whereas that of IP for PGI_2 remained relatively constant. Collectively, these results suggest the predominant role of endogenous PGI_2 in the stimulatory effect of the pretreatment of

cultured preadipoccytes with AA during the differentiation phase without IBMX on adipogenesis after the maturation phase.

My laboratory has previously shown that cultured adipocytes have the ability to biosynthesize PGI_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, I 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 PGI_2 and the related selective agonists for the IP receptor including MRE-269 and treprostinil rescued the storage of fats attenuated by aspirin, a COX 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 PGI_2 and MRE-269 together with troglitazone, an activator for PPARγ, 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 PPARγ 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 PGI_2 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.

Summary in Japanese

今回の研究は、培養脂肪細胞の異なるステージでの脂肪細胞形成に対する、別名、プロスタグランジン（PG）I_2と呼ばれるプロスタサイクリンの特異的な作用を、２つの観点から解明することを試みた。最初に、cAMP 濃度を増加させる因子のない条

件下で、分化誘導期の培養前駆脂肪細胞をアラキドン酸で前処理したときの成熟期の脂肪細胞形成に対する効果を研究した。そして、次の研究は、培養脂肪細胞の成熟期における脂肪蓄積に対するプロスタサイクリンやプロスタノイド IP 受容体の選択的アゴニストが脂肪細胞の成熟期での脂肪蓄積に及ぼす影響を解明するために行ったものである。

アラキドン酸（AA）とその関連物質のプロスタノイド類は、培養条件やライフステージより複雑な作用を及ぼす。ここでは,3-イソブチル-1-メチルキサンチン(IBMX)を含まない条件での分化誘導期における培養 3T3-L1 前駆脂肪細胞を外因性の AA で前処理したときの成熟期の脂肪蓄積に対する効果を検討した。このアラキドン酸による前処理は、脂肪細胞形成の遺伝子マーカーの発現の亢進で明らかなように、成熟期の脂肪細胞形成を有意に促進した。この AA での前処理の促進効果は、シクロオキシゲナーゼ（COX）阻害剤を共存させることにより抑制された。外因性のプロスタノイドやその関連物質のなかで、PGI_2 の IP 受容体に選択的なアゴニストである MRE-269 は、脂肪細胞での脂肪蓄積を著しく促進した。アラキドン酸 COX 経路の遺伝子発現の解析により、誘導性 COX-2、膜結合性 PGE 合成酵素-1、および PGF 合成酵素は、AA で処理した培養前駆脂肪細胞でより大きく減少した。対照的に、COX-1、細胞質可溶性の PGE 合成酵素、および PGI 合成酵素は、常在性で一定のままであった。AA で培養前駆脂肪細胞を処理すると、分化誘導期において脂肪細胞形成に促進的な PGI_2 の生合成は促進されたが、脂肪細胞形成に抑制的な PG 類として作用する PGE_2 と $PGF_{2\alpha}$の生成が減少するに至った。さらに、PGE_2 と $PGF_{2\alpha}$のプロスタノイド受容体のそれぞれに対応する EP4 と FP の遺伝子発現レベルは、AA の添加により徐々に抑制されたが、対照的に PGI_2 の IP 受容体は比較的に一定であった。従って、これらの結果より、IBMX のない条件で分化誘導期に培養前駆脂肪細胞を AA で前処理したときの成熟期後の脂肪細胞形成に対する促進効果において、内因性 PGI_2 の主要な役割を暗示している。

私の研究室では、以前に培養脂肪細胞が、PGI 合成酵素とプロスタノイド IP 受容体の遺伝子発現の正の調節作用により、プロスタサイクリンとも呼ばれる PGI_2 を生合成する能力を持つことを示している。いかにプロスタサイクリンが脂肪細胞形成を制御しているかを解明するために、私は、培養脂肪細胞の成熟期の脂肪蓄積に対するプロスタサイクリンと IP 受容体の特異的アゴニストもしくはアンタゴニストの効果を探究した。外因性の PGI_2 と、MRE-269 やトレプロスチニルのような IP 受容体に対する選択的なアゴニスト類は、COX 阻害剤のアスピリンによる脂肪蓄積の阻害を回復した。一方、CAY10441 や CAY10449 のような IP 受容体に対する選択的な

アンタゴニストは、ペルオキシソーム増殖剤応答性受容体(PPAR)γに特異的なアンタゴニストのGW9662のように脂肪蓄積を抑制するのに効果的であった。従って、プロスタサイクリンの脂肪細胞形成の促進作用は、脂肪細胞の成熟期に発現されるIP受容体を介する作用により説明できる。培養脂肪細胞をPPARγの活性剤であるトログリタゾンとともに、PGI_2とMRE-269のいずれかと培養すると、それぞれの化合物そのものよりも脂肪蓄積を付加的に促進することが示された。そのMRE-260とトログリタゾンの相加的な効果は、CAY10441ではなくてGW9662とともに培養することで、ほとんど抑制された。MRE-269はGW9662により抑制された脂肪細胞形成を回復することができなかったが、トログリタゾンの濃度を増加させると、CAY10441の阻害効果を濃度依存的に回復することが見出された。このことは、IP受容体を介する脂肪細胞形成の促進のための下流因子としてのPPARγの重要な役割を示している。培養脂肪細胞を細胞透過性の安定なcAMP類縁体あるいはcAMPレベルを高める試薬としてのフォルスコリンで処理すると、アスピリンによる阻害効果が部分的に解除された。しかし、フォルスコリンにより増加した過剰のcAMPレベルは脂肪細胞形成を低下した。プロテインキナーゼA(PKA)の細胞透過性阻害剤のH-89の添加は、脂肪蓄積に対するPGI_2もしくはMRE-269の促進効果に効果がなかった。これにより、IP受容体により仲介される脂肪細胞形成の促進にはPKAの活性化が必要でないことが示唆される。

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List of publications

Chapter 2

Ferdous Khan, Pinky Karim Syeda, Michael Nii N. Nartey, Mohammad Shahidur Rahman, Mohammad Safiqul Islam, Kohji Nishimura, Mitsuo Jisaka, Fumiaki Shono, Kazushige Yokota. (2016) Pretreatment of cultured preadipocytes with arachidonic acid during the differentiation phase without a cAMP-elevating agent enhances fat storage after the maturation phase. ***Prostaglandins and Other Lipid Mediators*****,** 123:16-27 doi:10.1016/j.prostaglandins.2016.02.003

Chapter 3

Ferdous Khan, Pinky Karim Syeda, Michael Nii N. Nartey, Mohammad Shahidur Rahman, Mohammad Safiqul Islam, Kohji Nishimura, Mitsuo Jisaka, Fumiaki Shono, Kazushige Yokota. (2016) Stimulation of fat storage by prostacyclin and selective agonists of prostanoid IP receptor during the maturation phase of cultured adipocytes. ***Cytotechnology***, in press doi: 10.1007/s10616-016-9960-7