Role of cyclooxygenase-1 in adipogenesis and biosynthesis of prostacyclin at the maturation phase of adipocytes

[脂肪細胞形成におけるシクロオキシゲナーゼ-1の役割と脂肪細胞の成熟期でのプロスタサイクリンの生合成]

Mohammad Sharifur Rahman

2014

Index

Chapter 3 Endogenous synthesis of prostacyclin was positively regulated during the maturation phase of cultured adipocytes

Abbreviations

AA	Arachidonic acid
ACS	Acyl-CoA synthetase
ADD1	Adipocyte determination and differentiation factor-1
aP2	Adipocyte fatty acid binding protein P2
C/EBP	CCAAT/enhancer binding protein
COX	Cyclooxygenase
CREB	cAMP response element binding protein
DME-HEPES	Dulbecco's modified Eagle's medium with 25mM HEPES;
DM	Differentiation medium
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
G418	Geneticin disulfate
GH	Growth hormone
GLUT	Glucose transporter
GM	Growth medium
GPCR	G-protein-coupled receptor
IBMX	3-isobutyl-1-methylxanthine
IGF-1	Insulin-like growth factor-1
IP	Prostacyclin receptor
LPL	Lipoprotein lipase
MAPK	Mitogen-activated protein kinase
MM	Maturation medium
PAI	Plasminogen activator inhibitor
PBS (−)	Phosphate-buffered saline without Ca^{2+}, and Mg^{2+}
PCR	Polymerase chain reaction
PG	Prostaglandin
PGDS	Prostaglandin D synthase
PGES	Prostaglandin E synthase
PGFS	Prostaglandin $F_{2\alpha}$ synthase
PGIS	Prostaglandin I synthase

15d-PGJ_2	15-deoxy-$\Delta^{12,14}$-PGJ_2
PMA	Phorbol 12-myristate 13-acetate
pref-1	Preadipocytes factor-1
PPAR	Peroxisome proliferator-activated receptor
PPRE	PPARγ response element
PVDF	Polyvinylidene difluoride
RT	Reverse transcriptase
RXR	Retinoid X receptor
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SREBP	Sterol regulatory element binding protein
TG	Triacylglycerol
TNFα	Tumor necrosis factor alpha
TXB_2	Thromboxane B_2
WAT	White adipose tissue

Chapter 1

Introduction

1.1. Obesity- a serious health concern

Obesity is a metabolic dysfunction appearing as a major cause of morbidity and mortality in the present era. It happens due to the over-calorie intake for a longer period of time. In obese individuals, the capacity for adipose tissue to accommodate excess lipid becomes very high, resulting in the abnormal accumulation of lipid in other tissues. It brings complicacy like non-insulin dependent diabetes, hypertension, cancer, osteoarthritis, gout, gallbladder disease, atherosclerosis, stroke and many other problems [1]. Therefore, the development of obesity and the factors related with it should be properly understood to prevent and counteract the disorders. Some progresses have already been made to explore the cellular and molecular events of adipogenesis using animal model and cell cultures [2]. More comprehensive research is still required.

1.2. Origin of adipose tissues and experimental adipocytes

The origin and development of adipose tissues are still a matter of study. Mature adipocytes, the main cellular component of WAT, are uniquely equipped to function in energy storage and balance under tight hormonal control. WAT formation starts before birth and expansion happens rapidly after the birth with increased fat cell size and increased fat cell number. Fat cell precursors isolated from adult WAT of various species, including humans, can be differentiated *in vitro* into mature adipocytes. The potential to acquire to new fat cells from fat cell precursors throughout the life span is undisputed. Several studies on multipotent clonal cell lines have suggested that the adipocyte lineage derives from an embryonic stem cell precursors with the capacity to differentiate into the mesodermal cell types of adipocytes, chondrocytes, osteoblasts and myocytes [2].

However, some details of the adipogenic programs have been explored by using various *in vitro* cell culture systems including preadipocytes cell lines and primary culture of adipose-derived stromal vascular precursor cells. The most popular cell lines are 3T3-F442A and 3T3-L1 which were clonally prepared from Swiss 3T3 cells developed from disaggregated 17- to 19-day mouse embryos [3-7].

1.3 Adipogenic program in adipocytes

Preadipose cell lines or primary adipocytes have to withdraw from the cell cycle and require growth arrest for their differentiation [8]. The growth-arrested preadipocytes should be stimulated by a proper combination of mitogenic and adipogenic signals for committing differentiation. The nature of the induction depends on the specific cell culture model used because the responsiveness to inducing agents may vary considerably between preadipocyte cell lines and primary preadipocytes. Usually, in serum-containing medium, the standard adipogenic cocktail contains dexamethasone, 3-isobutyl-1-methylxanthine (IBMX) and high concentration of insulin. Post-confluent preadipocytes undergo at least one round of DNA replication and cell doubling, leading to the clonal amplification of committed cells [9]. However, primary preadipocytes derived from human adipose tissue do not require cell division to enter the differentiation process [10].

At the growth phase, both preadipocytes lines and primary preadipocytes are morphologically similar to fibroblasts. During confluence, the induction of differentiation causes the preadipocyte to convert into a spherical shape, accumulate lipid droplets, and acquire the morphological and biochemical characteristics of the mature adipocytes gradually [2]. 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).

The process of cellular differentiation represents a remarkably coordinated regulation of gene expression that directs multipotential stem cell precursors down various lineages into fully mature and functionally distinct cell types. Numerous investigators have demonstrated that many genes are regulated in a differentiation-dependent manner [11-13].

1.4 Factors modulating adipogenesis

Adipocyte differentiation requires the cell to process a variety of combinatorial inputs during the decision to undergo differentiation. Hormones and growth factors with a role in adipocyte differentiation act via specific receptor to transduce external growth and differentiation signals through a cascade of intracellular events [14]. Extracellular matrix (ECM) proteins may play an important role in modulating adipocyte differentiation by pertaining the morphological changes and adipocyte-specific gene expression that accompany differentiation [15].

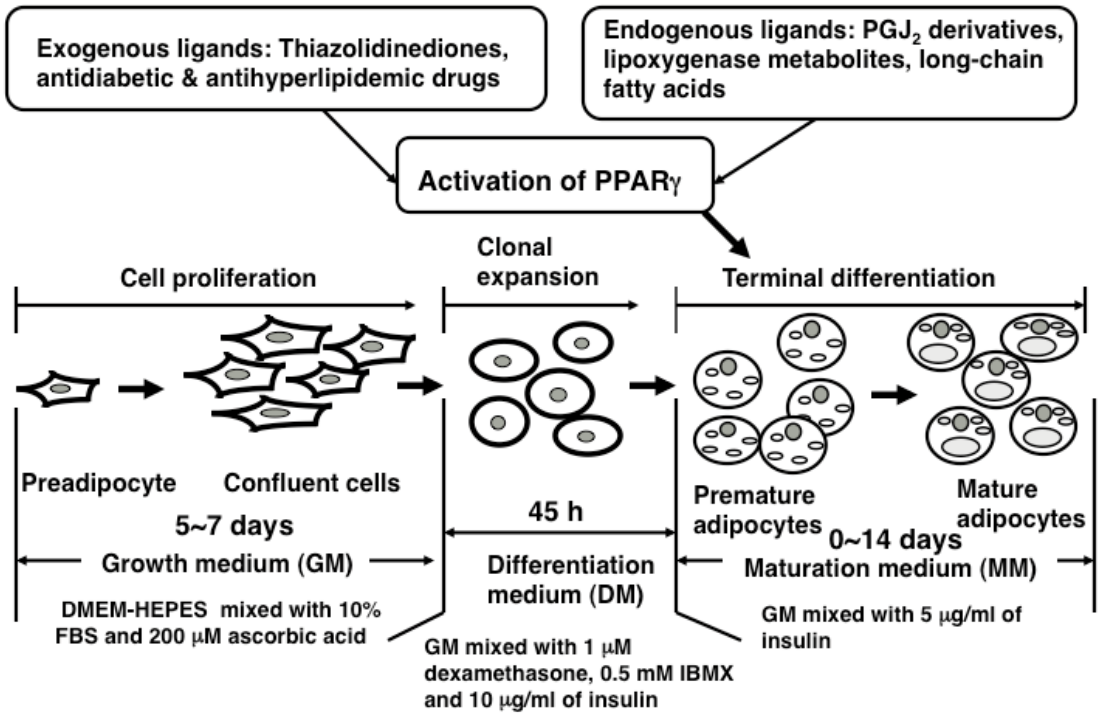


Fig. 1-1. Scheme of life cycle of preadipogenic mouse embryonic 3T3-L1 cells and the regulation by ligand-activated transcription factors.

A role for growth hormone (GH) in adipocyte differentiation was first reported by Green et al. [16]. It has been suggested that GH promotes differentiation and sensitizes the cells to the mitogenic effects of IGF-I for clonal expansion [16-18]. Rubin and colleagues first reported that IGF-I is an essential factor for 3T3-L1 adipocyte differentiation, using fetal calf serum depleted of GH, insulin, and IGF-I by charcoal and ion-exchange resin treatment [19, 20]. Unlike IGF-I, other growth factors and cytokines are generally considered as inhibitors of adipocyte differentiation. This is perhaps because of the their mitogenic effects, since cell growth and differentiation are usually mutually exclusive [21, 22]. IBMX accelerates the differentiation of preadipose cell lines and primary preadipocytes. As with glucocorticoids, IBMX is routinely used for the differentiation of a variety of preadipocytes. Cell adhesion molecules and ECM components modulate the interaction of cells with their environment in a manner that influences cell differentiation and migration. This may lead to cytoskeletal network rearrangement and an intracellular cascade of signal transduction that influences differentiation [23-26].

1.5 Transcription factors essential for adipocyte differentiation

Adipocyte differentiation involves communication of extracellular signals and those of the ECM environment to the nucleus. This leads to a coordinate regulation of adipocyte-specific gene expression resulting in the mature adipocyte. Many classes of molecules transduce inductive and inhibitory signals from the environment. Although the full complement of proteins involved in this process remains to be determined, ultimately the PPAR and C/EBP family of transcription factors must function cooperatively to transactivate adipocyte genes and thereby bring about adipocyte differentiation [27, 28].

The PPARs belong to type II nuclear hormone receptor family and form heterodimers with the RXR [29, 30]. Peroxisome proliferator-activated receptor-γ is the most adipose specific of the PPARs, and it is induced before transcriptional activation of most adipocyte genes. The expression of PPAR-γ has been shown to be sufficient to induce growth arrest as well as to initiate adipogenesis in exponentially growing fibroblast cell lines, demonstrating its critical role in the regulation of adipocyte differentiation [31-33]. Both synthetic and natural ligands of PPAR-γ have recently been identified. Thiazolidinediones can directly bind and activate PPAR-γ and have been shown to stimulate adipose conversion (Fig. 1-2) [34-36]. Peroxisome proliferator-activated receptor-α was also reported to be able to induce significant adipocyte differentiation in response to strong PPAR activators. Because this isoform is only weakly expressed in adipocytes and has been reported to be less adipogenic than PPAR-γ, its precise role in triggering and/or maintaining the adipocyte phenotype remains unclear [37]. Although PPAR-δ is not adipocyte specific, this PPAR isoform is highly expressed in adipose tissue. It is upregulated very early during adipocyte differentiation and has been suggested to play an important role in adipogenesis [38].

Another important type of transcription factors are C/EBP. These transcription factors have a basic transcriptional activation domain and an adjoining leucine zipper motif, which provides the ability for homo- and heterodimerization. Studies of adipocyte differentiation, primarily in 3T3-L1 cells, provide extensive evidence for C/EPB-α function in adipocyte differentiation [39]. Although not strictly adipocyte specific, C/EPB-α is expressed just before the transcription of most adipocyte-specific genes is initiated. CCAAT/enhancer binding protein-α binds and transactivates the promoters of several adipocyte genes, including aP2, SCD1, GLUT-4, PEPCK, leptin, and the insulin receptor.

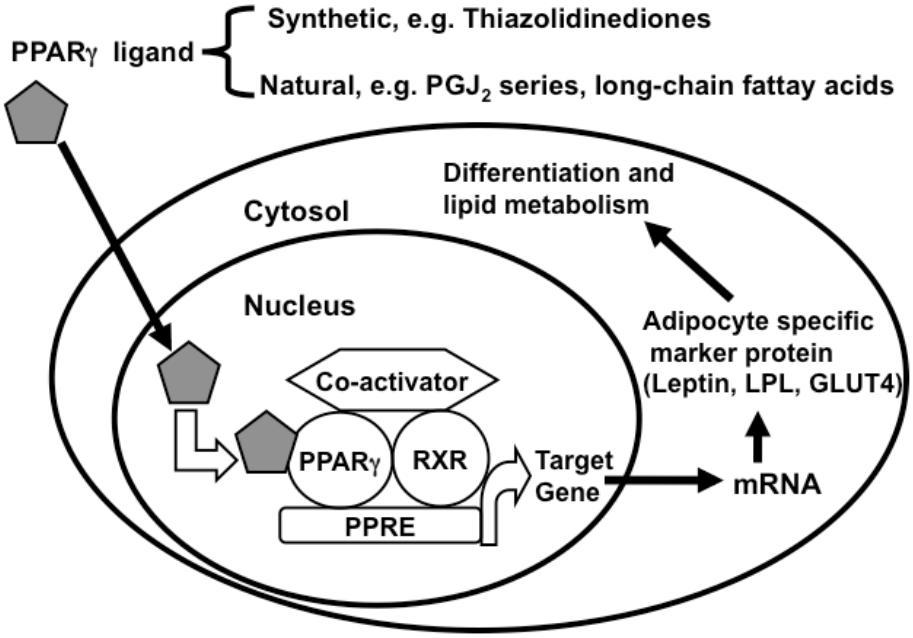


Fig. 1-2. Regulation of PPAR-γ. Ligands after entering into the cell, bind to PPAR-γ. The ligand bound PPARγ then forms a heterodimer with the nuclear retinoid X receptor (RXR) to recognize PPAR-response elements (PPRE) in the promoter region of target genes and stimulate transcription.

In some instances, constitutive expression of C/EPB-α is sufficient to induce differentiation of 3T3-L1 cells in the absence of hormonal agents, and expression of antisense C/EPB-α mRNA in 3T3-L1 preadipocytes prevents differentiation [40]. Moreover, C/EPB-α can efficiently promote adipogenesis in a variety of mouse fibroblastic cells, including those that have little or no spontaneous capacity to develop into adipocytes [41]. C/EPB-α has also been indicated to function in the termination of the mitotic clonal expansion that occurs early in the differentiation program and seems to be involved in the maintenance of the adipocyte phenotype through autoactivation of the C/EBP-α gene [12, 27, 28, 37]. Together, these findings provide evidence that C/EPB-α is both required and sufficient to induce adipocyte differentiation. Fine-tuning of the control of adipocyte gene expression by C/EBP proteins involves homo- and heterodimerization between the C/EBP-α, C/EBP-β, and C/EBP-δ isoforms. C/EBP-α expression occurs relatively late in differentiation, the β- and δ-isoforms of C/EBP are present in preadipocytes, and their levels increase transiently early in differentiation [42, 43]. C/EBP homologous protein-10

(CHOP-10) is another member of the C/EBP family. It displays overall sequence similarity to C/EBP proteins in the DNA binding and dimerization domain.

Sterol regulatory element binding proteins (SREBP) is another key transcription factor known to modulate adipocyte differentiation [44]. The SREBP family consists of three isoforms designated as SREBP-1a, SREBP-1c and SREBP-2. In human and mice SREBP-1a and SREBP-1c are produced from a single gene through the use of alternative transcription sites. Adipocyte determination and differentiation dependent factor 1 (ADD 1), cloned independently from a rat adipocyte cDNA library, is homologous to human SREBP-1c. Adipose tissue expresses predominantly ADD1/SREBP-1c in comparison to other isoforms. Overexpression studies with SREBP-1c in cultured preadipocytes activated genes involved in fat cell differentiation and lipid accumulation. This indicates the involvement of SREBP-1c in differentiation of adipocytes [45]. Recent studies showed that ADD-1 exerts its adipogenic action by modulating both expression and activation of PPAR-γ [46].

Several other transcription factors including GATA-binding transcrition factors GATA-2 and GATA-3 and cAMP response element binding protein (CREB), play a critical role in the molecular control of the preadipocyte-adipocyte transition. Constitutive expression of GATA-2 and GATA-3 suppress adipocytes differentiation thorugh direct suppression of the activity of PPAR-γ. On the other hand, CREB is expressed prior to and during adipogenesis and overexpression in 3T3-L1 preadipocytes is necessary and sufficient to initiate adipogenesis [47].

Moreover, other signaling molecule such as pref-1 and Wnts regulate adipocyte differentiation. Pref-1 is an inhibitor of adipocyte differentiation and its down regulation is required for adipose conversion. Wnt signaling is appears to be a molecular switch that governs adipogenesis. Wnt signaling maintains preadipocytes in an undifferentiated state that is mediated through inhibition of C/EBPα and PPAR-γ [45, 48].

1.6. Mature adipocytes act as specialized endocrine cells

Recent findings revealed that adipocytes act as a specialized endocrine cell. Immune system-related proteins produced by adipocytes include adipsin, acylation stimulatin protein (ASP), adipocyte complement-related protein (Acrp30/AdipoQ), tumor necrosis factor alpha (TNFα) and macrophage migration inhibitory factor [37]. ASP may

be involved in regulating energy storage by stimulating triacylglycerol synthesis and glucose transport. Acrp30/AdipoQ might be involved in signaling pathway and regulation of energy homeostasis. TNFα levels are elevated in WAT obese rodent and human. It inhibits adipocyte differentiation and may contribute to the insulin resistance that accompanies obesity and non-insulin dependent diabetes mellitus [49, 50]. On the other hand, leptin, a hormone produced by adipocytes, level increases with obesity. Leptin binds to its receptor in the hypothalamus of brain and inhibits food intake. Loss of fat stores decreases leptin levels that increase the neuropeptide Y level; that leads to increase food intake. Leptin has a role on modulation of insulin action in liver, production of steroids in ovary, effect on adrenocorticoid action, in reproductive physiology, in hematopoietic and immune system development [51, 52].

Some vascular function related proteins such as angiotensinogen and plasminogen activator inhibitor type 1(PAI-1) are secreted by adipocytes. Angiotensinogen play a role in regulating adipose tissue blood supply and fatty acid efflux from fat. Angiotensin II, the cleavage product of angiotensinogen, has been implicated adipose tissue growth by stimulating production of prostacyclin by matured fat cells and thereby promoting adipocyte differentiation via paracrine/autocrine manner [2]. Plasma PAI-1 levels are closely correlated with visceral fat area but not with subcutaneous fat area in human subjects. Enhanced expression of the PAI-1 gene in visceral fat may increase plasma levels and may have a role in the development of vascular disease in visceral obesity [53].

In short, it can be said that adipocytes act as an endocrine as well as paracrine/autocrine cell. Along with the active role in regulating energy balance, they are also involved in a variety of other physiological processes, including the auto-regulation of adipose tissue growth and development.

1.7 Biosynthesis of prostaglandin in adipocytes

Prostaglandins (PGs) and their related compounds such as prostacyclins, thromboxane, leukotrienes etc. that are collectively known as eicosanoids. They are synthesized from the polyunsaturated fatty acid, arachidonate providing specific effects on target cells close to their site of formation. They are also considered as local hormones. Prostaglandins are found in most tissues and organs. They are produced by almost all nucleated cells.

PGs are synthesized through the arachidonate cyclooxygenase (COX) pathway (Fig. 1-3) in which the enzymatic reaction of PGH synthase alternatively called COX is the rate-limiting step for the generation of PGs after the release of free arachidonic acid (AA) [54-56]. The COX enzyme is known to occur as two types of isoforms, including the constitutive COX-1 and the inducible COX-2. COX-1 is constitutively expressed in most of the cells and is mainly utilized in the immediate PG biosynthesis important for homeostasis and certain physiological functions, which occurs within several minutes after stimulation with Ca^{2+} mobilizers [57]. On the other hand, COX-2 is expressed inducibly and is utilized for delayed PG biosynthesis, which last for several hours after proinflammatory stimuli [58].

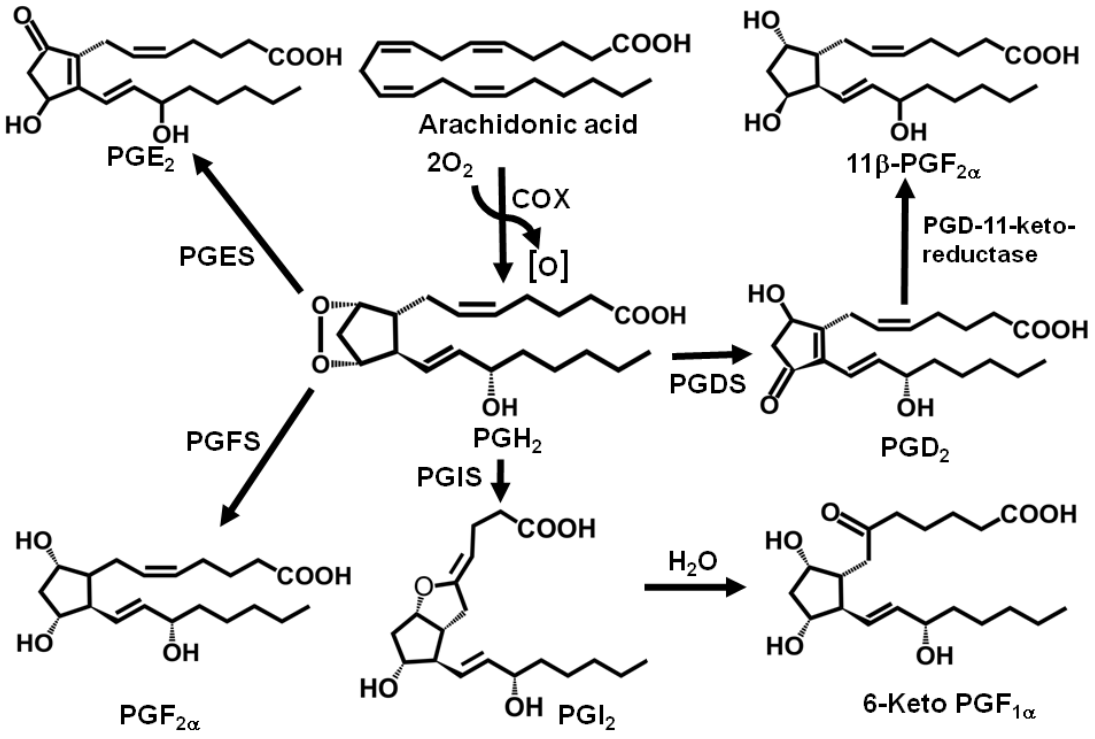


Fig. 1-3. Biosynthesis of prostaglandins by arachidonate cyclooxygenase pathway.

Generation of PGs involves the oxidation and subsequent isomerization of membrane-derived arachidonic acid (AA), polyunsaturated fatty acid, via three sequential enzymatic reactions. The process is initiated through the release of AA from membrane phospholipids, a reaction catalysed by phospholipases A_2 that catalyzes the hydrolysis of

arachidonic acid from *sn*-2 position of phospholipids. The released AA is then subsequently metabolized to prostaglandin (PG) H_2 by the action of cyclooxygenase (COX) enzyme.

Finally, the intermediate PGH_2 is converted into various bioactive derivatives such as PGE_2, $PGF_{2\alpha}$, PGD_2 and PGI_2 by terminal synthase enzymes PGES, PGFS, PGDS and PGIS, respectively. The J_2 series of PGs (PGJ_2, Δ^{12} -PGJ_2 and 15d-PGJ_2) are derived non-enzymatically from PGD_2, through dehydration and isomerisation [59, 60]. PGD_2 is further converted to 11β-$PGF_{2\alpha}$ by the action of enzyme PGD-11-keto reductase [61]. PGI_2 (prostacyclin) is a very unstable compound which is very rapidly hydrolyzed into stable 6-Keto-$PGF_{1\alpha}$ [62].

1.8 Cyclooxygenases - structures and functions

The cyclooxygenase isoforms (COX-1 and COX-2) are the integral part of the arachidonate COX pathway. They catalyze the bis-dioxygenation and subsequent reduction of arachidonic acid (AA) to PGG_2 and PGH_2 (Fig. 1-3). This PGH_2 goes through metabolism by downstream enzymes yielding the family of PGs, each member of which exerts a range of physiologic effects through specific G-protein-coupled receptors [63, 64].

Human COX-1 and COX-2 are homodimers and they contain 576 and 581 amino acids, respectively. Both enzymes contain three high mannose oligosaccharides, one of which facilitates protein folding. A fourth oligosaccharide, present only in COX-2, regulates its degradation. Considering the 60% identity in sequence between COX-1 and COX-2, it is not surprising that their three-dimensional structures are nearly superimposable. Each subunit of the dimer consists of three domains, the epidermal growth factor domain (residues 34-72), the membrane binding domain (residues 73-116), and the catalytic domain comprising the bulk of the protein, which contains the cyclooxygenase and peroxidase active sites on either side of the heme prosthetic group [65-68]. On the opposite side of the protein from the membrane binding domain, the peroxidase active site consists of the heme positioned at the bottom of a shallow cleft. This structure provides considerable solvent accessibility to the heme with the exception of a cluster of several hydrophobic amino acids that form a dome over part of the cleft. The structure of the active site helps to explain the promiscuous substrate specificity of the COX peroxidase, which reduces a wide range of primary and secondary organic hydroperoxides [69]. Although the

hydrophobic dome would appear to explain the preference of the peroxidase for organic peroxides over H_2O_2, mutation of the dome residues to alanine has little effect on peroxidase activity or substrate specificity [70].

The cyclooxygenase active site lies on the opposite side of the heme from the peroxidase active site at the top of an L-shaped channel that originates in the membrane binding domain. The mouth of the channel consists of the lobby, a large volume that narrows to a constriction that must open before substrates or inhibitors can pass deeper into the channel. Above the constriction, the channel is surrounded by hydrophobic residues, which outline the nearly right angle bend and the narrow terminus. When AA binds in the cyclooxygenase active site, it lies with its carboxyl group at the constriction and its ω-methyl group at the narrow terminus of the channel. This places carbon-13 of AA at the bend in the channel in close proximity to Tyr-385, which is the critical catalytic amino acid for the cyclooxygenase reaction [65, 66, 68].

The first step in the conversion of AA to the hydroperoxy-endoperoxide, PGG_2, is abstraction of the pro-S hydrogen atom from carbon-13. The steps that follow are consistent with the mechanism of nonenzymatic lipid peroxidation, so the main contributions of COX to PGG_2 formation are to restrict the options for hydrogen abstraction and dictate reaction stereochemistry. Cyclooxygenase catalysis requires that the enzyme first be activated, a process dependent on the peroxidase activity. Two-electron reduction of a peroxide substrate results in the oxidation of the ferric heme to an oxo-ferryl porphyrin radical cation. Transfer of an electron to the heme from Tyr-385 of the protein generates a tyrosyl radical in the cyclooxygenase active site. This radical, as noted above, is positioned perfectly to abstract the pro-S hydrogen from carbon-13 of AA, initiating the cycloxygenase reaction. The final step of the reaction, reduction of the peroxyl radical to the hydroperoxide to form PGG_2, regenerates the tyrosyl radical. Thus, activated COX can carry out multiple turnovers without need to repeat the activation step. After initiating the cyclooxygenase reaction, the primary function of the peroxidase is to reduce the 15-hydroperoxy of PGG_2 to the corresponding alcohol of PGH_2 [65, 66, 71].

COX-1 is widely distributed and constitutively expressed in most tissues where it is found. Its gene, *Ptgs-1*, codes for a 2.8 kb mRNA, which is relatively stable. By contrast, the gene for COX-2, *Ptgs-2*, is an immediate early gene that is activated by a wide variety of inflammatory and proliferative stimuli, and the 4 kb COX-2 mRNA turns over rapidly

due to the presence of instability sequences in the 3′-untranslated region [65, 66]. The difference in the pattern of gene expression provides an obvious explanation for the existence of the two COX isoforms, suggesting that COX-1 provides PGs that are required for homeostatic functions, including gastric cytoprotection and hemostasis, whereas COX-2 plays the predominant role in PG formation during pathophysiologic states, such as inflammation and tumorigenesis [72].

In case of adipogenesis, the role of COX-2 has been explored in both animal model and cell lines to a significant extent [73, 74]. However, the adipogenic role of COX-1 in adipocytes has not been evaluated well.

1.9 Prostacyclin- generation and functions

Prostacyclin (PGI_2) is an end product derived from the sequential metabolism of arachidonic acid via COX pathway (Fig. 1-3) and was first reported by Needleman and Vane in 1976. The enzyme prostacyclin synthase (PGIS) is responsible for converting arachidonate metabolite PGH_2 to an end product PGI_2. This biologically active PGI_2 is a very unstable compound and very rapidly hydrolyzes into inactive 6-keto-$PGF_{1\alpha}$ (Fig. 1-3) [74, 75].

Several types of cells have been shown to express PGIS and they include fibroblasts, follicular dendritic cells, endothelial cells, smooth muscle cells, and thymic nurse cells. The contributory role of PGIS in the production of PGI_2 is decreased through the inhibition of PGIS by tyrosine-nitrating agents such as peroxynitrite and tetranitromethane [77, 78]. Besides, PGIS can be limited by substrate-dependent suicide inactivation if there is adequate conversion of PGH_2, the substrate for PGIS, which causes accumulation of inactivated enzyme [79]. PGI_2 has been understood to play a role in cardiovascular health specifically inhibiting platelet aggregation and having powerful vasodilatory effects via relaxation of smooth muscle [80]. Many PGI_2 analogues have been successfully used for therapy in pulmonary arterial hypertension, peripheral occlusive disease, vascular complication of diabetes mellitus, and treatment of reperfusion injury [81].

The cell surface receptor for prostacyclin is a seven transmembrane G-protein-coupled receptor termed IP. It is coupled to a guanosine nucleotide-binding α-stimulatory protein. When activated by PGI_2, IP stimulates adenyl cyclase leading to increased intracellular cyclic AMP (cAMP). Increased cAMP then leads to activation of

protein kinase A (PKA) and further phosphorylation of key proteins [81]. These actions culminate in relaxation of smooth muscle, reduced cell proliferation, and other inhibitory mechanisms. Human IP receptors are present on multiple cell types including platelets, medullary thymocytes, neutrophils, dendritic cells, eosinophils, T regulatory cells, and activated T cells [82, 84]. IP receptors are also found on many cell types in the lung such as macrophages, pneumocytes, smooth muscle cells, and fibroblasts [82, 85]. PPARs, expressed in adipose tissue, spleen, and large intestines predominately, are thought to be a downstream of the activated IP membrane receptor and can be stimulated via stable PGI_2 analogues [81].

In case of adipocytes, several works have been reported regarding the role prostacyclin in differentiation of Ob1771 and 3T3-F442A cells [86-90]. However, the proadipogenic action of PGI_2 in 3T3-L1 cells is still ambiguous.

1.10 Biological and pathophysiological roles of prostaglandins

Different PGs are produced in cells under the stimulation of metabolic and external signaling factors and exert biological effects in a paracrine or autocrine manner. They ligate a sub-family of cell surface seven-transmembrane receptors, G-protein-coupled receptors. These receptors are termed DP1-2, EP1-4, FP, IP1-2, and TP, corresponding to the ligated prostaglandin [91]. Depending on the cell type, the activated G protein may stimulate or inhibit formation of cAMP, or may activate a phosphatidyl inositol signal pathway leading to intracellular Ca^{2+} release. Instead, PGJ_2 derivatives act via nuclear receptor PPARγ, a transcriptional regulator of adipocytes differentiation [92-94].

PGD_2 has marked effect on sleep induction, platelet aggregation, relaxation of vascular and nonvascular smooth muscle and nerve cell function [75, 95]. PGE_2 activity influences inflammation, fertility, gastric mucosal integrity and immune modulation, fever generation and nociception [96, 97]. Both PGE_2 and PGI_2 contribute to the development of inflammatory erythema and pain. PGI_2 is a major prostanoids secreted by endothelial cells and binds to the IP receptors on vascular smooth muscles cells and inhibits vascular contraction. The IP receptor couples to the Gs proteins and increases intracellular cAMP concentrations, thus antagonizing the contractile agonists and inhibiting the mitogen-activated protein kinase (MAPK) pathway. Therefore, PGI_2 produced by the COX pathways is important in normal control of vascular homeostasis and thrombosis [98].

$PGF_{2\alpha}$ causes contraction of vascular, bronchial, intestinal, and myometrial smooth muscle, and also exhibits potent luteolytic activity [99].

1.11 Prostaglandins modulate adipocyte differentiation

Several lines of evidence indicate that arachidonate metabolites play an important physiological role in adipose tissue metabolism and development [100]. $PGF_{2\alpha}$ inhibits differentiation of various preadipose cell lines and primary rat preadipocytes through FP receptor. In 3T3-L1 cells, FP receptor stimulation causes a transient rise in intracellular calcium ion concentration, activation of a calcium/calmodulin-dependent protein kinase (CaM kinase) and an increase in DNA synthesis that is associated with the inhibition of differentiation [64] and through activation of mitogen-activated protein kinase (MAPK), resulting in the inhibitroy phosphorylation of of peroxisome proliferators activated receptor [101]. It also inhibits differentiation of preadipocytes by stimulating synthesis of transforming growth factor-alpha (TGFα) [102]. While PGE_2 inhibits the differentiation of preadipocytes by interacting with EP receptors, mainly EP4 receptor. The differentiation associated responses in genes such as adipocytokines and enzymes related to lipid metabolism were largely weakened upon PGE_2 treatment. In particular, the expression of PPARγ and CEBPα, genes playing a central role in adipogenesis, was greatly suppressed [103]. PGD_2, an unstable product of lipocaline-type prostaglandin D synthase (LPGDS) enzyme in the COX pathway, is degraded nonenzymatically to form PGJ_2 series such as 15d-PGJ_2, and Δ^{12}-PGJ_2. PGJ_2 series prostanoids are thought to be natural ligand of PPARγ, expressed at high levels in adipose tissues and is thought to play an important role in the controlling the differentiation of preadipocytes to adipocytes [60].

1.12 The issues remain to be addressed and aim of the study

COX isoforms include the constitutive COX-1 and the inducible COX-2. COX-1 is generally associated with the immediate production of PGs to execute house keeping functions, such as platelet aggregation and vascular homeostasis, while COX-2 is mostly responsible for the delayed synthesis of PGs by the induction of the de novo synthesis of the new enzyme in response to mitogenic and inflammatory factors in pathological tissues. Adipocyte produces varieties of PGs which might regulate the adipogenic development of

them [104]. The specificity for the synthesis of PG species depends on the functional coupling of either COX with the downstream PG synthases expressed in mammalian cells. A previous study have described that obesity is induced more highly in mice heterozygous for COX-2 than COX-1 and COX-2 deficient homozygotes and their control littermates [105]. This observation suggests that COX-2 is implicated in the negative regulation of adipogenesis in whole body. However, mice heterozygous for COX-1 have not been described, so that it remains elusive as for the role of COX-1 in adipogenesis and obesity *in vivo*.

In addition, the endogenous prostanoids have complicated role on adipogenesis and many studies have been conducted on different PGs using different cell lines [106-110]. But, the PGI_2 has not been studied to a greater extent. This metabolite is a very unstable and has a very short half-life ranging from 30 seconds to a few minutes. It is rapidly hydrolyzed to produce stable 6-Keto-$PGF_{1\alpha}$ [75, 95]. It should be noted that different cell types and alterations in the culture conditions of cultured adipocytes and precursor cells would gave contradictory results on adipogenesis. For example, $PGF_{2\alpha}$ serves as anti-adipogenic factor in cultured 3T3-L1 cells in contrast to the stimulatory effect on the adipocyte differentiation of cultured Ob1771 pre-adipose cells as described above [111-114]. In addition, a previous study observed that the repeated addition of exogenous PGI_2 to cultured 3T3-L1 cells inhibited insulin- and indomethacin-mediated adipocyte differentiation [115]. Earlier, cultured 3T3-L1 cells have been described to generate PGI_2 as an immediate response to calcium ionophore A23187 for 5 min or by the incubation with extracellular arachidonic acid [102]. However, until now the biosynthesis of PGI_2, which is usually quantified as the levels of stable 6-keto-$PGF_{1\alpha}$, has not been monitored comprehensively at different life stages of adipocytes through the growth, differentiation, and maturation phases. To clarify theses issues, initiative should be taken to quantify prostacyclin as 6-Keto-$PGF_{1\alpha}$ and shed light on the role of endogenously synthesized PGI_2 for causing the differentiation and subsequent adipogenesis in 3T3-L1 preadipocytes.

References

1. Camp, H. S., Ren, D., and Leff, T. (2002) Adipogenesis and fat-cell function in obesity and diabetes. Trends Mol. Med. 8, 442-447
2. Gregoire, F. M., Smas, C. M., and Sul, H. S. (1998) Understanding adipocyte

differentiation. Physiol. Rev. 78, 783-809

3. Green, H., and Kehinde, O. (1974) Sublines of mouse 3T3 cells that accumulate lipid. Cell 1, 113-116
4. Green, H., and Kehinde, O. (1976) Spontaneous heritable changes leading to increased adipose conversion in 3T3 cells. Cell 7, 105-113
5. Green, H., and Kehinde, O. (1975) An established preadipose cell line and its differentiation in culture. II. Factors affecting the adipose conversion. Cell 5, 19-27
6. Green, H., and Meuth, M. (1974) An established pre-adipose cell line and its differentiation in culture. Cell 3, 127-133
7. Kuri-Harcuch, W., and Green, H. (1978) Adipose conversion of 3T3 cells depends on a serum factor. Proc. Natl. Acad. Sci. USA 75, 6107-6109
8. Pairault, J., and Green, H. (1979) A study of the adipose conversion of suspended 3T3 cells by using glycerophosphate dehydrogenase as differentiation marker. Proc. Natl. Acad. Sci. USA 76, 5138-5142
9. Cornelius, P., Macdougald, O. A., and Lane, M. D. (1994) Regulation of adipocyte development. Annu. Rev. Nutr. 14, 99-129
10. Entenmann, G., and Hauner, H. (1996) Relationship between replication and differentiation in cultured human adipocyte precursor cells. Am. J. Physiol. Cell Physiol. 270, C1011-C1016
11. Richon, V. M., Lyle, R. E., and McGehee, R. E., Jr. (1997) Regulation and expression of retinoblastoma proteins p107 and p130 during 3T3-L1 adipocyte differentiation. J. Biol. Chem. 272, 10117-10124
12. MacDougald, O. A., and Lane, M. D. (1995) Transcriptional regulation of gene expression during adipocyte differentiation. Annu. Rev. Biochem. 64, 345-373
13. Cowherd, R. M., Lyle, R. E., and McGehee, R. E., Jr. (1999) Molecular regulation of adipocyte differentiation. Semin. Cell Dev. Biol. 10, 3-10
14. Aubert, J., Ailhaud, G., and Negrel, R. (1996) Evidence for a novel regulatory pathway activated by (carba)prostacyclin in preadipose and adipose cells. FEBS Lett. 397, 117-121
15. Bachmeier, M., and Loffler, G. (1995) Influence of growth factors on growth and differentiation of 3T3-L1 preadipocytes in serum-free conditions. Eur. J. Cell Biol. 68, 323-329
16. Green, H., Morikawa, M., and Nixon, T. (1985) A dual effector theory of

growth-hormone action. Differentiation 29, 195-198

17. Corin, R. E., Guller, S., Wu, K. Y., and Sonenberg, M. (1990) Growth hormone and adipose differentiation: growth hormone-induced antimitogenic state in 3T3-F442A preadipose cells. Proc. Natl. Acad. Sci. USA 87, 7507-7511
18. Guller, S., Corin, R. E., Yuan-Wu, K., and Sonenberg, M. (1991) Up-regulation of vinculin expression in 3T3 preadipose cells by growth hormone. Endocrinology 129, 527-533
19. Schmidt, W., Poll-Jordan, G., and Loffler, G. (1990) Adipose conversion of 3T3-L1 cells in a serum-free culture system depends on epidermal growth factor, insulin-like growth factor I, corticosterone, and cyclic AMP. J. Biol. Chem. 265, 15489-15495
20. Smith, P. J., Wise, L. S., Berkowitz, R., Wan, C., and Rubin, C. S. (1988) Insulin-like growth factor-I is an essential regulator of the differentiation of 3T3-L1 adipocytes. J. Biol. Chem. 263, 9402-9408
21. Luetteke, N. C., Lee, D. C., Palmiter, R. D., Brinster, R. L., and Sandgren, E. P. (1993) Regulation of fat and muscle development by transforming growth factor α in transgenic mice and in cultured cells. Cell Growth Differ. 4, 203-213
22. Serrero, G., and Lepak, N. (1996) Endocrine and paracrine negative regulators of adipose differentiation. Int. J. Obes. Relat. Metab. Disord. 20 Suppl 3, S58-64
23. Chapman, A. B., Knight, D. M., Dieckmann, B. S., and Ringold, G. M. (1984) Analysis of gene expression during differentiation of adipogenic cells in culture and hormonal control of the developmental program. J. Biol. Chem. 259, 15548-15555
24. Gaillard, D., Wabitsch, M., Pipy, B., and Negrel, R. (1991) Control of terminal differentiation of adipose precursor cells by glucocorticoids. J. Lipid Res. 32, 569-579
25. Grigoriadis, A. E., Heersche, J. N., and Aubin, J. E. (1988) Differentiation of muscle, fat, cartilage, and bone from progenitor cells present in a bone-derived clonal cell population: effect of dexamethasone. J. Cell Biol. 106, 2139-2151
26. Rubin, C. S., Hirsch, A., Fung, C., and Rosen, O. M. (1978) Development of hormone receptors and hormonal responsiveness *in vitro*. Insulin receptors and insulin sensitivity in the preadipocyte and adipocyte forms of 3T3-L1 cells. J. Biol. Chem. 253, 7570-7578
27. Mandrup, S., and Lane, M. D. (1997) Regulating adipogenesis. J. Biol. Chem. 272, 5367-5370

28. Lane, M. D., Lin, F. T., MacDougald, O. A., and Vasseur-Cognet, M. (1996) Control of adipocyte differentiation by CCAAT/enhancer binding protein alpha (C/EBPα). Int. J. Obes. Relat. Metab. Disord. 20 Suppl 3, S91-96
29. Juge-Aubry, C. E., Gorla-Bajszczak, A., Pernin, A., Lemberger, T., Wahli, W., Burger, A. G., and Meier, C. A. (1995) Peroxisome proliferator-activated receptor mediates cross-talk with thyroid hormone receptor by competition for retinoid X receptor. Possible role of a leucine zipper-like heptad repeat. J. Biol. Chem. 270, 18117-18122
30. Schoonjans, K., Staels, B., and Auwerx, J. (1996) The peroxisome proliferator activated receptors (PPARS) and their effects on lipid metabolism and adipocyte differentiation. Biochim. Biophys. Acta 1302, 93-109
31. Altiok, S., Xu, M., and Spiegelman, B. M. (1997) PPARgamma induces cell cycle withdrawal: inhibition of E2F/DP DNA-binding activity via down-regulation of PP2A. Genes Dev. 11, 1987-1998
32. Hu, E., Tontonoz, P., and Spiegelman, B. M. (1995) Transdifferentiation of myoblasts by the adipogenic transcription factors PPARγ and C/EBPα. Proc. Natl. Acad. Sci. USA 92, 9856-9860
33. Tontonoz, P., Hu, E., and Spiegelman, B. M. (1995) Regulation of adipocyte gene expression and differentiation by peroxisome proliferator activated receptor γ. Curr. Opin. Genet. Dev. 5, 571-576
34. Kletzien, R. F., Clarke, S. D., and Ulrich, R. G. (1992) Enhancement of adipocyte differentiation by an insulin-sensitizing agent. Mol. Pharmacol. 41, 393-398
35. Lehmann, J. r. M., Moore, L. B., Smith-Oliver, T. A., Wilkison, W. O., Willson, T. M., and Kliewer, S. A. (1995) An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor γ (PPAR γ). J. Biol. Chem. 270, 12953-12956
36. Sandouk, T., Reda, D., and Hofmann, C. (1993) Antidiabetic agent pioglitazone enhances adipocyte differentiation of 3T3-F442A cells. Am. J. Physiol. 264, C1600-1608
37. Brun, R. P., Kim, J. B., Hu, E., Altiok, S., and Spiegelman, B. M. (1996) Adipocyte differentiation: a transcriptional regulatory cascade. Curr. Opin. Cell Biol. 8, 826-832
38. Torti, F. M., Dieckmann, B., Beutler, B., Cerami, A., and Ringold, G. M. (1985) A macrophage factor inhibits adipocyte gene expression: an *in vitro* model of cachexia.

Science 229, 867-869

39. Brun, R. P., Tontonoz, P., Forman, B. M., Ellis, R., Chen, J., Evans, R. M., and Spiegelman, B. M. (1996) Differential activation of adipogenesis by multiple PPAR isoforms. Gene Dev. 10, 974-984
40. Amri, E.-Z., Bonino, F. d. r., Ailhaud, G. r., Abumrad, N. A., and Grimaldi, P. A. (1995) Cloning of a protein that mediates transcriptional effects of fatty acids in preadipocytes. Homology to peroxisome proliferator-activated receptors. J. Biol. Chem. 270, 2367-2371
41. Grimaldi, P. A., Teboul, L., Inadera, H., Gaillard, D., and Amri, E. Z. (1997) Trans-differentiation of myoblasts to adipoblasts: triggering effects of fatty acids and thiazolidinediones. Prostaglandins Leukot. Essent. Fatty Acids 57, 71-75
42. Lin, F. T., and Lane, M. D. (1992) Antisense CCAAT/enhancer-binding protein RNA suppresses coordinate gene expression and triglyceride accumulation during differentiation of 3T3-L1 preadipocytes. Genes Dev. 6, 533-544
43. Lin, F. T., and Lane, M. D. (1994) CCAAT/enhancer binding protein alpha is sufficient to initiate the 3T3-L1 adipocyte differentiation program. Proc. Natl. Acad. Sci. USA 91, 8757-8761
44. Osborne, T. F. (2000) Sterol regulatory element-binding proteins (SREBPs): key regulators of nutritional homeostasis and insulin action. J. Biol. Chem. 275, 32379-32382
45. Kim, J. B., and Spiegelman, B. M. (1996) ADD1/SREBP1 promotes adipocyte differentiation and gene expression linked to fatty acid metabolism. Genes Dev. 10, 1096-1107
46. Fajas, L., Miard, S., Briggs, M. R., and Auwerx, J. (2003) Selective cyclo-oxygenase-2 inhibitors impair adipocyte differentiation through inhibition of the clonal expansion phase. J. Lipid. Res. 44, 1652-1659
47. Kim, J. B., Spotts, G. D., Halvorsen, Y. D., Shih, H. M., Ellenberger, T., Towle, H. C., and Spiegelman, B. M. (1995) Dual DNA binding specificity of ADD1/SREBP1 controlled by a single amino acid in the basic helix-loop-helix domain. Mol. Cell Biol. 15, 2582-2588
48. Ericsson, J., Jackson, S. M., Kim, J. B., Spiegelman, B. M., and Edwards, P. A. (1997) Identification of glycerol-3-phosphate acyltransferase as an adipocyte determination and differentiation factor 1- and sterol regulatory element-binding

protein-responsive gene. J. Biol. Chem. 272, 7298-7305

49. Reusch, J. E., Colton, L. A., and Klemm, D. J. (2000) CREB activation induces adipogenesis in 3T3-L1 cells. Mol. Cell Biol. 20, 1008-1020
50. Tong, Q., Dalgin, G., Xu, H., Ting, C. N., Leiden, J. M., and Hotamisligil, G. S. (2000) Function of GATA transcription factors in preadipocyte-adipocyte transition. Science 290, 134-138
51. Ross, S. E., Hemati, N., Longo, K. A., Bennett, C. N., Lucas, P. C., Erickson, R. L., and MacDougald, O. A. (2000) Inhibition of adipogenesis by Wnt signaling. Science 289, 950-953
52. Sul, H. S., Smas, C., Mei, B., and Zhou, L. (2000) Function of pref-1 as an inhibitor of adipocyte differentiation. Int. J. Obes. Relat. Metab. Disord. 24 Suppl 4, S15-19
53. Petruschke, T., and Hauner, H. (1993) Tumor necrosis factor-alpha prevents the differentiation of human adipocyte precursor cells and causes delipidation of newly developed fat cells. J. Clin. Endocrinol. Metab. 76, 742-747
54. Pelleymounter, M. A., Cullen, M. J., Baker, M. B., Hecht, R., Winters, D., Boone, T., and Collins, F. (1995) Effects of the obese gene product on body weight regulation in ob/ob mice. Science 269, 540-543
55. Cohen, B., Novick, D., and Rubinstein, M. (1996) Modulation of insulin activities by leptin. Science 274, 1185-1188
56. Bennett, B. D., Solar, G. P., Yuan, J. Q., Mathias, J., Thomas, G. R., and Matthews, W. (1996) A role for leptin and its cognate receptor in hematopoiesis. Curr. Biol. 6, 1170-1180
57. Darimont, C., Vassaux, G., Ailhaud, G., and Negrel, R. (1994) Differentiation of preadipose cells: paracrine role of prostacyclin upon stimulation of adipose cells by angiotensin-II. Endocrinology 135, 2030-2036
58. Shimomura, I., Funahashi, T., Takahashi, M., Maeda, K., Kotani, K., Nakamura, T., Yamashita, S., Miura, M., Fukuda, Y., Takemura, K., Tokunaga, K., and Matsuzawa, Y. (1996) Enhanced expression of PAI-1 in visceral fat: possible contributor to vascular disease in obesity. Nat. Med. 2, 800-803
59. Xu, L., Miyoshi, H., Nishimura, K., Jisaka, M., Nagaya, T., and Yokota, K. (2007) Gene expression of isoformic enzymes in arachidonate cyclooxygenase pathway and the regulation by tumor necrosis factor alpha during life cycle of adipocytes. Prostaglandins Other Lipid Mediat. 83, 213-218

60. Chowdhury, A. A., Rahman, M. S., Nishimura, K., Jisaka, M., Nagaya, T., Ishikawa, T., Shono, F., and Yokota, K. (2011) 15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 interferes inducible synthesis of prostaglandins E_2 and $F_{2\alpha}$ that suppress subsequent adipogenesis program in cultured preadipocytes. Prostaglandins Other Lipid Mediat. 95, 53-62
61. O'Neill, G. P., and Ford-Hutchinson, A. W. (1993) Expression of mRNA for cyclooxygenase-1 and cyclooxygenase-2 in human tissues. FEBS Lett. 330, 156-160
62. Fu, J. Y., Masferrer, J. L., Seibert, K., Raz, A., and Needleman, P. (1990) The induction and suppression of prostaglandin H_2 synthase (cyclooxygenase) in human monocytes. J. Biol. Chem. 265, 16737-16740
63. Hata, A. N., and Breyer, R. M. (2004) Pharmacology and signaling of prostaglandin receptors: multiple roles in inflammation and immune modulation. Pharmacol. Ther. 103, 147-166
64. Samuelsson, B. (1978) Prostaglandins and thromboxanes. Recent Prog. Horm. Res. 34, 239-258
65. Smith, W. L., DeWitt, D. L., and Garavito, R. M. (2000) Cyclooxygenases: structural, cellular, and molecular biology. Annu. Rev. Biochem. 69, 145–182
66. Rouzer, C. A., and Marnett. L. J. (2003) Mechanism of free radical oxygenation of polyunsaturated fatty acids by cyclooxygenases. Chem. Rev. 103, 2239–2304
67. Mbonye, U. R., Yuan, C., Harris, C. E., Sidhu, R. S., Song, I., Arakawa, T., and Smith, W. L. (2008) Two distinct pathways for cyclooxygenase-2 protein degradation. Biol. Chem. 283, 8611–8623
68. Garavito, R. M., Malkowski, M. G., and DeWitt, D. L. (2002) The structures of prostaglandin endoperoxide H synthases-1 and -2. Prostaglandins Other Lipid Mediat. 68–69, 129–152
69. Kulmacz, R. J., van der Donk, W. A., and Tsai, A. L. (2003) Comparison of the properties of prostaglandin H synthase-1 and -2. Prog. Lipid Res. 42, 377–404
70. DuBois, R. N., Abramson, S. B., Crofford, L., Gupta, R. A., Simon, L. S., Van de Putte, B. A., and Lipsky, P. E. (1998) Cyclooxygenase in biology and disease. FASEB J. 12, 1063–1073
71. van der Donk, W. A., Tsai, A. L. and Kulmacz, R. J. (2002) The cyclooxygenase reaction mechanism. Biochemistry. 41, 15451–15458
72. Rouzer, C. A. and Marnett, L. J. (2009) Cyclooxygenases: structural and functional

insights. J. Lipid Res. 50, (Supplement) S29-S34

73. Chu, X., Nishimura, K., Jisaka, M., Nagaya, T., Shono, F., and Yokota, K. (2010) Up-regulation of adipogenesis in adipocytes expressing stably cyclooxygenase-2 in the antisense direction. Prostaglandins Other Lipid Mediat. 91, 1-9
74. Fajas, L., Miard, S., Briggs, M. R., and Auwerx, J. (2003) Selective cyclo-oxygenase-2 inhibitors impair adipocyte differentiation through inhibition of the clonal expansion phase. J. Lipid Res. 44, 1652-1659
75. Stehle, R. G. (1982) Physical chemistry, stability, and handling of prostaglandins E_2, $F_{2\alpha}$, D_2, and I_2: A critical summary. Methods Enzymol. 86, 436-458
76. Gryglewski, R. J. (2008) Prostacyclin among prostanoids. Pharmacol. Rep. 60, 3-11
77. Zou, M. H. and Ullrich, V. (1996) Peroxynitrite formed by simultaneous generation of nitric oxide and superoxide selectively inhibits bovine aortic prostacyclin synthase. FEBS Lett. 382, 101–104
78. Zou, M., Martin, C and Ullrich, V. (1997) Tyrosine nitration as a mechanism of selective inactivation of prostacyclin synthase by peroxynitrite. Biol. Chem. 378, 707–713
79. Wade, M. I., Voelkel, N. F., and Fitzpatrick, F. A. (1995) Suicide' inactivation of prostaglandin I_2 synthase: characterization of mechanism-based inactivation with isolated enzyme and endothelial cells. Arch. Biochem. Biophys. 321, 453–458
80. A. Mohite, A., Chillar, A., So, S. P., Cervantes, V., and Ruan, K. H. (2011) Novel mechanism of the vascular protector prostacyclin: regulating microRNA expression, Biochemistry. 50, 1691–1699
81. Dorris, S. L. and Peebles, R .S. (2012) PGI_2 as a Regulator of Inflammatory Diseases, Mediators Inflamm. Article ID 926968, 9 pages
82. Chillar, M. A., So, S. P., Cervantes, V. and Ruan, K. H. (2011) Novel mechanism of the vascular protector prostacyclin: regulating microRNA expression. Biochemistry 50, 1691–1699
83. Lovgren, A. K., Jania, L. A., Hartney, J. M., Parsons, K. K., Audoly, L. P., FitzGerald, G. A., Tilley, S. L., and Koller, B. H. (2006) COX-2-derived prostacyclin protects against bleomycin-induced pulmonary fibrosis. Am. J. Physiol. Lung Cell Mol. Physiol. 291, L144-L156
84. Gurgul-Convey, E., and S. Lenzen, S. (2010) Protection against cytokine toxicity through endoplasmic reticulum and mitochondrial stress prevention by prostacyclin

synthase overexpression in insulin-producing cells. J. Biol. Chem. 285, 11121–11128

85. Jaffar, Z., Wan, K. S., and Roberts, K. (2002) A key role for prostaglandin I_2 in limiting lung mucosal Th2, but not Th1, responses to inhaled allergen. J. Immunol. 169, 5997–6004
86. Falcetti, E., Flavell, D. M., Staels, B., Tinker, A., Haworth, S. G., and Clapp, L. H. (2007) IP receptor-dependent activation of PPARγ by stable prostacyclin analogues. Biochem. Biophys. Res. Commun. 360, 821-827
87. Massiera, F., Saint-Marc, P., Seydoux, J., Murata, T., Kobayahis, T., Narumiya, S., Guesnet, P., Amri, E. Z., Negrel, R., and Ailhaud, G. (2003) Arachidonic acid and prostacyclin signaling promote adipose tissue development: a human health concern? J. Lipid Res. 44, 271-279
88. Aubert, J., Saint-Marc, P., Belmonte, N., Dani, C., Négrel, R., and Ailhaud, G. (2000) Prostacyclin IP receptor up-regulates the early expression of C/EBPβ and C/EBPδ in preadipose cells. Mol. Cell Endocrinol. 160,149-156
89. Ailhaud. G. (1999) Cell surface receptors, nuclear receptors and ligands that regulate adipose tissue development. Clin. Chim. Acta. 286,181-190
90. Vassaux, G., Gaillard, D., Ailhaud, G., and Négrel, R. (1992) Prostacyclin is a specific effector of adipose cell differentiation. Its dual role as a cAMP- and Ca^{2+}-elevating
91. Shibata, T., Kondo, M., Osawa, T., Shibata, N., Kobayashi, M., and Uchida, K. (2002) 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2. A prostaglandin D_2 metabolite generated during inflammatory processes. J. Biol. Chem. 277, 10459-10466
92. Liston, T. E., and Roberts, L. J., 2nd. (1985) Transformation of prostaglandin D_2 to 9α, 11β-(15S)-trihydroxyprosta-(5Z,13E)-dien-1-oic acid (9α, 11β-prostaglandin F_2): a unique biologically active prostaglandin produced enzymatically *in vivo* in humans. Proc. Natl. Acad. Sci. USA 82, 6030-6034
93. Watanabe, K., Iguchi, Y., Iguchi, S., Arai, Y., Hayaishi, O., and Roberts, L. J., 2nd. (1986) Stereospecific conversion of prostaglandin D_2 to (5Z,13E)-(15S)-9α-11β,15-trihydroxyprosta-5,13-dien-1-oic acid (9α,11β-prostaglandin F_2) and of prostaglandin H_2 to prostaglandin $F_{2\alpha}$ by bovine lung prostaglandin F synthase. Proc. Natl. Acad. Sci. USA 83, 1583-1587
94. Urade, Y., Watanabe, K., Eguchi, N., Fujii, Y., and Hayaishi, O. (1990) 9α,11β-prostaglandin F_2 formation in various bovine tissues. Different isozymes of

prostaglandin D_2 11-ketoreductase, contribution of prostaglandin F synthetase and its cellular localization. J. Biol. Chem. 265, 12029-12035

95. Whittaker, N., Bunting, S., Salmon, J., Moncada, S., Vane, J. R., Johnson, R. A., Morton, D. R., Kinner, J. H., Gorman, R. R., McGuire, J. C., and Sun, F. F. (1976) The chemical structure of prostaglandin X (prostacyclin). Prostaglandins 12, 915-928
96. Miller, C. W., Casimir, D. A., and Ntambi, J. M. (1996) The mechanism of inhibition of 3T3-L1 preadipocyte differentiation by prostaglandin $F_{2\alpha}$. Endocrinology 137, 5641-5650
97. Tsuboi, H., Sugimoto, Y., Kainoh, T., and Ichikawa, A. (2004) Prostanoid EP4 receptor is involved in suppression of 3T3-L1 adipocyte differentiation. Biochem. Bioph. Res. Commun. 322, 1066-1072
98. Giles, H., and Leff, P. (1988) The biology and pharmacology of PGD_2. Prostaglandins 35, 277-300
99. Fitzpatrick, F. A., and Wynalda, M. A. (1983) Albumin-catalyzed metabolism of prostaglandin D_2. Identification of products formed *in vitro*. J. Biol. Chem. 258, 11713-11718
100. Simmons, D. L., Botting, R. M., and Hla, T. (2004) Cyclooxygenase isozymes: the biology of prostaglandin synthesis and inhibition. Pharmacol. Rev. 56, 387-437
101. Yan, H., Kermouni, A., Abdel-Hafez, M., and Lau, D. C. (2003) Role of cyclooxygenases COX-1 and COX-2 in modulating adipogenesis in 3T3-L1 cells. J. Lipid. Res. 44, 424-429
102. Hyman, B. T., Stoll, L. L., and Spector, A. A. (1982) Prostaglandin production by 3T3-L1 cells in culture. Biochim. Biophys. Acta 713, 375-385
103. Sugimoto, Y., Tsuboi, H., Okuno, Y., Tamba, S., Tsuchiya, S., Tsujimoto, G., and Ichikawa, A. (2004) Microarray evaluation of EP4 receptor-mediated prostaglandin E_2 suppression of 3T3-L1 adipocyte differentiation. Biochem. Biophys. Res. Commun. 322, 911-917
104. Lu, S., Nishimura, K., Hossain, M. A., Jisaka, M., Nagaya, T., and Yokota, K. (2004) Regulation and role of arachidonate cascade during changes in life cycle of adipocytes. Appl. Biochem. Biotechnol. 118, 133-153
105. Fain, J. N., Ballou, L. R., and Bahouth, S. W. (2001) Obesity is induced in mice heterozygous for cyclooxygenase-2. Prostaglandins Other Lipid Mediat. 65, 199-209
106. Lepak, N. M., and Serrero, G. (1995) Prostaglandin $F_{2\alpha}$ stimulates transforming

growth factor α expression in adipocyte precursors. Endocrinology 136, 3222-3229

107. Forman, B. M., Tontonoz, P., Chen, J., Brun, R. P., Spiegelman, B. M., and Evans, R. M. (1995) 15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 is a ligand for the adipocyte determination factor PPARγ. Cell 83, 803-812
108. Kliewer, S. A., Lenhard, J. M., Willson, T. M., Patel, I., Morris, D. C., and Lehmann, J. M. (1995) A prostaglandin J_2 metabolite binds peroxisome proliferator-activated receptor gamma and promotes adipocyte differentiation. Cell 83, 813-819
109. Chowdhury, A. A., Hossain, M. S., Rahman, M. S., Nishimura, K., Jisaka, M., Nagaya, T., Shono, F., and Yokota, K. (2011) Sustained expression of lipocalin-type prostaglandin D synthase in the antisense direction positively regulates adipogenesis in cloned cultured preadipocytes. Biochem. Biophys. Res. Commun. 411, 287-292
110. Syeda, P. K., Hossain, M. S., Chowdhury, A. A., Rahman, M. S., Nishimura, K., Jisaka, M., Nagaya, T., Shono, F., and Yokota, K. (2012) Generation of monoclonal antibody for 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 and development of enzyme-linked immunosorbent assay for its quantification in culture medium of adipocytes. Appl. Biochem. Biotechnol. 167, 1107-1118
111. Hertz, R., Berman, I., Keppler, D., and Bar-Tana, J. (1996) Activation of gene transcription by prostacyclin analogues is mediated by the peroxisome-proliferators-activated receptor (PPAR) Eur. J. Biochem. 235, 242-247
112. Gaillard, D., Negrel, R., Lagarde, M., and Ailhaud, G. (1989) Requirement and role of arachidonic acid in the differentiation of pre-adipose cells. Biochem. J. 257, 389-397
113. Negrel, R., Gaillard, D., and Ailhaud, G. (1989) Prostacyclin as a potent effector of adipose-cell differentiation. Biochem. J. 257, 399-405
114. Catalioto, R. M., Gaillard, D., Maclouf, J., Ailhaud, G., and Negrel, R. (1991) Autocrine control of adipose cell differentiation by prostacyclin and $PGF_{2\alpha}$. Biochim. Biophys. Acta 1091, 364-369
115. Hopkins, N., and Gorman, R. R. (1981) Regulation of 3T3-L1 fibroblast differentiation by prostacyclin (prostaglandin I_2). Biochim. Biophys. Acta 663, 457-466

Chapter 2

Cultured preadipocytes undergoing stable transfection with cyclooxygenase-1 in the antisense direction accelerate adipogenesis during the maturation phase of adipocytes

1. Introduction

Fat mass in the animal body is progressively enhanced by adipogenesis through excess food intake, resulting in the increases in the cell number and the size of mature adipocytes in adipose tissue. The process for the development of mature adipocytes includes distinct steps, such as the growth phase for the proliferation of preadipocytes, the differentiation phase for the induction of adipogenesis program, and the terminal maturation phase for the accumulation of fats due to the stimulated adipogenesis [1-3]. Obesity is characterized by the pathological abundance of hypertrophic adipocytes in visceral and subcutaneous adipose tissues and the changes in the quality of adipocytes leading to the onset of insulin resistance and altered secretion patterns of adipocytokines [4, 5]. Hence, understanding of the cellular mechanism underlying the control of the adipocyte differentiation and adipogenesis by bioactive modulators is important in terms of obesity and the related diabetes.

Prostaglandins (PGs) and the related compounds classified as autacoids or local hormones are known to be produced at different life stages of adipocytes and exert their opposite effects on adipogenesis depending on the type of PG species [6-9]. For example, PGE_2 and $PGF_{2\alpha}$ can be generated predominantly by undifferentiated preadipocytes in the growth phase of cultured 3T3-L1 cells [7, 10]. Both compounds have been shown to act as anti-adipogenic factors for the differentiation and maturation of adipocytes through the specific cell surface receptors of the EP4 and FP receptors for PGE_2 and $PGF_{2\alpha}$, respectively [6, 7]. On the other hand, recent studies have revealed that PGs of J_2 series including 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2) and Δ^{12}-PGJ_2, the dehydration products of PGD_2, promote adipogenesis in cultured adipocytes as pro-adipogenic factors by the activation of peroxisome proliferator-activated receptor γ (PPARγ), a nuclear hormone

receptor known as a master regulator of adipogenesis [11, 12]. Especially, 15d-PGJ_2 is known to be one of the most potent high-affinity ligand for the PPARγ. We have previously described that cultured 3T3 -L1 cells can enhance the ability to endogenous PGs of J_2 series during the later maturation phase and contribute to the up-regulation of adipogenesis in an autocrine manner [8, 9]. Nevertheless, the effects of PGs on adipogenesis and related functions of adipocytes remain yet to be elucidated because those actions are complicated due to the differences in the used cell type and the culture condition among various approaches.

PGs are synthesized through the arachidonate cyclooxygenase (COX) pathway in which the enzymatic reaction of PGH synthase alternatively called COX is the rate-limiting step for the generation of PGs after the release of free arachidonic acid (AA) [13]. The COX enzyme is known to occur as two types of isoforms, including the constitutive COX-1 and the inducible COX-2. While COX-1 is generally associated with the immediate production of PGs to execute house keeping functions, such as platelet aggregation and vascular homeostasis, COX-2 is mostly responsible for the delayed synthesis of PGs by the induction of the de novo synthesis of the new enzyme in response to mitogenic and inflammatory factors in pathological tissues. The specificity for the synthesis of PG species depends on the functional coupling of either COX with the downstream PG synthases expressed in mammalian cells. A previous study have described that obesity is induced more highly in mice heterozygous for COX-2 than COX-1 and COX-2 deficient homozygotes and their control littermates [14]. This observation suggests that COX-2 is implicated in the negative regulation of adipogenesis in whole body. However, mice heterozygous for COX-1 have not been described, so that it remains elusive as for the role of COX-1 in adipogenesis and obesity *in vivo*.

Cultured mouse preadipogenic 3T3-L1 cells have been useful for the studies on the regulation of the arachidonate COX pathway and the role of the products in the control of adipogenesis and other functions of adipocytes because of the established culture conditions consisted of the growth, differentiation, and maturation phases [15, 16]. Therefore, we have been studying the gene expression of the biosynthetic enzymes of the COX pathway and the roles for endogenous and exogenous PGs at different life stages [7-9]. The delayed synthesis of PGE_2 and $PGF_{2\alpha}$ by cultured preadipocytes can be simulated by the addition of a mixture of active phorbol diester and calcium ionophore,

which was accompanied by the induction of COX-2 [7]. Alternatively, our study has shown that cultured adipocytes during the maturation phase enhance the ability to generate endogenous PGJ_2 derivatives and contribute to the up-regulation of adipogenesis [8, 9]. Since both specific inhibitors for COX-1 and COX-2 were found to be effective in the inhibition of the generation of endogenous PGJ_2 derivatives, both COX isoforms could be responsible for the production of them. Some of other studies have reported the selective inhibitors for COX-1 and COX-2 similarly accelerated the differentiation of 3T3-L1 adipocytes in the modified differentiation medium without dexamethasone in the presence of a PPARγ agonist [17] or in another culture medium supplemented with free arachidonic acid [18]. Thus, COX inhibitors sometimes exert different effects on adipogenesis by the use of modified differentiation medium with varied hormone cocktails. As well, it should be noted that some of COX inhibitors by themselves act as active ligands for PPARγ at higher concentrations [19].

On the basis of the findings and uncertainties as described above, we attempted to investigate the contribution of the constitutive COX-1 to adipogenesis during the maturation phase of adipocytes. For this analysis, we undertook the approach to selectively suppress the expression of COX-1 by making use of cultured preadipocytes transfected stably with COX-1 oriented in the antisense direction. The resulting methods have been shown to be effective for the selective attenuation of COX-2 and lipocalin-type PG synthase in our recent studies [20, 21]. In this study, we show that sustained expression of antisense COX-1 selectively block the expression of sense COX-1 and inhibited the immediate PG synthesis without affecting the induction of COX-2. The resulting transfectants can promote adiopogenesis at higher levels during the maturation phase than untransfected parent cells and the transfectants with the vector only.

2. Materials and methods

2.1 Materials

Dulbecco's modified Eagle's medium with 25 mM HEPES (DME-HEPES), penicillin G potassium salt, streptomycin sulfate, dexamethasone, phorbol 12-myristate 13-acetate (PMA), calcium ionophore A23187, and recombinant human insulin were obtained from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from MP

Biomedicals (Solon, OH, USA), and Plasmid Mini Kit and PolyFect Transfection Reagent were from Qiagen (Valencia, CA, USA). 3-Isobutyl-1-methylxanthine (IBMX), the *Xba*I restriction enzyme, geneticin disulfate (G418), and Triglyceride E-Test Kit were supplied by Wako (Osaka, Japan). AA, PGs, COX inhibitors, and rabbit polyclonal antibodies against murine COX-1 and COX-2 were provided by Cayman Chemical (Ann Arbor, MI, USA). M-MLV reverse transcriptase (RT) (ribonuclease H minus) and Polymerase Chain Reaction (PCR) MasterMix were commercial products of Promega (Madison, WI, USA). Oligonucleotides used for the reactions of RT and PCR were supplied by Sigma Genosys Japan (Ishikari, Japan). 96-Well microplates for enzyme-liked immunosorbent assay (ELISA) were purchased from BD Falcon (Durham, NC, USA). Other chemicals and materials used for cell culture, RT-PCR, and ELISA were obtained as described earlier [7, 20, 22].

2.2 Cell culture of preadipocytes and the differentiation into mature adipocytes

The preadipogenic mouse 3T3-L1 cells (JCRB9014) were plated at 5×10^4 cells/ml in the growth medium (GM) containing DME–HEPES, 10% FBS, 100 μg/ml streptomycin sulfate, 100 U/ml penicillin G, and 200 μM ascorbic acid, and then cultured at 37 °C under 7% CO_2 until confluence. The resulting monolayer cells were exposed to the differentiation medium (DM) corresponding to GM supplemented with 1 μM dexamethasone, 0.5 mM IBMX, and 10 μg/ml insulin for 45 h for the induction of the differentiation phase. Then, the culture medium was replaced with the maturation medium (MM) consisting of GM with 5 μg/ml insulin, and refed every 2 days to promote the accumulation of fats in adipocytes during the maturation phase as described earlier [7-9, 23]. For the determination of fat storage, the cultured cells were usually harvested after 6 days of the maturation phase. Furthermore, MM was collected during the corresponding period of 8-10 days for the quantification of J_2 series PGs as described below.

2.3 Construction of expression vector for COX-1 oriented in the antisense direction

Total RNA was extracted from cultured murine MC3T3-E1 cells and used for the amplification of mouse COX-1 cDNA coding for the open reading frame by RT-PCR using specific 5′- and 3′-primers with the *Xba*I recognition site as described previously [20, 24].

The amplified DNA fragments were cut with *Xba*I and ligated to the same site of the mammalian expression vector, pcDNA3.1(+) with neomycin-resistant gene (Invitrogen, Carlsbad, CA, USA). To obtain the recombinant plasmid, *E. coli* DH5α was transformed with the ligation product along with ampicillin. After extraction of the recombinant DNA from the independent single colonies, it was subjected to the analysis of the orientation of COX-1 cDNA by digesting with unique restriction enzymes. Finally, the desired DNA fragment was extracted and purified by the Plasmid Mini Kit. The isolated plasmid DNA was analyzed for the confirmation of the cDNA insert of COX-1 oriented in the antisense direction by the DNA sequence using a ABI Prism 3100 Genetic Analyzer and the BigDye Terminator v.1.1 Cycle Sequence Kit (Applied Biosystems, Foster City, CA, USA) [7, 20, 24].

2.4 Isolation of cloned stable transfectants with antisense COX-1

Parent 3T3-L1 preadipocytes were transfected with the mammalian expression vector, pcDNA3.1(+), harboring the cDNA insert of mouse COX-1 oriented in the antisense direction or the expression vector only as a control using the PolyFect Transfection Reagent according to the manufacturer's instructions. The cultured cells were grown in GM supplemented with 500 μg/ml G418 as described previously [20, 21, 24]. The resulting stable transfectants were employed for the isolation of cloned cells from single cells by the limiting dilution method in 96-well microplates or using cloning glass rings in 100-mm Plastic culture dishes. The isolated independent clones were propagated and analyzed for the transcript levels of sense and antisense mRNA of COX-1 and protein levels of COX isoforms as described below.

2.5 Analysis of transcript and protein expression levels

Total RNA was extracted from the cultured parent 3T3-L1 cells and the stable transfectants at the indicated stages by the method of acid guanidium thiocyanate/phenol/chloroform mixture [25]. For the specific detection of mRNA levels of desired mouse genes, total RNA (1 μg) was used for RT-PCR with M-MLV reverse transcriptase (ribonuclease H minus) and 1×PCR Master Mix as described earlier [7, 8]. Most of the RT reaction was performed using a mixture of the oligo-$(dT)_{15}$ primer and a

random primer (9 mer) (Takara, Kyoto, Japan) for the synthesis of single strand cDNA. To distinguish sense and antisense mRNA levels of mouse COX-1, 5'-TCAGAGCTCAGTGGAGCGTCT-3' as a complementary sequence of terminal sense COX-1 mRNA and 5′- ATGAGTCGAAGGAGTCTCTCG-3′ as a complementary sequence of terminal antisense COX-1 mRNA were used instead of the oligo-$(dT)_{15}$ primer. The amplification of target genes was conducted using a combination of 5′-primer and 3′-primer specific for COX-1, COX-2, neomycin-resistant gene, PPARγ, CCAAT/enhancer-binding protein α (C/EBPα), glucose transporter-4 (GLUT-4), leptin, lipoprotein lipase (LPL), adipocyte protein 2 (aP2), adiponectin, and β-actin as listed previously [7, 8, 20, 24]. The amplified DNA fragments were separated by 1.5% agarose gel electrophoresis and stained with ethidium bromide. Moreover, the DNA sequences of the PCR products were confirmed as reported earlier.

Parent cultured 3T3-L1 cells and transfectants with antisense COX-1 or the vector only were cultured until 100% confluence in GM. The confluent cells were harvested for the determination of the protein levels of COX-1 by Western blot analysis using a polyclonal rabbit antibody raised specifically against mouse COX-1 as reported previously [7, 20, 24]. For the determination of inducible COX-2 protein levels, the confluent cells were stimulated for 24 h with a mixture of 0.1 μM PMA and 0.1 μM A23187 in GM. After the incubation, the cultured cells were harvested for Western blot analysis using a polyclonal rabbit antibody specific for mouse COX-2 according to our previous methods [7, 20, 24].

2.6 ELISA for PGE_2, 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2) and Δ^{12}-PGJ_2

To monitor the immediate synthesis of PGE_2, parent preadipocytes and the transfectants were grown to 100% confluence in GM and stimulated for 30 min with either 10 μM free AA or 10 μM calcium ionophore A23187. After the incubation, the culture medium was collected for the quantification of PGE_2 by ELISA using a monoclonal antibody specific PGE_2 as described before [22]. Alternatively, for the determination of endogenous synthesis of J_2 series of PGs, parent preadipocytes and the transfectants were allowed to undergo the differentiation and maturation as described above. During 8-10 days of the maturation phase, MM was collected and subjected to the quantification of 15d-PGJ_2 and Δ^{12}-PGJ_2 by ELISA specific for each of the compounds using specific antibodies raised

for 15d-PGJ_2 and Δ^{12}-PGJ_2, respectively as we reported recently [8, 9].

2.7 Other methods

The content of triacylglycerols in adipocytes was measured by using Triglyceride E-Test Kit [7, 23]. The amount of cellular proteins in adipocytes was determined according to the method of Lowry et al. [26] following the precipitation of proteins with cold trichloroacetic acid to remove the interfering substances [27]. The lipid droplets accumulated in cultured 3T3-L1 adipocytes were stained with Oil Red O for macroscopic and microscopic views in culture dishes as reported earlier [23, 28]. Cell number of cultured cells attached to dishes was counted using a hepatocytometer after treatment with a mixture of trypsin and EDTA. Quantification of transcript and protein levels of target genes was performed as described previously [29]. Statistical analysis was evaluated by the Student's *t* test, and the values of $p<0.05$ were used for the consideration of significant difference.

3. Results

3.1 Isolation of cloned stable transfectants from cultured preadipoytes with antisense COX-1

To suppress specifically the expression of COX-1, the constitutive isoform, cultured 3T3-L1 preadipocytes were transfected with the mammalian expression vector, pcDNA3.1(+), having the insert of mouse COX-1 oriented in antisense direction. Eventually, cloned stable transfectants with antisense COX-1 were independently isolated after the continued growth by the selection in the presence of G418, a neomycin analogue. Among these cloned transfectants, we made use of four types of cloned cells termed C-3, C-7, C-11, and C-21 as representative ones for further characterization of them by the comparison with untransfected parent cells and the transfectants with the vector only. The neomycin-resistant gene was consistently expressed in all the isolated clones of the stable transfectants (Fig. 2-1). By contrast, the gene was not expressed in the untransfected parent preadipocytes.

We attempted to distinguish the gene expression levels of sense and antisense COX-1

mRNA by RT-PCR using primers specific for mouse COX-1 sequences after the RT reaction was performed with unique oligonucleotides as primers complementary to the respective terminal sequences of sense and antisense COX-1 mRNA. The analysis revealed that the expression of sense COX-1 was markedly blocked in the transfectants with antisense COX-1 while its expression was not affected in both untransfected parent preadipocytes and the transfectants with the vector only (Fig. 2-1).

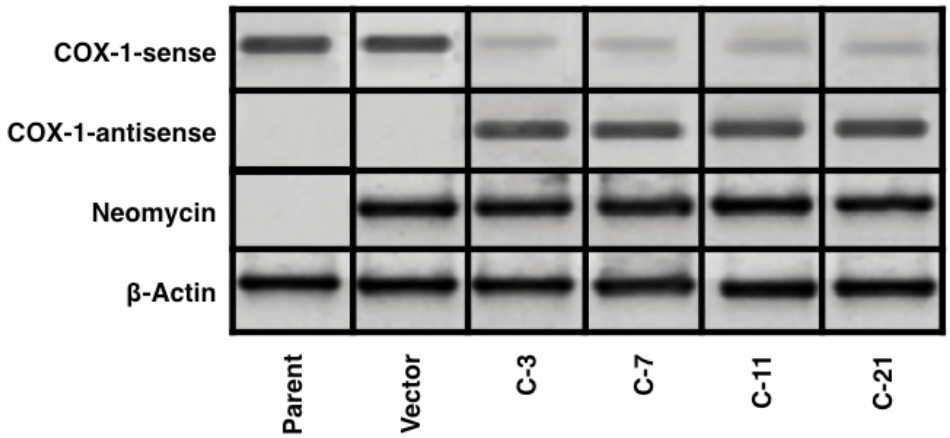


Fig. 2-1. Transcript levels of sense and antisense COX-1 in untransfected parent 3T3-L1 cells and the transfectants with COX-1 orientated in the antisense direction. Parent 3T3-L1 preadipocytes and the stable transfectants with either the mammalian expression vector only or the vector having the insert of murine COX-1 in the antisense direction were plated at 5×10^4 cells/ml in a 60-mm Petri dish containing 4 ml of GM and grown to until confluence. Total RNA was extracted from cultured cells for the analysis for mRNA levels of sense or antisense COX-1, a neomycin-resistant gene in the vector, and β-actin as a control. The gene expression levels of sense and antisense COX-1 were specifically detected by RT-PCR using the corresponding specific primer complementary to the terminal sequence of either sense or antisense COX-1 mRNA. The amplified DNA fragments were separated by 1.5% agarose gel electrophoresis and stained with ethidium bromide. Data are shown from a representative one done in triplicate experiments. Labels: Parent, untransfected cells; Vector, cloned transfectants with the expression vector only; C-3, C-7, C-11, and C-21, cloned transfectants with antisense COX-1.

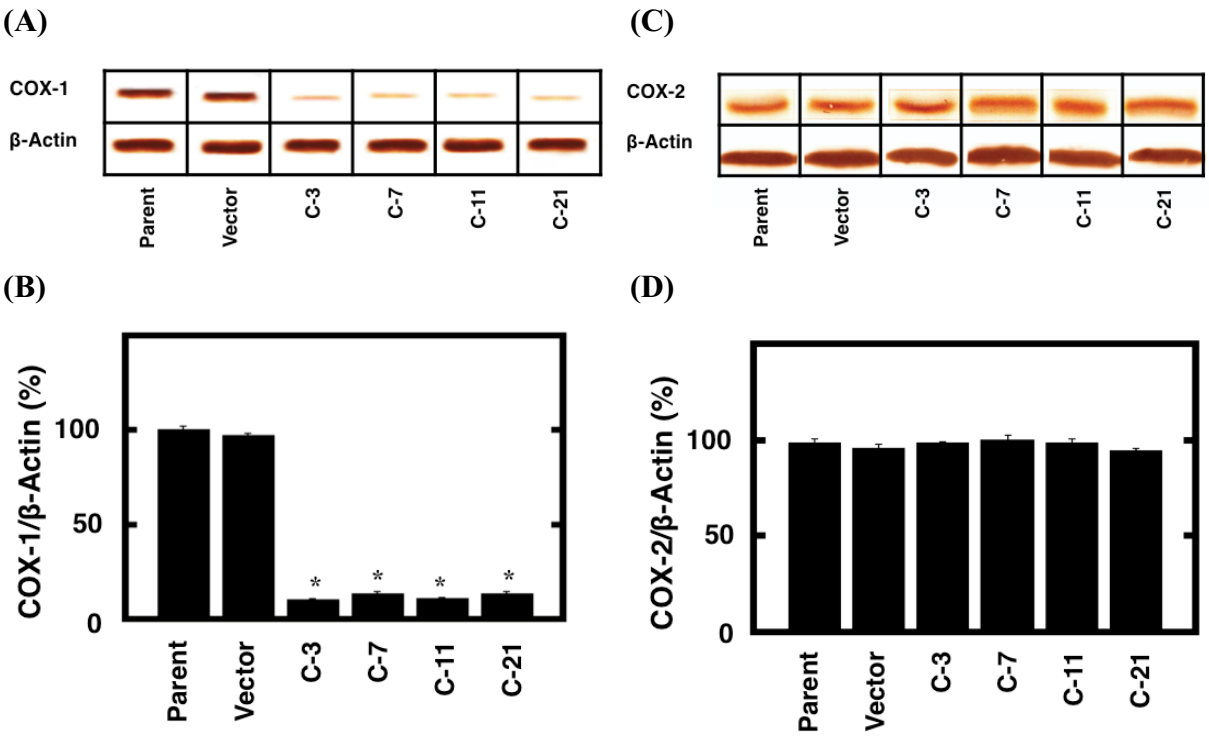


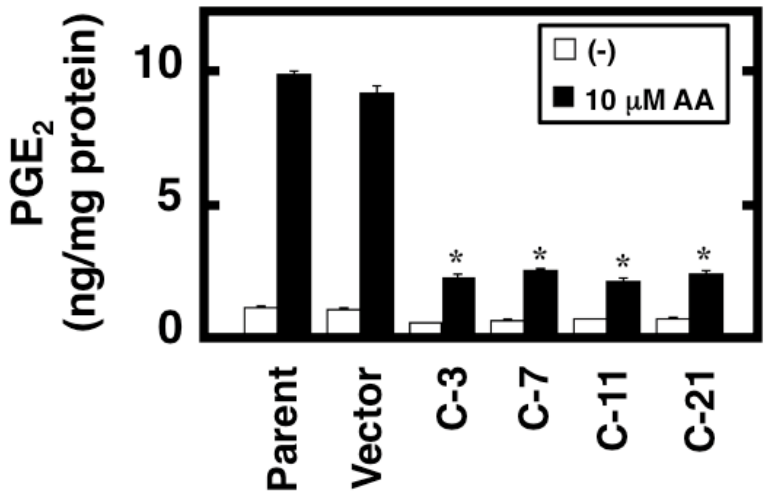
Fig. 2-2. Protein expression levels of COX-1 and COX-2 in cultured parent cells and the transfectants with antisense COX-1. Parent preadipocytes and the transfectants were plated and grown to confluence as described in Fig. 2-1. (A) Western blot analysis of COX-1. After the confluent cells were harvested, the resulting lysates (20 μg in each lane) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 7.5% separation gel, and then transferred onto polyvinylidene difluoride (PVDF) membrane. The blotted membrane was used for the detection by a rabbit polyclonal antibody specific murine COX-1. Data are shown from a representative one done in triplicate experiments. (B) Quantification of the expressed protein levels of COX-1. The results of (A) were employed for the quantification by the image analysis. Data represent the mean ± S.E.M. of three independent experiments relative to the average value of parent cells. *p<0.05 compared with the transfectants with the vector only. (C) Western blot analysis of COX-2. After the stimulation of the confluent cells for 24 h with a mixture of 0.1 μM PMA and 0.1 μM A23187, the cell lysates (20 μg in each lane) were harvested, separated by SDS-PAGE, and transferred on the PVDF membrane as described above. The blotted membrane was subjected to the detection by a polyclonal antibody specific for murine COX-2. Data are shown from a representative one done in triplicate experiments. (D) Quantification of the expressed protein levels of COX-2. The results of (C) were analyzed for the quantification by the image analysis. Data represent the mean ± S.E.M. of three independent experiments relative to the average value of parent cells.

On the other hand, all of the stable transfectants with antisense COX-1 showed clearly the expression of antisense COX-1 mRNA that were not detectable in other control cells. Moreover, we investigated the protein expression levels of COX isoforms in those cultured cells by Western blot analysis (Fig. 2-2). Consistent with the results of the transcription, the protein expression levels of COX-1 were significantly suppressed in the transfectants with antisense COX-1 (Figs. 2-2A and 2-2B). However, the transfection of cultured preadipocytes with antisense COX-1 had no effect on the protein expression of the inducible COX-2 in response to a mixture of PMA and A23187 (Figs. 2-2C and 2-2D). All of cultured cells showed much lower basal levels of COX-2 without the stimuli, which were almost similar among the used cultured cells. The basal expression levels of COX-2 were much lower and These findings indicated that we established successfully cloned stable transfectants unable to specifically express the constitutive COX-1 without the influence on the expression of the inducible COX-2.

3.2 Immediate generation of PGE_2 by parent preadipocytes and the transfectants with antisense COX-1

The immediate synthesis of PGs is known to involve the enzymatic reaction of the constitutive COX-1 in response to acute cellular stimuli [13]. To determine whether the suppressed gene expression of COX-1 could affect the immediate production of PGE_2 by the cloned stable transfectants, the cultured cells during the growth phase were allowed to react for 30 min with 10 μM free AA (Fig. 2-3A) or 10 μM calcium ionophore A23187 serving as an activator of phospholipase A_2 through the elevation of intracellular Ca^{2+} levels to release free AA from membrane phospholipids (Fig. 2-3B). The quantification of endogenous PGE_2 by its specific ELISA provided the evidence that the immediate synthesis of PGE_2 from both exogenous and endogenous AA was substantially attenuated in the stable transfectants with antisense COX-1 under the same conditions as compared with the parent preadipocytes and the transfectants with the vector only. These results were consistent with those of the specifically reduced gene expression of COX-1 as described above.

(A)



(B)

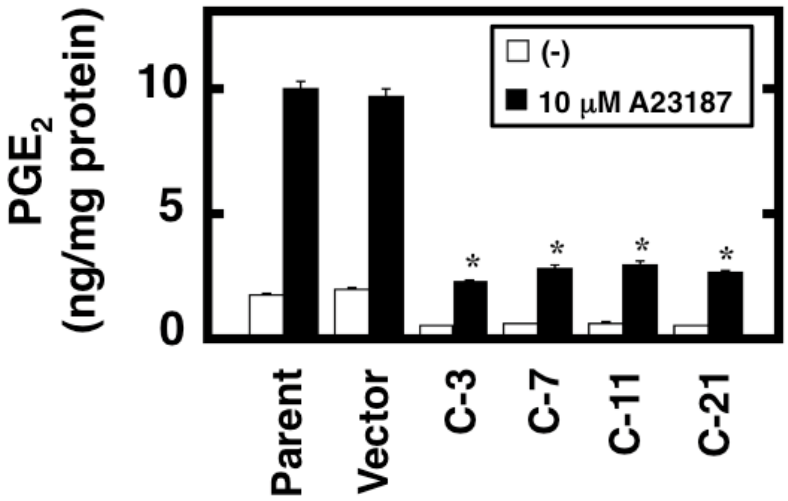


Fig. 2-3. Immediate PGE_2 synthesis by cultured transfectants with antisense COX-1. (A) PGE_2 synthesis from exogenous free AA. Parent preadipoctyes and the transfectants were plated at 5×10^4 cells/ml in a 35-mm dish Petri dish containing 2 ml of GM and grown to confluence. The resulting cultured cells were incubated for 30 min with 10 μM free AA (A) or 10 μM A23187 (B). The culture medium was collected for the quantification of endogenously synthesized PGE_2 by its specific ELISA. Data represent the mean ± S.E.M. of three experiments. *p< 0.05 compared with the transfectants with the vector only after incubation with free AA or A23187.

3.3 Stimulated storage of fats in transfectants with antisense COX-1 during the maturation phase

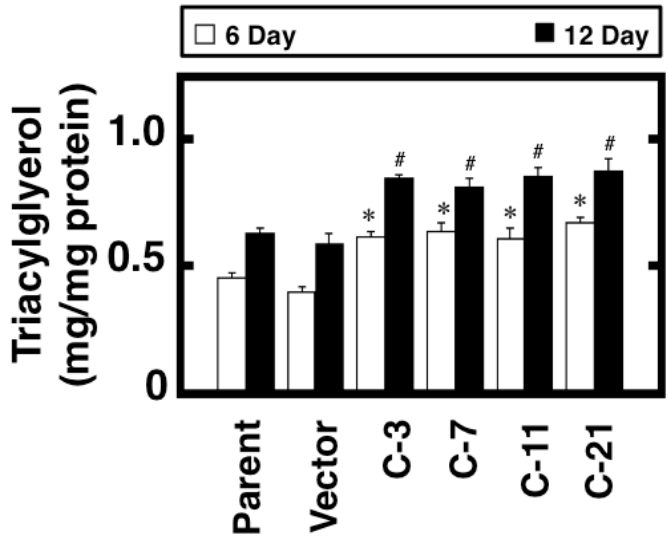
The accumulation of triacylglycerols was determined in the cloned stable transfectants and the related control cells after 6 and 12 days of the maturation phase (Fig. 2-4A). The quantitative analysis revealed that the stored levels of fats were significantly higher during both periods of the maturation phase in all the cloned stable transfectants with antisense

COX-1 than in the parent adipocytes and the vector-transfected cells. On the other hand, no appreciable difference of cell proliferation was observed between the cloned stable transfectants and other related control cells when the cell number was monitored after 6 and 12 days of the maturation phase (Fig. 2-4B). Moreover, the observation of microscopic and macroscopic views of cultured cells before or after staining of fat droplets with Oil Red O confirmed the elevated levels of stored fats in cloned stable transfectants with antisense COX-1 (Fig. 2-5A). In addition, the quantitative analysis revealed that the size of fat droplets in individual cloned transfectants was found to be significantly larger than that in the parent adipocytes and the vector-transfected cells (Fig. 2-5B). Thus, these findings obviously confirmed that the stable transfection of cultured preadipocytes with antisense COX-1 enhance fat storage in cultured adipocytes during the maturation phase.

3.4 Gene expression of adipogenesis markers

Next, we investigated whether the increased accumulation of fats in the cloned stable transfectants could be associated with the positive regulation of adipogenesis program after the maturation phase. To determine this involvement, the transcript levels of well-known adipogenesis markers were evaluated in cultured adipocytes and the related transfectants after 6 days of the maturation phase (Fig. 2-6). All of the cloned transfectants with antisense COX-1 expressed higher transcript levels of typical adipogenesis markers, including C/EBPα, GLUT-4, leptin, LPL, aP2, and adiponectin, under the control of PPARγ as a master regulator of adipogenesis as compared with the parent adipocytes and the transfectants with vector only (Fig. 2-6A). The quantitative determination confirmed

(A)



(B)

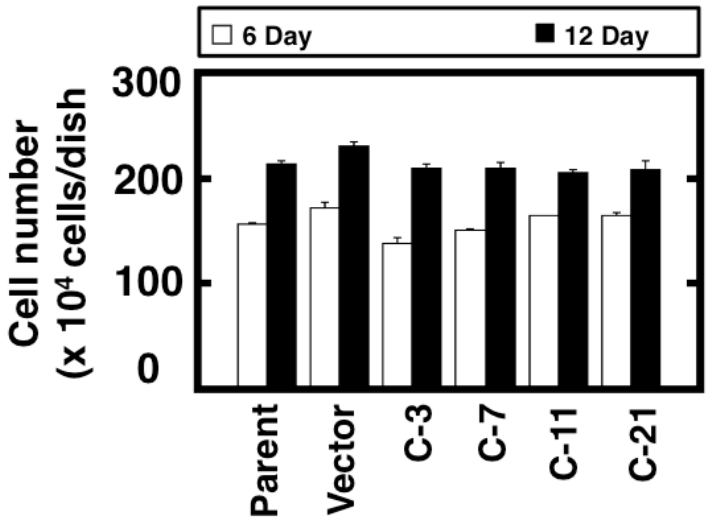
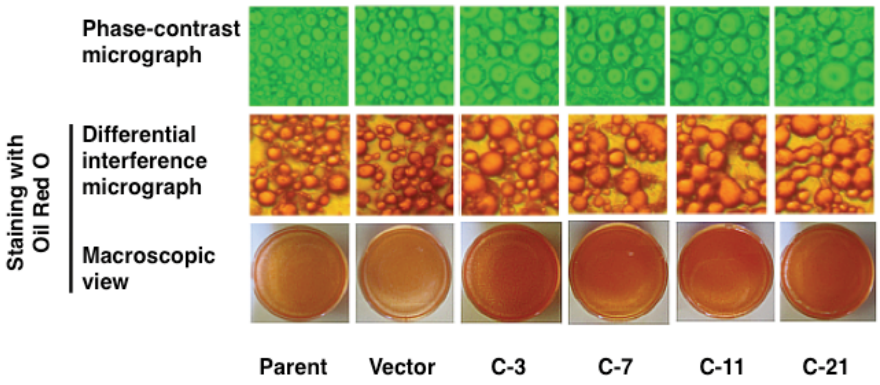


Fig 2-4. Storage of fats and cell growth during the maturation phase in cultured transfectants with antisense COX-1. Parent preadipocytes and the transfectants were plated and grown to confluence as described in Fig. 2-3. The resulting monolayer cells were allowed to undergo the differentiation and maturation in DM and MM, respectively. The amount of accumulated triacylglycerols (A) and the cell number of cultured cells attached to the dishes (B) were quantified after 6 and 12 days of the maturation phase. Data represent the mean ± S.E.M. of three experiments. *p< 0.05 compared with the transfectants with the vector only after 6 days of the maturation phase. #p< 0.05 compared with the transfectants with the vector only after 12 days of the maturation phase.

(A)



(B)

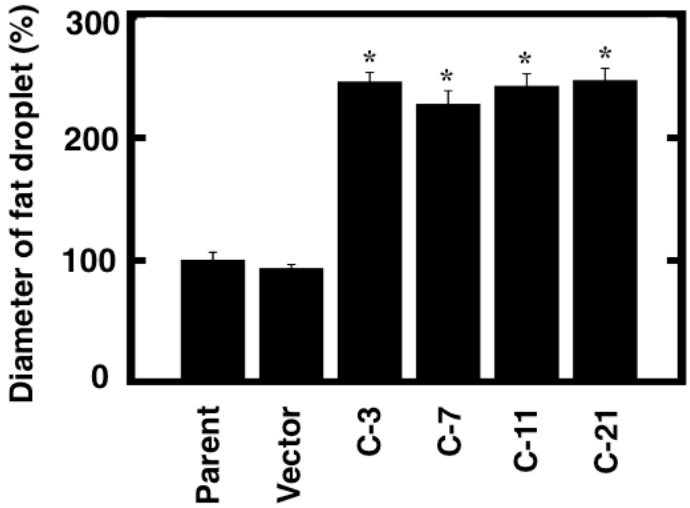


Fig. 2-5. Observation of cultured transfectants with antisense COX-1. Parent preadipocytes and the transfectants were plated and grown to confluence as described in Fig. 2-3. The resulting monolayer cells were allowed to undergo the differentiation and maturation. (A) Microscopic and macroscopic views of cultured cells. After 6 days of the maturation phase, the cultured cells were viewed by phase-contrast microscopy at 200× magnification (upper panels). Alternatively, the cultured cells under the same conditions were observed as microscopic views by differential-interference microscopy (middle panels) or macroscopic views (lower panels) after staining with Oil Red O. Data are shown from a representative one done in triplicate experiments. (B) Determination of the size of oil droplets. The average diameter of oil droplets in different cells was quantified by the image analysis of the cells stained with Oil Red O. Data represent the mean ± S.E.M. (n=30). *p< 0.05 compared with the transfectants with the vector only after 6 days of the maturation phase.

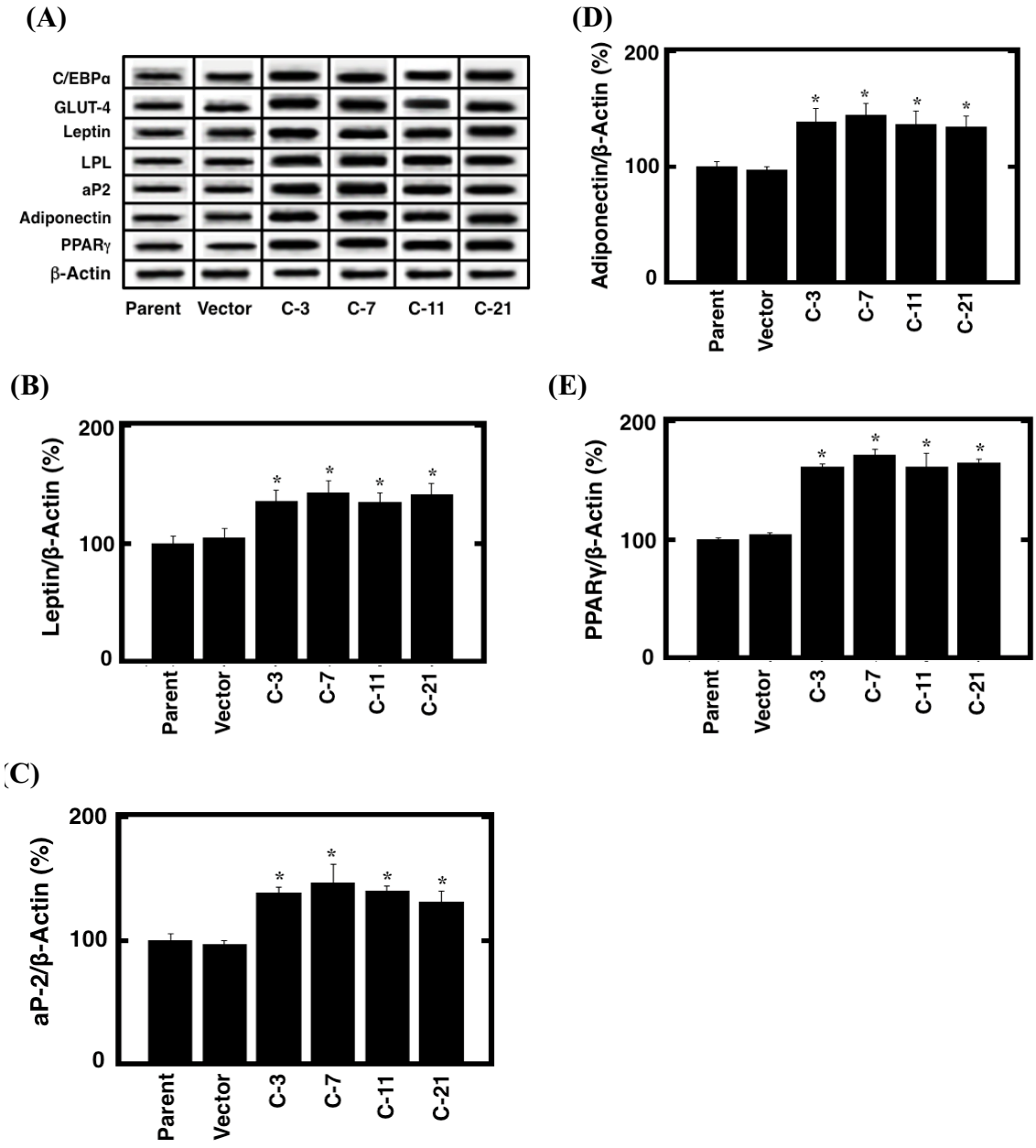


Fig. 2-6. Gene expression levels of adipocyte-specific genes in cultured transfectants with COX-1 orientated in the antisense direction. Parent preadipocytes and the transfectants were plated and grown to confluence as described in Fig. 2-1. The resulting monolayer cells were allowed to undergo the differentiation and maturation. After 6 days of the maturation phase, total RNA was extracted from the cultured cells as described in Fig. 2-1 and subjected to the gene expression analysis of adipocyte-specific markers including C/EBPα, GLUT-4, leptin, LPL, aP2, adiponectin, PPARγ and β-actin (A). Data are shown from a representative one performed in triplicate experiments. The quantified transcript levels of leptin (B), aP-2 (C), adiponectin (D), and PPARγ (E) were also represented with the data showing the mean ± S.E.M. of three experiments for

each target gene. *p< 0.05 compared with the transfectants with the vector only after 6 days of the maturation phase.

that the transcript levels of leptin (Fig. 2-6B), aP-2 (Fig. 2-6C), adiponectin (Fig. 2-6D), and PPARγ (E) were significantly higher than in the closed cells transfected with antisense COX-1 than in other control cells. These results indicate that the stimulated fat storage was tightly linked with the up-regulation of adipogenesis program during the maturation phase in the stable transfectants with antisense COX-1.

3.5 Endogenous production of J_2 series PGs by cultured adipocytes and the transfectants during the maturation phase

PGD_2 has been shown to undergo the non-enzymatic dehydration to generate PGJ_2 derivatives including 15d-PGJ_2 and Δ^{12}-PGJ_2 through PGJ_2 as an unstable intermediate [30]. These J_2 series PGs are known to serve as pro-adipogenic factors in cell culture systems of adipocytes [8, 9, 11, 12]. Here, to assess whether the stimulation of fat storage in the transfectants with antisense COX-1 could be accompanied by the increases in the endogenous levels of PGJ_2 derivatives, we evaluated the ability of cultured cells during the maturation phase to generate endogenous 15d-PGJ_2 and Δ^{12}-PGJ_2 by ELISA specific for each of them. The cloned stable transfectants with antisense COX-1 generated endogenous 15d-PGJ_2 (Fig. 2-7A) and Δ^{12}-PGJ_2 (Fig. 2-7B) between 8 and 10 days of the maturation phase at significantly higher levels than parent adipocytes and the transfectants with the vector only. The increased ability of the transfectants with antisense COX-1 was also recognized after 2 days of the maturation phase and found to be evident up to 14 days of it. These findings suggest the contribution of endogenous PGJ_2 derivatives to the up-regulation of adipogenesis in the transfectants with antisense COX-1. .

4. Discussion

The preadipogenic cultured 3T3-L1 cells have been widely used for the monitoring a series of cellular processes of cell proliferation, the induction of the differentiation, and the

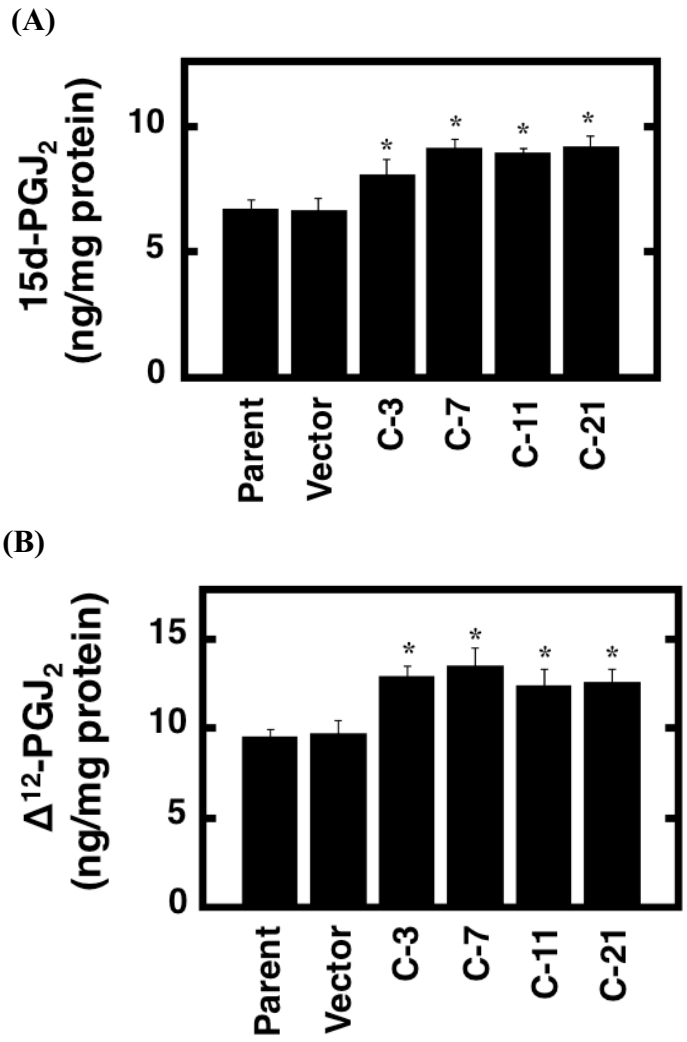


Fig. 2-7. Endogenous synthesis of 15d-PGJ_2 and Δ^{12}-PGJ_2 by cultured transfectants with antisense COX-1 during the maturation phase. Parent preadipocytes and the transfectants were plated and grown to confluence as described in Fig. 2-3. The resulting monolayer cells were allowed to undergo the differentiation and maturation. The culture medium was collected between 8 and 10 days of the maturation phase for the quantification of 15d-PGJ_2 (A) and Δ^{12}-PGJ_2 (B) by ELISA specific for each compound. Data represent the mean ± S.E.M. of three experiments. *p< 0.05 compared with the transfectants with the vector only at the corresponding stage of the maturation phase

maturation process associated with adipogenesis under the controlled conditions [10, 15, 16]. Earlier studies have shown that cultured adipocytes and the precursor preadipocytes are capable of generating endogenously several types of PGs with opposite effects on

adipogenesis at different life stage of adipocytes [7-9, 23]. Hence, the roles of PGs in the function of adipocytes are complicated in addition to the existence of multiple receptors for each PG species. Cultured 3T3-L1 cells express two types of COX isoforms, the constitutive COX-1 and the inducible COX-2, as the rate-limiting enzymes for the arachidonate COX pathway [13]. To evaluate the contribution of each of COX isoforms to the biosynthesis of PGs in cultured preadipocytes, we have previously shown that the sustained expression of antisense mRNA of COX-2 can selectively suppress the expression levels of sense transcript of COX-2 [20]. In this study, we alternatively attempted to apply this approach to the specific attenuation of the gene expression of COX-1 in the cultured preadipocytes. The following lines of evidence supported the successful isolation of cloned stable transfectants with antisense COX-1 to block specifically the expression of sense COX-1 without affecting the expression of another isoform, the inducible COX-2. At first, the expression levels of sense mRNA of COX-1 were appreciably reduced and accompanied by the increased expression of antisense COX-1 mRNA in the desired clones of the transfectants with antisense COX-1. In contrast, the gene expression of sense COX-1 was not influenced by the stable transfection with the vector only and occurred at the same levels as that by the parent cells, indicating the exclusion of the artifacts due to the transfection. Furthermore, the protein expression levels of COX-1 in all the trasfectants with antisense COX-1 were substantially reduced in agreement with the decreased transcript levels of COX-1 mRNA. On the other hand, we did not detect a significant change in the protein expression levels of the inducible COX-2 in response to PMA and A23187 in all the parent cells and the transfectants.

Previous studies have reported that cultured 3T3-L1 preadipocytes can synthesize PGE_2 as one of major cyclooxygenase products through both the immediate and delayed responses [7, 20, 24, 29]. The immediate synthesis of PGs within 30 min is known to include the generation of PGs from exogenous 10 μM free AA by the action of the constitutive COX-1 [13, 20, 24, 31]. As well, we can monitor the immediate production of PGs by COX-1 after stimulating cultured cells with 10 μM calcium ionophore A23187 to raise the intracellular concentration of Ca^{2+} which activates cytosolic phospholipase A_2 to release free AA from membrane phospholipids [13, 20, 24, 31]. Our study confirmed that all the immediate synthesis of PGs by the cloned stable transfectants with antisense COX-1 with exogenous AA and A23187 occurred at significantly lower levels than the

untransfected parent preadipocytes and the control cells transfected with the vector only. These results reflect the suppressed gene expression of the constitutive COX-1 in the desired stable transfectants with antisense COX-1 as expected. Recently, we have shown that cultured 3T3-L1 preadipocytes are capable of stimulating the delayed synthesis of PGE_2 involving de novo synthesis of the inducible COX-2 after 24 h of the incubation in response to a mixture of 0.1 μM PMA and 0.1 μM A23187 [7, 29]. The present study ascertained that the sustained transfection of preadipocytes with antisense COX-1 did not affect the delayed synthesis of PGE_2 as well as the induction of COX-2 under the conditions described above. These combined findings firmly support the selective suppression of the gene expression of the constitutive COX-1 without the artificial interference with the inducible COX-2 in the cloned stable transfectants.

The growth-arrested cultured preadipocytes can respond to a mixture of IBMX, dexamethasone, and insulin to induce the differentiation phase characterized as clonal expansion phase [32, 33], after which the accumulation of fats proceeds due to the up-regulation of adipogenesis program during the maturation phase under the established conditions. The present study demonstrated that all the cloned stable transfectants with antisense COX-1 accumulated triacylglycerols at significantly higher levels than the parent and the control cells after both 6 and 12 days of the maturation phase. According to the microscopic and macroscopic views of the cultured cells during the maturation phase, we obviously observed the increased size of fat droplets inside the cells of cloned transfectants with antisense COX-1, which became evident within 6 days of the maturation phase. However, we did not detect any other phenotypic aberrance, suggesting the promotion of normal adipogenesis program. This notion was supported by the current study revealing the enhanced gene expression of typical adipogenesis markers, such as GLUT-4, leptin, LPL, aP2, and adiponectin under the control of PPARγ and C/EBPα, the nuclear transcription factors serving as master regulators of the differentiation of adipocytes [1-3]. On the other hand, we did not observe any discernible difference between the cloned stable transfectants with antisense COX-1 and the control cells with respect to the cell growth after the maturation phase, indicating that the sustained transfection had no effect on the clonal expansion involving the mitogenic cell cycle prior to normal adipogenesis. Furthermore, it is also unlikely that the unusual inhibition of basal lipolysis was responsible for the increased levels of fats stored in the stable transfectants with antisense COX-1.

PGJ_2 derivatives including 15d-PGJ_2 and Δ^{12}-PGJ_2 can be formed by the non-enzymatic dehydration of PGD_2, one of classical PGs that are biosynthesized by the COX pathway [30, 34]. These PGs of J_2 series have been shown to serve as pro-adipogenic factors in cultured adipocytes during the maturation phase when being administered exogenously [8, 9]. As well, we have recently reported the ability of cultured adipocytes to produce increasingly the endogenous synthesis of PGJ_2 derivatives after the maturation phase [8, 9, 23]. Our present study also confirmed that the stable transfectants with antisense COX-1 produced both endogenous PGJ_2 derivatives at significantly higher levels compared with the parent cells and the vector-transfected cells. The resulting up-regulation of the biosynthesis of J_2 series PGs is more likely to be linked closely with the promotion of a series of adipogenesis program leading to the maturation of adipocytes. According to earlier studies, the endogenous synthesis of PGJ_2 derivatives and the storage of fats have been shown to be attenuated by both selective inhibitors for COX-1 and COX-2, respectively, as well as non-selective aspirin and indomethacin if those compound are added to the culture medium during the maturation phase [8, 23]. These findings imply that both COX isoforms could contribute to the generation of the endogenous PGJ_2 derivatives serving as pro-adipogenic factors in untransfected parent cells. On the basis of the preceding information, we initially predicted that the suppressed expression of COX-1 in the stable transfectants with antisense COX-1 could result in the attenuation of fat storage in adipocytes after the maturation phase. Nevertheless, our cloned stable transfectants with antisense COX-1 positively regulated the storage of fats and the endogenous synthesis of J_2 series PGs. It is conceivable that the inducible COX-2 can compensate for the suppressed expression of the constitutive COX-1 in the stable transfectants with antisense COX-1 and is involved in the generation of PGJ_2 derivatives during the maturation phase. Interestingly, more recent studies have reported the role of COX-2 in the production of PGD_2 and PGJ_2 derivatives acting as modulators of adipocyte differentiation using COX-2-deficient mice [35].

It remains unclear about the detailed cellular mechanism by which the stable transfection of cultured preadipocytes with antisense COX-1 caused the stimulation of adipogenesis in adipocytes during the maturation phase. Previous studies have revealed that cultured 3T3-L1 preadipocytes synthesize anti-adipogenic PGs, such as PGE_2 and $PGF_{2\alpha}$,

by the COX pathways through the immediate and delayed responses [7, 20, 24, 29]. In particular, EP4, a subtype of the prostanoid receptor for PGE_2, is predominantly expressed during the differentiation phase before the commitment to cultured adipocytes and is involved in the inhibition of adipocyte differentiation [6]. Therefore, it is plausible that the decreased levels of PGE_2 and $PGF_{2\alpha}$, synthesized endogenously by our stable transfectants with antisense COX-1 during the growth phase or the differentiation phase would be associated with the up-regulation of adipogenesis program during the maturation phase. Consistent with our idea, Yan et al. [17] have earlier reported that selective inhibitors for COX-1 and COX-2 accelerate the differentiation of cultured 3T3-L1 cells by adding each of them in the differentiation medium without dexamethasone, suggesting the role of both COX isoforms in the negative regulation of adipocyte differentiation. However, it should be noted that higher concentrations of some COX inhibitors act as active ligands for PPARγ, a master regulator for adipocyte differentiation [19]. As another potential cellular mechanism for the influence of COX isoforms on adipogenesis independent of endogenous PGs, the suppression of the constitutive COX-1 might rescue the inhibitory action of COX-1 on adipogenenesis program through the activation of NF-κB due to the peroxidase activity of COX-1 in response to cellular reactive oxygen species [36]. More extensive studies need to be conducted for the dissection of the specific role of COX-1 in the control of adipogenesis in adipose tissues.

In conclusion, we were able to specifically suppress the gene expression of the constitutive COX-1 by isolating cloned preadipocytes transfected stably with antisense COX-1. The resulting stable transfectants exhibited the marked reduction in the gene expression of the sense transcript of COX-1, which was followed by the substantial decrease in the immediate synthesis of PGE_2, an anti-adipogenic factor. In sharp contrast, the gene expression of the inducible COX-2 was not affected. Fat storage was stimulated in the stable transfectants with antisense COX-1 due to the positive regulation of adipogenesis program, which was accompanied by the higher production of J_2 series PGs known as pro-adipogenic PGs. These findings indicate that remaining COX-2 can contribute to the endogenous synthesis of those PGJ_2 derivatives by the COX pathway by compensating for the suppressed expression of COX-1. The understanding of the cellular mechanism for the up-regulation of adipogenesis awaits more extensive investigation.

References

1. MacDougald, O. A., and Lane M. D. (1995) Transcriptional regulation of gene expression during adipocytes differentiation. Annu. Rev. Biochem. 64, 535-559
2. Gregoire, F. M., Smas, C. M., and Sul, H. S. (1998) Understanding adipocyte differentiation. Physiol. Rev. 78, 783-809
3. Rangwala, S. M., and Lazar, M. A. (2000) Transcriptional control of adipogenesis. Annu. Rev. Nutr. 20, 535-559
4. Guilherme, A., Virbasius, J. V., Puri, C., and Czech, M. P. (2008) Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes. Nat. Rev. Mol. Cell Biol. 9, 367-377
5. Wajchenberg, B. L. (2000) Subcutaneous and visceral adipose tissue: their relation to the metabolic syndrome. Endocr. Rev. 21, 697-738
6. Tsuboi, H., Sugimoto, Y., Kainoh, T., and Ichikawa, A. (2004) Prostanoid EP4 receptor is involved in suppression of 3T3-L1 adipocyte differentiation. Biochem. Biophys. Res. Commun. 322, 1066-1072
7. Xu, L., Nishimura, K., Jisaka, M., Nagaya, T., and Yokota, K. (2006) Gene expression of arachidonate cyclooxygenase pathway leading to the delayed synthesis of prostaglandins E_2 and $F_{2\alpha}$ in response to phorbol 12-myristate 13-acetate and action of these prostanoids during life cycle of adipocytes. Biochim. Biophys. Acta 1761, 434-444
8. Mazid, M. A., Chowdhury, A. A., Nagaya, K., Nishimura, K., Jisaka, M., Nagaya, T., and Yokota, K. (2006) Endogenous 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 synthesized by adipocytes during maturation phase contributes to upregulation of fat storage. FEBS Lett. 580, 6885-6890
9. Hossain, M. S., Chowdhury, A. A., Rahman, M. S., Nishimura, K., Jisaka, M., Nagaya, T., Shono, F., and Yokota, K. (2011) Development of enzyme-linked immunosorbent assay for Δ^{12}-prostaglandin J_2 and its application to the measurement of the endogenous product generated by cultured adipocytes during the maturation phase. Prostaglandins Other Lipid Mediat. 94, 73-80

10. Hyman, B. T., Stoll, L. L., and Spector, A. A. (1995) Prostaglandin production by 3T3-L1 cells in culture. Biochim. Biophys. Acta 713 (1982) 375-385
11. Forman, B. M., Tontonoz, P., Chen, J., Brun, R. P., Spiegelman, B. M., and Evans, R. M. (1995) 15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 is a ligand for the adipocyte determination factor PPARγ. Cell 83, 803-812
12. Kliewer, S. A., Lenhard, J. M., Wilson, T. M., Patel, I., Morris, D. C., and Lehman, J. M. (1995) A prostaglandin J_2 metabolite binds peroxisome proliferator-activated receptor γ and promotes adipocyte differentiation. Cell 83, 813-819
13. Smith, W. L. DeWitt, D. L. and Garavito, R. M. (2000) Cyclooxygenases: structural, cellular, and molecular biology. Annu. Rev. Biochem. 69, 145-182
14. Fain, J. N., Ballou, L. R., and Bahouth, S. W. (2001) Obesity is induced in mice heterozygous for cyclooxygenase-2. Prostaglandins Other Lipids Mediat. 65, 199-209
15. Green, H., and Kehinde, O. (1974) Sublines of mouse 3T3 cells that accumulate lipid. Cell 1, 113-116
16. Green, H., and Kehinde, O. (1975) An established preadipose cell line and its differentiation in culture. II. Factors affecting the adipose conversion. Cell 5, 19-27
17. Yan, H. Kermouni, A. Abdel-Hafez, M., and Lau, D. C. W. (2003) Role of cyclooxygenases COX-1 and COX-2 in modulating adipogenesis in 3T3-L1 cells. J. Lipid Res. 44, 424-429
18. Petersen, R. K., Jørgensen, C., Rustan, A. C., Frøyland, L., Muller-Decker, K., Furstenberger, G., Berge, R. K., Kristiansen, K., and Madsen, L. (2003) Arachidonic acid-dependent inhibition of adipocyte differentiation requires PKA activity and is associated with sustained expression of cyclooxygenases. J. Lipid Res. 44, 2320-2330
19. Lehmann, J. M., Lenhard, J. M., Oliver, B. B., Ringold, G. M., and Kliewer, S. A. (1997) Peroxisome proliferator-activated receptors alpha and gamma are activated by indomethacin and other non-steroidal anti-inflammatory drugs. J. Biol. Chem. 272, 3406-3410
20. Chu, X., Nishimura, K., Jisaka, M., Nagaya, T., Shono, F., and Yokota, K. (2010) Up-regulation of adipogenesis in adipocytes expressing stably cyclooxygenase-2 in the antisense direction. Prostaglandins Other Lipid Mediat. 91, 1-9
21. Chowdhury, A. A., Hossain, M. S., Rahman, M. S., Nishijima, K., Jisaka, M., Nagaya, T., Shono, F., and Yokota, K. (2011) Sustained expression of lipocalin-type

prostaglandin D synthase in the antisense direction positively regulates adipogenesis in cloned cultured preadipocytes. Biochem. Biophys. Res. Commun. 411, 287-292

22. Yokota, K., Morishima, T., Nagaya, T., Jisaka, M., and Takinami, K. (1996) Modification of cultured Madin-Darby canine kidney cells with dietary unsaturated fatty acids and regulation of arachidonate cascade reaction. Biosci. Biotechnol. Biochem. 60, 1096-1103
23. Lu, S., Nishimura, K., Hossain, M.A., Jisaka, M., Nagaya, T., and Yokota, K. (2004) Regulation and role of arachidonate cascade during changes in life cycle of adipocytes. Appl. Biochem. Biotechnol. 118, 133-153
24. Chu, X., Xu, L., Nishimura, K., Jisaka, M., Nagaya, T., Shono, F., and Yokota, K. (2009) Suppression of adipogenesis program in cultured preadipocytes transfected stably with cyclooxygenase isoforms. Biochim. Biophys. Acta 1791, 273-280
25. Chomczynski, P., and Sacchi, N. (1987) An improved method for isolating RNA from porcine adipose tissue. Anal. Biochem. 162, 156-159
26. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. L. (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265-275
27. Markwell, M. A., Hass, S. M., Toleret, N. E., and Bieber, L. I. (1981) Protein determination in membrane and lipoprotein samples: manual and automated procedures. Methods Enzymol. 72, 296-303
28. Kuri-Harcuch, W., and Green, H. (1978) Adipose conversion of 3T3 cells depends on a serum factor. Proc. Natl. Acad. Sci. USA 75, 6107-6109
29. Chowdhury, A. A., Rahman, M. S., Nishimura, K., Jisaka, M., Nagaya, T., Ishikawa, T., Shono, F., and Yokota, K. (2011) 15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 interferes inducible synthesis of prostaglandins E_2 and $F_{2\alpha}$ that suppress subsequent adipogenesis program in cultured preadipocytes. Prostaglandins Other Lipid Mediat. 95, 53-62
30. Shibata, T., Kondo, M., Osawa, T., Shibata, N., Kobayashi, M., and Uchida, K. (2002) 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2. A prostaglandin D_2 metabolite generated during inflammatory processes. J. Biol. Chem. 277, 10459-10466
31. Murakami, M., Kambe, T., Shimbara, S., and Kudo, I. (1999) Functional coupling between various phospholipase A_2s and cyclooxygenases in immediate and delayed prostanoid biosynthetic pathways. J. Biol. Chem. 274, 3103-3115

32. Cornelius, P., MacDougald, O. A., and Lane, M. D. (1994) Regulation of adipocyte development. Annu. Rev. Nutri. 14, 99-129
33. Mandrup, S., and Lane, M. D. (1997) Regulating adipogenesis. J. Biol. Chem. 272, 5367-5370
34. Fitzpatrick, F. A., and Wynalda, M. A. (1983) Albumin-catalyzed metabolism of prostaglandin D_2. Identification of products formed *in vitro*. J. Biol. Chem. 258, 11713-11718
35. Ghoshal, S., Trivedi, D. B., Graf, G. A., and Loftin, C. D. (2011) Cyclooxygenase-2 deficiency attenuates adipose tissue differentiation and inflammation in mice. J. Biol. Chem. 286, 889-898
36. Munroe, D. G., Wang, E. Y., MacIntyre, J. P., Tam, S. S. C., Lee, D. H. S., Taylor, G. R., Zhou, L. B., Plante, R. K., Kazmi, S. M. I., Bäuerle, P. A., and Lau, C. Y. (1995) Novel intracellular signaling function of prostaglandin H synthase-1 in NF-κB activation. J. Inflamm. 45, 260–268

Chapter 3

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

1. Introduction

Adipose tissue serves as fat depot for the storage and mobilization of fats depending on the necessity of energy requirement. Excess accumulation of fats is well known to generate obesity characterized by the increases in the number and size of mature adipocytes due to the enhanced energy intake and the reduced energy expenditure. Therefore, the body mass is controlled by adipogenesis through the differentiation of adipocytes from undifferentiated preadipocytes in adipose tissue *in vivo*. As well, adipocytes function as an endocrine organ to secrete a variety of bioactive adipocytokines, such as leptin and adiponection, and are involved in the suppression of food intake and the maintenance of insulin sensitivity, respectively [1]. By contrast, hypertrophic adipocytes in obese tissues have been shown to secrete other types of adipocytokines including monocyte chemoattractant protein-1, interleukin-6, and tumor necrosis factor-α, which are inflammatory factors to promote macrophage infiltration into adipose tissue with obesity and generate adipocyte inflammation and insulin resistance [2-4]. For these functional changes and the control of adipogenesis, peroxisome proliferator-activated receptor γ (PPARγ), a nuclear hormone receptor and ligand-activated transcription factor, functions as a master regulator [5, 6]. Since prostaglandins (PGs) of J_2 series, a member of bioactive eicosanoids, have been shown to be the potent natural ligand for the activation of PPARγ [7, 8], much attention has been paid to the role of PGs in the control of adipocyte function. PGs are generally regarded as local hormones or autacoids that exert their effects on the neighboring cells in an autocrine or paracrine manner.

Adipocytes and the precursor cells can synthesize different types of prostanoids with opposite effects on adipogenesis through the arachidonate cyclooxygenase (COX) pathway [9-12]. In addition, the biosynthesis of individual PG species is specifically

regulated at different stages leading to adipocytes from undifferentiated preadipocytes. Mouse preadipogenic 3T3-L1 cells, an established cell line, have been employed as a useful model system for the study on adipogenesis [13, 14]. The cells can be induced to undergo spontaneous morphologic and biochemical differentiation into adipocytes in culture under the controlled culture conditions involving the growth, differentiation, and maturation phases. PGs of J_2 series including 15-deoxy-$\Delta^{12,14}$-PGJ_2 and Δ^{12}-PGJ_2 formed through non-enzymatic dehydration of PGD_2 serve as members of pro-adipogenic factors because of their capability of activating PPARγ and the effectiveness of exogenous PGJ_2 derivatives to promote adipogenesis [7, 8, 15, 16]. We have recently shown that those PGs of J_2 series can be synthesized endogenously during the maturation phase of cultured 3T3-L1 cells and contribute to up-regulation of adipogenesis through the activation of PPARγ [15, 16]. Moreover, our studies have revealed that parent PGD_2 for PGJ_2 derivatives can rescue the storage of fats reduced in the presence of COX inhibitors after the maturation phase. On the other hand, another types of prostanoids exhibit anti-adipogenic effects through the cell-surface membrane receptors. For example, PGE_2 inhibited adipocyte differentiation of cultured 3T3-L1 cells through the mediation of the EP4 receptor, one of the PGE_2 receptor subtypes when PGE_2 was added to both the differentiation and maturation phases [17]. Alternatively, $PGF_{2\alpha}$ has been reported to inhibit the differentiation of preadipocytes into adipocytes through the specific FP receptor, which transmits the cellular signal to phosphorylate PPARγ by mitogen-activated protein kinase [18].

Earlier studies have reported that carbaprostacyclin, a stable analogue of prostacyclin also called PGI_2, stimulates terminal differentiation of Ob1771 mouse pre-adipose cells in serum-free hormone-supplemented medium [19, 20]. Furthermore, under the same conditions, they recognized the pro-adipogenic effect of arachidonic acid, which was blocked by COX inhibitors. According to the ability of those cells to 6-keto-$PGF_{1\alpha}$, a stable hydrolysis product of unstable parent PGI_2, and $PGF_{2\alpha}$, those prostanoids are considered as autocrine mediators in the process of adipose conversion.

Nevertheless, the pro-adipogenic action of endogenous PGI_2 remains obscure due to the instability of PGI_2 and its uncertainty of effective concentration of the parent form. Biologically active PGI_2 is rapidly hydrolyzed to inactive 6-keto-$PGF_{1\alpha}$ in most biological fluids [21]. It should be noted that different cell types and alterations in the culture conditions of cultured adipocytes and precursor cells would gave contradictory results on adipogenesis. For example, $PGF_{2\alpha}$ serves as anti-adipogenic factor in cultured 3T3-L1 cells in contrast to the stimulatory effect on the adipocyte differentiation of cultured Ob1771 pre-adipose cells as described above. In addition, a previous study observed that the repeated addition of exogenous PGI_2 to cultured 3T3-L1 cells inhibited insulin- and indomethacin-mediated adipocyte differentiation [9]. Earlier, cultured 3T3-L1 cells have been described to generate PGI_2 as an immediate response to calcium ionophore A23187 for 5 min or by the incubation with extracellular arachidonic acid [10]. However, until now the biosynthesis of PGI_2, which is usually quantified as the levels of stable 6-keto-$PGF_{1\alpha}$, has not been monitored comprehensively at different life stages of adipocytes through the growth, differentiation, and maturation phases.

In this study, we obtained an antiserum specific for 6-keto-$PGF_{1\alpha}$ and used for the development of a sensitive, convenient solid-phase enzyme-linked immunosorbent assay (ELISA) for the quantification of 6-keto-$PGF_{1\alpha}$. After the confirmation of the precision and accuracy of our ELISA, this immunological assay was applied to the determination of 6-keto-$PGF_{1\alpha}$ reflecting the endogenous levels of PGI_2 synthesized by cultured 3T3-L1 cells at different life stages of adipogenesis. We here provided the evidence that cultured adipocytes at the maturation phase have the highest ability to generate endogenous PGI_2 as determined by 6-keto-$PGF_{1\alpha}$, which was accompanied by the coordinated gene expression of PGI synthase (PGIS) and the IP receptor for PGI_2 and then followed by the continuous promotion of adipogenesis.

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, recombinant human insulin, and ExtrAvidin-peroxidase conjugate were supplied by Sigma (St. Louis, MO, USA). Biotin-conjugated rabbit anti-mouse IgG antibody was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Fetal bovine serum (FBS) was obtained from MP Biomedicals (Solon, OH, USA). Authentic PGs were purchased from Cayman Chemical (Ann Arbor, MI, USA). M-MLV reverse transcriptase (RT) (Ribonuclease H minus, point mutant) and polymerase chain reaction (PCR) MasterMix were supplied by Promega (Madison, WI, USA). 3-isobutyl-1-methylxanthine (IBMX), and Triglyceride E-Test Kit were purchased from Wako (Osaka, Japan). Oligonucleotides used for the PCR reaction were provided by Sigma Genosys Japan (Ishikari, Japan). 96-Well microplates for ELISA were purchased from BD Falcon (Durham, NC, USA), and other Petri dishes and multiwell plates with the Iwaki brand for tissue culture were from Asahi Glass (Tokyo, Japan). All other chemicals used are of reagent or tissue culture grade. Other materials and apparatus used for the experiments of cell culture, ELISA and gene expression were obtained as described previously [12, 15, 16]

2.2 Cell culture of 3T3-L1 cells and adipocyte differentiation

Mouse preadipogenic 3T3-L1 cells (JCRB9014) were plated at 5×10^4 cells/ml in growth medium (GM) containing DMEM-HEPES, 10% FBS, 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, and 200 μM ascorbic acid, and then cultured at 37 °C under 7% CO_2. After growth until confluence, the monolayer cells were exposed to the differentiation medium (DM) composed of GM supplemented with 1 μM dexamethasone, 0.5 mM IBMX, and 10 μg/ml insulin for 45 h. Then, DM was replaced with by maturation medium (MM) consisting of GM and 5 μg/ml of insulin, and refed every 2 days with fresh MM as described earlier [11, 13-15]. At the indicated life stage, the resulting culture medium was collected and used for the determination of 6-keto-$PGF_{1\alpha}$ by ELISA, whereas the cells were harvested for determination of triacylglycerol and cellular proteins as well as the staining with Oil Red O as described below.

2.3 Solid-phase ELISA for 6-keto-$PGF_{1\alpha}$

A conjugate of 6-keto-$PGF_{1\alpha}$ and bovine serum albumin was formed chemically and employed for the immunization of female BALB/c mice to prepare antisera specific for 6-keto-$PGF_{1\alpha}$ according to our previous methods regarding the generation of antibodies for other prostanoids [22-24]. For conducting solid-phase ELISA for 6-keto-$PGF_{1\alpha}$, another conjugate of 6-keto-$PGF_{1\alpha}$ and bovine γ-globulin was prepared and used as an immobilized antigen attached on the surface of the bottom in a 96-well ELISA microplate. The resulting immobilized antigen was then allowed to react competitively with 50 μl of a 2 x 10^5-fold diluted solution of a mouse antiserum specific for 6-keto-$PGF_{1\alpha}$ and 50 μl of a solution containing various amounts of standard 6-keto-$PGF_{1\alpha}$ or samples to be tested. The immunocomplex was furthermore incubated with biotin-conjugated rabbit anti-mouse IgG antibody followed by ExtrAvidin-peroxidase conjugate after washing at each step. The newly formed immunocomplex was finally detected spectrophotometrically by the measuring peroxidase activity using *o*-phenylenediamine as a substrate as detailed previously [22-24].

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

2.4 Application of ELISA to the determination of endogenous synthesis of 6-keto-$PGF_{1\alpha}$

To determine 6-keto-$PGF_{1\alpha}$ in different culture media used for the growth, differentiation and maturation phases from undifferentiated preadipocytes to mature adipocytes, the calibration curve was generated separately using each of GM, DM, and MM. Namely, authentic 6-keto-$PGF_{1\alpha}$ was serially diluted using an equal volume of each fresh culture medium and the ELISA buffer containing phosphate-buffered saline without Ca^{2+} and Mg^{2+} ions, 0.5% bovine serum albumin and 0.02% NaN_3. For the analysis of samples,

the corresponding culture medium was collected at the indicated time of cell cultures and diluted 2-fold with the ELISA buffer followed by further dilution by 4-fold and 8-fold with an equal volume of the respective fresh culture medium and the ELISA buffer. The resulting diluted samples were placed individually in 96-well ELISA microplates at three different dilutions in duplicate and processed for the quantification of 6-keto-$PGF_{1\alpha}$ by the specific ELISA. To obtain the final concentration of 6-keto-$PGF_{1\alpha}$ in the corresponding culture medium for an unknown sample, the values read within the range of 10–90% of the binding from the calibration curve were used for calculation of the average value of each sample after the multiplication by dilution folds.

For the evaluation of the accuracy of our ELISA in the quantification of 6-keto-$PGF_{1\alpha}$ in the respective culture medium, each of fresh GM, DM, and MM was fortified with known amounts of authentic 6-keto-$PGF_{1\alpha}$, and then subjected to ELISA for 6-keto-$PGF_{1\alpha}$ after serial dilutions (2-fold, 4-fold, and 8-fold) to give an equal-volume mixture of the same culture medium and the ELISA buffer as described above. The resulting samples were subjected to the quantification of 6-keto-$PGF_{1\alpha}$ by ELISA and the subsequent regression analysis between added values and measured ones.

2.5 Gene expression analysis

Total RNA was extracted at different stages of adipocytes using a mixture of acid guanidium thiocyanate, phenol and chloroform [12, 15, 16]. For the analysis of gene expression of a target gene, total RNA (1 μg) was subjected to amplification of each desired DNA fragment by RT-PCR with M-MLV reverse transcriptase (Ribonuclease H minus, point mutant) and 1× PCR Master Mix as reported previously [12, 15, 16]. In order to perform the RT reaction for producing single stranded cDNA, oligo-$(dT)_{15}$ and a random 9 mer (Promega) were used as primers. The cDNA fragments for the target genes were amplified by PCR in a semi-quantitative manner. Oligonucleotides used for detecting mRNA levels of PGIS were 5′-ATGAAGCCGACGCTCATGCAC-3′ as 5′- primer and 5′-GAAACAGGCTGCTCTGCTTG-C-3′ as 3′-primer. The transcription levels of IP receptor were detected using 5′-TGAGCCCTGCAGTGTTTGTGG-3′ as 5′- primer and 5′-GAAGCCTCGGATCATGAGAGG-3′ as 3′-primer. The amplified DNA fragments were separated by electrophoresis on a 1.5% agarose gel and stained with ethidium bromide. For

confirming the target gene, the DNA sequences of the PCR products were determined using ABI Prism 3100 Genetic Analyzer following the reaction with BigDye Terminator v.1.1 Cycle Sequence Kit (Applied Biosystems, Foster City, CA, USA) as reported earlier [12, 25].

2.6 Other procedures

For quantification of the amounts of triacylglycerols in cultured adipocytes, the homogenates from the cells harvested at different stages were subjected to the assay of triacylglycerols using Triglyceride E-Test Kit as reported earlier [12, 15, 16]. Cellular proteins were determined after precipitating with cold trichloroacetic acid to remove interfering substance according to the method reported previously [11, 12, 25]. The accumulated lipid droplets were observed as macroscopic and microscopic views by staining cultured cells with Oil Red O as described previously [11, 12, 15, 16].

3. Results

3.1 Development of a solid-phase ELISA specific for 6-keto-$PGF_{1\alpha}$

PGI_2 is known to undergo rapid conversion to 6-keto-$PGF_{1\alpha}$ as the stable hydrolysis product in neutral solutions [26, 27]. Therefore, to monitor specifically the endogenous synthesis of PGI_2 in cultured cells, the target for the measurement should be 6-keto-$PGF_{1\alpha}$. Here, we attempted to develop a sensitive solid-phase ELISA for 6-keto-$PGF_{1\alpha}$ using the immobilized antigen in 96-well microplates. Among several mice used for the immunization with the BSA conjugate of 6-keto-$PGF_{1\alpha}$, a mouse polyclonal antiserum highly reactive with the immobilized antigen was obtained and used for the development of the ELISA for 6-keto-$PGF_{1\alpha}$. After extensive optimization of the conditions of the ELISA procedures, a standard calibration curve was established allowing us to determine the amounts of 6-keto-$PGF_{1\alpha}$ from 0.8 pg to 7.7 ng in an assay corresponding to 90% and 10% of the maximal binding of the immobilized antigen, respectively (Fig. 3-1). This assay also gave a standard value of 38 pg at the 50% displacement in the typical calibration curve.

To validate the precision of our solid-phase ELISA for 6-keto-$PGF_{1\alpha}$, we determined the intraassay and interassay coefficients of variation under the established standard assay conditions. The analysis of the intraassay coefficients of variation provided

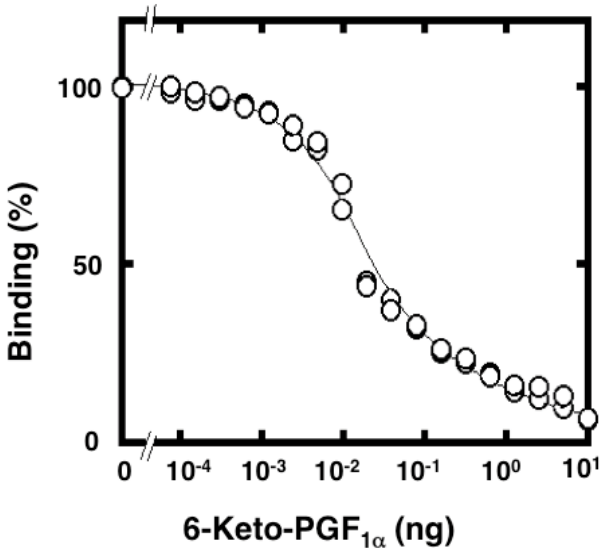


Fig. 3-1. Calibration curve of solid-phase ELISA for 6-keto-$PGF_{1\alpha}$. Binding percentage of the immobilized antigen is plotted against increasing amounts of authentic 6-keto-$PGF_{1\alpha}$

values ranging from 2.2% to 8.6% with five groups of ten replicate assays for each group in the range of 0-10 ng/well of authentic 6-keto-$PGF_{1\alpha}$. In addition, the interassay coefficients of variations were evaluated by repeating the measurements five times using five groups of five replicate assays for each group containing the range of 0-10 ng/well, giving values ranging from 2.2% to 7.2%. The specificity of mouse polyclonal antibody used for the current ELISA was examined by the immunoreaction with increasing amounts of other prostanoids and free arachidonic acid (Fig. 3-2 and 3-3). Our ELISA for 6-keto-$PGF_{1\alpha}$ exhibited almost no cross-reaction, with values of less than 0.1% with other prostanoids except $PGF_{2\alpha}$, with a cross-reaction of 1.5% (Table 3-1). The findings indicated that our antiserum used for the development of our solid-phase ELISA was specific for 6-keto-$PGF_{1\alpha}$ and useful for the quantification of the target molecule.

3.2 Endogenous synthesis of 6-keto-$PGF_{1\alpha}$ and fat storage at different life stages of adipocytes

To monitor the endogenous synthesis of 6-keto-$PGF_{1\alpha}$ reflecting the biosynthesis of PGI_2 at different life stages of adipocytes-the growth, differentiation, and maturation

phases, our solid-phase ELISA specific for 6-keto-$PGF_{1\alpha}$ was applied to the quantification of 6-keto-$PGF_{1\alpha}$ in the corresponding culture medium of each phase. Initially, we evaluated the accuracy for the determination of 6-keto-$PGF_{1\alpha}$ by our solid-phase ELISA by fortifying with increasing concentrations of authentic 6-keto-$PGF_{1\alpha}$ in the fresh culture medium using either GM, DM, or MM. The resulting immunological assays of all of the different culture media showed a satisfactory, linear proportionality between added amounts and read values. Accordingly, the least-regression analysis of the MM supplemented known amounts of 6-keto-$PGF_{1\alpha}$ in a range of 0-200 pg/ml revealed the relationship with a calculated recovery of 103% (y= 1.03x – 0.0965) (Fig. 3-4). The resulting methods were employed for the determination of endogenous synthesis of 6-keto-$PGF_{1\alpha}$ at different life stages of cultured 3T3-L1 cells from undifferentiated preadipocytes to the adipocytes after 14 days of the maturation phase (Fig. 3-5). The ability of cultured 3T3-L1 cells to generate endogenous

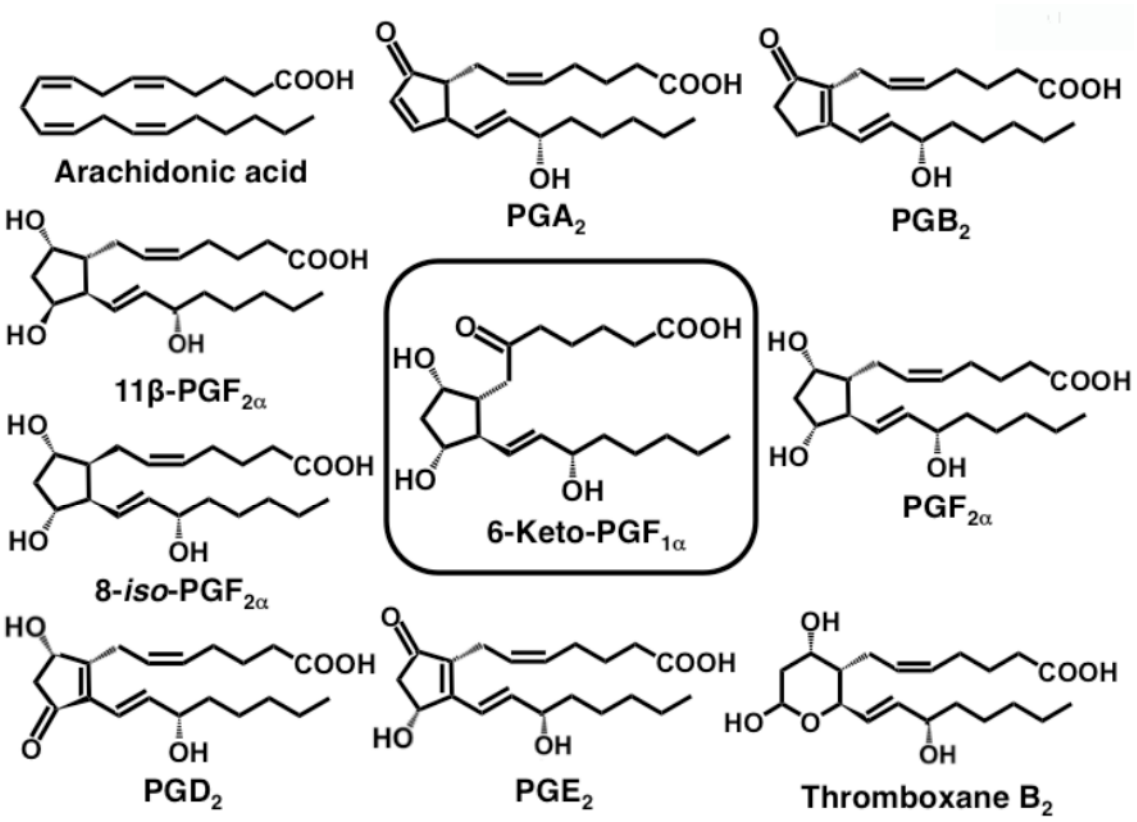


Fig. 3-2. Chemical structures of prostanoids and arachidonic acid used for determining the cross-reaction with them of mouse antiserum raised for 6-keto-$PGF_{1\alpha}$

6-keto-$PGF_{1\alpha}$ was found to be much higher during the maturation phase of adipocytes than other growth and differentiation phases. The highest levels were observed in the MM harvested during the periods of 4-6 days after the maturation phase of adipocytes.

Table 3-1. Cross-reaction of mouse antiserum for 6-keto-$PGF_{1\alpha}$ with other prostanoids and arachidonic acid

Compound	Cross-reaction (%)[a]
6-Keto-$PGF_{1\alpha}$	100
$PGF_{2\alpha}$	1.5
PGE_2	0.1
PGA_2	<0.1
PGB_2	<0.1
PGD_2	<0.1
11-β-$PGF_{2\alpha}$	<0.1
8-*iso*-$PGF_{2\alpha}$	<0.1
Arachidonic acid	<0.1
Thromboxane B_2	<0.1

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

When we monitored the progression of adipogenesis at different life stages of adipocytes under the same culture conditions as described above, the storage of fats was found to increase gradually up to 14th day of the maturation phase as revealed by the increased amounts of triacylglycerol (Fig. 3-6A) and by the marked staining of lipid droplets with Oil Red O at microscopic and macroscopic views (Fig. 3-6B). The observation raised the possibility that the endogenous synthesis of parent PGI_2 as determined by the levels of 6-keto-$PGF_{1\alpha}$ might contribute to the promotion of adipogenesis of adipocytes during the maturation phase since the production of 6-keto-$PGF_{1\alpha}$ preceded the stimulated accumulation of fats in adipocytes, which was more evident after 6 days of the maturation phase.

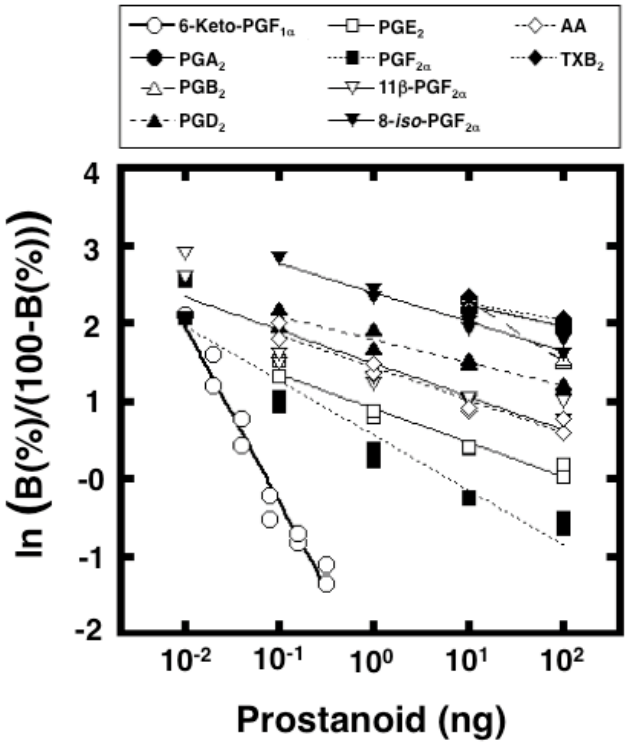


Fig. 3-3. Cross-reaction of mouse antiserum for 6-keto-$PGF_{1\alpha}$ with other prostanoids and arachidonic acid. A fixed amount of immobilized antigen in each 96-well ELISA microplate was incubated with a 2 x 10^5-fold diluted solution of a mouse antiserum for 6-keto-$PGF_{1\alpha}$ in a total volume of 100 μl together with each of increasing amounts of each authentic prostanoid and free arachidonic acid under the established condition. The values of the binding percentage were subjected to the logit transformation in the ordinate to give the linearized straight lines. All of the data are plotted in duplicates. AA, arachidonic acid; TXB_2, thromboxane B_2

3.3 Gene expression of PGIS and IP receptor during the maturation phase

Cultured 3T3 cells have been shown to express the arachidonate COX pathway involving two types of COX isoforms, constitutive COX-1 and inducible COX-2, which are involved in the biosynthesis of different prostanoids with opposite effects on adipogenesis [12, 15]. For the endogenous synthesis of PGI_2, the arachidonate COX pathway requires PGIS, the biosynthetic enzyme using PGH_2 as the substrate, which is the product of COX isoforms. In addition, PGI_2 exerts its biological activity through the specific PGI_2 receptor, the IP receptor [28, 29]. Here, we investigated the gene expression

profiles of PGIS and IP receptor during the maturation phase of adipocytes (Fig. 3-7). The analysis revealed that the transcript levels of both PGIS and IP receptor increased gradually and reached the highest levels after 6 days of the maturation phase although the gene expression of them was detectable at earlier stages including growth and differentiation phases. However, their mRNA levels at the differentiation phase were found to be much lower than other phases, presumably due to the presence of dexamethasone or IBMX in DM. These results suggest that the enhanced gene expression of PGIS was responsible for the stimulation of the PGI_2 synthesis as determined by the production of 6-keto-$PGF_{1\alpha}$ during the maturation phase as described in Fig. 3-5. As well, a co-expression of IP receptor in a similar way could effectively contribute to the action of PGI_2 synthesized endogenously by adipocytes during the process of adipogenesis.

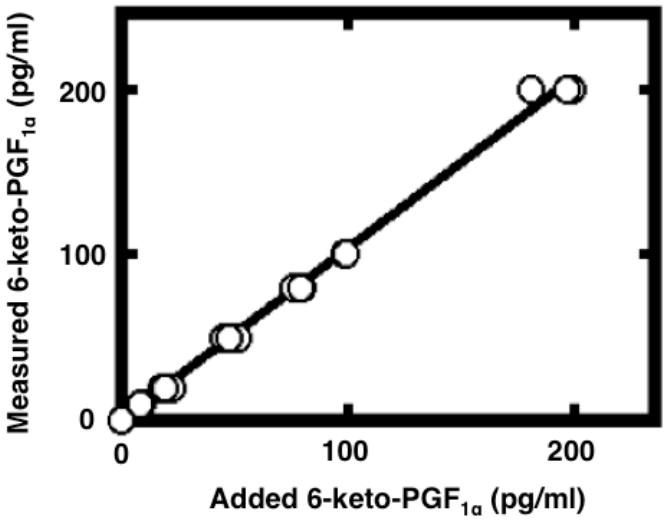


Fig. 3-4. The accuracy for the quantification of 6-keto-$PGF_{1\alpha}$ in the maturation medium of cultured adipocytes. The fresh maturation medium to be used for cultured adipocytes was fortified with increasing amounts of authentic 6-keto-$PGF_{1\alpha}$ in a range of 0-200 pg/ml. The resulting samples were diluted and applied to the quantification of 6-keto-$PGF_{1\alpha}$ by ELISA

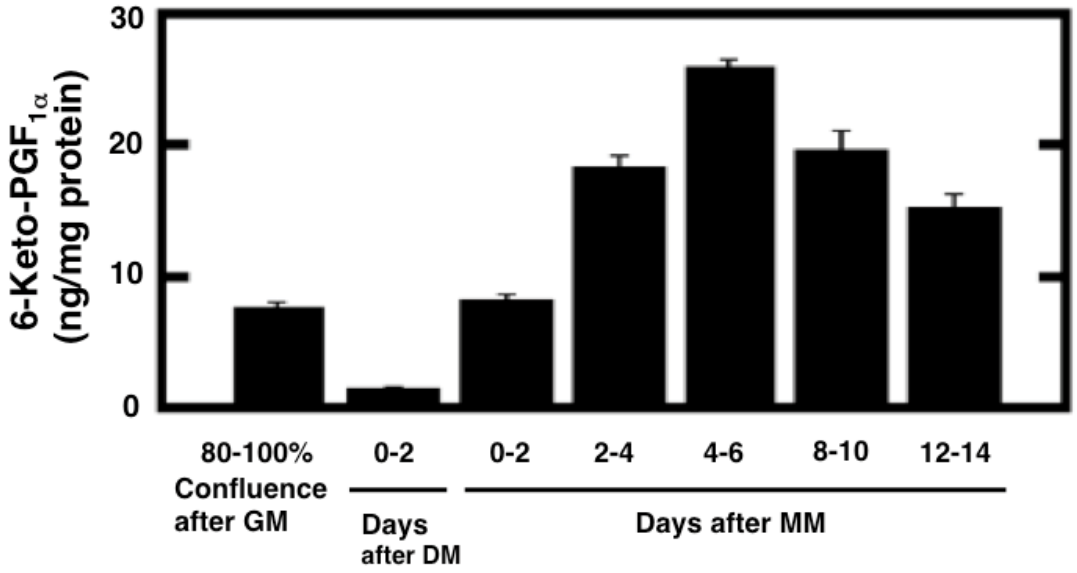


Fig. 3-5. Endogenous synthesis of 6-keto-$PGF_{1\alpha}$ by cultured adipocytes at different life stages of adipocytes. 3T3-L1 cells were plated at 5×10^4 cells/ml in a 35-mm Petri dish containing 2 ml of GM, and grown to the confluence. The confluent cells were exposed to DM for the induction of the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

4. Discussion

A role of prostacyclin PGI_2 as a potent inhibitor of platelet aggregation and a powerful vasodilator is well established in the blood vascular system [21]. In adipose tissue, earlier studies have described that carbaprostacyclin, a stable analogue of prostacyclin, promotes terminal differentiation of cultured mouse OB1771 preadipose cells and 3T3-F442A cells under serum-free culture conditions [19, 20]. Nevertheless, until now the endogenous synthesis of PGI_2 has not been determined quantitatively at different life stages of adipocytes from the growth, differentiation, and maturation phases. PGI_2 is a

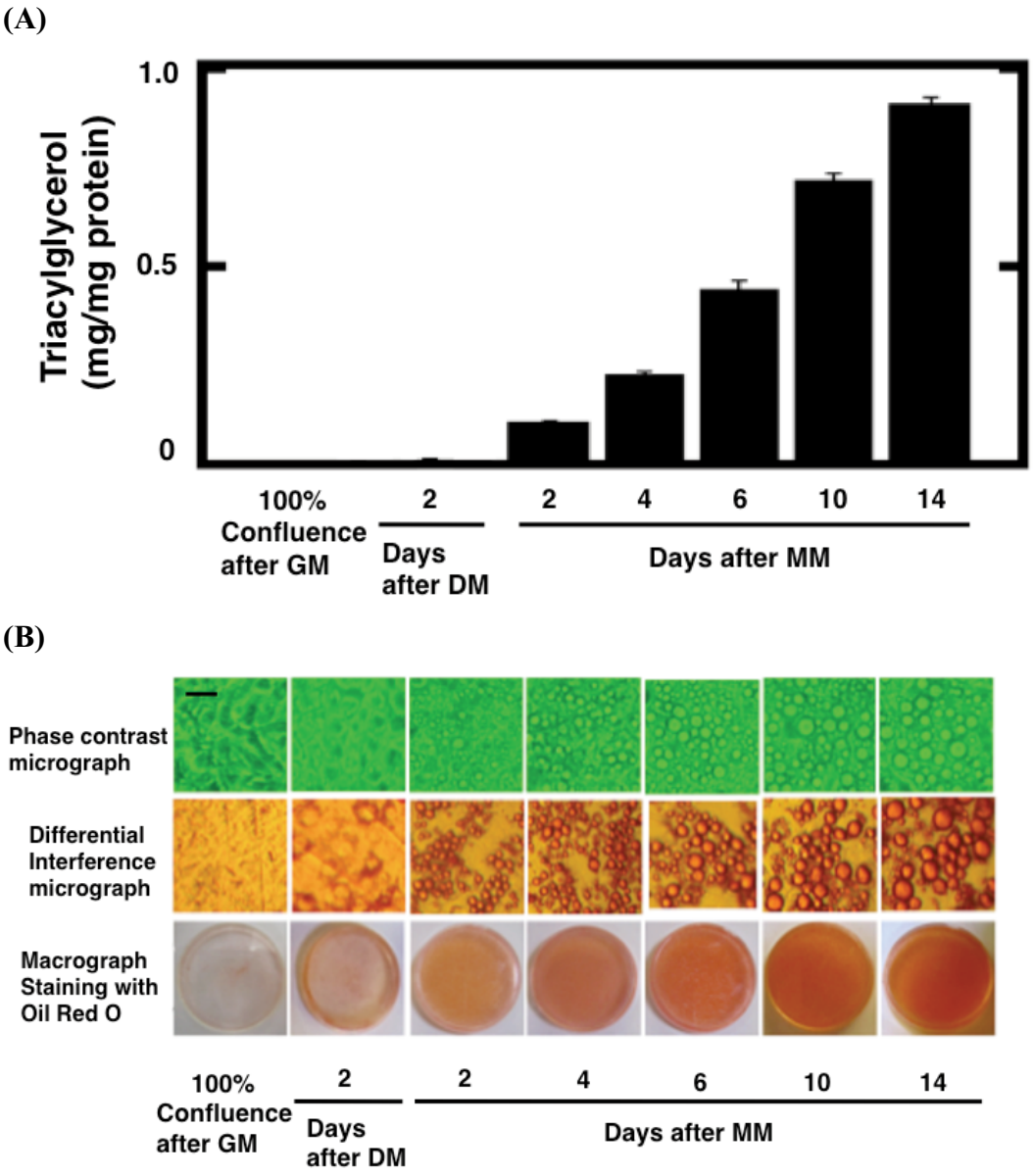


Fig. 3-6. Accumulation of fats by cultured adipocytes during the maturation phase. 3T3-L1 cells were plated and grown to the confluence as described in Fig. 3-5. The confluent cells were exposed to DM for the induction of the differentiation phase, and then matured to terminal differentiation up to the 14th day of the maturation phase by changing MM every 2 days. At the indicated time, the cultured cells were harvested to determine the accumulation of triacylglycerols (A). Data represent the mean ± S.E.M. of three independent experiments. Alternatively, the unstained cultured cells were viewed by phase-contrast microscopy (upper panels), or the cultured cells after staining with Oil Red O were observed by differential-interference microscopy (middle panels) as well as macroscopic views (lower panels) (B). Data are shown from a representative one done in replicate experiments. Scale bar, 50 μm

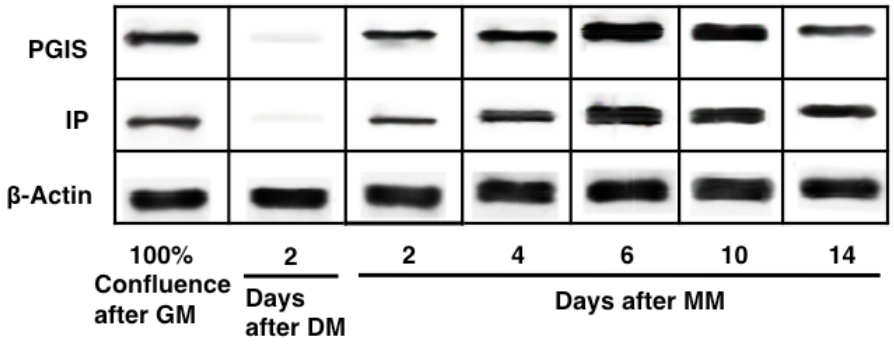


Fig. 3-7. Gene expression of PGIS and IP receptor at different life stages of adipocytes. 3T3-L1 cells were plated at 5×10^4 cells/ml in a 60-mm Petri dish containing 4 ml of GM, and grown to the confluence. The resulting cultured cells were differentiated and matured to terminal differentiation up to the 10th day of the maturation phase by changing MM every 2 days. At the indicated time, total RNA was extracted from the cultured cells at different life stages of adipocytes and subjected to the analysis of mRNA levels of PGIS, IP receptors and β-actin (reference). Data are shown from a representative one done in replicate experiments

well-known unstable product generated by the arachidonate COX pathway and converted rapidly to 6-keto-$PGF_{1\alpha}$ by spontaneous hydrolysis [27]. Hence, to monitor the endogenous synthesis of PGI_2, the target of the measurement should be a stable 6-keto-$PGF_{1\alpha}$ although this compound is biologically inactive. In order to apply the quantification of 6-keto-$PGF_{1\alpha}$ as a measure of the endogenous levels of PGI_2 in different culture media used for adipogenesis of cultured 3T3-L1 cells, we currently attempted to develop a sensitive solid-phase ELISA specific for 6-keto-$PGF_{1\alpha}$ in biological fluids. This immunological assay using the immobilized antigen is a convenient and useful method that can be used for the measurement of many samples at the same time with low-cost instruments. Our solid-phase ELISA was found to be sensitive enough to quantify the amount of over 1 pg in an assay that corresponds to the concentration of 20 pg/ml in a biological fluid in our system. In this study, we also demonstrated that our immunological assay was specific for 6-keto-$PGF_{1\alpha}$ due to with a very low cross-reaction of the antibody with other prostanoids

and the related compounds, with values of less than 0.1%. Thus, the sensitivity and specificity of our assay were almost comparable with other ELISA for each of 15d-PGJ_2 and Δ^{12}-PGJ_2 using its specific antiserum as reported recently [15, 16]. As long as cultured 3T3-L1 cells are used for the generation of PGI_2, the end product is more likely to be 6-keto-$PGF_{1\alpha}$ without further metabolism to other products, since there are no further oxidation systems in adipocytes and the related precursor cells, which is different from blood circulation system *in vivo*.

To monitor the endogenous production of 6-keto-$PGF_{1\alpha}$ derived from PGI_2 at different life stage of adipocytes, we employed three types of culture media including the GM, DM, and MM corresponding to the growth, differentiation, and maturation phases, respectively. Hence, it is crucial to demonstrate that non-specific interference can be excluded in individual assays with different culture media. We tried to verify the accuracy of our ELISA for the quantification of 6-keto-$PGF_{1\alpha}$ by generating the calibration curve using an ELISA buffer mixed equally with the desired culture medium. The resulting analysis provided the evidence that the measured values of 6-keto-$PGF_{1\alpha}$ in the culture medium were linearly correlated with those fortified with increasing amounts of the authentic compound in the respective one. The findings indicated that we successfully confirmed the accuracy of our ELISA for the determination of the levels of 6-keto-$PGF_{1\alpha}$ in the individual culture media used for life stages leading to adipogenesis in cultured adipocytes. The combined results led us to know that non-specific interference was not detectable in our immunological assays for 6-keto-$PGF_{1\alpha}$ due to the presence of various components in different culture media.

After confirming the validity of our developed solid-phase ELISA, we applied our developed solid-phase ELISA to the determination of endogenous synthesis of 6-keto-$PGF_{1\alpha}$ at different life stages of cultured 3T3-L1 cells leading to adipocytes from undifferentiated preadipocytes. In this study, we showed that preadipocytes at the growth phase produced 6-keto-$PGF_{1\alpha}$ reflecting the endogenous synthesis of PGI_2. However, when the cultured cells were induced to undergo the differentiation phase, the synthesis of

6-keto-$PGF_{1\alpha}$ was suppressed substantially. This suppression is considered to be due to the presence of dexamethasone in DM, a well-known anti-inflammatory synthetic compound acting through the glucocorticoid receptor [30]. Once DM was removed and cells were refed with MM every 2 days, adipocytes were found to recover the ability to synthesize 6-keto-$PGF_{1\alpha}$ after the maturation phase of adipocytes, exhibiting the highest level during the periods of 4-6 days. Then, the capability of cultured adipocytes to generate 6-keto-$PGF_{1\alpha}$ was gradually attenuated at later maturation phase while the stimulation of fat storage was more evident in later maturation phase. This up-regulation of the endogenous synthesis of 6-keto-$PGF_{1\alpha}$ was consistent with our current data on the gradual increase in gene expression levels of PGIS responsible for the synthesis of PGI_2 from PGH_2 with the highest level after 6 days of the maturation phase. We have already reported the gene expression of biosynthetic enzymes in the arachidonate COX pathway necessary for the formation of PGH_2 from arachidonic acid in cultured 3T3-L1 adipocytes [15]. These include cytosolic phospholipase $A_2\alpha$ for the release of free arachidonic acid from membrane phospholipids and COX isoforms for the oxygenation of free arachidonic acid to generate PGH_2, such as the constitutive COX-1 and the inducible COX-2. Thus, both COX isoforms appear to be involved in the generation of PGI_2 during the maturation phase.

Previous studies have described the synthesis of PGI_2 by cultured 3T3-L1 cells as determined by the amount of 6-keto-$PGF_{1\alpha}$. For example, an earlier study has described that rapidly growing cultured 3T3-L1 preadipocytes can produce PGI_2 as an acute response to the stimulation with the calcium ionophore A23187 or incubation with exogenous arachidonic acid [10]. According to this study, the capability to form PGI_2 was lower in cultured adipocytes than preadipocytes. Alternatively, Xie et al. [31] have documented the reduced levels of 6-keto-$PGF_{1\alpha}$ in 3T3-L1 adipocytes compared with preadipoctyes. However, the development stages of cultured cells and the culture conditions used were not specified in that study. In contrast, [32] have reported gradual increase in the generation of 6-keto-$PGF_{1\alpha}$ after cultured 3T3-L1 cells had been induced to the differentiation phase for two days in the presence of IBMX, dexamethasone, and insulin. The reason of the

discrepancy might be explained by differences in their culture conditions, sampling periods, or assay methods. Neither of the above studies determined the endogenous synthesis of 6-keto-$PGF_{1\alpha}$ at different life stages continuously from undifferentiated preadipoctyes to mature adipocytes. On the other hand, we found that the ability of cultured cells to generate 6-keto-$PGF_{1\alpha}$ was reduced when the cells were shifted from the growth phase to the differentiation phase. Instead, the up-regulation of the endogenous synthesis of 6-keto-$PGF_{1\alpha}$ was observed markedly during the maturation phase, which was accompanied by the enhanced gene expression of PGIS. As a separate cell culture system, Ob1771 pre-adipose cells have been used for the synthesis of prostanoids including PGI_2 although the changes in the capability of cells to form PGI_2 were not monitored at the different stages of adipogenesis [31]. In this case, cultured cells were maintained in serum-free hormone-supplemented medium. Therefore, the culture conditions are largely different from our culture conditions of 3T3-L1 cells.

We found the highest capability of cultured adipocytes to endogenously synthesize 6-keto-$PGF_{1\alpha}$ reflecting PGI_2 around the periods of 4-6 days during the maturation phase. Following this endogenous synthesis of PGI_2, the accumulation of fats occurred more increasingly at later maturation phase. Moreover, the gene expression of the cell surface IP receptor was closely linked with the generation of PGI_2 and the transcript levels of PGIS. Considering these findings, endogenously synthesized PGI_2 appears to potentially contribute to the positive regulation of adipogenesis in an autocrine manner. In support of this idea, we have recently reported that well-known COX inhibitors including aspirin and indomethacin attenuated fat storage during the maturation phase. However, we have recently shown that other pro-adipogenic prostanoids including J_2 series PGs were generated during the maturation phase [15, 16]. Hence, the up-regulation of adipogenesis is not explained solely by the action of PGI_2. Furthermore, since biologically active PGI_2 is an unstable compound in biological fluids, the effectiveness of intact PGI_2 remains yet to be determined during the maturation phase of adipocytes. Nonetheless, it is conceivable that the endogenous PGI_2 could partly be efficacious to up-regulate adipogenesis through

its IP receptor. Just recently, we have noticed that specific IP agonists can partly rescue the inhibition of adipogenesis in the presence of indomethacin or aspirin. As well, specific antagonists for the IP receptor were able to attenuate fat storage during the maturation phase under our experimental conditions (data not shown). Although the cell line and the culture conditions are different from the ones used in the present study, earlier studies have reported that carbaprostacyclin, a stable analogue of prostacyclin, stimulated the terminal differentiation of Ob1771 pre-adipose cells in serum-free hormone-supplemented medium, indicating the pro-adipogenic action through the IP receptor [19, 20]. In these studies, they did not show any results using a natural PGI_2 itself presumably due to the instability of this prostanoids. Interestingly, a recent study using a cell-based reporter gene assay in HEK-293 cell lines 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 [33]. Another study has described that fat mass gain is suppressed in IP-deficient mice compared with wild-type mice when mother mice were fed high-fat diet rich in linoleic acid, indicating the contribution of the signaling through the IP receptor to adipose tissue development [34]. Thus, the modulation of adipogenesis by PGI_2 analogues through the IP receptor is more likely to occur in cultured cells or adipose tissues. In addition, the present study support the potential idea that the increased synthesis of endogenous prostacyclin by adipocytes during the maturation phase can stimulate adipogenesis in an autocrine manner by interacting with its specific IP receptor, the expression of which is regulated coordinately with PGIS. The resulting signaling process would involve the indirect activation of PPARγ, a master regulator of adipogenesis. However, much work remains to be done regarding the role of naturally occurring PGI_2 itself in adipogenesis in terms of an autocrine or paracrine control.

In conclusion, we have developed a sensitive solid-phase ELISA specific for 6-keto-$PGF_{1\alpha}$, a stable hydrolysis product of an unstable parent PGI_2 called also prostacyclin. Our ELISA was validated for the application to the quantification of 6-keto-$PGF_{1\alpha}$ reflecting the biosynthesis of PGI_2 at different life stages of adipogenesis. We

revealed that biosynthesis of PGI_2 was up-regulated at the highest levels around after 4-6 days after the maturation phase of adipocytes, which was accompanied by the gene expression of PGIS and the IP receptor. Following these responses, the accumulation of fats occurred increasingly at later maturation phase. These findings provided the useful information on the generation of endogenous thesis of PGI_2 and raised the potential role of this prostanoid as one of the pro-adipogenic factors in an autocrine manner in cultured adipocytes. Thus, the biosynthesis of prostacyclin can be used as a biomarker of adipocyte differentiation.

References

1. Antuna-Puente, B., Feve, B., Fellahi, S., and Bastard, J. P. (2008) Adipokines: the missing link between insulin resistance and obesity. Diabetes Metab. 34, 2-11
2. Sethi, J. K., and Hotamisligil, G. S. (1999) The role of TNF alpha in adipocyte metabolism. Semin. Cell Dev. Biol. 10, 19-29
3. Sartipy, P., and Loskutoff, D. J. (2003) Monocyte chemoattractant protein 1 in obesity and insulin resistance. Proc. Natl. Acad. Sci. USA 100, 7265-7270
4. Permana, P. A., and Menge, C., Reaven, P. D. (2006) Macrophage-secreted factors induce adipocyte inflammation and insulin resistance. Biochem. Biophys. Res. Commun. 341, 507-514
5. Tontonoz, P., Hu, E., and Spiegelman , B. M. (1994) Stimulation of adipogenesis in fibroblasts by PPARγ2, a lipid-activated transcription factor. Cell 79, 1147-1156
6. Chawla, A., Schwarz, E. J., Dimaculangan, D. D., and Lazar, M. A. (1994) Peroxisome proliferator-activated receptor (PPAR) gamma: adipose-predominant expression and induction early in adipocyte differentiation. Endocrinology 135, 798-800
7. Forman, B. M., Tontonoz, P., Chen, J., Brun, R. P., Spiegelman, B. M., and Evans, R. M. (1995) 15-Deoxy-$\Delta^{12, 14}$-prostaglandin J_2 is a ligand for the adipocyte determination factor PPARγ. Cell 83, 803-812
8. Kliewer, S. A., Lenhard, J. M., Willson, T. M., Patel, I., Morris, D. C., and Lehmann, J. M. (1995) A prostaglandin J_2 metabolite binds peroxisome proliferator-activated

receptor γ and promotes adipocyte differentiation. Cell 83, 813-819

9. Hopkins, N., and Gorman, R. R. (1981) Regulation of 3T3-L1 fibroblast differentiation by prostacyclin (prostaglandin I_2). Biochim. Biophys. Acta 663, 457-466
10. Hyman, B. T., Stoll, L. L., and Spector, A. A .(1982) Prostaglandin production by 3T3-L1 cells in culture. Biochim. Biophys. Acta 713, 375-385
11. Lu, S., Nishimura, K., Hossain, M. A., Jisaka, M., Nagaya, T., and Yokota, K. (2004) Regulation and role of arachidonate cascade during changes in life cycle of adipocytes. Appl. Bichem. Biotechnol. 118, 133-153
12. Xu, L., Nishimura, K., Jisaka, M., Nagaya, T., and Yokota, K. (2006) Gene expression of arachidonate cyclooxygenase pathway leading to the delayed synthesis of prostaglandins E_2 and $F_{2\alpha}$ in response to phorbol 12-myristate 13-acetate and action of these prostanoids during life cycle of adipocytes. Biochim. Biophys. Acta 1761, 434-444
13. Green, H., and Kehinde, O. (1974) Sublines of mouse 3T3 cells that accumulate lipid. Cell 1, 113-116
14. Green, H., and Kehinde, O. (1975) An established preadipose cell line and its differentiation in culture. II. Factors affecting the adipose conversion. Cell 5, 19-27
15. Mazid, M. A., Chowdhury, A. A., Nagao, K., Nishimura, K., Jisaka, M., Nagaya, T., and Yokota, K. (2006) Endogenous 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 synthesized by adipocytes during maturation phase contributes to upregulation of fat storage. FEBS Lett. 580, 6885-6890
16. Hossain, M. S., Chowdhury, A. A., Rahman, M. S., Nishimura, K., Jisaka, M., Nagaya, T., Shono, F., and Yokota, K. (2011) Development of enzyme-linked immunosorbent assay for Δ^{12}-prostaglandin J_2 and its application to the measurement of the endogenous product generated by cultured adipocytes during the maturation phase. Prostaglandins Other Lipid Mediat. 94, 73-80
17. Tsuboi, H., Sugimoto, Y., Kainoh, T., and Ichikawa, A. (2004) Prostanoid EP4 receptor is involved in suppression of 3T3-L1 adipocyte differentiation. Biochem. Biophys. Res. Commun. 322, 1066-1072
18. Reginato, M. J., Krakow, S. L., Bailey, S. T., and Lazar, M. A. (1998) Prostaglandins promote and block adipogenesis through opposing effects on peroxisome

proliferator-activated receptor gamma. J. Biol. Chem. 273, 1855-1858

19. Négrel, R., Gaillard, D., and Ailhaud, G. (1989) Prostacyclin as a potent effector of adipose-cell differentiation. Biochem. J. 257, 399-405
20. Catalioto, R. M., Gaillard, D., Maclouf, J., Ailhaud, G., and Negrel, R. (1991) Autocrine control of adipose cell differentiation by prostacyclin and $PGF_{2\alpha}$. Biochim. Biophys. Acta 1091, 364-369
21. Kelton, J. G., and Blajchman, M. A. (1980) Prostaglandin I_2 (prostacyclin). Can. Med. Assoc. J. 122, 175-179
22. Shono, F., Yokota, K., Horie, S., Yamamoto, K., Yamashita, K., Watanabe, H., Miyazaki, and H. (1988) A heterologous enzyme immunoassay of prostaglandin E_2 using a stable enzyme-labeled hapten mimic. Anal Biochem. 168, 284-291
23. Yokota, K., Morishima, T., Naagya, M., Jisaka, M., and Takinami, K. (1996) Modification of cultured Madin-Darby canine kidney cells with dietary unsaturated fatty acids and regulation of arachidonate cascade reaction. Biosci. Biotechnol. Biochem. 60, 1096-1103
24. Syeda, P. K., Hossain, M. S., Chowdhury, A. A., Rahman, M. S., Nishimura, K., Jisaka, M., Nagaya, T., Shono, F., and Yokota, K. (2012) Generation of monoclonal antibody for 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 and development of enzyme-linked immunosorbent assay for its quantification in culture medium of adipocytes. Appl. Biochem. Biotechnol. 167, 107-1118
25. Chu, X., Xu, L., Nishimura, K., Jisaka, M., Nagaya, T., Shono, F., and Yokota, K. (2009) Suppression of adipogenesis program in cultured preadipocytes transfected stably with cyclooxygenase isoforms. Biochim. Biophys. Acta 1791, 273-280
26. Moncada, S., Gryglewski, R., Bunting, S., and Vane, J. R. (1976) An enzyme isolated from arteries transforms prostaglandin endoperoxides to an unstable substance that inhibits platelet aggregation. Nature 263, 663-665
27. Stehle, R. G. (1982) Physical chemistry, stability, and handling of prostaglandins E_2, $F_{2\alpha}$, D_2, and I_2: A critical summary. Methods Enzymol. 86, 436-458
28. Narumiya, S., Sugimoto, Y., and Ushikubi, F. (1999) Prostanoid receptors: structures, properties, and functions. Physiol. Rev. 79, 1193-1226
29. Wise, H. (2003) Multiple signalling options for prostacyclin. Acta Pharmacol. Sin. 24, 625-630

30. Goppelt-Struebe, M., Wolter, D., and Resch, K. (1989) Glucocorticoids inhibit prostaglandin synthesis not only at the level of phospholipase A_2 but also at the level of cyclo-oxygenase/PGE isomerase. Br. J. Pharmacol. 98, 1287-1295
31. Xie, Y., Kang, X., Ackerman, W. E. T., Belury, M. A., Koster, C., Rovin, B. H., Landon, M. B., Kniss, D. A. (2006) Differentiation-dependent regulation of the cyclooxygenase cascade during adipogenesis suggests a complex role for prostaglandins. Diabetes Obes. Metab. 8, 83-93
32. Fajas, L., Miard, S., Briggs, M. R., and Auwerx, J. (2003) Selective cyclo-oxygenase-2 inhibitors impair adipocyte differentiation through inhibition of the clonal expansion phase. J. Lipid Res. 44, 1652-1659
33. Falcetti, E., Flavell, D. M., Staels, B., Tinker, A., Haworth, S. G., and Clapp, L. H. (2007) IP receptor-dependent activation of PPARγ by stable prostacyclin analogues. Biochem. Biophys. Res. Commun. 360, 821-827
34. Massiera, F., Saint-Marc, P., Seydoux, J., Murata, T., Kobayahis, T., Narumiya, S., Guesnet, P., Amri, E. Z., Negrel, R., and Ailhaud, G. (2003) Arachidonic acid and prostacyclin signaling promote adipose tissue development: a human health concern? J. Lipid Res. 44, 271-279

Chapter 4

Conclusion

We have conducted a comprehensive study on the role of COX-1 in adipogenesis. Here, we were able to specifically suppress the gene expression of the constitutive COX-1 by isolating cloned preadipocytes transfected stably with antisense COX-1. The resulting stable transfectants exhibited the marked reduction in the gene expression of the sense transcript of COX-1, which was followed by the substantial decrease in the immediate synthesis of PGE_2, an anti-adipogenic factor. In sharp contrast, the gene expression of the inducible COX-2 was not affected. Fat storage was stimulated in the stable transfectants with antisense COX-1 due to the positive regulation of adipogenesis program, which was accompanied by the higher production of J_2 series PGs known as pro-adipogenic PGs. These findings indicate that remaining COX-2 can contribute to the endogenous synthesis of those PGJ_2 derivatives by the COX pathway by compensating for the suppressed expression of COX-1. The understanding of the cellular mechanism for the up-regulation of adipogenesis awaits more extensive investigation.

Besides, we performed another study on Prostacyclin alternatively called prostaglandin (PG) I_2. Earlier the biosynthesis of PGI_2 has not been determined comprehensively at different life stages of adipocytes. We have developed a sensitive solid-phase ELISA specific for 6-keto-$PGF_{1\alpha}$, a stable hydrolysis product of an unstable parent PGI_2 called also prostacyclin. Our ELISA was validated for the application to the quantification of 6-keto-$PGF_{1\alpha}$ reflecting the biosynthesis of PGI_2 at different life stages of adipogenesis. We revealed that biosynthesis of PGI_2 was up-regulated at the highest levels around after 4-6 days after the maturation phase of adipocytes, which was accompanied by the gene expression of PGIS and the IP receptor. Following these responses, the accumulation of fats occurred increasingly at later maturation phase. These findings provided the useful information on the generation of endogenous thesis of PGI_2 and raised the potential role of this prostanoid as one of the pro-adipogenic factors in an autocrine manner in cultured adipocytes. Thus, the biosynthesis of prostacyclin can be used as a biomarker of adipocyte differentiation.

Summary

The present study was undertaken to conduct cell science studies regarding role of cyclooxygenase (COX)-1, the constitutive COX isoform, in adipogenesis and biosynthesis of prostacyclin called also prostaglandin (PG) I_2 at the maturation phase of adipocytes using cultured preadipogenic cells with the ability to differentiate into adipocytes. PGs are synthesized through the arachidonate COX pathway in which the enzymatic reaction of PGH synthase alternatively called COX is the rate-limiting step for the generation of PGs after the release of free arachidonic acid. The COX enzyme is known to occur as two types of isoforms, including the constitutive COX-1 and the inducible COX-2. Cultured mouse preadipogenic 3T3-L1 cells have been useful for the studies on the regulation of the arachidonate COX pathway and the role of the products in the control of adipogenesis and other functions of adipocytes because of the established culture conditions consisted of the growth, differentiation, and maturation phases. Therefore, I have been studying the gene expression of the biosynthetic enzymes of the COX pathway and the roles for endogenous and exogenous PGs at different life stages.

The arachidonate COX pathway is involved in the generation of several types of endogenous PGs with opposite effects on adipogenesis at different life stages of adipocytes. However, specific role of COX isoforms, the rate limiting enzymes for the pathway, remains elusive in the regulation of the endogenous synthesis of PGs. This study was aimed at the selective suppression of the constitutive COX-1 in cultured preadipocytes by the isolation of cloned preadipocytes transfected stably with a mammalian expression vector harboring cDNA encoding mouse COX-1 in the antisense direction. The gene expression analysis revealed that the transcript and protein levels of the constitutive COX-1 were substantially suppressed in the isolated cloned transfectants with antisense COX-1. By contrast, the expression of the inducible COX-2 was not affected in the stable transfectants with antisense COX-1. All the cloned stable transfectants with antisense COX-1 exhibited the significant reduction in the immediate synthesis of PGE_2 serving as an anti-adipogenic factor. The sustained expression of COX-1 at the antisense direction induced the appreciable stimulation of fat storage in adipocytes during the maturation phase, which was associated with the higher expression levels of adipocyte-specific genes, indicating the positive regulation of adipogenesis program. Moreover, the up-regulation of adipogenesis is accompanied by higher production of J_2 series PGs including 15-deoxy-$\Delta^{12,14}$-PGJ_2 and

Δ^{12}-PGJ_2 known as pro-adipogenic factors by the transfectants with antisense COX-1. The results suggest that the inducible COX-2 can contribute to the endogenous synthesis of PGJ_2 derivatives acting as autocrine mediators to simulate adipogenesis during the maturation phase by way of compensation for the suppressed expression of the constitutive COX-1.

Prostacyclin alternatively called PGI_2 is an unstable metabolite synthesized by the arachidonate COX pathway. Earlier studies have suggested that prostacyclin analogues can act as a potent effector of adipose differentiation. However, biosynthesis of PGI_2 has not been determined comprehensively at different life stages of adipocytes. PGI_2 is rapidly hydrolyzed to the stable product, 6-keto-$PGF_{1\alpha}$, in biological fluids. Therefore, the generation of PGI_2 can be quantified as the amount of 6-keto-$PGF_{1\alpha}$. In this study, I attempted to develop a solid-phase ELISA using a mouse antiserum specific for 6-keto-$PGF_{1\alpha}$. According to the typical calibration curve of the current enzyme-linked immunosorbent assay (ELISA), 6-keto-$PGF_{1\alpha}$ can be quantified from 0.8 pg to 7.7 ng in an assay. The evaluation of this ELISA revealed the higher specificity of my antiserum without the cross-reaction with other related prostanoids while it exhibited only the cross-reaction of 1.5% with $PGF_{2\alpha}$. The resulting ELISA was applied to the quantification of 6-keto-$PGF_{1\alpha}$ generated endogenously by cultured 3T3-L1 cells at different stages. The cultured cells showed the highest capability to generate 6-keto-$PGF_{1\alpha}$ during the maturation phase of 4-6 days, which was consistent with the coordinated changes in the gene expression of PGI synthase and the IP receptor for PGI_2. Following these events, the accumulation of fats was continuously promoted up to 14 days. Thus, the present immunological assay specific for 6-keto-$PGF_{1\alpha}$ is useful for monitoring the endogenous levels of the unstable parent PGI_2 at different life stages of adipogenesis and for further studies on the potential association with the up-regulation of adipogenesis in cultured adipocytes.

Summary in Japanese

今回の研究は、脂肪細胞へ分化する能力を持つ前駆脂肪細胞株を用いて、脂肪細胞形成におけるシクロオキシゲナーゼ（COX）-1、すなわち、構成的なCOXアイソフォームの役割と脂肪細胞の成熟期におけるプロスタグランジン（PG）I_2とも呼ばれるプロタサイクリンの生合成に関する細胞科学研究を行うことを試みた。PGsは、別名COXとも呼ばれるPGH合成酵素の酵素反応が遊離脂肪酸の放出後のPG類の生成の律速段階であるアラキドン酸COX経路を介して生合成される。そのCOX酵素は、常在性のCOX-1と誘導性のCOX-2のような２つの種類のアイソフォームとして存在することが知られている。培養細胞のマウス前駆脂肪細胞である3T3-L1細胞は、生育期、分化誘導期、成熟期からなる確立された培養条件のため、アラキドン酸COX経路の調節と脂肪細胞形成や他の脂肪細胞の機能の制御における代謝産物の役割を研究するのに有用であった。それゆえ、私は、COX経路の生合成酵素の遺伝子発現や異なるライフステージでの内因性及び外因性PG類の役割を研究してきた。

アラキドン酸COX経路は、脂肪細胞の異なるライフステージで脂肪細胞形成に異なる効果を示す数種類の内因性PG類の生成に関与する。しかし、内因性PG類の生合成調節における、この経路の律速段階の酵素であるCOXアイソフォームの特異的な役割は不明のままである。本研究のねらいは、アンチセンス方向に位置したマウスCOX-1を暗号化するcDNAを組み込んだ哺乳動物の発現ベクターで安定発現したクローン化前駆脂肪細胞を単離することで、培養前駆脂肪細胞での常在性のCOX-1を選択的に抑制することであった。その遺伝子発現の解析により、常在性COX-1の転写産物とタンパク質の発現レベルは、アンチセンスCOX-1で形質転換して単離されたクローン化細胞で有意に抑制された。対照的に、誘導型COX-2の発現は、アンチセンスCOX-1で安定な形質転換細胞で影響を受けなかった。アンチセンスCOX-1で安定に形質転換されたすべての細胞は、脂肪細胞形成の抑制因子として作用するPGE_2の即時的な生成反応の著しい低下を示した。アンチセンス方向にCOX-1を安定に発現すると、成熟期での脂肪細胞での脂肪蓄積の有意な促進が誘導され、それは、脂肪細胞特異的な遺伝子のより高い発現レベルに関係していた。そのことは、脂肪細胞形成プログラムの正の調節を示している。さらに、脂肪細胞形成の調節の促進は、アンチセンスのCOX-1による形質転換細胞による脂肪細胞形成の促進因子として知られる15-デオキシ-$\Delta^{12,14}$-PGJ_2やΔ^{12}-PGJ_2のようなJ_2シリーズのPG類のより高い生成を伴っていた。それらの結果より、誘導性の

COX-2 が、常在性の COX-1 の発現抑制を補償することにより、成熟期の脂肪細胞形成を促進する自己分泌性のメディエーターとして作用する内因性 PGJ_2 誘導体の生成に貢献できることを示唆している。

別名、PGI_2 と呼ばれるプロスタサイクリンは、アラキドン酸 COX 経路により生合成される不安定な代謝産物である。以前の研究により、プロスタサイクリンの類縁体が脂肪細胞の分化の強力な効果因子として作用できることが示唆されている。しかし、PGI_2 の生合成は、脂肪細胞の異なるライフステージで詳細に検討されていない。PGI_2 は、生体の溶液中で安定産物の 6-ケト-$PGF_{1\alpha}$ に急速に加水分解される。それゆえ、PGI_2 の生成は、6-ケト-$PGF_{1\alpha}$ の量として定量することができる。本研究では、私は、6-ケト-$PGF_{1\alpha}$ に対する特異的なマウス抗血清を用いて固相化免疫測定法（ELISA）を開発することを試みた。今回の ELISA の典型的な標準曲線で、6-ケト-$PGF_{1\alpha}$ は測定あたり 0.8 pg から 7.7 ng まで定量できる。この ELISA の評価により、私の使用した抗血清は、他のプロスタノイド関連物質とほとんど交差反応性を示さず、$PGF_{2\alpha}$ に対して 1.5%の交差反応性を示すのみであることが明らかになった。その結果、今回の ELISA は、異なる段階の 3T3-L1 培養細胞により生成される内因性 6-ケト-$PGF_{1\alpha}$ の定量に適用した。本培養細胞により、成熟期の 4-6 日の間で 6-ケト-$PGF_{1\alpha}$ を生成する能力が最も高いことを示された。この結果は、PGI 合成酵素や PGI_2 に対する IP 受容体の遺伝子発現の協調的な変動に一致していた。これらの現象の後、脂肪蓄積は、14 日まで連続的に促進された。従って、6-ケト-$PGF_{1\alpha}$ に対する特異的な今回の免疫学的測定法は、脂肪細胞形成の異なるライフステージでの不安定な元の PGI_2 の内因性の生成レベルを調べ、培養脂肪細胞の脂肪細胞形成での正の調節との潜在的な関連についてさらなる研究をするのに有用である。

Acknowledgement

I would like to express my heartfelt thanks to Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan for providing me the financial support for the doctoral course.

My research is the outcome of the planning and guiding of Professor Kazushige Yokota. Whatever I am, he made me. Without him, this research would never become true. So, I am very much grateful to him. I feel happy when I think about his time to time monitoring, step by step advancement, session after session scientific lectures and overall providing multicultural and multinational environment for research. He implanted into me the proper way of thinking and reviewing scientific issues.

In addition, special thanks to Dr. Tsutomu Nagaya, Dr. Mitsuo Jisaka, Dr. Kohji Nishimura, Prof. Ishikawa and Dr. Akihiro for their continuous laboratory support.

Besides, I cannot forget the help of Mr. Abu Asad Chowdhury, Mr. Salim Hossain, Ms. Shimoda, Ms. Pinky, Ms. Yoshida, Ms. Ikeda, Mr. Tabata, Mr. Nagata, Ms. Ita, Mr. Ogawa, Ms. Hanabara, Mr. Ferdous Khan and other members in the laboratory and other laboratories for their friendly and essential support during my research period.

List of publications

Chapter 2

Mohammad Sharifur Rahman, Pinky Karim Syeda, Ferdous Khan, Kohji Nishimura, Mitsuo Jisaka, Tsutomu Nagaya, Fumiaki Shono, Kazushige Yokota. (2013) Cultured preadipocytes undergoing stable transfection with cyclooxygenase-1 in the antisense direction accelerate adipogenesis during the maturation phase of adipocytes. ***Applied Biochemistry and Biotechnology,*** Volume 171, Issue 1, pp 128-144.
DOI 10.1007/s 12010-013-0347-3

Chapter 3

Mohammad Sharifur Rahman, Ferdous Khan, Pinky Karim Syeda, Kohji Nishimura, Mitsuo Jisaka, Tsutomu Nagaya, Fumiaki Shono, Kazushige Yokota. (2013) Endogenous synthesis of prostacyclin was positively regulated during the maturation phase of cultured adipocytes. ***Cytotechnology,*** in press
DOI: 10.1007/s10616-013-9616-9