Studies on serum parameters associated with metabolic syndrome and pro-adipogenic effects of prostaglandin D_2 and its stable analogue

［代謝症候群に関連する血清パラメーターとプロスタグランジン D_2 及びその安定類縁体の脂肪細胞形成の促進効果に関する研究］

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Abbreviations

AA	Arachidonic acid
AT	Adipose tissue
BSA	Bovine serum albumin
C/EBP	CCAAT/enhancer binding protein
COX	Cyclooxygenase
$cPLA_2$	Cytosolic PLA_2
DM	Differentiation medium
DME-HEPES	Dulbecco's modified Eagle medium with 25 mM HEPES
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FFA	Free fatty acids
GC-MS	Gas chromatography-mass spectrometry
GM	Growth medium
H-PGDS	Hematopoietic-type prostaglandin D synthase
IBMX	3-isobutyl-1-methylxanthine
LC-MS/MS	Liquid chromatography-mass spectrometry/mass spectrometry
L-PGDS	Lipocalin-type prostaglandin D synthase
MM	Maturation medium
NFκB	Nuclear factor-κB
NSAIDs	Nonsteroidal anti-inflamatory drugs
PBS (-)	Phosphate-buffered saline without Ca^{2+} and Mg^{2+} ions
PG	Prostaglandin
PGDS	PGD synthase
15d-PGJ_2	15-deoxy-$\Delta^{12,14}$-prostaglandin J_2
PL	Phospholipase
PLA_2	Phospholipase A_2
PPAR	Peroxisome proliferator-activated receptor
RP-HPLC	Reverse-phase high-performance liquid chromatography
TAG	Triacylglycerol
TNFα	Tumor necrosis factor α

Chapter 1

Introduction

1.1 General Introduction

Obesity is defined as an increase in adipose tissue mass. An increase in adipose tissue mass can be the result of the production of new fat cells through the process of adipogenesis and/or the deposition of increased amounts of cytoplasmic triglyceride per cell. Thus, the biological events leading to obesity are characterized by changes in the cell properties of adipocytes as represented by an increase in cell numbers, size or both. Obesity is the most prevalent nutritional and metabolic disorder in industrialized countries and is a growing problem in developing countries [1]. Obesity is closely associated with a number of pathological disorders, including non-insulin dependent diabetes, hypertension, cancer, gallbladder disease and atherosclerosis [2]. With regard to this wide range of health implications, the need to develop new and effective strategies in controlling obesity has become more acute. Therefore, complete understanding the cellular and molecular basis of the adipose tissue growth in physiological and pathophysiological states is an important area of research that may lead to the development of innovative therapies to treat obesity related disorders.

1.2 Adipose Tissue, Adipocyte Dysfunction and Metabolic Syndrome

For many years, it has been postulated that when energy intake exceeds energy expenditure, the excess metabolic fuel is stored passively in the adipocytes in much the same way that oil can store its content. Normally, adipocytes are organized in a multidepot organ called adipose tissue. Only one-third of the adipose tissues contain mature adipocytes. Remaining is composed of preadipocytes, blood vessels, nerve cells and fibroblasts. There are two types of adipose tissue. One is brown adipose tissue, mainly responsible for transferring energy from food into heat [3]. The other one is white adipose tissue, which is involved in lipid metabolism, glucose metabolism and endocrine functions [4]. The origins of the adipose cell and adipose tissue are still poorly understood, and the molecular events leading to the commitment of the embryonic stem cell precursor to the adipose lineage remain to be characterized. Recent studies have suggested that adipocytes lineage derives from an embryonic stem cell precursor with the capacity to differentiate into mesodermal cell types of adipocytes, chondrocytes and myocytes [5]. Various cell culture models have been extensively used to study the adipocyte differentiation. This has led to a dissection of the molecular and cellular events taking place during the transition from fibroblast-like preadipocytes into mature round fat cells though still the whole mechanism for this conversion is unknown.

Metabolic syndrome is a serious health condition and its prevalence is increasing globally. It is a group of risk factors that raises risk of heart disease, diabetes, stroke, and other health problems and is diagnosed when risk factors such as high blood glucose, low levels of HDL ("good") cholesterol in the blood, high levels of triglycerides in the blood, large waist circumference or "apple-shaped" body and high blood pressure are present. Obesity is frequently associated with chronic inflammation, metabolic and vascular alterations that lead to the development of the metabolic syndrome. However, the individual obesity-related risk for the metabolic syndrome is not determined by increased fat mass alone. Heterogeneity of body composition, fat distribution and adipose tissue (AT) function may underly the variable risk to develop metabolic and cardiovascular diseases associated with increased body fat mass. People who have the metabolic syndrome can reduce their risk for cardiovascular disease and type 2-diabetes by controlling risk factors. The best way is often for them to lose weight, eat a healthy diet and increase their physical activity.

1.3 Adipogenesis: from preadipocyte to mature adipocyte

Recent studies revealed that committed preadipocytes have to withdraw from the cell cycle before undergoing adipose conversion. For preadipocytes, growth arrest is required for adipocyte differentiation and is normally achieved through contact inhibition. Following growth arrest, preadipocytes must receive an appropriate combination of mitogenic and adipogenic signals to continue through subsequent differentiation steps, leading to the progressive acquisition of the morphological and biochemical characteristics of the mature adipocytes [5]. 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. Overall, in serum containing medium, the standard adipogenic cocktails that induce differentiation contain supraphysiological concentration of insulin, dexamethasone (DEX) and isobutylmethylxanthine (IBMX). Post-confluent adipocytes undergo at least one-round of DNA replication and cell doubling, leading to the clonal amplication of committed cells [6]. In contrast, data collected from 3T3-L1 preadipocytes revealed that DNA synthesis and and mitotic expansion are not required steps for preadipocyte differentiation into adipocytes [7]. The first hallmark of the adipogenesis process is the dramatic alteration in cell shape as the cells convert from fibroblast to spherical shape. After differentiation, the preadipocytes become matured, the main cellular component of WAT, and uniquely equipped to function in energy storage and balance under tight hormonal control.

Many of the changes that occur during preadipocyte differentiation take place at the gene expression level. Several reports have attempted to schematize the stages of adipocyte differentiation into a simple hierarchy of molecular events. Genes differentially regulated during adipogenesis have

been categorized into early, intermediate and late mRNA/protein markers [8-12]. However, obtaining an accurate chronology of the molecular events that take place during differentiation is a difficult task. Growth arrest and clonal expansion are accompanied by complex changes in the pattern of gene expression that can differ with cell culture models and the specific differentiation protocols employed. Moreover, progressive acquisition of the adipocyte phenotype is associated with changes in the expression of over 200 genes [13]. Over 100-expressed sequence tags representing uncharacterized genes were expressed only in preadipocytes, clearly indicating that further research is required to fully understand the adipogenesis process.

1.4 Factors Modulating Adipogenesis

The growth and differentiation of animal cells are controlled by communication between individual cells or between cells and the extra-cellular environment. Adipocyte differentiation therefore, 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]. Identification of agents or molecules that modulate the process in either a positive or negative manner provides insight into the signal transduction pathway involved. Extracellular matrix proteins may play an important role in modulating adipocyte differentiation by pertaining the morphological changes and adipocyte-specific gene expression that accompany differentiation [15].

Several transcription factors are key regulator of the adipogenesis process. The role of peroxisome proliferator activator receptor (PPAR)-γ and CCAAT-enhancer binding proteins, C/EBPs have been intensively investigated. The PPARs are ligand-activated transcription factors that belong to the nuclear hormone receptor family. There are three isoforms of PPARs (α, γ and δ). Among different isoforms, PPAR-γ is the most adipose specific and it is induced before transcriptional activation of most adipocyte gene. The amino-terminal domain is poorly conserved among the isoforms and contains a lignd-independent transactivation function. The highly conserved central domain brings the capacity of DNA binding (DNA binding domain, DAB). The carboxyl-terminal domain contains the ligand dependent tarnsactivation function (Ligand dependent domain, LBD). PPARs heterodimerize with the cis-retinoid receptors (RXR) (Fig 1-1). The transcription complex then binds with peroxisome proliferator response element (PPRE), which is an imperfect direct repeat of six nucleotides with one spacing base (DR-1, direct repeat-1 base spacer) and stimulates transcription of target genes such as FAS, acyl CoA synthase etc. [16]. On the other hand, members of C/EBP family were the first transcription factors demonstrated to play a major role in adipocyte differentiation. These transcription factors have a basic transcriptional activation domain and an adjoining leucine zipper motif, which

provides the ability for homo- and heterodimerization. C/EBP binds and transactivates the promoters of several adipocyte genes, including aP2, SCD1, GLUT-4, leptin and insulin receptor. Mutation of the C/EBP- α site in these genes abolishes transactivation [17-18]. Studies on adipocyte differentiation, primarily in 3T3-L1 cells, provide extensive evidence for C/EBP-α function in adipocyte differentiation. Members of the C/EBP family and PPAR-γ are involved in the terminal differentiation by their subsequent transactivation of adipocyte-specific genes. Exposure of confluent preadipocytes to adipogenic cocktail induce expression of C/EBPβ and C/EBPδ, which in turn activate PPAR-γ and C/EBPα [9-10]. Production of appropriate ligand of PPAR-γ by the differentiating preadipocyte is likely a limiting step in this transcription cascade.

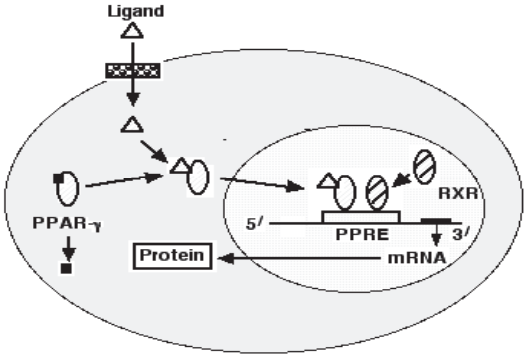

Fig 1-1. Regulation of PPARγ. After entering into the cell by an unknown mechanism, ligands bind to PPARγ, resulting in PPARγ dissociation from a co-repressor (black square) and translocation of the ligand binding PPARγ to the nucleus. The ligand binding PPARγ forms a heterodimer with the nuclear retinoid X receptor (RXR) to recognize PPAR-response elements (PPRE) in the promoter region of target genes and stimulate transcription.

Sterol regulatory element binding proteins (SREBP) is another key transcription factor known to modulate adipocyte differentiation [19]. 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 [20]. Recent studies showed that ADD-1 exerts its adipogenic action by modulating both expression and activation of PPARγ [21]. In addition to C/EBPs, PPAR-γ and ADD1/SREBP-1c, 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 PPARγ. 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 [22,23]. In addition to transcription factors, 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 PPARγ [24,25].

1.5 Adipose tissue as an endocrine organ

Mature adipocytes are the main cellular component of WAT. Adipocytes secret several factors which are known to play a vital role in immunological responses, vascular diseases, appetite regulation etc. 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 migation inhibitory factor [5]. With the exception of TNF-a, their physiological functions remain to be elucidated. 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. TNF-α 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 [26]. 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 [27-29]. 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 [30]. 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 [31].

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.

1.6 Biosynthesis of Prostaglandin by adipocytes

Prostaglandins (PGs) and related compounds are called "local hormones" that are synthesized from the polyunsaturated fatty acid, arachidonate and provide specific effects on target cells close to their site of formation. Almost all the mammalian cells except red blood cells produce PGs and their related compounds such as prostacyclins, thromboxane, leukotrienes etc. that are collectively known as eicosanoids.

Biosynthesis of PGs through the cyclooxygenase (COX) pathway involves 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 A2 that catalyzes the hydrolysis of fatty acids 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. There are two isoforms of cyclooxygenase. 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 [32]. On the other hand, COX-2 is expressed inducibly and is utilized for delayed PG biosynthesis, which last for several hours after proinflammatory stimuli [33]. Expression of COX-1 increases in cell lines that undergo differentiation and thus mimic developmental process. Where as, elevated level of COX-2 expression is responsible for inflammatory mediators such as IL-1β, TNFα, lipopolysaccaride but glucocorticoids and anti-inflammatory cytokines suppress COX-2 expression. Selective inhibitors of COX-2 have confirmed that this enzyme plays a crucial role in inflammation, pain and fever [34,35]. However, the precise molecular mechanisms underlying the functional segregation between the two COXs are still obscure.

The final step for the biosynthesis of PG is the conversion of PGH_2 to various bioactive derivatives such as PGE_2, $PGF_{2\alpha}$, PGD_2 etc. by terminal synthase enzymes PGES, PGFS and PGDS respectively. The J_2 series of PGs (PGJ_2, Δ^{12} -PGJ_2 and 15-d-PGJ_2) are derived non-enzymatically from PGD_2, through dehydration and isomerisation [36]. Different PGs are produced in adipocytes at different stages of the life cycles under the stimulation of metabolic and external signaling factors and exert biological effects in a paracrine or autocrine manner. Among the PGs, some such as PGE_2, $PGF_2\alpha$, PGD_2 etc. act via plasma membrane derived G-protein coupled receptors. Depending on the

cell type, the activated G protein may stimulate or inhibit formation of cAMP, or may activate a phosphatidylinositol signal pathway leading to intracellular Ca^{2+} release. Instead, PGJ_2 derivatives act via nuclear receptor PPARγ, a transcriptional regulator of adipocytes differentiation [37,38].

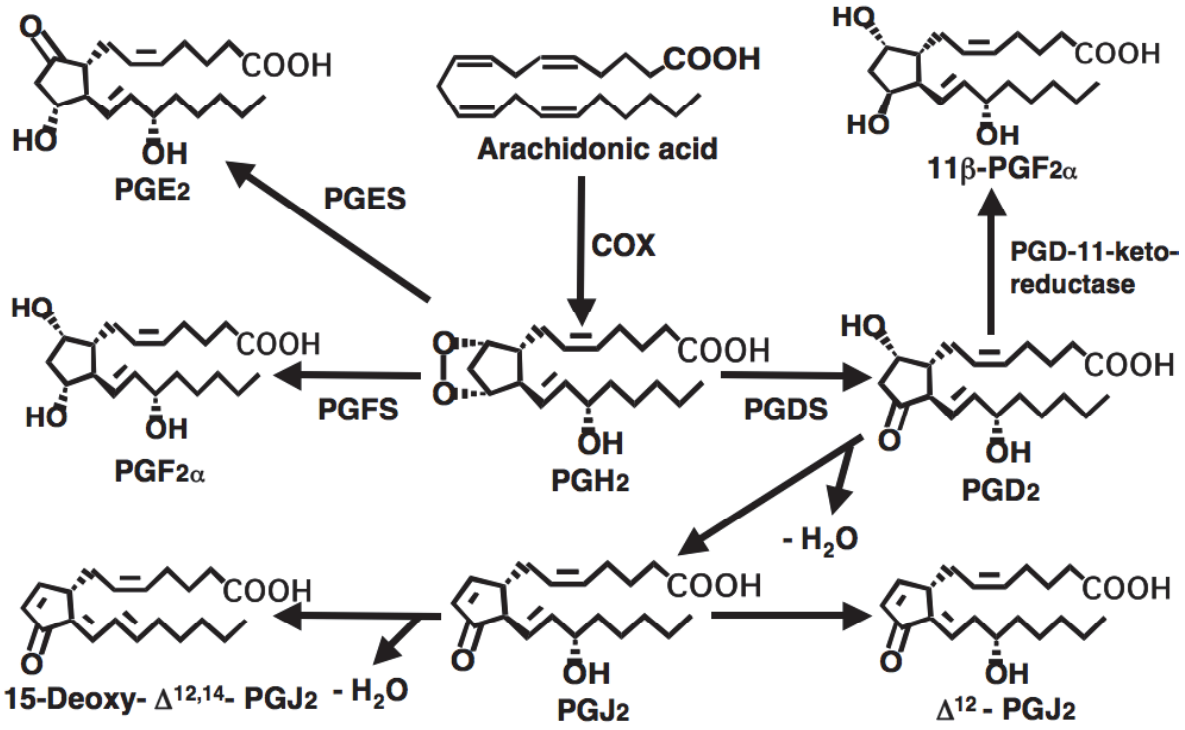

Fig. 1-2. Biosynthesis of prostaglandins by arachidonate cyclooxygenase pathway.

1.7 Biological and pathophysiological role of Prostaglandins

Adipocytes and precursor cells can synthesize large amount of PGs at different stages of their life cycle under the stimulation of metabolic and external signaling factors. These PGs play important roles in physilogocal and pathophysiological events. They exert a wide range of actions in the body, which are mainly mediated thorough plasma membrane-derived G-protein coupled receptors (GPCRs). They have been shown to affect the level of second messengers through interaction with GPCRs with seven trans-membrane domains [38]. The receptors include DP, EP, FP, and IP that preferentially respond to PGD_2, PGE_2, PGF_{2a} ad PGI_2, respectively. Furthermore, EP is subdivided into four subtypes, EP1, EP2, EP3, and EP4 [39]. In addition to classical prostanoids that act via plasma membrane-derived GPCRs, several COX products such as PGJ_2, 15d-PGJ_2 and PGA_2 can activate nuclear receptors of PPAR family [29].

PGD_2 has marked effect on a number of biological process including sleep induction, platelet aggregation, relaxation of vascular and nonvascular smooth muscle and nerve cell function [40]. It rapidly undergoes dehydration *in vivo* and *in vitro* to yield additional biologically active PGs of J_2

series [41]. Members of J_2 series have been reported to have their own unique spectrum of biological effects, including the effect of cell cycle progression, the super expression of viral replication, the induction of heat shock protein expression, and the stimulation of osteogenesis [42]. Recent studies show that in addition to stimulating the PPARγ receptors, PGJ_2 derivatives also inhibit the IκB kinase (IKK) activity and thereby block nuclear factor kB (NFkB) transcription factor pathway [43]. PGE_2 activity influences inflammation, fertility and perturation, gastric mucosal integrity and immune modulation, fever generation and nociception [44]. 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, PGI2 produced by the COX pathways is important in normal control of vascular homeostasis and thrombosis [44].

1.8 Prostaglandins modulate adipocyte differentiation

Studies revealed that arachidonate metabolites may play an important role in adipose tissue metabolism and development. Mature adipocytes and preadipocytes produce significant amount of prostaglandins, including PGE_2, $PGF_{2\alpha}$, PGD_2 and PGI_2 [45]. Prostaglandin E_2 is a strong antilipolytic compound and PGF_{2a} and PGJ_2 have been shown to modulate preadipocyte differentiation. Prostaglandin D_2 and 15-deoxy-J_2 derivatives may be analogous ligands of PPARγ and thereby act as adipogenic signals. Prostaglandin $F_{2\alpha}$ 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 [37]. It also inhibits differentiation of preadipocytes by stimulating synthesis of transforming growth factor alpha (TGFα) [46]. 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 [47].

In contrast to PGE_2 and $PGF_2\alpha$, prostacyclin is a potent adipogenic compound. It has been reported to be an activator of PPARs and to be most effective activator for PPARγ [48]. The role of PGD_2 is not clear. Most of the biological effects of PGD_2 is mediated through PGD_2 receptors. It is reported that PGD_2 is non-enzymatically degraded to PGJ_2 series, among them 15d-PGJ_2 is a natural

ligand for PPARγ through which, 15d-PGJ_2 causes adipogenesis [49].

1.9 Role of antioxidants for health

Free radicals (produced by the body due to normal metabolism, radiation exposure and environmental pollutants) are highly reactive, can damage cellular components and are implicated in a variety of diseases. Free radicals are normally neutralized by efficient systems in the body that include the antioxidant enzymes (superoxide dismutase, catalase, and glutathione peroxidase) and the nutrient-derived antioxidant small molecules (vitamin E, vitamin C, carotenes, flavonoids, glutathione, uric acid, and taurine) [50]. In healthy individuals, a delicate balance exists between free radicals and antioxidants. In some pathologic conditions such as diabetes, and in critically ill patients, oxidative stress causes the level of antioxidants to fall below normal. Antioxidant supplements for such conditions are expected to be of benefit. As a preventive measure against certain diseases, the best approach for healthy individuals is to regularly consume adequate amounts of antioxidant-rich foods, e.g., fruits and vegetables.

1.10 Role of trace elements for health

Trace elements are minerals present in living tissues in small amounts and function primarily as catalysts in enzyme systems; some metallic ions, such as iron and copper, participate in oxidation-reduction reactions in energy metabolism. Iron, as a constituent of hemoglobin and myoglobin, also plays a vital role in the transport of oxygen. In animal studies, iron-deficient rats given 1,2-dimethylhydrazine developed neoplastic liver lesions within 4 months, compared to 6 months in an iron-sufficient group [51]. The authors noted that severe lack of iron appeared to promote carcinogenesis. Also zinc, a constituent of more than 200 enzymes, plays an important role in nucleic acid metabolism, cell replication, tissue repair, and growth through its function in nucleic acid polymerases. Zinc has many recognized and biologically important interactions with hormones and plays a role in production, storage, and secretion of individual hormones. Severe, moderate, and marginal zinc deficiencies have been reported in the United States [52]. The richest sources of zinc are shellfish (oysters), beef, and other red meats. Poultry, eggs, hard cheeses, milk, yogurt, legumes, nuts, and whole-grain cereals are also good sources.

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Chapter 2

Patho-physiological status of serum antioxidant, macro-minerals and trace elements in patients with metabolic syndrome in Bangladesh

1. Introduction

Metabolic syndrome is a bunch of several hazardous heart attack risk factors: diabetes and raised fasting glucose in plasma, high cholesterol, abdominal obesity and hypertension [1-4]. It is found 20-25 percent of the world's adult population is still suffering by metabolic syndrome with three times greater risk of developing a heart attack compared to people without the syndrome. In addition, these patients are five time's prone to risk of developing type-2 diabetes while 230 million of people already world widely live with diabetes [5, 6], so it is become one of the most ubiquitous chronic diseases globally and take fourth or fifth place of narrative disorder leading to death in the developed country. That is why metabolic syndrome is become considering the engine of a new epidemic of cardiovascular disease because this pathological condition clustering cardiovascular disease (CVD) risk factors that intensified abnormal heart condition. In Pacific and Middle East nations it is confirmed as many as one in four deaths in adults (35-64 years) is susceptible to this disease with greater risk of occurring type 2-diabetes. Type 2 diabetes encounter 90 percent of all diabetes and become one of the leading causes of illness, premature death and cardiovascular disease, which in turn responsible for up to 80 percent of these deaths globally [7, 8].

Most of the people with impaired glucose tolerance or type 2 diabetes status showed multiple set of risk factors that often occur together what is now literally figure out under the name of "metabolic syndrome". Mostly in same individual "clustering" of metabolic abnormalities occur and precipitated to confer substantial additional cardiovascular risk with the clustering of the risk consociated with each abnormality [9, 10]. However, it is found that when a person diagnosed with diabetes, hyperglycemia and related changes in blood lipids (increased triglycerides and decreased HDL- C) showed high blood glucose levels and are high enough for increase the risk of cardiovascular disease [9]. The more components of metabolic syndrome that are evident, the higher is the rate of cardiovascular mortality [11]. Cardiovascular complications of diabetes, which is also the cardinal cause of blindness, amputation and kidney failure, account for much of the social and economic burden of the disease [12]. The prediction, "the incidence of diabetes will double by 2025" indicates a parallel increase in cardiovascular disease and death associated with an inevitable and profound impact on health systems worldwide. It was calculated that in 2003 for the 25 countries of the European Union, the overall medical costs of all diabetes in 20 and 79 years old was up to 64.9 billion Dollars

(ID), equivalent to 7.2 percent of total health expense in these countries [7, 13].

In recent years, there has been a notable increase in the number of people with the metabolic syndrome. It is become an epidemic disease and acknowledge globally with other life style disease such as type-2 diabetes and cardiovascular disease. It is with an increasing prevalence and leading cause of death in adults and children world widely and considered as the one of the most health squeezing disease for 21st century. Not much work has been done to find out the possible reasons for the development of metabolic syndrome, which is counted to be very significant for the treatment of this syndrome. Thus an attempt has been made to examine serum antioxidant levels (vitamin C); macro minerals (Ca, Na, K) and trace element concentrations (Zn, Fe) s, and to clinch the correlation between the serum levels of these components of this metabolic patient.

2. Methods and materials

2.1 Materials and chemicals

This study was conducted with some well-organized instrumental setup, which was HPLC machine, flame atomic absorption spectroscopy, pH meter, ultrapure water system, and some other auxiliary instrument to facilitate this whole experiment. All of these instruments were run and handled by following the SOP provided by respective manufacturer.

Chemicals were used for the determination of serum antioxidant, trace elements and macromolecules level was maintained in standard grade and purchased mostly from Merck, Germany. For antioxidant determination metaphosphoric acid, N-(1-napthyl) ethylene diamine dihydrochloride was purchased from Loba Chemie, India, while trichloroacetic acid (TCA) and copper sulphate was purchased from Guangdong & Uni-chem laboratory of China respectively. Standards for macromolecule and trace element determination were sourced from Buck Scientific, USA.

2.2 Study design

This case-control study was carried out in Laxmipur Diabetic Society, Noakhali, Bangladesh. Ethical permission was taken from ethical committee of the respective hospital. For the study purpose, 100 patients with metabolic syndrome were recruited as cases and for comparison 65 healthy volunteers were selected as control subjects.

2.3 Data collection

Detailed patient history was taken with a well-designed questionnaire by regularly attending to Laxmipur Diabetic Society. Following data's were collected from patients with metabolic syndrome

and healthy volunteers: age, sex, blood pressure, plasma glucose level, serum triglyceride level (TG), serum high-density lipoprotein (HDL) cholesterol level and body mass index (BMI).

2.4 Blood sample collection and processing

5 ml venous blood samples was drawn from each patient and control in a metal-free sterile tube. The blood sample was kept at room temperature for about 30 minutes to clot and centrifuged at 3000 rpm for 15 minutes to extract the serum. Then the serum was taken in eppendorf tube and was stored at -80°C until the study day. These samples were then used for determining the serum level of antioxidant (vitamin C), macro-minerals (Na, K, Ca) and trace elements (Zn, Fe).

2.5 Determination of serum antioxidant (vitamin C) level

Serum Vitamin C was estimated by phenyl-hydrazine spectrophotometry method [14]. Absorbance of sample and standard were read against reagent blank at 520 nm in the spectrophotometer (UV-1800, Shimadzu Corporation, Japan). The concentration of ascorbic acid in the serum was calculated by using the formula used by M. S. Sarwar *et al*. [15].

2.6 Determination of serum macro-minerals and trace elements

By using Czuprynetal method, macro-minerals (Na, K, Ca) and trace elements (Fe, Zn) were determined through flame atomic absorption spectrometry (Shimadzu AA 6800) as well as graphite furnace [16]. By using deionized water, samples were diluted by a dilution factor of 10. Different concentrations (0.5, 1.0, 2.0, 5.0, and 10.0 mg/L) of trace elements were used for calibration of standard graphs. For zinc, calcium, iron, sodium and potassium determination, absorbances at 213.9 nm, 422.7 nm, 248.3 nm, 589.0 nm and 766.5 nm were taken respectively. To verify the assay accuracy and to maintain quality, the standard solutions were run for every ten-test sample. A software package (Wizard AA software) was used to calculate the concentration of zinc, calcium, iron, sodium and potassium.

2.7 Statistical analysis

All values were expressed in the form of mean ± SEM (standard error mean). Statistical analysis was calculated by using the statistical software package named SPSS, version 16.0 (SPSS Inc., Chicago, IL). Independent sample t-test was undertaken to determine the level of significance of various parameters between studied and control groups. Finally, correlation among the study parameters was done by using Pearson's correlation analysis.

3. Results

3.1 Socio-demographic profile of the study population

This study comprised of 100 patients with metabolic syndrome as cases and 65 normal healthy adults as controls. All data are expressed as mean ± SEM. Socio-demographic profile of the patients and controls are represented in Table 3-1. It was observed that mean age of the patients with metabolic syndrome and controls were 53.43 ± 1.28 and 54.75 ± 1.63 years respectively and difference between age of this two group was not found statistically significant (p = 0.522). In this study, it was observed that women with metabolic syndrome had higher prevalence than men. The relative percentages of women and men with metabolic syndrome were 54% and 46% respectively.

Table 2-1. Socio-demographic profile of the study population

Variables	Patient group	Control group	p value
	Value ± SEM		
Age (years)	53.43 ± 1.28	54.75 ± 1.63	0.522^{NS}
	Value, n (%)		
Sex			
Male	46 (46%)	38 (58.46%)	
Female	54 (54%)	27 (41.54%)	

NS = Not significant

3.2 Anthropometric, clinical and biochemical evaluations of the study population

Anthropometric, clinical and biochemical characteristics of the patients and controls are represented in Table 2-2. Statistical analysis of below parameters showed that the patients (38.64 ± 0.45 kg/m^2) with metabolic syndrome had significantly ($p<0.05$) higher level of BMI in comparison to control subjects (21.67 ± 0.26 kg/m^2). The patients had significantly higher level of blood glucose (14.09 ± 0.46 mmol/L) in comparison to control subjects with a mean value of 6.46 ± 0.09 mmol/L ($p<0.05$). Mean value of triglyceride level of the patients and controls were found to be 312.28 ± 17.50 and 161.63 ± 5.04 mg/dl respectively, where patients with metabolic syndrome had significantly higher level than control subjects ($p<0.05$). Finally the patients group showed significantly lower level of HDL (44.35 ± 0.72 mg/dl) when compared to control group having mean value of HDL as 54.09 ± 0.86 mg/dl ($p<0.05$).

Table 2-2. Anthropometric, clinical and biochemical characteristics of the study population

Parameters	Values (Mean ± SEM)		
	Patient group	Control group	p value
BMI (kg/m^2)	38.64 ± 0.45	21.67 ± 0.26	0.000^{**}
Blood Glucose level (mmol/L)	14.09 ± 0.46	6.46 ± 0.09	0.000^{**}
Triglyceride (mg/dl)	312.28 ± 17.50	161.63 ± 5.04	0.004^{**}
HDL (mg/dl)	44.35 ± 0.72	54.09 ± 0.86	0.006^{**}

**p < 0.05 (Significant difference between patient and control groups at 95% confidence interval)

3.3 Antioxidant status (vitamin C)

The patients and controls serum level of vitamin C are represented in Table 2-3. Serum level of vitamin C in patient and control groups was found to be 13.26 ± 0.45 and 16.47 ± 0.98 µmol/L respectively. Statistical analysis reveals that the patients showed significantly lower serum concentration of vitamin C in comparison to control subjects ($p<0.05$).

Table 2-3. Serum level of vitamin C in the study population

Parameter	Values (Mean ± SEM)		
	Patient group	Control group	p value
Vitamin C (µmol/L)	13.26 ± 0.45	16.47 ± 0.98	0.001**

**P < 0.05 (Significant difference between patient and control groups at 95% confidence interval)

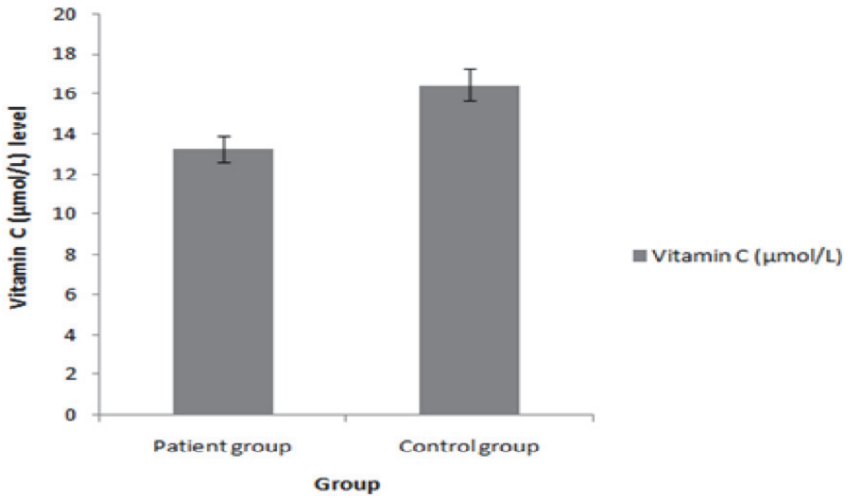

Fig. 2-1. Serum level of vitamin C in patient and control groups

3.4 Macro minerals status (Ca, K, Na)

Serum level of macro minerals (Ca, K, Na) in patients and control groups are represented in Table 2-4. Statistical analysis reveals that the patients had significantly ($p<0.05$) lower level of Ca with a mean value of 45.39 ± 2.19 mg/L in comparison to control subjects (86.24 ± 2.49 mg/L). The mean value of K level was found to be 61.23 ± 3.03 and 167.41 ± 2.80 mg/L in patient and control groups respectively. Similar to K, lower level of Na was also found in patients with metabolic disorder while control subjects had higher serum level for these two macro-minerals (K and Na) and they were found to be statistically significant ($p<0.05$). But in patient group alone the serum level of Na was found to be higher in value when compared to other two macro-minerals (Ca and K).

Table 2-4. Serum level of Ca, K and Na in the study population

Parameters	Values (Mean ± SEM)		
	Patient group	Control group	p value
Ca (mg/L)	45.39 ± 2.19	86.24 ± 2.49	0.000**
K (mg/L)	61.23 ± 3.03	167.41 ± 2.80	0.000**
Na (mg/L)	2821.2 ± 75.78	3210.8 ± 30.66	0.000**

**P < 0.05 (Significant difference between patient and control groups at 95% confidence interval)

3.5 Trace elements status (Zn, Fe)

Serum level of trace elements (Zn, Fe) in both the patient group and control group are represented in Table 2-5. Mean value of Zn and Fe levels in patient were 0.30 ± 0.01 and 0.36 ± 0.01 mg/L respectively and their levels were found to be significantly lower than that in control groups having Zn and Fe levels of 0.79 ± 0.03 and 0.78 ± 0.03 mg/L respectively. Among the groups, the levels of Zn and Fe were shown to be of more prominent value in controls than in the patients.

Table 2-5. Serum level of Zn and Fe in the study population

Parameters	Values (Mean ± SEM)		
	Patient group	Control group	p value
Zn (mg/L)	0.30 ± 0.01	0.79 ± 0.03	0.000**
Fe (mg/L)	0.36 ± 0.01	0.78 ± 0.03	0.000**

**P < 0.05 (Significant difference between patient and control groups at 95% confidence interval).

3.6 Correlation of age and BMI with serum vitamin C, macro minerals and trace elements in the patient and control groups

Pearson's correlation analysis was carried out to determine the relationship between different variables (Table 2-6). There was found an inverse relationship while age of patient and control groups were compared with HDL and BMI. A positive relationship was noticed for both of these groups when age was compared with Vitamin C, Zn, Fe and Na. Triglyceride level of patient group showed a negative correlation with age unlike the control group. Control group depicted a significant negative correlation of age with Ca, as well as a negative correlation of age for this control group with blood glucose level that was not statistically significant.

Table 2-6. Correlation of age and BMI with serum vitamin C, macro minerals and trace elements in the patient and control groups

Correlation Parameters	Patient group		Control group	
	R	P	R	P
Age and BMI	-0.049	0.629	-0.020	0.876
Age and Blood glucose	0.000	0.999	-0.237	0.058
Age and Triglyceride	-0.088	0.381	0.041	0.748
Age and HDL	-0.205	0.808	-0.085	0.500
Age and Vitamin C	0.123	0.224	0.054	0.671
Age and Zn	0.145	0.150	0.102	0.418
Age and Fe	0.078	0.441	0.090	0.474
Age and Ca	0.041	0.688	-0.264	0.034**
Age and K	0.027	0.787	-0.191	0.128
Age and Na	0.087	0.392	0.041	0.743
BMI and Blood glucose	-0.159	0.114	0.038	0.766
BMI and Triglyceride	0.087	0.391	-0.015	0.904
BMI and HDL	-0.073	0.472	0.191	0.127
BMI and Zn	0.028	0.781	0.045	0.724
BMI and Fe	0.016	0.872	0.242	0.052
BMI and Ca	-0.066	0.513	0.014	0.914
BMI and K	0.113	0.264	0.023	0.854
BMI and Na	0.134	0.183	-0.084	0.504
BMI and Vitamin C	0.050	0.623	-0.154	0.248

r = Correlation co-efficient; p = Significance; Values with negative sign indicate an inverse correlation;

**Correlation is significant at 0.05 level (two-tailed).

Relationship of BMI with serum vitamin C, macro minerals and trace elements in the patient and control groups showed totally different features. In this case, BMI for both groups depict positive correlation with Zn, Fe and K. Here, we also found a negative correlation of BMI with blood glucose, HDL and Ca level in the patient group while that of the control group showed a positive correlation of BMI with serum blood glucose, HDL and Ca levels. On the other hand, a negative correlation of BMI of the control group only with Na, serum vitamin C and triglyceride levels was shown. However, the patient group showed a positive BMI and triglyceride correlation ship. None of these data was found to be statistically significant.

3.7 Correlation of blood Glucose level and triglyceride with serum vitamin C, macro minerals and trace elements in the patient and control groups

Pearson's correlation data of blood glucose level and triglyceride with serum vitamin C, macro minerals and trace elements in the patient and control groups are shown in the Table 2-7. In the patient group, blood glucose level showed a negative correlation with vitamin C, Zn, Fe, K and Na, but depict a positive correlation ship of blood glucose level only with HDL and Ca levels. Contrary to

this, blood glucose level of the control group showed a negative correlation with HDL, vitamin C, Zn, and Fe (except Ca, Na, K) where only the negative correlation of blood glucose level with the trace element Fe was found to be statistically significant. Interestingly, the result of correlation study of blood triglyceride level with other parameters was different from the previous case when compared with both the patient and control groups. A negative correlation was found in the patient group when blood serum triglyceride level was compared with serum macro minerals of Ca, K and Na as well as trace element Zn. However, patient group showed a positive correlation of blood serum triglyceride level with HDL, vitamin C and trace element Fe levels. The data was found to be different in the case of the control group where only the serum Ca, K and Na levels showed a positive correlation with blood serum triglyceride level, whereas all other parameters in control group showed a negative correlation of blood serum triglyceride level and only a statistical significant negative correlation between triglyceride and Zn level alone.

Table 2-7. Correlation of blood glucose level and triglyceride with serum vitamin C, macro minerals and trace elements in the patient and control groups

Correlation Parameters	Patient group		Control group	
	R	P	R	P
Blood Glucose and Triglyceride	0.119	0.240	0.199	0.112
Blood Glucose and HDL	0.068	0.505	-0.021	0.867
Blood Glucose and Vitamin C	-0.094	0.354	-0.145	0.248
Blood Glucose and Zn	-0.143	0.155	-0.179	0.153
Blood Glucose and Fe	-0.156	0.122	-0.245	0.049**
Blood Glucose and Ca	0.102	0.310	0.226	0.070
Blood Glucose and K	-0.015	0.885	0.181	0.149
Blood Glucose and Na	-0.021	-0.837	0.000	0.998
Triglyceride and HDL	0.016	0.871	-0.043	0.733
Triglyceride and Vitamin C	0.039	0.698	-0.085	0.502
Triglyceride and Zn	-0.045	0.658	-0.280	0.024**
Triglyceride and Fe	0.036	0.720	-0.134	0.289
Triglyceride and Ca	-0.003	0.976	0.009	0.940
Triglyceride and K	-0.023	0.822	0.123	0.330
Triglyceride and Na	-0.021	0.837	0.087	0.488

r = Correlation co-efficient; p = Significance; Values with negative sign indicate an inverse correlation;

**Correlation is significant at 0.05 level (two-tailed)

3.8 Inter-element-correlations between macro minerals and trace elements

The present study also depicts inter-element-correlations for the analyzed elements (macro minerals and trace elements) between the patient and control subjects, which exhibited either a positive (direct) or negative (inverse) correlation for selected element. The correlation coefficient and the statistical confidence levels at which the correlations were determined are presented in Table 2-8.

Here, Zn ion showed a positive inter-elemental correlation with other macro minerals like Ca and Na and trace element Fe in the patients group, whereas a positive correlation of Zn ion only with Fe in the control group. However the patient group showed a negative inter-elemental correlation of Zn ion with K ion and similarly the control group also depicts a negative correlation of Zn ion but with Ca, K and Na ions. While in the case for both the patient and control groups, Ca ion showed a negative correlation with both K and Na ions (except for Fe as positive correlation with Ca ion for patient). On the other hand, Fe showed a positive and negative correlation with Na and K ions respectively in patients, while correlation of K with Na ion itself depicts a negative correlation ship for both the patient and control groups, but none of these data was found to be statistically significant.

Table 2-8. Inter-element-correlations between macro minerals and trace elements

Correlation Parameters	Patient group		Control group	
	R	P	R	P
Zn and Fe	0.024	0.813	0.101	0.425
Zn and Ca	0.056	0.582	-0.036	0.778
Zn and K	-0.094	0.354	-0.026	0.835
Zn and Na	0.156	0.122	-0.076	0.547
Ca and Fe	0.030	0.769	-0.024	0.849
Ca and K	-0.057	0.573	-0.070	0.580
Ca and Na	-0.010	0.924	-0.035	0.784
Fe and K	-0.003	0.975	0.007	0.958
Fe and Na	0.085	0.403	-0.190	0.130
K and Na	-0.052	0.608	-0.082	0.517

r = Correlation co-efficient; p = Significance; Values with negative sign indicate an inverse correlation.

4. Discussion

Conceptualized as a grouping of several metabolic and cardiovascular conditions [17], metabolic syndrome can include all of the following such as high blood pressure, abdominal obesity, hyperglycemia, fasting TG and low HDL-cholesterol (HDL-C) as two types of dyslipidemia [18]. Other conditions for metabolic syndrome include elevated level of C-reactive protein, high homeostatic model assessment insulin resistance (HOMA-IR), high hemostasis and hyperuricemia. Metabolic syndrome has found a stationary association with escalated risk of type 2 diabetes, cardiovascular disease and these two-disease state is responsible for mortality [19-24].

Present study was conducted to understand the role and status of antioxidants, minerals, macro and trace elements in metabolic patients, by comparing the serum level of vitamin C, calcium (Ca), potassium (K), sodium (Na), Zinc (Zn) and iron (Fe) levels in the normal group and patient. A

recent study found that FRAP (Ferric reducing ability of plasma) levels were significantly found in lower level in the study group compared to controls (p=0.001) [25]. FRAP is a measure of the antioxidant potency, based on ferrous ions reduced by the effect of the reducing power of plasma constituents, produced by low molecular weight antioxidants of a hydrophilic and hydrophobic character particularly vitamins C and E, serum bilirubin and uric acid in serum. Therefore, one can say that FRAP can provide more biologically and clinically apropos information on antioxidant capacity than that provided by individual antioxidants measurements. This depicts the dynamic equilibrium between pro- and antioxidants in plasma [26]. Metabolic patients showed to have depleted levels of antioxidant vitamins [27, 28]. This depletion has been shown to be more in obese patients compared with non-obese patients with metabolic syndrome [28, 29]. Furthermore, the high concentration of serum uric acid observed in metabolic patients, can result in pro-oxidant effects, causing a further depletion in the antioxidant capacity of plasma [30].

Our present finding showed depleted level of vitamin C in metabolic patients than the healthy subjects, which endorses the hypothesis that the oxidation may be a causative factor in the pathogenesis of metabolic syndrome. Antioxidant supplementation may be recommended to scavenge the action of free radicals, which may be useful as secondary therapy to prevent oxidative damage in the tissue of metabolic patients. Handsome number of trace elements (Fe, Zn, Cu, Se, I, Mn, Mb, Cr, Co etc) has been weighed to be important for the nutritional requirement of human. High undersupply of these trace elements catalyze a number of diseases such as breast, colon, lung and prostate cancers, leukemia, multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), arteriosclerosis, osteoporosis, arthritis, diabetes, edema, Alzheimer's disease, Attention Deficit Disorder (ADD), bi-polar, influenza, heart and cardiovascular diseases, many allergies and birth defects [30, 31].

Trace elements are acting as a substrate molecule for enzymes, which attract and facilitate conversion of particular component to specific end products. Some donate or accept electrons in the reduction and oxidation reactions resulting in the generation and use of metabolic energy. Trace elements are usually absorbed in the gastrointestinal tract freely. Homeostasis of trace elements are regulated by excretion through bile, sweat, breathing and most frequently through urine, storage of trace element in inactive sites or inactive forms is another mechanism that prevents inappropriate quantities of reactive trace elements to be present, for example, storing as ferritin iron.

On the other hand, releasing a trace element from a storage site may also be important in the prevention of deficiency. The interest in clinical and biochemical consequence of the metabolism of

trace elements has been constantly increasing. Trace elements when present at low concentrations have shown significant physiological impacts other than those associated with classical toxicity. There is evidence that the chemical modification of several trace elements is altered in diabetes [33, 34]. Zinc is an essential trace element that is required for normal cells processing (cell division and apoptosis). Zn participates in many biochemical pathways such as transcription, translation and cell division [35]. More than 300 enzymes require zinc for their catalytic activities. Moreover, the elimination of Zn from catalytic site leads to loss of enzyme activity [36]. About 70% of Zn is bound to albumin and any pathological change of albumin alters the serum zinc levels [37]. Several disorders such as dermal disorders, gastrointestinal, neurological and immunological disorders are manifested due to mal-absorption of zinc [38].

Recently published studies show that type-2 diabetic (T2D) patients have substandard zinc status in the blood due to increased urinary depletion [38]. As a result, hypozincemia and hyperzincemia develop in diabetics [38, 39]. Zinc plays a key role in the storage and secretion of insulin, which then boosts the uptake of glucose [39, 40]. Decreased plasma zinc level adversely affects the competency of the islet cells to produce and secrete insulin [40, 41]. It is well established that the zinc conveyer (ZnT8) is the main protein for the regulation of insulin secretion from pancreatic β-cells. Recently, a mutation in ZnT8 conveyer has partnered with T2D [42]. Concisely all these evidences show the magnitude of zinc in the sustentation and integration of insulin hexamer and its role in metabolic regulation. Fe is indispensable for proper metabolism, including normal cell function and thyroid activation. Obesity-induced inflammation increases a hormone called hepcidin, which has the damning side effect of altering iron metabolism in our body. Hepcidin is a hormone produced in the liver that regulates iron homeostasis.

One of the ways that hepcidin regulates the iron level is by determining how much iron to let into our body. High levels of hepcidin reduce or prevent iron intake in the diet, regardless of the iron content of the food. A new study shows the serious metabolic consequences of low iron status [43]. This shows that low iron turns on genes in the liver and muscles that elevate fat storage and cause unusual blood sugar elevation, precisely what is wrong with the metabolism leading to metabolic syndrome. Our study explored that metabolic patients have low serum concentration of zinc and Fe than the normal individuals. Statistical analysis reveals that the patients had significantly lower level of Zn and Fe in comparison to control subjects ($p < 0.05$), which supports the hypothesis that depleted level of trace elements, may be a causative factor in the pathogenesis of metabolic syndrome. There was found an inverse relationship between consuming a higher intake of potassium with blood

pressure and cardiovascular disease [44]. In line with this, thiazide-induced hypokalemia and glucose intolerance in thiazide-treated subjects showed a muscular relationship with each other. Another effect is related to the participation of potassium in glucose metabolism, where in the potassium depletion resulting from a low potassium diet impairs insulin secretion, which in turn induces glucose intolerance [45].

Another study revealed that potassium ion is responsible for the regulation or increasing of insulin secretion in humans [46]. It is confirmed that essentially hypertension as well as type-2 diabetes is a common feature of potassium deficiency [47]. Low potassium intake is reported to be significantly associated with increased systolic blood pressure and diastolic blood pressure in hypertensive Japanese patients. Low potassium intake is also associated with the prevalence of metabolic syndrome in Japanese women [48].

Our study explored that metabolic patients have low serum concentration of K than the normal individuals. Statistical analysis reveals that the patients had significantly lower level of K in comparison to control subjects ($p<0.05$) which supports the hypothesis that depleted level of trace elements may be a causative factor in the pathogenesis of metabolic syndrome. So, potassium supplementation may be recommended to reduce the risk of metabolic syndrome.

Potassium supplements were found to reduce salt-induced high blood pressure [49, 50], and to improve salt-induced insulin resistance [51] in patients with hypertension. In parallel with these findings, the DASH (Dietary Approaches to Stop Hypertension) diet has been considered to lower lipid-induced oxidative stress in obese people and to lower blood pressure and fasting glucose in hypertensive patients related to the composition of the diet composed of fruits and vegetables, which are rich sources of potassium [52, 53]. Based on these findings, it can be said that salt restriction with a diet rich in food sources of potassium is the first-line therapy for the treatment of metabolic patients [52]; when combined with physical activity, a further improvement in insulin resistance and salt sensitive hypertension can be gained. A research on a group of Korean women enforced this assumption that a higher intake of dietary potassium was found associated with a lower risk of insulin resistance and metabolic syndrome [54].

Calcium is an all-round intracellular messenger that is used throughout the life cycle of an organism to control various biological processes [55]. Diabetes and cardiovascular diseases are linked by a common defect and this defect could be divalent cation metabolism, which may include Ca [56].

A recent study found that changes in calcium metabolism, even within the physiological range, were associated with hypertension, hyperlipidemia and glucose intolerance [57]. A survey in the population of Newfoundland, Canada indicated that altered calcium homeostasis correlated with impaired fasting serum glucose, insulin resistance, and the function of B cells and interestingly, this relationship remained even after adjustment for 25-OH vitamin D and PTH [58].

A study suggested that serum calcium levels were involved in all metabolic syndrome components except HDL-cholesterol [59]. So when finding and interpreting serum calcium levels, metabolic syndrome should be taken into account. Our study reveals that the patients had significantly lower level of Ca and Na in comparison to control subjects ($p<0.05$), which indicates clear involvement of Ca and Na level in metabolic syndrome.

5. Conclusion

The present study suggests a strong association between the pathogenesis of the metabolic syndrome with the level of depletion of vitamin C, Zn, Fe, Ca, K and Na. Dietary supplementation with antioxidants, macro-minerals and trace elements may drive the treatment of metabolic syndrome and thus reduce its complications. Moreover, changes in lifestyle and therapeutics may reduce adiposity and could provide the benefit of preventing obesity-related morbidity and mortality.

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Chapter 3

Comparison of pro-adipogenic effects between prostaglandin (PG) D_2 and its stable, isosteric analogue, 11-deoxy-11-methylene-PGD_2, during the maturation phase of cultured adipocytes

1. Introduction

The differentiation of preadipocytes into mature adipocytes is regulated by a cascade of transcriptional factors including the CCAAT enhancer-binding proteins (C/EBPs) and peroxisome proliferator-activated receptors (PPARs) [1,2]. Among these nuclear factors, PPARγ, a member of the nuclear hormone superfamily, is abundantly expressed in adipocytes and regarded as a master regulator of adipogenesis [3]. The activation of PPARγ requires active ligands because this nuclear hormone receptor is a ligand-dependent transcription factor. PPARγ can be activated by a variety of lipophilic ligands such as polyunsaturated fatty acids and their metabolites as natural ligands [4]. Although the true endogenous ligands for this receptor have not yet been established with certainty in vivo, one of natural potent activators for PPARγ is 15-deoxy-$\Delta^{12,14}$- prostagladin (PG) J_2 (15d-PGJ_2) [5,6], which is formed by non-enzymatic dehydration of unstable PGD_2 [7,8]. Therefore, PGD_2 and the related PGJ_2 derivatives including 15d-PGJ_2 and Δ^{12}-PGJ_2 serves as pro-adipogenic factors in cultured adipocytes expressing PPARγ [9,10]. Alternatively, PGI_2 is known to be a pro-adipogenic factor, but its action is mediated through the cell-surface membrane receptor, the prostanoid IP receptor [11,12]. By contrast, other prostanoids such as PGE_2 and $PGF_2\alpha$ exhibit anti-adipogenic effects through their specific cell-surface membrane receptors of the EP_4 and FP receptors [13,14]. Thus, different classes of PGs have opposite effects on adipogenesis through their specific nuclear hormone receptors or cell- surface membrane receptors. Like some prostanoids, PGD_2 can exert its biological activities in certain immune cells through two types of G protein-coupled membrane receptors, the DP_1 receptor and the DP_2 receptor [15]. DP_2 is also called the chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) [16,17]. Little is known about the role for these cell-surface membrane receptors for PGD_2 in the control of adipogenesis in adipose tissues.

Mouse preadipogenic cultured 3T3-L1 cells are useful for monitoring adipogenesis stages including the growth, differentiation, and maturation phases [18,19]. The cultured preadipocytes are grown in the differentiation medium until confluence. The growth-arrested cells are generally exposed to a hormonal mixture of insulin, dexamethasone, and 3-isobutyl-1-methylxanthine (IBMX) in the

differentiation medium, which can activate adipogenesis program by inducing expression of some C/EBPs. Following the induction of the differentiation phase, the treated cells start to accumulate fats during the maturation phase of adipocytes expressing PPARγ [20–22]. The arachidonate cyclooxygenase (COX) pathway is responsible for the biosynthesis of PGD_2, which undergoes readily its non-enzymatic dehydration to form PGs of J_2 series [7,8]. Using cultured 3T3-L1 cells, previous studies have shown the enhanced expression of lipocalin-type PGD synthase (L-PGDS) during the maturation phase of adipocytes [23,24]. We have also reported that the cultured adipocytes have the ability to increasingly synthesize endogenous PGJ_2 derivatives including 15d-PGJ_2 and Δ^{12}-PGJ_2 known as the activators of PPARγ [9,10]. As well, exogenous PGD_2 and PGJ_2 derivatives are effective pro-adipogenic factors to rescue the accumulation of fats suppressed in the presence of COX inhibitors during the maturation phase of adipocytes. However, the biological activity of PGD_2 is complicated by its instability in biological fluids and the action through different types of nuclear and cell-surface receptors, such as PPARγ, DP_1 and CRTH2.

Earlier, we were able to successfully prepare a monoclonal antibody specific for PGD_2 using 11-deoxy-11-methylene-PGD_2 (11d-11m-PGD_2), a chemically stable, isosteric analogue of PGD_2, as a hapten mimic to develop a highly sensitive and specific immunological assay for PGD_2 [25]. Hence, we hypothesized that 11d-11m-PGD_2 could be a useful analogue to mimic the biological action of PGD_2 in some biological systems. However, the biological activity of this stable analogue of PGD_2 remains still to be evaluated. An earlier study described that 11d-11m-PGD_2 did not exhibit appreciable DP_1 agonist activity on human platelets [26]. In this study, we attempted to evaluate pro-adipogenic effects of 11d-11m-PGD_2 during the maturation phase of cultured adipocytes and compared with those of natural PGD_2 and the related PGJ_2 derivatives with special reference to possible mediation of nuclear and cell-surface membrane receptors.

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, fatty acid-free bovine serum albumin, recombinant human insulin, and Oil Red O were obtained from Sigma (St. Louis, MO, USA). L-Ascorbic acid phosphate magnesium salt n-hydrate, IBMX and Triglyceride E-Test Kit were supplied by Wako (Osaka, Japan). Fetal bovine serum (FBS) was purchased from MP Biomedicals (Solon, OH, USA). PGD_2, 11d-11m-PGD_2, PGE_2, $PGF_2\alpha$, 15d-PGJ_2, Δ^{12}-PGJ_2, indomethacin, aspirin,

troglitazone, GW9662, BW245C, 15R-15-methyl-PGD_2 (15R-15m-PGD_2), BWA868C and CAY10471 were products of Cayman Chemical (Ann Arbor, MI, USA). M-MLV reverse transcriptase (RT) (Ribonuclease H minus, point mutant) and polymerase chain reaction (PCR) MasterMix were obtained from Promega (Madison, WI, USA). Oligonucleotides used for the PCR amplification were provided by Sigma Genosys Japan (Ishikari, Japan). Petri dishes of Iwaki brand for tissue culture were supplied by Asahi Glass (Tokyo, Japan). All other chemicals used here are of reagent or tissue culture grade.

2.2 Cell culture of 3T3-L1 cells and storage of fats during the maturation phase

The mouse 3T3-L1 preadipogenic cell line (JCRB9014) was obtained from JCRB Cell Bank (Osaka, Japan). The cells were plated at 5×10^4 cells/ml in the growth medium (GM) containing DMEM- HEPES, 10% FBS, 100 units/ml penicillin G, 100 μg/ml streptomycin sulfate, and 200 μM ascorbic acid, and grown until confluence at 37°C under 7% CO_2. Under the standard culture conditions, the confluent monolayer cells were exposed to the differentiation medium (DM) consisting of GM supplemented with 1 μM dexamethasone, 0.5 mM IBMX, and 10 μg/ml insulin for 45 h to induce the differentiation phase as described earlier [14,24,27]. To promote the storage of fats in adipocytes during the maturation phase, the treated cells were cultured furthermore in the maturation medium (MM) containing GM and 5 μg/ml insulin up to a total of 12 days by replacing with the fresh MM every 2 days under the established conditions [9,10,18].

For the examination of the effects of various compounds, including exogenous PGs, inhibitors, agonists, and antagonists, on the storage of fats during the maturation phase, the cultured cells after the differentiation phase were cultured in MM supplemented with the compounds to be tested by replacing with fresh MM having the same compounds every 2 days until the indicated days. The test compounds were dissolved in ethanol as a vehicle and added to MM to allow the volume of ethanol to be 0.2%.

2.3 Quantification of cellular levels of triacylglycerols and proteins

For the determination of cellular levels of fats and proteins, cultured adipocytes were harvested as cell suspensions after incubation at 37 °C with 0.05% trypsin and 0.53 mM EDTA in phosphate-buffered saline without $Ca^{2+}$and Mg^{2+} (PBS (-)) for 5 min. The resulting cell suspensions were divided into two parts after washing with PBS (-). The divided ones were individually applied to the determination of the amounts of cellular triacylglycerols and proteins after homogenizing in 25 mM Tris–HCl buffer (pH 7.4) containing 1 mM EDTA, and 1 N NaOH, respectively. The accumulation of triacylglycerols in adipocytes during the maturation phase was

determined quantitatively using the Triglyceride E-Test Kit by assaying the released amount of glycerol through the action of microbial lipoprotein lipase and the related enzymes according to the procedures of the manufacturer as described previously [14,27–29]. Cellular proteins in cultured adipocytes were quantified using fatty acid-free bovine serum albumin as a standard by the method of Lowry *et al.* [30] after the proteins to be assayed were precipitated with ice-chilled 6% trichloroacetic acid to remove the interfering substances [31]. The data on the storage of fats were normalized to protein content and are expressed as the mg triacylglycerol per mg protein.

2.4 Microscopic and macroscopic observation of cultured adipocytes

The status of cultured adipocytes was observed by phase-contrast microscopy using a Nikon inverted microscope system, Eclipse TE300 (Nikon, Tokyo, Japan), and the micrographs were recorded by Nikon digital camera D-5200 attached to the inverted microscope system. Oil droplets stored in adipocytes were stained with Oil Red O for the observation by differential-interference microscopy and macroscopic views of cultured cells in 35-mm Petri dishes as described previously [24,25]. For the assessment of adipogenic efficiency, phase-contrast micrographs were used for the quantification of number of oil droplets in one cell, proportion of oil droplet area in one cell, and relative cell size by ImageJ 1.52a (Wayne Rasband, NIH, USA).

2.5 Gene expression analysis

Total RNA was extracted at different adipogenic stages of adipocytes by the method of acid guanidium thiocyanate/phenol/chloroform mixture [32]. For the analysis of gene expression of a target gene, total RNA (1 μg) was subjected to the reverse transcription and amplification of each desired DNA fragment by RT-PCR with M-MLV reverse transcriptase (Ribonuclease H minus, point mutant) and 1x PCR Master Mix as reported previously [10, 14, 24, 28, 29,]. For the RT reaction to synthesize single stranded cDNA, oligo-$(dT)_{15}$ and a random 9 mer (Promega) were used as primers. The cDNA fragments for the target genes from mouse were amplified by PCR in a semi-quantitative manner using a combination of 5′- and 3′-primers specific for each of PPARγ, lipoprotein lipase (LPL), glucose transporter 4 (GLUT4) and adiponectin as reported earlier [9,14,28,29].

Oligonucleotides used for detecting mRNA levels of other mouse genes by RT-PCR were 5′-GACAAGTTCCTGGGGCGCTG-3′ as 5′-primer and 5′-CTGCTGTAGAGGGTGGCCAT-3′ as 3′-primer for L-PGDS, 5′-CTATCGCTGT CAGACATCCAC-3′ as 5′-primer and 5′-TGGATGAAAC ACCAGGTGCCT-3′ as 3′-primer for DP_1, 5′-GGCGGTGCTCAACACAAT ACC-3′ as 5′-primer and 5′-CTGTC TTCTACCAGCACGCTT-3′ as 3′- primer for CRTH2, and 5′-GTTTGAGACCTTCAAC

ACCCC-3′ as 5′- primer and 5′-GGAGGAGCAATGATCTTGATC-3′ as 3′-primer for β-actin. The amplified DNA fragments were separated by 1.5% agarose gel electrophoresis and detected by staining with ethidium bromide. For the confirmation of the target genes, the nucleotide sequences of the amplified DNA fragments 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 [14,24,28]. The sizes of amplified cDNA fragments using total RNA from 3T3-L1 cells were as follows: PPARγ, 1032 bp; L-PGDS, 344 bp; GLUT4, 774 bp; LPL, 438 bp; adiponectin, 392 bp; DP_1, 543 bp; CRTH2, 519 bp; β-actin, 630 bp.

Quantitative real-time PCR was carried out to determine the tran- script levels of the PGD_2 receptors, L-PGDS, and adipogenic markers using a SYBR Premix Ex Taq (Tli RNaseH Plus) kit (Takara Bio Inc., Ohtsu, Japan) and analyzed by a Thermal Cycler Dice Real Time System Single instrument (Takara Bio Inc.) according to the manufacturer's recommended procedures using threshold cycle (C_T) and the $\Delta\Delta C_T$ method. Oligonucleotides used here were mouse sequences corresponding to 5′-GAGTCCTATCGCTGTCAGA-3′ as 5′-primer and 5′-CCA GAAGATTGCCCAGAAG-3′ as 3′-primer for DP_1, 5′-GCGCTATCCGACT TGTTAG-3′ as 5′-primer and 5′-GTAGCTTGCAGAAGGTAGTG-3′ as 3′-primer for CRTH2, 5′-CTTCGCTGATGCACTGCCTAT-3′ as 5′-primer and 5′-GGGTCAGCTCTTGTGAATGGA-3′ as 3′-primer for PPARγ, 5′-TCTGAAGGACGAGCTGAA-3′ as 5′-primer and 5′-AATGCACTTATCCGGTTGG-3′ as 3′-primer for L-PGDS, 5′-GGATTCCATCCCACAAGGCA-3′ as 5′-primer and 5′-CCAACACGGCCAAGACATTG-3′ as 3′-primer for GLUT4, 5′-AGCCGCTTATGTGTATCGCT-3′ as 5′-primer and 5′-GAGTC CCGGAATGTTGCAGT-3′ as 3′-primer for adiponectin, 5′-TTGCAGAGA GAGGACTCGGA-3′ as 5′-primer and 5′-GGAGTTGCACCTGTATGCCT-3′ as 3′-primer for LPL, and 5′ -GCGGGCGACGATGCT-3′ as 5′-primer and 5′-TGCCAGATCTTCTCCATGTCG-3′ as 3′-primer for β-actin. The reaction was performed at 95°C for 30 s and followed by 40 cycles of amplification at 95°C for 5 s and 60°C for 30 s. After further reaction at 95°C for 15 s and 60°C for 30 s, the transcript levels of each target gene were determined and normalized to those of β-actin of each sample as a control.

2.6 Other procedures

Data are expressed as the mean ± S.E.M. of three independent experiments. Statistical significance of mean differences was evaluated by the Student's t test. The difference was considered to be significant when $p < 0.05$.

3. Results

3.1 Stimulation of fat storage by PGD_2 and 11d-11m-PGD_2, a chemically stable, isosteric analogue of PGD_2, during the maturation phase of cultured adipocytes

To determine the effect of parent PGD_2 and its stable analogue, 11d-11m-PGD_2, on adipogenesis in adipocytes, cultured 3T3-L1 cells during the maturation phase were treated with increasing concentrations of either compound in the presence of 1 μM indomethacin, a well-known COX inhibitor (Fig. 3-1). As shown in Fig. 3-1A, both compounds effectively stimulated the accumulation of triacylglycerols in a dose-dependent manner. We obviously noticed that 11d-11m-PGD_2 at concentrations from 10 nM to 1 μM exhibited significantly more potent effect than natural PGD_2. The microscopic views of cultured adipocytes before or after staining with Oil Red O showed increased number of oil droplets inside the cells by the supplementation with higher concentrations of both compounds (Fig. 3-1B). It was evident that 11d-11m-PGD_2 had higher ability than PGD_2 to deposit oil droplets during the maturation phase of cultured adipocytes.

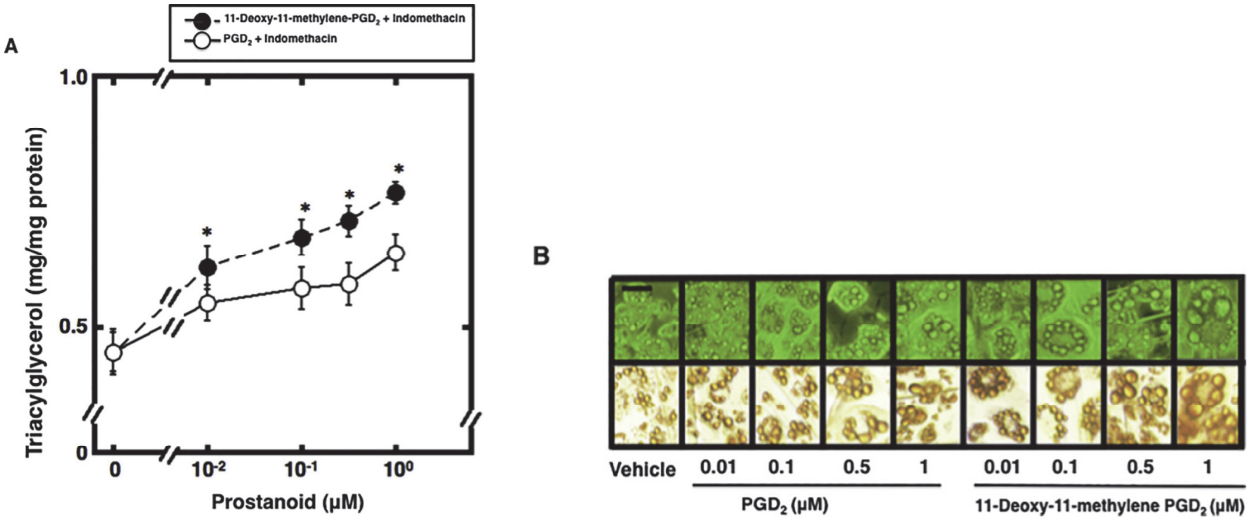


Fig. 3-1. Dose-dependence of fat storage on PGD_2 and 11d-11m-PGD_2 together with indomethacin during the maturation phase of cultured adipocytes. 3T3-L1 cells were plated at 5×10^4 cells/ml in a 35-mm Petri dish containing 2 ml of GM and grown to 100% confluence. The resulting cells were exposed to 2 ml of DM for 45 h to induce the differentiation phase. Then, the cells were further- more cultured for a total of 10 days during the maturation phase by replacing every 2 days with 2 ml of fresh MM containing increasing concentrations of PGD_2 or 11m-11d-PGD_2 in the presence of 1 μM indomethacin. After the treatments, the cultured mature adipocytes were harvested for the determination of the amounts of triacylglycerols and proteins (A). Data represent the mean ± S.E.M. of three independent

experiments. *p < 0.05 compared with the cells treated with PGD_2 at the same concentration. Separately, the cultured mature adipocytes after the treatments were observed by phase-contrast microscopy (upper panels) or the cells after staining with Oil Red O were viewed by differential-interference microscopy (lower panels) (B). Pictures are shown from a representative one performed in three experiments. Scale bar, 50 μm.

We next compared the efficacy of PGD_2 and 11d-11m-PGD_2 with that of other prostanoids to rescue the storage of fats during the maturation phase (Fig. 3-2). The accumulation of fats was appreciably suppressed during the maturation phase in the presence of indomethacin (Fig. 3-2A). The inhibitory effect of indomethacin can be explained by the reduced biosynthesis of endogenous pro-adipogenic prostanoids, such as PGJ_2 derivatives formed by the dehydration of PGD_2 [9] in addition to PGI_2 [27]. The attenuated storage of fats was significantly reversed by the treatment with each of exogenous PGD_2 and 11d-11m-PGD_2 as well as 15d-PGJ_2 and Δ^{12}-PGJ_2 known as pro-adipogenic factors (Fig. 3-2A). In contrast, the decreased storage of fats was not enhanced by the addition of PGE_2 and $PGF_2\alpha$ serving as anti-adipogenic prostanoids [13,14]. The microscopic observation of cultured adipocytes after treatment with either of PGD_2 and 11d-11m-PGD_2 together with indomethacin confirmed the increases in number of oil droplets, oil droplet area, and cell size as compared with indomethacin alone. The result was evident with both micrographs of phase-contrast views (Fig. 3-S1 and Fig. 3-2B) and stained ones with Oil Red O (Fig. 3-2C). The rescuing effects of PGD_2 and 11d-11m-PGD_2 were also confirmed in the presence of aspirin, another COX inhibitor (data not shown).

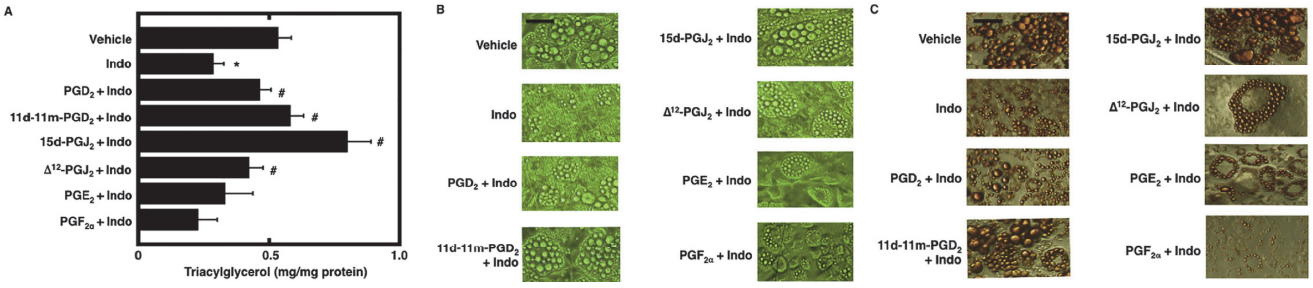


Fig. 3-2. Effect of various prostanoids together with indomethacin on the storage of fats during the maturation phase. 3T3-L1 cells were grown, differentiated, and matured to adipocytes as described in Fig. 3-1. During the maturation phase, the cultured cells were treated for a total of 12 days with either of prostanoids at 1 μM in the presence of 1 μM indomethacin. The resulting cultured adipocytes were harvested for the determination of the amounts of triacylglycerols and proteins (A). Data represent the mean ± S.E.M. of three independent

experiments. *p < 0.05 compared with the cells treated with vehicle. #p < 0.05 compared with the cells treated with indomethacin alone. Alternatively, the cultured cells treated as described above were observed by phase-contrast microscopy without staining (B) or by differential-interference microscopy after staining with Oil Red O (C). Label: Indo, indomethacin. Scale bar, 50 μm.

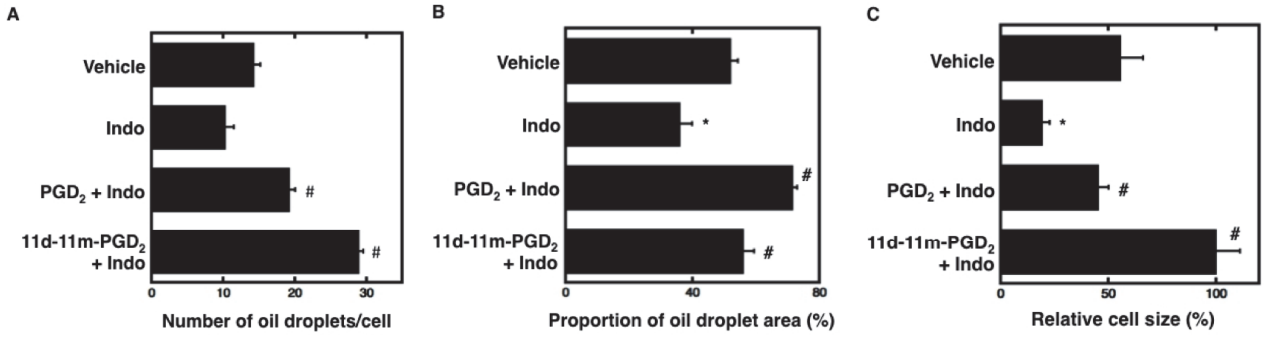


Fig. 3-S1. Assessment of adipogenic efficiency. The data of phase-contrast micrographs in Fig. 3-2B were used for the quantitative assessment of number of oil droplets in one cell (A), proportion of oil droplet area in one cell (B), and relative cell size (C). Data represent the mean ± S.E.M. of three independent experiments. *p<0.05 compared with the cells treated with vehicle. #p<0.05 compared with the cells treated with indomethacin alone.

3.2 Action of PGD_2 and 11d-11m-PGD_2 on fat storage suppressed by a specific PPARγ antagonist

Here, we investigated the contribution of the activated PPARγ to the pro-adipogenic effects of PGD_2 and 11d-11m-PGD_2 during the maturation phase of adipocytes. The treatment of cultured adipocytes with GW9662 [33], a potent antagonist for PPARγ, at 0.1 and 1 μM resulted in a significant suppression of fat storage (Fig. 3-3). The inhibitory effect of lower 0.1 μM GW9662 was appreciably rescued to the level of the cells with vehicle by the co-incubation with each of the PPARγ activators, such as troglitazone, 15d-PGJ_2, and Δ^{12}-PGJ_2 at 1 μM. As well, PGD_2 and 11d-11m-PGD_2 exhibited potent rescuing effects. By contrast, the PPARγ activators at the same concentration of 1 μM failed to fully reverse the attenuating effect of higher 1 μM GW9662 on the accumulation of fats. Nevertheless, PGD_2 and 11d-11m-PGD_2 were found to be more effective to rescue the storage of fats suppressed by the treatment with 1 μM GW9662 than the PPARγ activators. These findings support the idea that other receptors besides PPARγ are appreciably involved in the pro-adipogenic effects of PGD_2 and

11d-11m-PGD_2. In particular, the contribution of activated PPARγ to the promotion of adipogenesis by 11d-11m-PGD_2 appears to be negligible under our culture conditions.

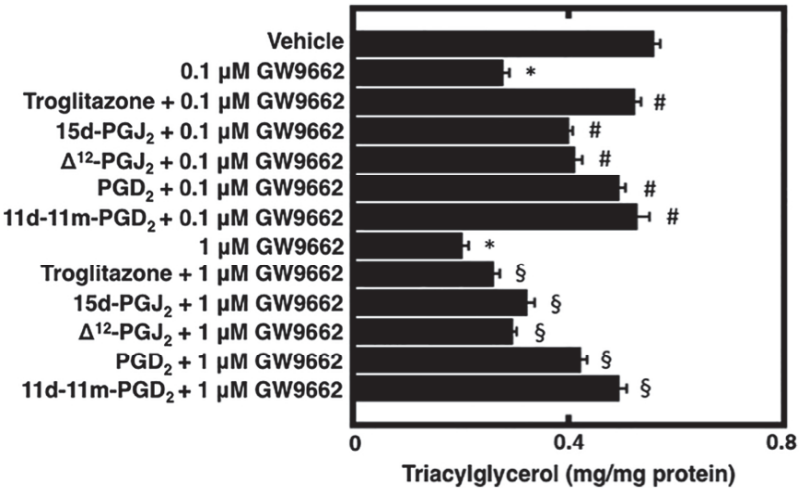


Fig. 3-3. Stimulation of fat storage by PGD_2, 11d-11m-PGD_2, and agonists for PPARγ in the presence of GW9662. 3T3-L1 cells were grown, differentiated, and matured to adipocytes as described in Fig. 3-1. During the maturation phase, the cultured cells were treated for a total of 10 days with either of vehicle, 1 μM troglitazone, 1 μM 15d-PGJ_2, 1 μM Δ^{12}-PGJ_2, 1 μM PGD_2, and 1 μM 11d-11m-PGD_2 in the presence of 0.1 μM or 1 μM GW9662. Data represent the mean ± S.E.M. of three independent experiments. *$p < 0.05$ compared with the cells treated with vehicle. $^{\#}p < 0.05$ compared with the cells treated with 0.1 μM GW9662 alone. $^{\S}p < 0.05$ compared with the cells treated with 1 μM GW9662 alone.

3.3 Gene expression of prostanoid DP receptors and PPARγ during the maturation phase

PGD_2 has been shown to exert its effects through cell-surface membrane prostanoid DP receptors including DP_1 [15] and DP_2 receptors. The prostanoid DP_2 receptor is alternatively called CRTH2 [16,17]. Here, cultured adipocytes during the maturation phase were subjected to the analysis of gene expression of DP_1, CRTH2, and L-PGDS (Fig. 3-4). The transcripts of all the genes were detectable from growth, differentiation, and maturation phases. Moreover, the mRNA levels of DP_1 and CRTH2 were determined by quantitative real-time PCR during the maturation phase and compared with those of L-PGDS and PPARγ (Fig. 3-5). The expression levels of DP_1 (Fig. 3-5A), CRTH2 (Fig. 3-5B), and PPARγ (Fig. 3-5C) tended to increase during the maturation phase and reached to the highest level after 6 days of the maturation phase. Then, their transcript levels declined after 10 days. By contrast, L-PGDS showed a gradual increase in the levels of mRNA up to 10 days (Fig. 3-5D).

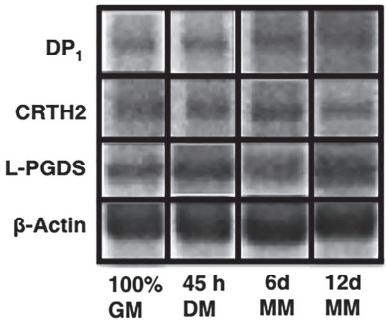


Fig. 3-4. Gene expression of DP receptors, DP_1 and CRTH2, at different adipogenic stages of adipocytes. 3T3 cells were plated at 5×10^4 cells/ml in a 60-mm Petri dish containing 4 ml of GM and grown to 100% confluence during the growth phase. The resulting confluent cells were treated for 45 h in 4 ml of DM during the differentiation phase and furthermore cultured for a total of 12 days by replacing every 2 days with 4 ml of fresh MM during the maturation phase. At the indicated time, the cultured cells were harvested for the extraction of total RNA. The resulting RNA was used for the RT-PCR analysis of mRNA levels of DP_1, CRTH2, L-PGDS, and β-actin (control).

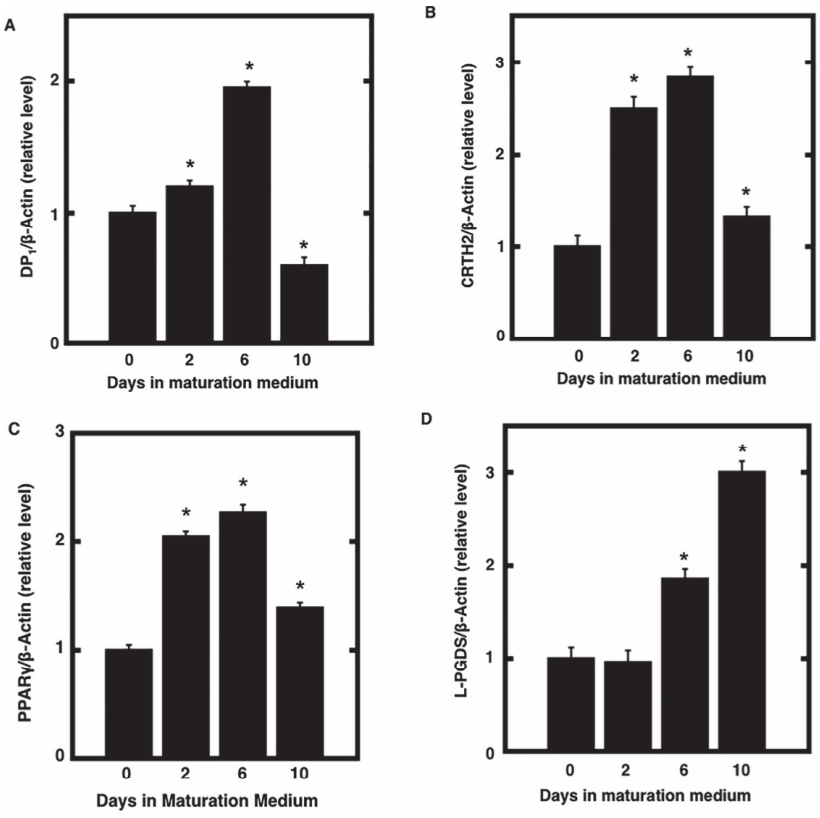


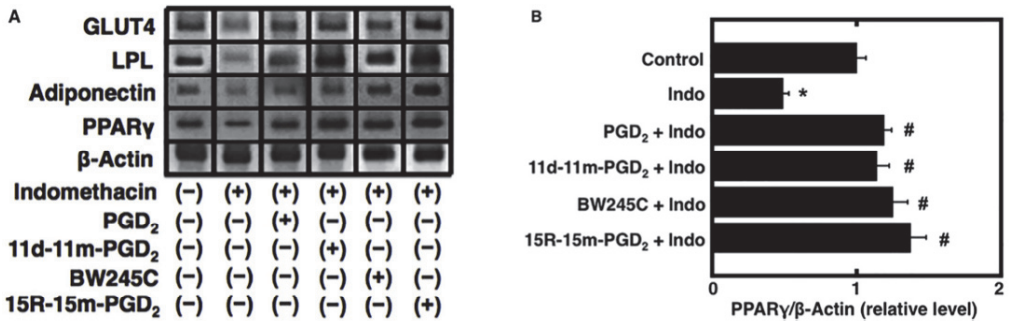
Fig. 3-5. Change in transcript levels of DP_1, CRTH2, PPARγ, and L-PGDS during the maturation phase of adipocytes. 3T3-L1 cells were grown, differentiated, and matured to adipocytes as

described in Fig. 3-4. At the indicated time, the culture cells after the maturation phase were harvested for the extraction of total RNA. The extracted RNA was subjected to quantitative real-time PCR analysis of the transcript levels of DP_1 (A), CRTH2 (B), PPARγ (C), and L-PGDS (D) against those of β-actin (control). Data represent the mean ± S.E.M. of three independent experiments. *$p < 0.05$ compared with the cells on day 0.

3.4 Gene expression of adipogenesis markers and fat storage in response to PGD_2, 11d-11m-PGD_2, and an agonist specific for DP_1 or CRTH2 receptor along with indomethacin

To define whether the enhanced accumulation of fats by PGD_2 and 11d-11m-PGD_2 in the presence of indomethacin could be due to the positive regulation of adipogenesis program, we examined the gene expression of typical adipocyte-specific markers in cultured adipocytes during the maturation phase (Fig. 3-6). The gene expression analysis of adipocyte-specific markers revealed that all of PGD_2, 11d-11m-PGD_2, BW245C [34], a selective agonist for DP_1, and 15R-15m-PGD_2 [35], a specific agonist for CRTH2, at 1 μM were clearly effective to rescue the inhibitory effect of indomethacin (Fig. 3-6A). The quantitative analysis of mRNA levels by real-time PCR led us to confirm significant increases in the expression levels of PPARγ (Fig. 3-6B), adiponectin (Fig. 3-6C), LPL (Fig. 3-6D), and GLUT4 (Fig. 3-6E). These results indicate that the stimulated storage of fats by PGD_2 and its stable analogue, 11d-11m-PGD_2, is brought about by the up-regulation of adipogenesis program during the maturation phase in a similar manner.

We also determined the accumulation of fats in cultured adipocytes during the maturation phase after exposure to an agonist specific for DP_1 or CRTH2 together with indomethacin (Fig. 3-7). Both BW245C and 15R-15m-PGD_2 at concentrations of 0.1 and 1 μM had appreciably stimulatory effects to reverse the action of indomethacin to attenuate the storage of fats. The effects of the DP_1 and CRTH2 agonists were comparable to those of PGD_2 and 11d-11m-PGD_2. The findings implicate the idea that PGD_2 and 11d-11m-PGD_2 can stimulate adipogenesis through DP_1 and CRTH2 receptors.



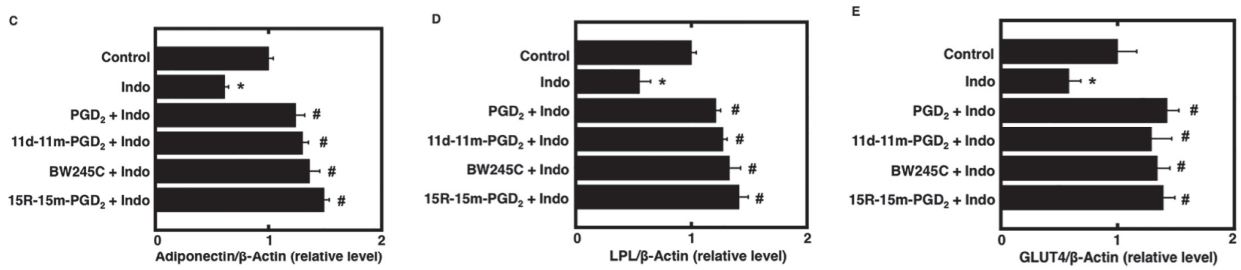


Fig. 3-6. Effect of PGD_2, 11d-11m-PGD_2, and an agonist specific for DP_1 or CRTH2 receptor on the gene expression of adipocyte-specific markers during the maturation phase in the presence of indomethacin. 3T3-L1 cells were grown, differentiated, and matured to adipocytes as described in Fig. 3-4. During the maturation phase, the cells were cultured for a total of 6 days with either of vehicle, 1 μM PGD_2, 1 μM 11d-11m-PGD_2, 1 μM BW245C and 1 μM 15R-15m-PGD_2 in the presence or absence of 1 μM indomethacin. After these treatments, the cultured cells were harvested for the extraction of total RNA. The extracted RNA was used for the RT-PCR analysis of mRNA levels of GLUT4, LPL, adiponectin, leptin, PPARγ, and β-actin (control) (A). Moreover, quantitative real-time PCR was performed to quantify the transcript levels of PPARγ (B), adiponectin (C), LPL (D), and GLUT4 (E) against those of β-actin. Data represent the mean ± S.E.M. of three independent experi- ments. *$p < 0.05$ compared with the cells treated with vehicle. $^{\#}p < 0.05$ compared with the cells treated with indomethacin alone.

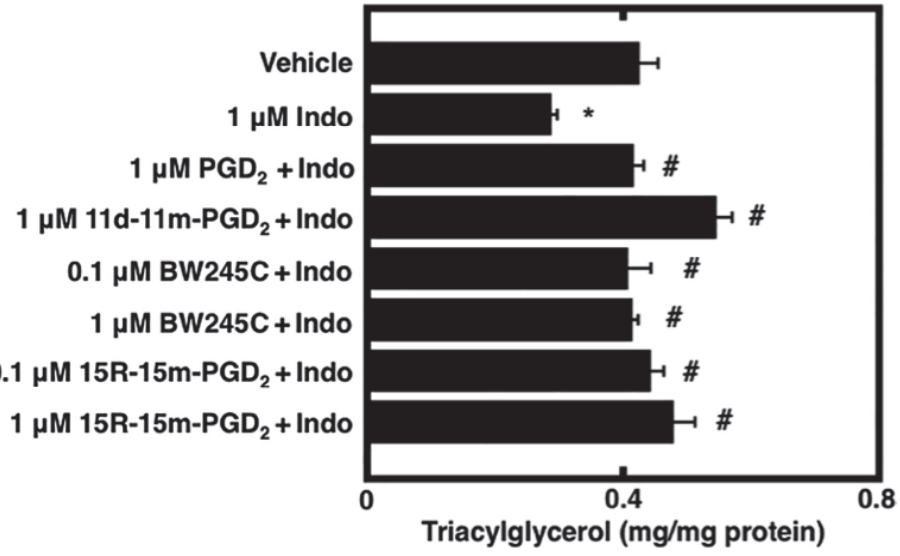
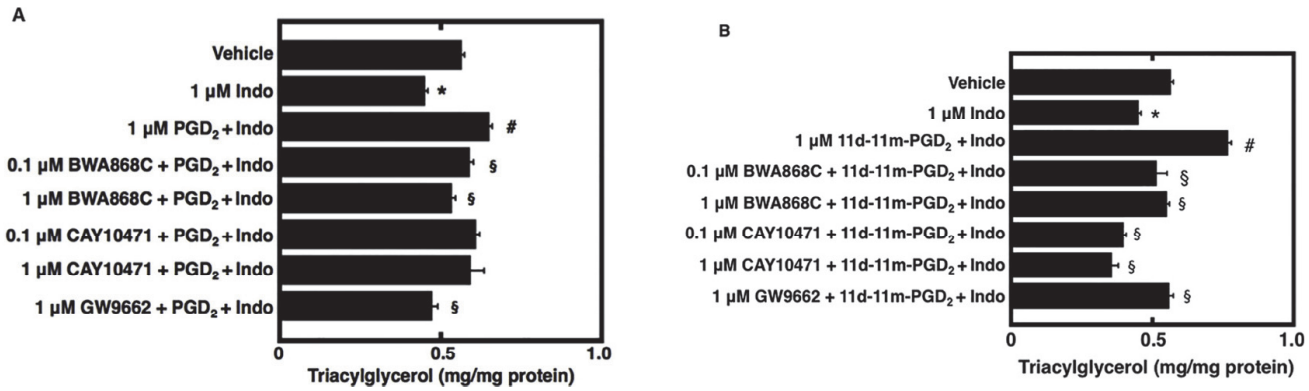


Fig. 3-7. Effect of PGD_2, 11d-11m-PGD_2, and an agonist specific for DP_1 or CRTH2 receptor on the storage of fats during the maturation phase. 3T3-L1 cells were grown, differentiated, and matured to adipocytes as described in Fig. 3-1. During the maturation phase, the cultured

cells were treated for a total of 6 days with either of vehicle, 1 μM PGD_2, 1 μM 11d-11m-PGD_2, 0.1 μM or 1 μM BW245C, and 0.1 μM or 1 μM 15R-15m-PGD_2 in the presence or absence of 1 μM indomethacin. The resulting cultured adipocytes were harvested for the determination of the amounts of triacylglycerols and proteins. Data represent the mean ± S.E.M. of three independent experiments. *$p < 0.05$ compared with the cells treated with vehicle. $^{\#}p < 0.05$ compared with the cells treated with indomethacin alone.

3.5 Effect of an antagonist specific for DP_1 or CRTH2 receptor on fat storage stimulated by PGD_2 or 11d-11m-PGD_2

To evaluate the preferential involvement of the DP_1 or CRTH2 receptor in the pro-adipogenic effects of PGD_2 and 11d-11m-PGD_2, we examined the effect of BWA868C [36], an antagonist for DP_1, or CAY10471 [37], a selective antagonist for CRTH2, on the accumulation of fats stimulated by PGD_2 or 11d-11m-PGD_2 in the presence of indomethacin (Fig. 3-8). The effect of PGD_2 to stimulate adipogenesis along with indomethacin was significantly suppressed by BWA868C at concentrations of 0.1 and 1 μM (Fig. 3-8A). However, CAY10471 at the same concentrations showed no appreciable effect on the storage of fats enhanced by PGD_2 in the presence of indomethacin. GW9662, a specific antagonist for PPARγ, was effective to attenuate the stimulatory effect of PGD_2. On the other hand, the storage of fats enhanced by 11d-11m-PGD_2 was blocked much more appreciably by CAY10471 than that by BWA868C and GW9662 (Fig. 3-8B). The selective reduction in the number of oil droplets by each antagonist was also confirmed by microscopic views of cultured adipocytes during the maturation phase (Fig. 3-8C). These results suggest that the pro-adipogenic effect of PGD_2 is mediated mainly through DP_1 and PPARγ. In contrast, 11d-11m-PGD_2 exerted a stimulatory effect on adipogenesis preferentially through the CRTH2 receptor.



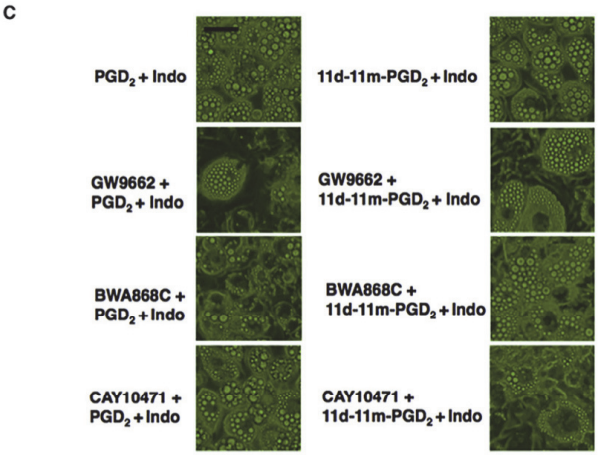


Fig. 3-8. Effect of an antagonist for specific for DP_1 or CRTH2 receptor and GW9662 on the storage of fats simulated by PGD_2 in the presence of indomethacin. 3T3-L1 cells were grown, differentiated, and matured to adipocytes as described in Fig. 3-1. During the maturation phase, the cultured cells were treated for a total of 10 days with either of vehicle, 0.1 μM or 1 μM BWA868C, 0.1 μM or 1 μM CAY10471, and 1 μM GW9662 in the presence or absence of 1 μM indomethacin together with 1 μM PGD_2 (A) or 11d-11m-PGD_2 (B). The resulting cultured adipocytes were harvested for the determination of the amounts of triacylglycerols and proteins. Data represent the mean ± S.E.M. of three independent experiments. *$p < 0.05$ compared with the cells treated with vehicle. $^{\#}p < 0.05$ compared with the cells treated with indomethacin alone. $^{\S}p < 0.05$ compared with the cells treated with a mixture of PGD_2 and indomethacin (A) or a mixture of 11d-11m-PGD_2 and indomethacin (B). Alternatively, the cultured cells were observed by phase-contrast microscopy after being treated as described above with either of vehicle, 1 μM GW9662, 1 μM BWA868C, 1 μM CAY10471, and in the presence or absence of 1 μM indomethacin together with 1 μM PGD_2 (left panels) or 11d-11m-PGD_2 (right panels) (C).
Scale bar, 50 μm.

4. Discussion

Previous studies have shown that cultured adipocytes have the ability to synthesize endogenous PGD_2 and the related PGJ_2 derivatives [9,10,24], which serve as pro-adipogenic factors during the maturation phase of adipocytes. PGJ_2 derivatives including 15d-PGJ_2 and Δ^{12}-PGJ_2 are well-known activators for the nuclear hormone receptor PPARγ, a master regulator of adipogenesis [5,6]. Since PGD_2 undergoes the non-enzymatic dehydration to form J_2 series of PGs in biological fluids [7,8], PGD_2 may exert a stimulatory effect on adipogenesis through PPARγ. However, PGD_2 is

chemically unstable, and its action and analysis are complicated by its short half-life in vivo. Therefore, in this study, we came to investigate the biological activity of a stable analogue of PGD_2. The current study used 11d-11m-PGD_2 as a chemically stable analogue of PGD_2 [26] although its biological activity remains unclear. Interestingly, our study revealed that 11d-11m-PGD_2 at concentrations from 10 nM to 1 μM was more potent that natural PGD_2 to stimulate adipogenesis in the presence of indomethacin or aspirin during the maturation phase of adipocytes. The stimulatory effects of PGD_2 and 11d-11m-PGD_2 at 1 μM were comparable to those of PGJ_2 derivatives. The similar results were also observed in the absence of the COX inhibitor. These findings made us curious for the cellular mechanism whereby the isosteric analogue exerted its pro-adipogenic effect. A plausible explanation might be the action of 11d-11m-PGD_2 through the mediation of PPARγ as PGD_2 and PGJ_2 derivatives. However, it is more unlikely that 11d-11m-PGD_2 activates PPARγ because this compound is a chemically stable analogue of PGD_2 with an exo-cyclic methylene in place of the 11-keto group. The present study using different concentrations of GW9662, a selective antagonist PPARγ, provided the evidence that the pro-adipogenic effect of 11d-11m-PGD_2 was almost independent of the activation of PPARγ. On the other hand, we confirmed that the stimulatory effect of 1 μM PGD_2 on adipogenesis was partially mediated by the action of PPARγ. However, we speculate that other mechanisms would be also responsible for the promotion of adipogenesis by PGD_2 besides the activation of PPARγ. Our results suggest an obvious difference between PGD_2 and 11d-11m-PGD_2 in the mode of the pro-adipogenic action.

PGD_2 is known to exert its inflammatory effects through the specific interactions with two types of cell-surface membrane receptors called the G protein-coupled receptors DP_1 and CRTH2 also known as DP_2 [38]. However, little is known about the involvement of DP_1 and CRTH2 in the pro-adipogenic action of PGD_2 and its stable analogue 11d-11m-PGD_2. Our current study revealed the gene expression of both DP_1 and CRTH2 in cultured 3T3-L1 adipocytes. Their transcript levels reached maximal levels around 6 days of the maturation phase, which was similar to that of the nuclear hormone receptor PPARγ. In contrast, the expression level of L-PGDS responsible for the biosynthesis of PGD_2 increased gradually up to 10 days. These finding raised the possibility that two types of the DP receptors could be associated with the up- regulation of adipogenesis at the terminal differentiation phase. Hence, we investigated the effects of BW245C, a specific DP_1 agonist, and 15R-15m-PGD_2, a selective CRTH2 agonist, on the gene expression of adipocyte-specific markers expressed during the maturation phase. The results showed that both compounds effectively promoted the gene expression of the adipogenesis markers, such as PPARγ, GLUT4, LPL and adiponectin, attenuated by treatment with indomethacin. Taking these into consideration, the DP_1 and CRTH2 receptors appreciably would

contribute to the pro-adipogenic effects of PGD_2 and 11d-11m-PGD_2 by the promotion of adipogenesis program during the maturation phase of adipocytes. Consistent with the observed gene expression, the present study also demonstrated the stimulatory effects of both DP_1 and CRTH2 agonists on the accumulation of triacylglycerols suppressed in the presence of indomethacin.

In the present study, we compared the difference between PGD_2 and 11d-11m-PGD_2 in the cellular mechanism for their pro-adipogenic effects during the maturation phase of adipocytes. The following lines of evidence support the notion that PGD_2 exerts its pro-adipogenic effect mainly through the mediation of DP_1 and PPARγ, whereas the stimulatory effect of 11d-11m-PGD_2 on adipogenesis occurs preferentially by the interaction with CRTH2. At first, the promoting effect of PGD_2 along with indomethacin was significantly attenuated by the co-incubation with the DP_1 antagonist BWA868C. In addition, the pro-adipogenic effect of PGD_2 was found to be partially sensitive to the PPARγ antagonist GW9662. However, the stimulatory effect of PGD_2 was not significantly sensitive to the selective CRTH2 antagonist CAY10471 at both concentrations of 0.1 and 1 μM in our cultured system. However, the action of other metabolites derived from PGD_2 is not excluded since different prostanoids of D_2 and J_2 series have been reported to retain the activity on CRTH2 in other systems [38,39]. On the other hand, the pro-adipogenic effect of 11d-11m-PGD_2 together with indomethacin was much more preferentially suppressed by CAY10471 than BWA868C.

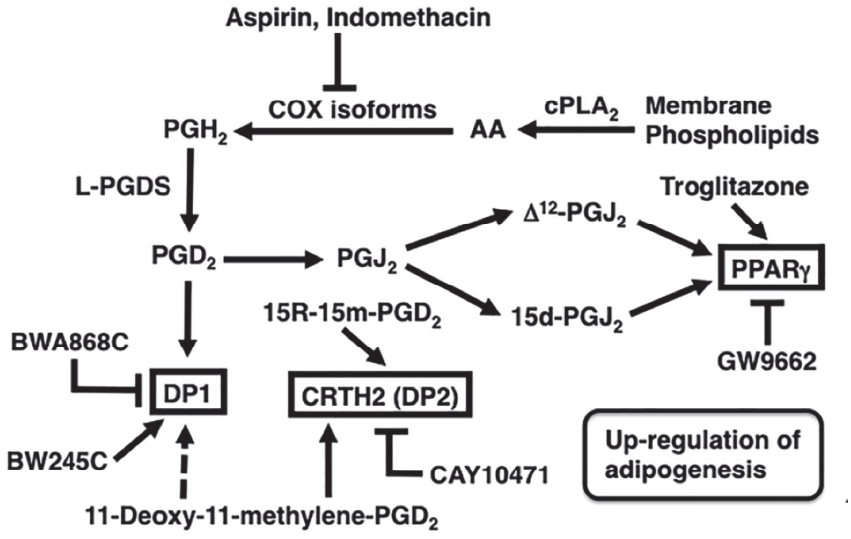


Fig. 3-9. Proposed action of PGD_2 and 11d-11m-PGD_2 on the stimulation of adipogenesis during the maturation phase of cultured adipocytes through the DP1 and CRTH2 receptors as well as PPARγ. PGD_2 exerts its pro-adipogenic effect mainly through the mediation of DP1 and

PPARγ, whereas the stimulatory effect of 11d-11m-PGD_2 on adipogenesis occurs preferentially by the interaction with CRTH2.

This finding indicates the predominant role of CRTH2 in the promotion of adipogenesis by 11d-11m-PGD_2. As well, we revealed that GW9662 failed to block the stimulatory effect of 11d-11m-PGD_2 on the storage of fats. These findings indicate an obvious contrast between natural PGD_2 and its isosteric analogue 11d-11m-PGD_2 in the way of the pro-adipogenic action (Fig. 3-9). Our study provided the evidence to show the efficacy and potency of 11d-11m-PGD_2 in the stimulation of adipogenesis in cultured adipocytes. Until now, the actions of 11d-11m- PGD_2 through the DP_1 and CRTH2 receptors have not been described in other biological systems as far as we know. Nevertheless, we have previously shown that 11d-11m-PGD_2 is useful as a hapten mimic to obtain a monoclonal antibody specific for PGD_2 and to develop its immunological assay with high sensitivity and specificity [25].

Our study showed that PGD_2 were able to stimulate adipogenesis partly through the DP_1 receptor. The DP_1 receptor is coupled to Gs proteins and increases intracellular levels of cAMP in immune cells [40,41]. The increased levels of cAMP could be responsible for the gene expression of adipogenic factors in cultured adipocytes through the control by a cascade of transcription factors starting with C/EBPβ [21]. Recently, we have reported that cAMP analogues and forskolin at certain concentrations rescue the storage of fats suppressed by aspirin during the maturation phase of cultured 3T3-L1 adipocytes [12]. Those rescuing effects of those compounds are partial, and higher concentrations tend to attenuate the positive effects on adipogenesis. It is possible that other signaling pathway specific for the DP_1 receptor by PGD_2 could act in this system. On the other hand, the CRTH2 receptor is known to decrease intracellular cAMP levels through Gi proteins and to enhance Ca^{2+} in immune cells, which mediate pro-inflammatory effects [17,41]. Moreover, based on the application of selective inhibitors, previous studies have implicated phospholipase Cβ and various kinases including phosphtidyinositol-3-kinase and Akt also known as protein kinase B in the signal propagation of CRTH2 [41,42]. Therefore, these signaling pathways might be involved in the pro-adipogenic action of 11d-11m-PGD_2 through CRTH2 in our system. More recently, a study has described that PGD_2 and the CRTH2 agonist 15R-15m-PGD_2 stimulates the expression level of adipogenic and lipogenic enzymes in adipocytes [43]. This report also suggests that PGD_2 enhances lipid accumulation through the suppression of lipolysis though CRTH2. Thus, the role of CRTH2 in adipogenesis and lipolysis has become an interesting subject. However, the action of PGD_2 is complex and different results can be obtained depending on the experimental culture conditions and cell lines of

adipocytes. More detailed studies are required to unravel the precise understanding of the cellular mechanism for the pro-adipogenic action of PGD_2 and the related derivatives through the DP receptors.

Adipose tissue is a major metabolic organ controlling systemic energy homeostasis in vivo through the storage of fats by lipogenesis and the mobilization of fatty acids by lipolysis. Our results suggest the potential usefulness of 11d-11m-PGD_2 and 15R-15m-PGD_2 as chemically stable synthetic analogues to stimulate adipogenesis of white pre-adipocytes through the preferential activation of the PGD_2 receptor CRTH2 when they are administered topically to some area of visceral or subcutaneous white adipose tissue. The efficacy of these compounds remains unknown since the metabolic stability of them has not been studied extensively. As the activation of CRTH2 is known to promote allergic responses in immune cells [38,41], the use of the synthetic analogues of PGD_2 to activate CRTH2 might raise the possibility of side effects in vivo. Further studies are required for the clarification of their control of adipogenesis in the body.

In conclusion, our study was undertaken to study the pro-adipogenic effects of PGD_2 and its isosteric analogue, 11d-11m-PGD_2, in the presence of indomethacin during the maturation phase of adipocytes. The dose-dependent study revealed that 11d-11m-PGD_2 was more potent than PGD_2 in stimulating the storage of fats. Their promoting effects of both compounds were due to up-regulation of adipogenesis. On the basis of the results with selective agonists and antagonists, we found that the action of 11d-11m-PGD_2 was mediated mainly through the CRTH2 receptor while PGD_2 exerted its effect through the DP_1 and PPARγ receptors. Our findings suggest a useful application of 11d-11m-PGD_2 to the adipogenesis study through CRTH2.

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Chapter 4

Conclusion

Here, we made a comparison between the action of natural PGD_2 and its stable analogue, 11-deoxy-11-methylene-PGD_2 (11d-11m-PGD_2) in adipogenesis program of cultured mouse 3T3-L1 adipocytes and we tried to know about their interaction with distinct classical cell surface membrane G-protein coupled DP receptors, also called as PGD_2 receptors. To clarify how the stable analogue of PGD_2 regulates adipogenesis, we investigated its effects and the specific agonists or antagonists for the DP receptor on the storage of fats during the maturation phase of cultured adipocytes. Both BW 245C (DP1 agonist) and 15(R)-15-methyl PGD_2 (CRTH2 agonist) were found to be actually efficacious in the stimulated adipogenesis. On the other hand, a selective antagonist for the DP1 receptor (BW A868C) more preferentially blocked the pro-adipogenic effect of PGD_2 than that of 11d-11m-PGD_2 while a selective antagonist for the CRTH2 receptor (CAY10471) was more preferentially inhibitory for the action of 11d-11m-PGD_2 as compared with PGD_2. These findings indicate that 11d-11m-PGD_2 exerts pro-adipogenic effect on cultured adipocytes during the maturation phase through cellular mechanism different from that of natural PGD_2. Thus, the present study showed that the treatment with PGD_2 and 11d-11m-PGD_2 during the maturation phase stimulated the storage of fats in mature adipocytes. The effects of these prostanoids were found to be dose-dependent and the stimulation was observed similarly for both these prostanoids when co-incubated with a non-specific COX-inhibitor, indomethacin. As expected, the up-regulation of adipogenesis was curtailed in the cultured cells treated with indomethacin when compared to control, thus attenuating the accumulation of triacylglycerols by this COX-inhibitor. Thus we observe that the addition of PGD_2 or its stable analogue in the presence of this COX-inhibitor was efficacious at reversing the effect of indomethacin alone. However, 11d-11m-PGD_2 was found to be significantly more potent than PGD_2 to stimulate the storage of fats during the maturation phase of adipocytes. We can say that this observation is fairly reasonable as we know that the parent compound, PGD_2, serve as a pro-adipogenic factor by converting to PGs of J_2 series, which are activators of PPARγ, a master regulator of adipogenesis. Overall this suggests the important role of membrane surface receptors for endogenous PGD_2 during maturation phase of adipocytes and giving a possibility that these PGs may act through interaction with intracellular protein targets by the DP1 and CRTH2 receptor pathway.

In conclusion, we demonstrated that natural PGD_2 or its analogue and their selective agonists for the PGD_2 receptor stimulated adipogenesis attenuated by indomethacin during the

maturation phase. Likely, the pro-adipogenic effect of PGD_2 was blocked by a specific antagonist for PPARγ as it is known that PGJ_2 derivatives obtained from parent PGD_2 are known as the direct activators of PPARγ. These findings suggest that the action of PGD_2 through the DP1 receptor maybe linked with the activation of PPARγ as a downstream factor. Also the suppression of adipogenesis by indomethacin was rescued by the treatment with 11d-11m-PGD_2 along with this COX inhibitor, indicating a pro-adipogenic effect of this stable PGD_2 analogue. In addition to this, we also found the stimulatory effect of 11d-11m-PGD_2 along with indomethacin, which was more preferentially inhibited in the presence of a specific CRTH2 antagonist than that by a specific DP1 antagonist. Also we found that the effect of stable analogue remained unchanged in presence of different concentrations of a specific antagonist for PPARγ unlike PGD_2. The results suggest the predominant role of the CRTH2 receptor that may not be linked with the activation of PPARγ in the pro-adipogenic effect of 11d-11m-PGD_2 during the maturation phase of adipogenesis.

Another research study was undertaken to elucidate the serum antioxidant (vitamin C), macro- minerals (Ca, Na, K) and trace elements (Zn, Fe) concentrations in patients with metabolic syndrome; and therefore, to find is there any patho-physiological correlation present or not. Metabolic syndrome was estimated by following the definition generated by the National Cholesterol Education Program Adult Treatment Panel III (NCEP ATPIII). Serum Vitamin C was estimated by phenyl-hydrazine spectrophotometry method, while Macro-minerals and trace elements were determined under coordination of two methods these was flame atomic absorption spectrometry (FAAS) and graphite furnace. This study found that vitamin C is lowering significantly ($p < 0.05$) in patients with metabolic syndrome while compared with control subjects. Analysis of serum trace elements (Zn, Fe) and macro minerals (Ca, Na, K) explored low serum concentrations in examined patients significantly either than control group ($P < 0.05$). Pearson's correlation analysis reviewed the existence of a negative correlation between Zn and K ($r = -0.094$, $p = 0.354$); Ca and K ($r = -0.057$, $p = 0.573$); Ca and Na ($r = -0.010$, $p = 0.924$); Fe and K ($r = -0.003$, $p = 0.975$); K and Na ($r = -0.052$, $p = 0.608$), while positive correlation was occurred for Zn and Fe ($r = 0.024$, $p = 0.813$); Zn and Ca ($r = 0.056$, $p = 0.582$); Zn and Na ($r = 0.156$, $p = 0.122$); Ca and Fe ($r = 0.030$, $p = 0.769$); Fe and Na ($r = 0.085$, $p = 0.403$). In conclusion, this inter-element-relationship found a noticeable disturbance in the element homeostasis in patients with metabolic syndrome. Based on this evidence our present study suggests there was found a strong association between the pathogenesis of the metabolic syndrome with the level of depletion of vitamin C, Zn, Fe, Ca, K and Na. So, dietary supplementation with antioxidants, macro-minerals and trace elements may boost the management of metabolic syndrome and thus reduce its complications.

Summary

Adipogenesis is a crucial aspect in regulating body fat mass and energy homeostasis. Recently, there has been a dramatic increase in the incidence of obesity with abnormal storage of fats in white adipose tissue in industrialized and newly developed countries. Obesity is recognized as a major risk factor for the development of insulin resistance and the related metabolic syndrome. In this study, I conducted health science studies regarding serum parameters associated with metabolic syndrome and pro-adipogenic effects of prostaglandin (PG) D_2 and its stable, isosteric analogue.

In recent years, there has been a notable increase in the number of people with the metabolic syndrome. It has become an epidemic disease and acknowledged globally other life style disease such as type-2 diabetes and cardiovascular disease. Not much work has been done to find out the possible reasons for the development of metabolic syndrome, which is counted to be very significant for the treatment of this syndrome. Thus an attempt has been made to examine serum antioxidant levels like vitamin C, macro minerals (Ca, Na, K), and trace element concentrations (Zn, Fe), and to clinch the correlation between the serum levels of these components of this metabolic patient. This study found that vitamin C was significantly lower ($p < 0.05$) in patients with metabolic syndrome as compared with control subjects. Analysis of serum trace elements (Zn, Fe) and macro minerals (Ca, Na, K) explored lower serum concentrations in examined patients than the control group at the significant levels ($p < 0.05$). Pearson's correlation analysis revealed negative correlations between blood glucose and Fe, and between triglyceride and Zn level were found statistically significant. On the basis of our present study it can be asserted that depletion of Vitamin C, Zn, Fe, Ca, K and Na levels is strongly associated with the metabolic syndrome pathogenesis. Dietary supplementation with antioxidants, macro-minerals and trace elements may drive the treatment of metabolic syndrome and thus reduce its complications. Moreover, changes in lifestyle and therapeutics may reduce adiposity and could provide the benefit of preventing obesity-related morbidity and mortality.

PGD_2 is relatively unstable and dehydrated non-enzymatically into PGJ_2 derivatives, which are known to serve as pro-adipogenic factors by activating peroxisome proliferator-activated receptor (PPAR) γ, a master regulator of adipogenesis. 11-Deoxy-11-methylene-PGD_2 (11d-11m-PGD_2) is a novel, chemically stable, isosteric analogue of PGD_2 in which the 11-keto group is replaced by an exocyclic methylene. Here I attempted to investigate pro-adipogenic effects of PGD_2 and 11d-11m-$PGD_2$and to compare the difference in their ways during the maturation phase of cultured adipocytes. The dose-dependent study showed that 11d-11m-PGD_2 was significantly more potent than

natural PGD_2 to stimulate the storage of fats suppressed in the presence of indomethacin, a cyclooxygenase inhibitor. These pro-adipogenic effects were caused by the up-regulation of adipogenesis as evident with higher gene expression levels of adipogenesis markers. Analysis of transcript levels revealed the enhanced gene expression of two subtypes of cell-surface membrane receptors for PGD_2, namely the prostanoid DP_1 and DP_2 (chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2)) receptors together with lipocalin-type PGD synthase during the maturation phase. Specific agonists for DP_1, CRTH2, and PPARγ were appreciably effective to rescue adipogenesis attenuated by indomethacin. The action of PGD_2 was attenuated by specific antagonists for DP_1 and PPARγ. By contrast, the effect of 11d-11m-PGD_2 was more potently interfered by a selective antagonist for CRTH2 than that for DP_1 while PPARγ antagonist GW9662 had almost no inhibitory effects. These results suggest that PGD_2 exerts its pro-adipogenic effect principally through the mediation of DP_1 and PPARγ, whereas the stimulatory effect of 11d-11m-PGD_2 on adipogenesis occurs preferentially by the interaction with CRTH2.

Summary in Japanese

脂肪細胞形成は、体脂肪量とエネルギーの恒常性を調節する点において重要な側面である。最近、産業の発達した国や新しく発展している国において白脂肪組織に異常に貯蔵された脂肪を伴う肥満の著しい増加がある。肥満は、インスリン抵抗性とその関連の代謝症候群の進行における主要な危険因子として認められる。本研究では、私は代謝症候群に関係する血清パラメーターとプロスタグランジン (PG) D_2とその安定で同じ立体性の類縁体の脂肪形成の促進効果に関する健康科学研究を行った。

近年、代謝症候群の人々の数の顕著な増加が認められる。それは、流行病であり、タイプ-2型糖尿病や心臓血管病のような他の生活習慣病を広くに認知する。代謝症候群の進行に関する可能な理由を見いだす研究はあまり行われていない。そのことは、この代謝症候群を治療するのに大変重要であると考えられる。従って、ビタミンCのような血清の抗酸化剤、主要な無機物（Ca, Na, K）及び微量元素濃度（Zn、Fe)を調べて、代謝疾患の患者の血清レベルの関係を明らかにすることを試みた。本研究で、ビタミンCは対照の被験者よりも代謝症候群の患者で有意に ($p < 0.05$) 低い濃度であることが見いだされた。血清の微量元素（Zn, Fe）と主要無機質（Ca, Na, K)の分析では、対照群に比較して代謝症候群の患者で有意なレベル ($p < 0.05$) で低濃度であることが分かった。ピアソンの相関関係の解析で、血液中のグルコースとFeの関係、およびトリグリセリドとZnのレベルの関係には負の相関関係あることが解明された。今回の研究に基づくと、ビタミンC、Zn、Fe、Ca、K及びNaのレベルの不足は、代謝症候群の病態発症と強く関係していることが確認された。抗酸化物、主要な無機質、及び微量元素の食事による供給は、代謝症候群の治療効果を推進して、その合併症を低減するかもしれない。さらに、生活習慣と治療法の変化は、脂肪蓄積を低下して、肥満に関係する罹患率や死亡率の増加を予防する利点を提供できると思われる。

PGD_2は比較的不安定で非酵素的に脱水されてPGJ_2誘導体に変換され、それらは脂肪細胞形成のマスター調節物質であるペルオキシソーム増殖剤応答性受容体 (PPAR)γを活性化することにより脂肪細胞形成の促進因子として役立つことが知られている。11-デオキシ-11-メチレン-PGD_2 (11d-11m-PGD_2)は、11-ケト基が外環のメチレン基に置き換わった新規で化学的に安定な同じ立体性のPGD_2類縁体である。ここでは、私は、PGD_2と11d-11m-PGD_2の脂肪細胞形成の促進効果を探究し、培養脂肪細胞の成熟期での作用様式の相違を比較することを試みた。用量依存性の研究により、11d-11m-PGD_2は天然のPGD_2よりもシクロオキシゲナーゼ阻害剤のインドメタシンの存在下で抑制された脂肪蓄積を促進するのに有意に強い作用であった。これらの脂肪細胞形成の促進効果は、脂肪細胞形成のマーカーのより高い遺伝子発現レベルで証明される様に脂肪細胞形成の正の調節により引き起こされた。転写レベルの解析で、リポカリン型PGD合成酵素と共にPGD_2に対する細胞表面受容体、すなわち、２つのサブタイプの

DP_1とDP_2（Th2細胞で発現される化学走化性受容体の相同性分子（CRTH2)）の遺伝子発現の亢進が明らかになった。DP_1, CRTH2、及びPPARγに対する特異的アゴニストは、インドメタシンにより低下する脂肪細胞形成を促進するのにかなり効果的であった。PGD_2の作用は、DP_1とPPARγに対する特異的なアンタゴニストにより抑制された。対照的に、11d-11m-PGD_2の効果は、DP_1に対するものよりもCRTH2に対する選択的なアンタゴニストにより強く阻害された。一方、PPARγアンタゴニストのGW9662は、ほとんど阻害効果がなかった。これらの結果は、PGD_2がDP_1とPPARγの作用を主に介して脂肪細胞形成の促進効果を及ぼすが、脂肪細胞形成に対する11d-11m-PGD_2の促進効果は、CRTH2との相互作用により優先的に起こることを示す。

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List of publications

Chapter 2

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Chapter 3

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