Role of Connexin 43 and Conjugated Linoleic Acid Isomers During Adipocyte Differentiation

by

Azadeh Yeganeh

A thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

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Of

MASTER OF SCIENCE

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Abstract

Obesity is major health problem in developed countries and is becoming a critical issue in developing countries. Obesity also is a major risk factor for a number of disorders, including hypertension, certain forms of cancer, cardiovascular disease, dyslipidemia and type 2 diabetes. In addition to storing fat, adipose tissue acts as an endocrine organ and secretes various humoral factors into the circulation called adipokines. Obesity, an excessive amount of in adipose tissue mass, results from an increase in both adipocyte number (hyperplasia) and size (hypertrophy). Thus, hyperplasia of adipocytes may have an important role in the development of obesity.

Connexin-43 (Cx43) is a membrane phosphoprotein expressed by numerous cell types. In addition to forming gap junctions and mediating direct inter-cellular communication, Cx43 is increasingly recognized for its potential to influence intra-cellular signaling, gene expression, differentiation and growth. Its role in adipogenesis is unknown.

Nutraceuticals such as conjugated linoleic acid (CLA) can influence adipogenesis, as evidenced by its effect on adipogeneic markers such as PPARγ (peroxisome proliferator-activated receptor-γ), C/EBPα (CCAAT/enhancer binding protein) and adiponectin. However, since the role of Cx43 in adipogenesis has not been investigated, the present study examined Cx43 expression and phosphorylation during differentiation of 3T3-L1 preadipocytes, which was induced with a cocktail of insulin, dexamethasone and methylisobutylxanthine.

Our study confirmed a previously reported finding that Cx43 expression is up-regulated in the early stages of differentiation and is down-regulated in the late stages

of differentiation of 3T3-L1 cells. We also demonstrated that in early differentiation, Cx43 is highly phosphorylated. As well, Cx43 maintained a membrane association at all stages of differentiation when subcellular fractions were examined by Western blotting, although microscopy revealed differences in the staining pattern suggestive of movement between membrane compartments such as the Golgi and endoplasmic reticulum.

We also investigated the effect of constitutive expression of Cx43 on adipogenesis in 3T3-L1 CARΔ adipocyte. Our results showed that over-expression of Cx43 during adipocyte differentiation has no apparent effect on adipokine production or lipid droplet formation. On the other hand, inhibition of PKC (protein kinase C) but not PKA (protein kinase A) prevented down-regulation of Cx43, although both treatments inhibited adipogenesis. These results suggest the possibility that phosphorylation of Cx43, an event that is required for its function, plays an important role in adipogenesis. Further analysis will be necessary to determine which phosphorylation sites are critical for adipocyte differentiation.

We also examined the role of cis9-trans11 and/or trans10-cis12 CLA isomers in regards to production of adipokine and lipid droplet formation as well as their effect on Cx43 expression. Our results showed that t10-c12 CLA but not c9-t11 CLA lowered production of adipokines such as adiponectin, adipsin and chemerin. Likewise, t10-c12 CLA reduced levels of lipid droplet proteins such as perilipin and A-FABP (adipocyte fatty acid binding protein). In addition, our results suggest adipokine production and amounts of lipid droplet protein are regulated through different pathways. In contrast, c9-t11 and/or t10-c12 CLA isomers did not influence Cx43

expression and phosphorylation. The lack of effect of CLA on Cx43 could be explained by the fact that CLA affects events in late and/or early differentiation, while the observed alterations in Cx43 occur during mitotic clonal expansion and therefore before differentiation begins.

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Abbreviations

ACRP30	Adipocyte complement related protein 30 kDa
ADD-1	Adipocyte determination and differentiation-dependent factor 1
ADRP	Adipose-differentiation related protein
A-FABP	Adipocyte fatty acid binding protein
AGA	18-α glycyrrhetinic acid
AMPK	Adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
BCA	Bicinchoninic acid
BMI	Body mass index
BSA	Bovine serum albumin
c9-t11CLA	Cis 9-trans 11 conjugated linoleic acid
CAD	coronary artery disease
cAMP	Cyclic adenosine monophosphate
CAR	Coxsackie and adenovirus receptor
cdk2	Cyclin-dependant kinase 2
C/EBP-β	CCAAT/ enhancer binding protein
CLA	Conjugated linoleic acid
CRT	Calreticulin
CREB	cAMP response element binding protein
CRP	C-reactive protein
Cx	Connexin
DEX	Dexamethasone

DMEM	Dulbecco's modified Eagle's medium
ER	Endoplasmic reticulum
ERK	Extracellular signaling-regulated kinases
FAS	Fatty acid synthase
FBS	Fetal bovine serum
GLUT	Glucose transporter
GSK-3	Glycogen synthase kinase-3
HDAC	Histone deacetylase
HEK	Human embryonic kidney cell line
HI-FBS	Heat inactivated fetal bovine serum
HLH	helix-loop-helix
HRP	horse radish peroxidases
ICAM-1	Intracellular cell adhesion molecule-1
IGF-1	Insulin-like growth factor-1
IRS	Insulin-receptor substrate
INS	Insulin
JNKs	c-Jun amino terminal kinases
kDa	Kilodalton
LXRα	Liver X receptor alpha
MAPK	Mitogen activated kinase
MKP-1	MAPK phosphatase-1
MIX	Methylisobutylxanthine
MOI	Multiplicity of infectivity

NHANES	National Health and Nutrition Examination Survey
PFU	Plaque formation unit
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PKB	Protein kinase B
Pref-1	Pre-adipocyte factor-1
PPARγ	Peroxisomal proliferator - activated receptor γ
PVDF	Polyvinylidene difluoride
RA	Rumenic acid
RXR	Retinioid X receptor
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SREBP-1	Sterol response element binding protein -1
SV	Stromal vascular
t10-c12 CLA	Trans 10-cis12 conjugated linoleic acid
TBST	Tris-buffered saline with Tween-20
TCID	Tissue culture infectious dose
TNF-α	Tumor necrosis factor-alpha
TZDs	Thiazolidinedione
UCP	Uncoupling protein
VA	Vaccenic acid
VCAM-1	Vascular cell adhesion molecule-1
ZDF	Zucker diabetic fatty

1 Literature Review

The role of connexin 43, a gap junction protein, in adipogenesis is still unclear. While Cx43 has been shown to influence the function of numerous cell types, little is known of its function in adipose tissue. Within this context, Cx43 has been shown to affect cell growth and differentiation, critical elements in the formation of adipose tissue and its ability to modulate lipid storage. Given this lack of information, research to address issues revolving around adipocyte differentiation and modulation of lipid metabolism by nutritional factors is needed.

1.1 Obesity

Obesity is a disorder associated with an excess of white adipose tissue. Normal values of fat mass are 9–18% of body weight in healthy males and 14–28% in females, but in obese individuals it may consist of 60–70% of body weight (1). For adults, overweight and obesity ranges are determined by using weight and height to calculate body mass index (BMI). BMI is used because, for most people, it correlates with the amount of body fat. An adult who has a BMI between 25 and 30 is considered overweight and a BMI more than 30 is obese. Between 1990 and 1999, the prevalence of obesity in the United States rose from 23% to 31% (2), and it is expected to reach to 39% by 2010. The latest National Health and Nutrition Examination Survey (NHANES) reported that roughly 127 million Americans are overweight, 60 million are obese and 10 million have life-endangering obesity. According to NHANES in 1999-2000, more than 64% of US adults were either overweight or obese. These data

show a 14% increase in the prevalence reported in NHANES III (1988-94) and a 36% increase from NHANES II (1976-80). The highest increase was seen in the obese group (BMI > 30 kg/m^2), which was double the number in 1976-80 (3). It has also been shown that obesity is responsible for approximately 325,000 deaths and 4.3-5.7% of direct death costs ($39–52 billion) each year in United States (4). According to Statistics Canada, about a quarter of the people 20 to 56 years old who were overweight in 1994-95 had become obese by 2002-03. Unfortunately, Statistics Canada also reported in 2004 that 26% of persons 2 to 17 years old were overweight or obese. Statistics Canada estimated that 5.5 million Canadian adults (23% of the population aged 18 or older) were obese in 2004. This increase in the incidence of obesity is estimated to contribute at least $1.8 billion to health care costs in Canada.

Obesity is a major risk factor for a number of disorders, including hypertension, certain forms of cancer, sleep apnea, coronary artery disease (CAD), dyslipidemia and type 2 diabetes. As a result of these complications, obesity exerts a tremendous burden on health care utilization and costs. This pandemic has been targeted by heath care and health services in order to minimize the risk of new cases of diabetes (especially type 2 diabetes), heart disease and other obesity-related complications.

1.2 Obesity and Type 2 Diabetes, critical risk factors for cardiovascular disease

Obesity is a primary risk factor for the development of type 2 diabetes, a disease characterized by a decrease in the response of tissues to insulin action. During the last 20 years, the incidence of diabetes in the United States increased to 25% of the

population and this increase has been linked to the elevated prevalence of obesity (5). Statistical data from the United States have shown that two thirds of adults diagnosed with type 2 diabetes have a BMI of 27 kg/m^2 or greater (6). Diabetes is responsible for approximately 41,500 Canadian deaths each year (7). Results from the World Health Organization Study of Vascular Disease in Diabetes demonstrated that cardiovascular disease is the most common cause (52%) of mortality in patients with type 2 diabetes (8). Type 2 diabetes or insulin resistance is also associated with atherosclerosis, hypertension, lipid abnormalities and abdominal obesity. When multiple risk factors are present, they contribute to a condition termed the metabolic syndrome (9, 10). Dysfunctional adipose tissue as a result of obesity is correlated with excessive fat deposition in muscle and liver as well as an increase in adipocyte size and number (11). Consequences of these changes include the release of fatty acids, hormones and pro/anti-inflammatory molecules from adipocytes, which can induce insulin resistance, type 2 diabetes, inflammation, dyslipidemia, and, possibly, endothelial dysfunction and vascular damage leading to hypertension and atherosclerosis.

Obesity has been recognized as an inflammatory state. Studies have shown that inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6) and C-reactive protein (CRP), are increased in the plasma of obese individuals (12-17). Furthermore, adipose tissue secretes most of these pro-inflammatory cytokines. Recent data show an accumulation of macrophages in the adipose tissue of obese subjects, which could be the source of these pro-inflammatory factors. It is suspected their presence can modulate the secretory activity of adipocytes (18, 19). These pro-inflammatory cytokines can promote vascular smooth muscle cell migration

and injure the endothelial layer, thus initiating the events that promote the development of atherosclerotic lesions (20). Another risk factor for cardiovascular disease is hypertension (21). The majority of patients with type 2 diabetes and hypertension are also obese (22). Results from the Framingham Offspring Study indicate that 78% of cases of hypertension in men and 64% in women were attributable to obesity (23). It has been shown by the long-term Nurses Health Study (24) that gaining weight after 18 years of age significantly increased the risk for hypertension. In 2002, cardiovascular disease caused 74,621 Canadian deaths and required $18 billion in heath care funding (25).

1.3 Adipokines

In addition to storing fat, adipose tissue acts as an endocrine organ and secretes various factors into the blood (26). These factors, termed adipokines, include adiponectin, leptin, resistin, adipsin, chemerin, omentin, lipin, angiotensinogen, prostaglandins, plasminogen activator inhibitor-1, tumor necrosis factor α and macrophage migration inhibitory factor.

1.3.1 Adiponectin

Thirty-kDa adipose-specific secreted protein, also known as adiponectin, ACRP30 (adipocyte complement related protein 30 kDa), AdipoQ and GBP28 (gelatin binding protein 28 kDa), was identified independently by four groups using a variety of different approaches (27-30). Adiponectin can be found as both the full-length protein and a shorter fragment called globular adiponectin. Almost all adiponectin appears to exist as full-length adiponectin in plasma, since only a small amount of globular adiponectin can be detected in human plasma (31). Adiponectin circulates in human serum at a concentration of 3-30 μg/ml, with circulating levels approximately two to three times higher in females than in males (27, 32). This is a high concentration when compared to other adipokines (eg. nanograms per milliliter for leptin). Subjects with obesity, type 2 diabetes, and coronary heart disease have a lower plasma concentration of adiponectin in comparison to healthy subjects (28, 33, 34). These results indicate that adiponectin could potentially serve as a link between obesity and obesity-related disorders such as coronary artery disease (35, 36),

hypertension (37), diabetes and metabolic syndrome (38). The mechanism by which the insulin-resistant condition is related with low levels of adiponectin is not clear. However, it has been shown that adiponectin stimulates glucose metabolism by promoting the phosphorylation and activation of adenosine monophosphate-activated protein kinase (AMPK) in skeletal muscle (39), liver (40) and adipocytes (41). Also, TNF- α, a proinflammetory cytokine that is increased in the white adipose tissue of obese subjects, might down-regulate adiponectin production (42, 43). On the other hand, it has been shown that adiponectin reduces the production and activity of TNF-α (44). Moreover, adiponectin inhibits IL-6 production accompanied by induction of the anti-inflammatory cytokines IL-10 and IL-1 receptor antagonist in human macrophages (45-47). Adiponectin influences the development of atherosclerosis by reducing induction of intracellular cell adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) by TNF-α or resistin (48, 49). Also, weight loss causes an increase in adiponectin levels, thus exhibiting a negative correlation with changes in body mass index (BMI), waist and hip circumference and plasma glucose levels.

1.3.2 Leptin

The *ob* gene codes for leptin, a 16-kDa protein which functions primarily to control appetite (50). Adipocytes are the most important source of leptin and circulating leptin levels (typical concentration in human serum is approximately 1–10 ng/ml) are positively correlated with BMI (51). Although leptin is predominately expressed in adipose tissue, it is found both in the circulation and in cerebrospinal fluid.

Extreme obesity has been seen in mice and humans with mutations in either the genes for leptin (ob/ob mice) or the leptin receptor (db/db mice) (52, 53). In addition to its main role in controlling food intake, leptin is capable of altering body weight and energy expenditure through secondary effects on skeletal muscle, liver, pancreas, adipose tissue, and numerous other cell types (54, 55). Leptin injection will reduce body weight and fat mass by increasing energy expenditure and decreasing food intake in rodents (56). Several studies have shown that leptin is able to activate AMPK in muscle and liver by acting directly on these tissues and by acting centrally through the central nervous system (57, 58). AMPK activation decreases anabolic ATP-consuming pathways such as glucose regulated transcription, protein synthesis, cholesterol synthesis and triglyceride synthesis. AMPK activation also increases ATP-producing catabolic pathways such as increased glucose transport, β-oxidation and glycolysis. Leptin influences immune function as well; o*b/ob* mice, in which the leptin gene is knocked out, have impaired T cell immunity (59).

1.3.3 Adipsin

Adipsin, a serine protease homologue, exhibits an apparent molecular weight of 44 and 37 kDa due to glycosylation of a core 28-kDa polypeptide (60-62). Adipsin is also known as complement factor D in human subjects. This adipokine is mostly synthesized in adipose tissue but it is detectable in the sciatic nerve and bloodstream (63, 64). Adipsin is produced in a non-glycosylated (28 kDa) form that is glycosylated (44-37 kDa) before secretion (65). Adipsin expression is different in rodents and humans; in mice it is mainly expressed by adipocytes but in human subjects both

adipocytes and monocytes-macrophages are sites of expression (66, 67). It has been reported that adipsin mRNA levels are significantly decreased in genetically obese mice (*db/db* and *ob/ob*) and also in a mouse model of chemically-induced obesity, but the changes are not as large when obesity is obtained by overfeeding normal rats. In addition, a 60-90% decrease in adipsin mRNA has been reported in WAT and BAT of $HSL^{-/-}$ mice (68). These results suggest adipsin has a role in lipogenesis. It also has been reported that adipsin causes the translocation of glucose transporters from the cytosol to the plasma membrane (69). In addition, it is known that insulin stimulates adipsin secretion by increasing the number of glucose transporters in 3T3-L1 cells, which enables binding with glycosylated adipsin and assists with its secretion (70).

1.3.4 Chemerin

Chemerin is a newly discovered chemoattractant protein with an active form of 16-kDa. A role in adaptive and innate immunity has been reported (71, 72). Recently, Goralsk et al (73) reported very high expression of chemerin in white adipose tissue. Their results showed a 60-fold increase in chemerin expression in differentiated 3T3-L1 (day 13) versus undifferentiated cells. This group showed that chemerin is a protein that is secreted by adipocytes so it has been classified as an adipokine. Another group showed that in mice chemerin and its receptor mRNA level increased in different regional fat depots with high fat diet (74). There is also an evidence that chemerin may be involved in regulating adipogenesis and adipocyte metabolism (73).

1.4 Lipid droplet proteins

Triglycerides are stored in the form of lipid droplets. The surface of lipid storage droplets are coated with proteins which play a critical role in structure and function of the lipid droplet. This family of proteins includes perilipin, caveolins and ADRP or adipophilin. Perilipin is the most abundant lipid droplet protein in adipocytes and is essential in regulating movement of lipids in and out of the droplets.

1.4.1 Perilipin

Perilipin is the major lipid-droplet protein in adipocytes, consisting of 0.25 to 0.5% of the total cell protein (75). Perilipin is found in 3 forms, perilipin A, B and C, which is result from alternative splicing of a single perilipin gene. Perilipin A is the most abundant perilipin in adipocytes. Brasamle et al (76) showed that over expression of perilipin in 3T3-L1 pre-adipocytes leads to an increase in the number of lipid droplets. Levels of triglycerol are increased as a result of a reduction in lipid hydrolysis, although there are no significant effects on the rate of lipid synthesis. Perilipin reduces lipid hydrolysis by blocking access of hormone sensitive lipase to the lipid droplet surface (76). On the other hand, lipolysis is stimulated by activation of PKA, which is responsible for phosphorylation of perilipin (77, 78).

1.5 Fatty acid binding proteins

Fatty acid binding proteins (FABPs) are members of larger family of lipid binding proteins. FABPs are found in different tissues such as liver, intestine, muscle, heart, skin, brain, testes, small intestine and adipose. The FABPs are divided into two groups, the plasma membrane FABPs ($FABP_{PM}$) and the cytoplasmic FABPs ($FABP_C$) (79). Clarke et al (80) showed that over-expression of $FABP_{PM}$ in mammalian tissue results in an increase of fatty acid transport. $FABP_{PM}$ proteins increase the absorption of fatty acids from the plasma membrane into the cytoplasm by increasing the solubility rate of fatty acids (81), and $FABP_C$ proteins accelerate fatty acid uptake by stimulating cytoplasmic diffusion of the fatty acids (82).

FABPs also regulate lipid metabolism by activating specific transcription factors (83). PPARs are one group of transcription factors that cooperate with FABPs. Consequently, FABPs may have an effect on ligand-dependent activation of PPARs (84).

1.6 Adipogenesis

Obesity, increased adipose tissue mass, results from both an increase in adipocyte number (hyperplasia) and an increase in adipocyte size (hypertrophy) (85). White adipose tissue forms shortly after birth as a result of increased size of accessible fat cells and proliferation of preadipocytes. Throughout life, new fat cells can be generated as a consequence of environmental factors, such as nutritional status (86-88).

Adipocytes are derived from multipotent mesenchymal stem cells. Although there have been many efforts to describe the cellular properties that distinguish these stem cells and mature adipocytes, specific intermediates have been difficult to characterize at the molecular level. Investigators in this field describe two phases of adipogenesis: determination (or commitment) and terminal differentiation. Determination involves the commitment of a pluripotent stem cell to the adipocyte lineage, which results in conversion of the stem cell to a pre-adipocyte. At this point there is no distinguishing morphological difference between pre-adipocytes and its precursor cell, but the stem cell has lost the potential to differentiate into other cell types. In the second phase, terminal differentiation, the pre-adipocyte differentiates into a mature adipocyte and displays the characteristics associated with lipid transport and synthesis, insulin sensitivity and the production of adipokines. The current understanding of the molecular regulation of terminal differentiation is more extensive than that for determination because most studies have used cell lines such as 3T3-L1 and 3T3-F442A, which are already restricted to the adipogenic lineage. These most commonly used pre-adipocyte cell lines are were cloned from heterogeneous Swiss 3T3 cells that had been derived from dissociated near-term mouse embryos (89-91).

1.7 Differentiation of pre-adipocytes

Studies of the molecular and cellular events that take place as pre-adipocytes differentiate into mature adipocyte have been conducted with various cell culture models, including pre-adipocyte cell lines and primary cultures of adipose-derived stromal vascular precursor cells. Adipogenesis of 3T3-L1 cells can be divided into four

separate stages: pre-confluent proliferation, confluence, hormonal induction, and terminal differentiation (92). Different pathways and factors are involved in each phase, and these will be discussed in the following sections. Specific genes are expressed in the different stages and the presence of respective mRNAs or proteins can be used as biochemical markers for adipogenesis.

1. Pre-confluent proliferation: In the proliferation stage, when the cells are growing, 3T3-L1 cells do not express adipocyte markers. Pref-1 is one of the proteins expressed in this phase and its constitutive expression inhibits differentiation (93, 94). Also, none of the adipogeneic transcription factors or adipokines are present. ERK-1/2 signaling is required for proliferation of pre-adipocytes during this phase (95).

2. Confluence: In this phase, cells are made permissive for differentiation by maintaining them in a confluent state for 2 days. This arrests the cells in the G0/G1 stage of the cell cycle and leads to cessation of growth. Even so, cell cycle regulators such as E2F, pRb and p130 are present, and they continue to be expressed during adipogenesis, although their phosphorylation states will be changed in different stages of differentiation.

3. Hormonal induction/Mitotic clonal expansion: Adipogenesis is induced by adding an adipogenic and mitogenic cocktail containing dexamethasone (DEX), insulin (INS) and methylisobutylxanthine (MIX) (96) that stimulates reentry of the confluent cells into the cell cycle. This is considered day 0 of the adipogenic program. This treatment leads to mitotic clonal expansion whereby cells undergo one or two rounds of mitosis over a 2 day period before terminally differentiating. However, the significance of mitotic clonal expansion for the adipogenic process is still controversial.

Several studies have indicated that DNA synthesis or mitotic clonal expansion is necessary for adipocyte differentiation (97-99). In contrast, Qiu et al (100) demonstrated that DNA synthesis and mitotic clonal expansion is not a required step for 3T3-L1 pre-adipocyte differentiation into adipocytes.

Each component of the adipogenic cocktail has a distinct function. DEX is a synthetic glucocorticoid agonist that affects the early stages of adipogenesis by promoting PPARγ ligand expression and suppressing Pref-1 (101, 102). Recently, Wiper-Bergeron et al (103, 104) showed that glucocorticoids mediate adipocyte differentiation by decreasing the interaction of C/EBP-β with histone deacetylase (HDAC)-1, thus leading to enhanced acetylation of C/EBP-β and an increase in its transcriptional activity. Insulin binds to the IGF-1 receptor and activates CREB, which is essential for the expression of PPARγ 2 (105). Insulin is also a component of the stimulation cocktail, and promotes mitotic clonal expansion. The role of insulin signaling in adipogenesis will be discussed in the following sections. MIX is a cAMP phosphodiesterase inhibitor (106) that enables cAMP levels to reach the threshold necessary for adipogenesis. cAMP plays an important role in the phosphorylation of C/EBP-β, an event that increases its transcriptional activity (107). Furthermore, cAMP dependent ligand activity can be detected on first two days of 3T3-L1 differentiation (108). Like insulin, cAMP is known to activate CREB phosphorylation (109, 110).

All of the three stimulants have a critical role in adipogenesis, with the order of importance being MIX ≥ DEX > INS. It has been reported that 3T3-L1 pre-adipocytes can differentiate into adipocytes in the absence of INS, although INS treatment enhances the number of differentiated cells (100).

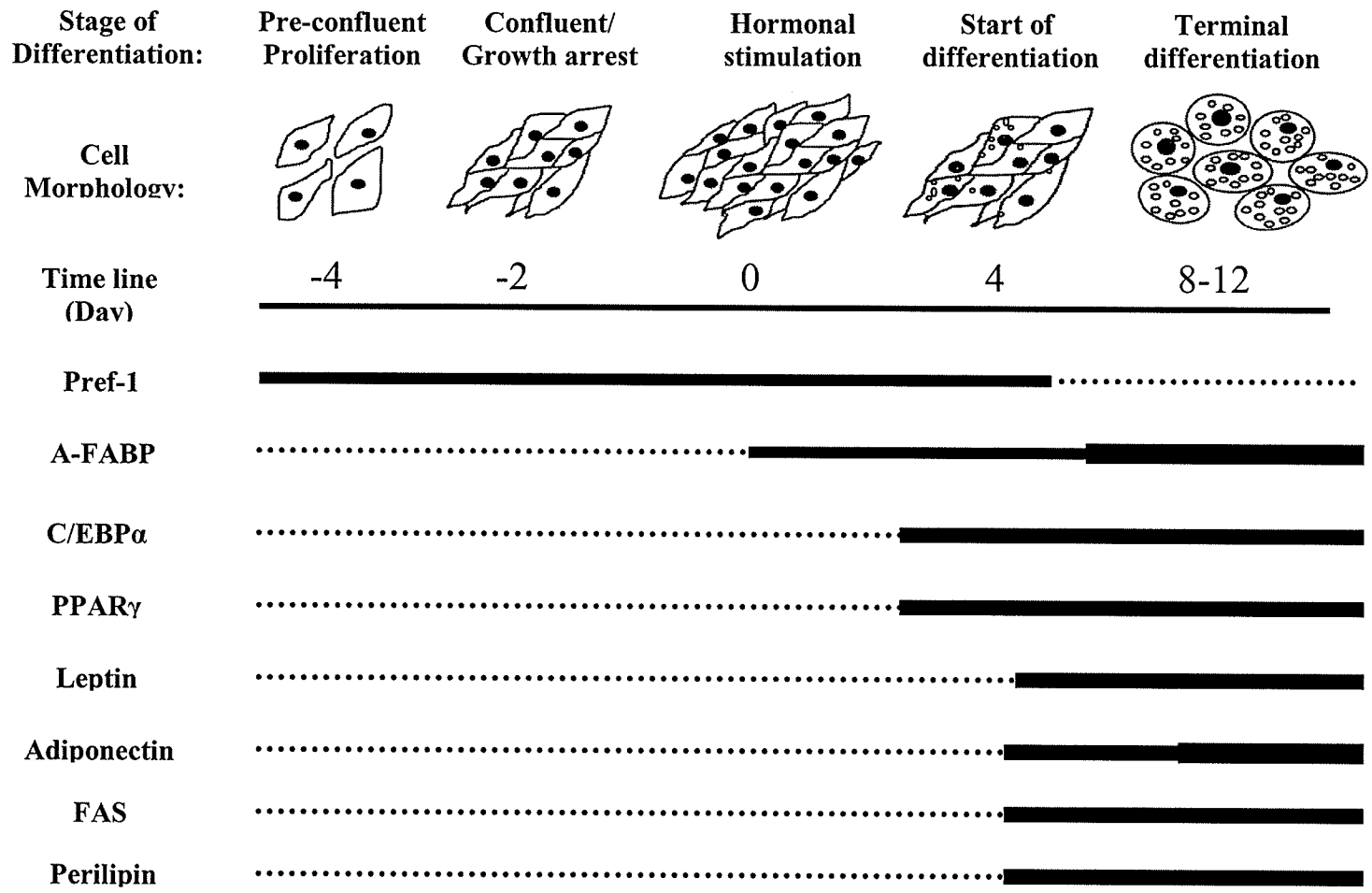
Following hormonal induction, the cell undergoes morphological changes that result in its conversion from a fibroblastic to a spherical shape. Prior to undergoing this change of shape, there are dramatic changes in the cytoskeleton such as decreases in tubulin and actin gene expression (111). The pathways involved behind this alteration or which component of the stimulation cocktail is responsible for this event is not known.

Overall, Pref-1 is still expressed in this phase, but after the addition of the adipogenic cocktail, pref-1 becomes undetectable. Addition of the adipogenic cocktail stimulates expression of SREBP-1 and C/EBP-δ and β (112, 113), which in turn trigger the production of PPARγ and C/EBP-α, the two master regulators of adipogenesis. Wnt signaling and MAPK activity, specifically the ERK-1 pathway, are decreased to reduce their effect on PPARγ activity. In contrast, insulin signaling is active throughout adipogenesis.

4. Terminal differentiation: Following the removal of the adipogenic cocktail on day two and completion of clonal expansion, the cells enter the final stage of differentiation. Insulin, however, is still retained in the culture media. Over the subsequent 4 days, the morphology of the cells changes from fibroblastic to spherical. The cells also fill with multiple lipid droplets. Activation of C/EBP-δ and β stimulates PPARγ and C/EBP-α, two key transcription factors. Activation of PPARγ and C/EBP-α leads to termination of mitotic clonal expansion through inhibition of cell cycle regulators such as cdk2 (114) and E2F (115). Over this time, some adipocytokines, including adiponectin, leptin, adipsin, omentin and chemerin, become detectable. As previously mentioned, cells also start to accumulate lipid in visible droplets, which is

indication of fatty acid synthase activity. The events that occur during adipocyte differentiation are summarized in Figure 1.

Figure 1: The events occur during 3T3-L1 differentiation



Adapted from: Robert M. et al.seminars in CELL & DEVELOPMENTAL BIOLOGY. Vol 10. 1999

1.8 Transcription factors involved in adipogenesis

Three transcription factor families that operate as adipogenic regulators are highlighted.

1.8.1 Peroxisomal Proliferator - Activated Receptor γ (PPARγ)

PPARγ, master regulator of adipogenesis, is a member of the nuclear hormone receptor family that includes retinoic acid receptors, thyroid hormone receptors and vitamin D_3 receptors (116). PPARγ 1 and PPARγ 2 are isoforms transcribed from a common gene but with different promoter start sites (117). PPARγ 2 has an extra 30 amino acids at its N-terminus, in comparison to PPARγ 1. PPARγ 2 is specific to adipocytes; whereas PPARγ 1 is found in other cell types besides adipocytes. Studies have shown that PPARγ expression is sufficient and necessary for adipogenesis (118, 119). Transfection of PPARγ 2 into PPARγ deficient cells restores adipogenesis, while over-expression of PPARγ 1 has almost no effect (120). PPARγ $^{-/-}$ mice have decreased adipose tissue mass, and embryonic fibroblasts isolated from these animals showed impaired adipogenesis (121).

PPARs are key factors for ensuring progression of the adipogenic program. PPAR response elements are present in the promoters of many adipocyte genes involved in fatty acid binding, such as aP2, also known as adipocyte-specific fatty acid-binding protein or 422 (122, 123), and fatty acid uptake, including those encoding lipoprotein lipase (124), phosphoenolpyruvate carboxykinase (123) and acyl-CoA synthetase (125).

Fatty acids and fatty acid derivatives are natural PPARγ ligands. Prostaglandins such as PGJ2 and PGD2 are able to activate PPARγ (126, 127). The retinoid X receptor (RXR) is the DNA binding partner and co-activator for PPAR (128), and RXR ligands, such as LG100268, promote 3T3-L1 pre-adipocyte differentiation (129). The most effective synthetic ligands for PPARγ are thiazolidinediones (TZDs), which are currently used for the treatment of type 2 diabetes (130). The endogenous PPARγ ligand is currently unknown, although sterol regulatory element binding protein-1c is involved in stimulating endogenous PPARγ ligand production (131). PPARγ 2, in turn, is responsible for induction of C/EBP-α.

1.8.2 CCAAT/ Enhancer Binding Protein (C/EBP)

Adipocytes express four of six members of the C/EBP family, including C/EBP-α, C/EBP-β, C/EBP-δ and C/EBP-ζ (also known as CHOP-10). The C/EBP family members have a similar ability to bind to the CCAAT motif found in several adipocyte gene promoters including stearoyl-CoA desaturase, GLUT-4, 422/aP2, phosphoenolpyruvate carboxykinase, and uncoupling protein (UCP) (132-135). The C/EBP family is involved in numerous activities such as cell growth and differentiation.

C/EBP-β and C/EBP-δ are early regulators of pre-adipocyte differentiation and over-expression of either factor in the pre-adipocyte enhances adipogenesis (113, 136). This result was supported by a study done with embryonic fibroblast cells derived from C/EBP-β or -δ deficient mice, which showed a marked reduction in adipogenic

activity compared to the wild type (137). Other studies have shown that C/EBP-β can increase PPARγ ligand production (131, 138).

C/EBP-β contains two cAMP response element binding protein (CREB) sequences in its promoter and phosphorylation of CREB maintains the transcriptional activity of C/EBP-β (139, 140). Elevated levels of CREB in the early phase of pre-adipocyte differentiation as well as CREB activation through phosphorylation are correlated with C/EBP-β expression.

The phosphorylation of C/EBP-β may play an important role in the clonal expansion stage of adipocyte differentiation. Many kinases can phosphorylate C/EBP-β including protein kinasecw A (141), protein kinase C (141), MAP kinase (142), Ca^{2+}calmodulin dependent kinase II (143), cyclin-dependant kinase 2 (cdk2) (144) and glycogen synthase kinase-3 (GSK-3) (145, 146). The phosphorylation of C/EBP-β enhances its DNA binding activity and consequently stimulates the process of pre-adipocyte differentiation. C/EBP-β and C/EBP-δ subsequently induce C/EBP-α.

Several investigators have shown that C/EBP-α expression is vital for adipogenesis (147). They also reported that conversion of fibroblasts to adipocytes is achievable by conditional expression of C/EBP-α, even without hormonal stimulation (148, 149). Ross et al (150) showed that phosphorylation of C/EBP-α on Thr222 and Thr226 by GSK-3, whose activity is antagonized by insulin via the phosphatidylinositol 3-kinase (PI3K) pathway, is required for adipogenesis.

C/EBP-ζ (CHOP-10), unlike the other C/EBPs, has anti-adipogenic activity, perhaps due to its ability to heterodimerize with the inactive C/EBP-β (136).

Despite the prominent role of the members of the C/EBP transcription factor family in adipogenesis, they are not functional in the absence of PPAR-γ. The induction of C/EBP-α expression by C/EBP-β is inhibited in the absence of PPAR-γ (151). In addition, adipogenesis in fibroblasts from PPAR-γ $^{-/-}$ is not restored by ectopic expression of C/EBP-α (152).

1.8.3 Sterol Response Element Binding Protein -1 (SREBP-1)

Adipocyte determination and differentiation-dependent factor 1 (ADD-1), a 98 kDa protein, belongs to the family of helix-loop-helix (HLH) transcription factors. SREBP-1 is the human homologue of ADD-1, whose expression is detected 24 h after adipogenic hormones are added (131). ADD-1 plays a positive role in adipocyte differentiation by regulating enzymes involved in the lipogenic pathway. It has been shown that a dominant-negative form of ADD1/SREBP-1 inhibits lipogenesis (153). It has also been proposed that SREBP-1 promotes adipogenesis by inducing the transcriptional activity of PPARγ/RXRα through an increase in expression of its endogenous ligand (154). One study reports that the activation of this transcription factor in response to insulin treatment in 3T3-L1 is elevated, which suggests that this protein is an insulin response factor (155).

1.9 Signaling molecules involved in adipogenesis

In addition to those important transcription factors discussed previously, other signaling molecules such as pre-adipocyte factor-1 (Pref-1), Wnts, MAPK (mitogen activated kinase) and insulin also regulate adipocyte differentiation (156, 157).

1.9.1 Pref-1/DLK signaling

Pref-1, a plasma membrane protein, inhibits adipocyte differentiation (93, 94). Pref-1 expression occurs in preadipocytes but it is not detected in mature adipocytes. Addition of dexamethasone to pre-adipocytes suppresses Pref-1 expression and makes the cells permissive for differentiation. This point is supported by the fact that preadipocytes that constitutively express Pref-1 fail to differentiate (93, 94). In contrast, over-expression of Pref-1 in adipose tissue causes lipolysis (93). It has been reported that Pref-1 has a supportive effect on Notch (a transmembrane receptor) signaling, since its activation inhibits adipocyte differentiation (158). Ross et al (159) showed that inhibition of some of the transcription factors such as HES1 and a bHLH, which regulate Notch and are down-regulated during adipogenesis (160), is dependent on Pref-1 suppression. It is also known that FOXA-2 inhibits adipocyte differentiation by activating transcription of the Pref-1 gene in pre-adipocytes (161).

1.9.2 Mitogen activated kinase (MAPK) signaling

The MAPK family includes extracellular signaling-regulated kinases (ERKs), p38 and c-Jun amino terminal kinases (JNKs). Each of these proteins is linked to intracellular signaling pathways that control many cellular processes, including proliferation and differentiation. The role of MAPK family members in adipogenesis is not completely clear due to conflicting results (162). In 1995, Sale et al (95) showed that in 3T3-L1 pre-adipocytes, ERK-1 and ERK-2 are required during the proliferative phase of differentiation. In contrast, other studies report that in the terminal phase of 3T3-L1 differentiation, phosphorylation of the adipocyte specific transcription factor PPAR-γ by ERK-1 inhibits adipocyte differentiation (163, 164). Altogether, the

literature findings demonstrate that ERK-1 activity is required for mitotic clonal expansion. Subsequently, its activity has to be reduced via MAPK phosphatase-1 (MKP-1) to enable the start of differentiation (165). It has been reported that the anti- or pro- adipogenic properties of p38 depend on the differentiation stage (162).

1.9.3 Insulin signaling

Insulin is one of the important ingredients of the adipogenic cocktail used to stimulate adipocyte differentiation in cell culture. In the early stages of differentiation, the insulin-like growth factor-1 (IGF1) receptor plays an important role, because pre-adipocytes have more receptors for IGF1 than for insulin (166). It has been shown that knocking down the insulin-receptor substrate (IRS) protein results in inhibition of adipogenesis (167). It is also known that IRS promotes phosphorylation of CREB which stimulates adipogenesis (168). Next in the insulin signaling cascade is PI3K following by protein kinase B (PKB)/AKT; inhibition of either protein prevents adipocyte differentiation (169). AKT/PKB regulates the phosphorylation of nuclear proteins, such as the forkhead family members FOXO1 and FOXA2 that have anti-adipogenic effects (161, 170). GATA2 is another nuclear protein that is phosphorylated by AKT; this nuclear protein can bind to PPARγ and the C/EBP promoter, and inhibit adipogenesis (171). The mediation of adipocyte differentiation signaling pathways by AKT was also reported by Kohn et al (172) who showed constitutive expression of AKT in 3T3-L1 cells results in their spontaneous differentiation into adipocytes, even in the absent of adipogenic cocktail.

1.10 Conjugated Linoleic Acid

CLA describes a mixture of positional and geometric isomers of octadecadienic (linoleic) acid. The most abundant CLA isomers in foods are *cis* 9-*trans* 11 followed by *trans* 7-*cis* 9; *cis* 11- *trans* 13; *cis* 8-*trans* 10; and *trans* 10-*cis* 12. CLAs are found in a number of different foods, with the highest concentrations found in the meats and milk of ruminant animals. Among the 21 possible geometric isomers of CLA, the c9-t11 and the t10-c12 isomers show the most biological activity of the isomers that have been studied in detail (173, 174). CLA isomers are produced in the rumen during the microbial biohydrogenation of linoleic acids (175). Biohydrogenation of dietary linoleic acid produces the c9-t11 isomer or rumenic acid (RA). Further hydrogenation results in t11-18:1 or vaccenic acid (VA). Both RA and VA can be absorbed, and taken up in tissues via the circulatory system. Once in tissues, VA may then be converted back to CLA. The conversion back to CLA occurs via the enzyme delta-9 desaturase (176). CLA is readily incorporated into the body's fat stores (triglycerides) and cell membranes (phospholipids) (173). In 1987, Ha et al (177) showed that synthetically produced CLA inhibits carcinogenesis in mice. Since then several physiological effects of CLA have been reported, including reduced adiposity in different animal models, modulation of immune function, and prevention of arteriosclerosis and Type 2 diabetes (178-183).

A number of recent articles have examined the effect of CLA on factors associated with adipogenesis. Ryder et al (184) observed beneficial effects of CLA-feeding in the Zucker diabetic fatty (ZDF) rat, a model of type 2 diabetes that, like the human disease, exhibits both insulin resistance (from a mutant leptin receptor causing

obesity) and inadequate pancreatic beta-cell compensation. Compared to controls, 6 week-old rats fed 1.5% CLA as a 50:50 mixture of the c9-t11 and t10-c12 isomers for 14 days showed reduced adiposity, fasting glucose, plasma insulin, and free fatty acids, as well as improved glucose tolerance and insulin sensitivity in muscle. These beneficial effects were not observed upon treatment with the c9-t11 isomer alone.

Furthermore, numerous studies have demonstrated that t10-c12 CLA treatment during adipocyte differentiation reduces lipid accumulation in both mouse and human adipocytes (185-188). House et al (189) investigated the effect of t10-c12 CLA on genes associated with lipid metabolism, adipokines and transcription factors involved in adipogenesis. This group reported a down-regulation of genes such as perilipin, caveolin, glycerol-3-phosphate acyltransferase, adiponectin, adipsin and PPARγ in mice fed t10-c12 CLA for 14 days, while genes such as phospholipase A2, uncoupling protein-1, caspase-3 and TNF-α were upregulated. In addition, Brown et al (190) showed with cultures of human stromal vascular (SV) cells containing newly differentiated adipocytes that chronic t10-c12 CLA treatment (18 days) decreases the expression of markers of adipocyte differentiation including adiponectin, aP2, LPL, perilipin, GLUT4, PPARγ 1/2 and C/EBP-α. In contrast, t10-c12 CLA treatment increased leptin mRNA. c9-t11 CLA treatment showed the opposite results on all of the examined genes. The results of this study also showed that c9-t11 CLA treatment rapidly activated MEK/ERK signaling, leading to phosphorylation of PPARγ, followed by a decrease in PPARγ target gene expression. One year later, the same group reported an effect of t10-c12 CLA on adipocyte lipolysis (191). This group also elucidated the role of this specific isomer on lipid droplet protein expression and the

size of droplets in SV cells. t10-c12 CLA treated cultures had smaller lipid droplets compared to c9-t11 treated cultures. t10-c12 CLA treatment also caused an increase in cytosolic perilipin. Furthermore, the small lipid droplets created by t10-c12 isomer treatment contain higher levels of adipose-differentiation related protein (ADRP). In contrast perilipin is more abundant on the larger droplets (191).

In support of these observations, Granlund et al (192) showed that inhibition of lipid accumulation induced by t10-c12 CLA treatment during adipocyte differentiation is associated with tight regulatory cross-talk between early (PPARγ and C/EBPα) and late (LXRα, aP2 and CD36) adipogenic marker genes. This finding has been confirmed in a microarray analysis described by House et al (189) who examined the effect of t10-c12 CLA on genes associated with lipid metabolism and adipocyte biology.

Claims that CLA is beneficial for human health remain unclear because of controversial results have been obtained from studies with rodent models in comparison to humans (193). In a 12 week study where mice were fed a high fat diet while receiving different dietary doses of CLA, body fat was lowered significantly in with doses ≥0.5% CLA (194). Growing female rats fed a diet containing 0.5% CLA showed a reduction in adipose tissue mass, but this change was far less than observed with the mice (195). In human studies, the results of CLA feeding have been inconsistent, with some studies showing a reduction in body fat mass (196) while in other studies CLA caused no significant change in body composition (197).

In addition, studies that have used pure preparations of single isomers have shown that some of the effects of CLA may be isomer-specific (185, 198). Also,

further investigation is needed to clarify the mechanism of how CLA influences adipogenesis and metabolism.

1.11 Connexin

In 1986, Paul and colleagues cloned and sequenced the first connexin (Cx), Cx 32 (199). Since then, 19 connexins have been cloned from mice and 20 from humans (200). Connexins are designated with the molecular weight of the deduced sequence in kilodaltons (e.g., connexin 43 or Cx43 is 43 kDa) (201). There are some sequence and structural similarities in the Cx gene family. For instance, all connexins have four transmembrane regions and two extracellular loops, and both amino and carboxyl termini are located in the cytoplasm (202, 203). These features are critical for functional assembly of gap junction channels (204). At the same time, connexins differ in the size of the cytoplasmic loop region and the length of the carboxyl terminal tail (201, 205, 206).

Gap junctions coordinate cell-cell communication and the transfer of molecules less than 1000 Da in size between cells. These include ions, amino acids, nucleotides, second messengers (e.g., Ca^{2+}, cAMP, cGMP, IP3) and various metabolites (200, 201, 207). Generally, gap junction channels between two cells contain the same connexin (homocellular gap junctions), although there have been reports of gap junction that consist of different Cx types (hetrocellular gap junctions) (208-210). Connexin expression varies in different tissues, with some being significantly expressed in only a few tissues and some, like Cx43, being more ubiquitous. Gap junction formation takes place by oligomerization of six connexin proteins into a hexameric hemi-channel, or

connexon, in the endoplasmic reticulum. This complex is then transported through the Golgi and exported to the plasma membrane, where hemi-channels are available for gap junction assembly; most of the Cx family including Cx43 follows this pattern but there are some exceptions. (211). Formation of gap junctions occurs when a connexon from one cell pairs with a connexon from an adjacent cell (212, 213). Since cell death could take place as a result of unrestricted connections between the cytoplasm and extracellular spaces, hemichannel function has to be tightly regulated. Various stimuli are responsible for gating these channels including voltage changes, pH and connexin phosphorylation (200, 201, 207, 214). Gap junctions play vital roles in embryonic development, apoptosis, differentiation, tissue homeostasis and metabolic transport in non-vascularized tissue (200, 215). Recent studies indicate that there is an association between deficient or improper gap junction function and diseases such as neuropathy, hereditary deafness, cataracts, skin disease, heart disease, and cancer (201).

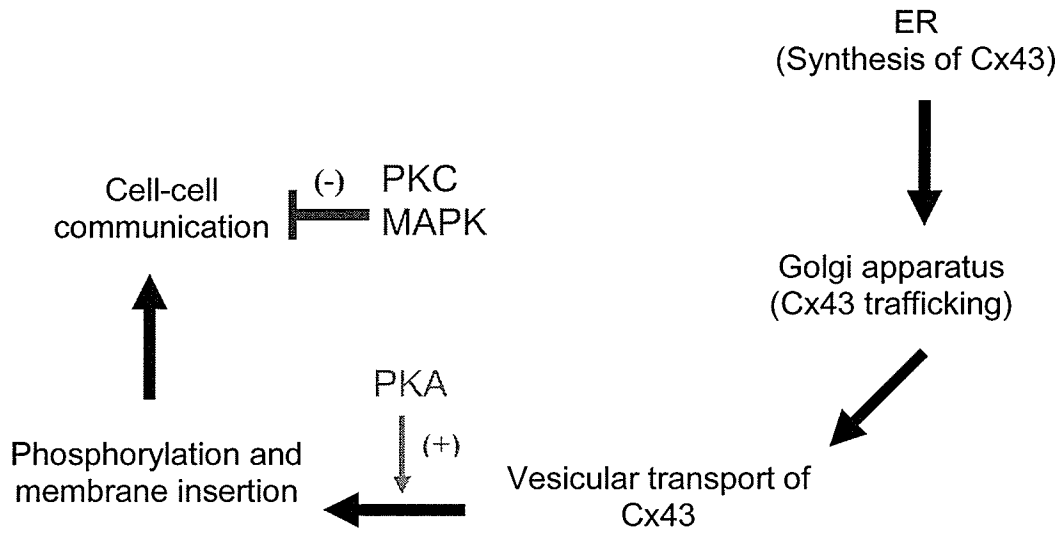
1.12 Connexin 43

Connexin 43 has a half-life of 1.5-3.5 h in cultured cells or in tissue (216-218). The C-terminal region of Cx43 appears to be the primary site for phosphorylation (217, 219-221) since N-terminal phosphorylation has not been reported yet. At least five different kinases target 12 or more serine and tyrosine residues within this region, including Src kinase, casein kinase 1 and $p34^{cdc2}$. In addition, mitogen activated protein kinase (MAPK) and protein kinase C (PKC) are two kinases which phosphorylate Cx43 to close the hemichannels (215).

Another kinase which targets the Cx43 C-terminus is protein kinase A (PKA), which is activated by cAMP, one of the small molecules that can pass through gap junctions. It has been reported that PKA activation by cAMP causes Cx43 phosphorylation in rat cardiomyocytes (222), but it was not the same in mouse fibroblasts (223). In addition, Dowling-Wariner et al (224) showed that Cx43 phosphorylation is necessary for differentiation of the human neural-glial cell line SVG. They showed that treatment of SVG with PKA activators such as forskolin or forskolin + 3-isobutyl-1-methylxanthine (MIX) can induce Cx43 phosphorylation along with SVG differentiation. Likewise, up-regulation of gap junctional coupling by agents that stimulate cAMP has been demonstrated in a number of cell types including hepatocytes (225, 226), fibroblasts (227), lung epithelial cells (228), sympathetic neurons (229) and gonadotropin-releasing hormone neurons. The role of different protein kinases in the life cycle of Cx43 is shown in Figure 2.

Even gap junction assembly seems to be a process that is correlated with Cx43 phosphorylation (230, 231). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) resolves multiple electrophoretic isoforms of Cx43, including a non-phosphorylated (P0 or NP) form, which migrates the fastest, and at least two slower migrating phosphorylated forms, commonly termed P1 and P2 (219, 232). Without a doubt, phosphorylation of Cx43 can occur before it reaches the plasma membrane, but phosphorylation of Cx43 may be transient or simply unnecessary for transport to the plasma membrane; the presence of the non-phosphorylated Cx43 at the plasma membrane is evidence in support of this statement (219, 230). Although phosphorylation of Cx43 in nonjunctional plasma membrane regions is not necessary,

it has been reported that Cx43 located in gap junctions is typically phosphorylated to the P2 form and resistant to solubilization by Triton X-100 (230). In contrast, phosphorylated Cx 43 in nonjunctional plasma membranes is soluble in 1% Triton X-100 (230).



Adapted from: J.L. Solan, P.D. Lampe / Biochimica et Biophysica Acta 1711 (2005) 154–163

Figure 2. Role of different protein kinases in the life cycle of Cx43

Cx43 produced in the endoplasmic reticulum (ER) assembles into a hexameric connexon and is then transported to the Golgi apparatus. After export to the plasma membrane, single Cx43 connexons (hemichannels) will accumulate at new gap junctions. The actions of different protein kinases on Cx43 transport are indicated (green = increase, red = reduction).

2 Rationale

Little research has been done to examine the link between adipogenesis and connexins as indicated by the fact there are just three general publications on this topic. In 1985, Azarnia et al (233) reported that 3T3-L1 cells lose their ability to communicate via gap junctions as they differentiate. Then, in 1992, Umezawa et al (234) showed that expression of Cx43 is down-regulated at the transcriptional level during the adipogenesis in H-1/A cells, a marrow stromal cell line that differentiates into adipocytes. Finally, Yanagiya et al (235) reported in 2007 that functional gap junctions are required for mitotic clonal expansion of 3T3-L1 pre-adipocytes. Since Cx43 is the major connexin in these cells, it is evident that both Cx43 levels are important for adipogenesis.

Many factors involved in adipogenesis are altered by nutrients. CLA is one of the nutraceuticals that can affect adipogenesis and has been investigated previously. These reports indicate that CLA affects the expression of several adipogenic markers. Consequently, the present study therefore proposes to examine the effect of CLA on expression of Cx43, since its expression is also modulated during the early stages of adipocyte differentiation. This represents a novel extension of previous experiments studying the relationship between diet and the generation of new adipose tissue.

3 Hypotheses

In light of the above observations, we hypothesize that:

1. Constitutive expression of Cx43 will inhibit adipocyte differentiation, since the level of Cx43 decreases through adipocyte differentiation.
2. Changes in Cx43 phosphorylation during adipogenesis are required for adipocyte differentiation.
3. Nutraceuticals such as conjugated linoleic acid (CLA) will modulate the level of Cx43 and its phosphorylation state during adipogenesis.

3.1 Aims of the study

1. Examine the effect of constitutive Cx43 expression on adipocyte differentiation.
   - Will it inhibit differentiation completely or merely reduce it?
   - Does over-expression have any effect on lipid droplet accumulation or lipid droplet proteins such as perilipin?
   - How will Cx43 over-expression affect differentiation markers such as perilipin, A-FABP and PPARγ?
2. Investigate the effect of constitutive Cx43 expression on adipokines such as adiponectin and adipsin.
3. Determine Cx43 phosphorylation status during adipogenesis.
4. Investigate the role of each element of the adipogenic cocktail (INS, DEX, MIX) in mediating the phosphorylation of Cx43.
5. Investigate the subcellular localization of Cx43 during adipogenesis.

6. Investigate the effect of nutraceuticals such as conjugated linoleic acid (CLA) in modulating the level of Cx43 and its phosphorylation state during adipogenesis, as well as in the adipose tissue of rats given a diet containing CLA.

3.2 Experimental Approaches

A variety of different experimental methods were used to complete the aims of the study. Immunoblotting was used to measure levels of Cx43 protein, transcription factor and adipokines. Because of a lack of suitable antibodies, only PPARγ was used to represent the various adipogenic transcription factors. This method was also valuable for monitoring Cx43 phosphorylation. Constitutive expression of Cx43 was achieved by adenovirus infection in 3T3-L1 CARΔ. Immunofluorescence was employed to examine localization of Cx43 and confirm the immunoblotting results. Immunofluorescence also enabled us to view the morphological changes that occur during adipogenesis. Cx43 localization was also monitored by subcellular fractionation. Finally, the effects of nutraceutical treatments on 3T3-L1 differentiation were possible via direct addition of CLA into the culture medium. Previous studies showed that t10-c12 CLA affects adipocyte differentiation and inhibits lipid droplet formation, however, the effect of CLA isomers on Cx43 expression has not been examined. In this study, the effect of the CLA isomers t10-c12 and c9-t 11 on expression of Cx43 expression during 3T3-L1 differentiation and rat adipose tissue was investigated. In parallel, Cx43 expression in the adipose tissue of rats fed a diet containing CLA was also examined.

4 Materials and Methods

4.1 Cell Culture and Differentiation

3T3-L1 and 3T3-L1 CARΔ are two different pre-adipocyte cell lines that were used in this study. The 3T3-L1 pre-adipocyte cell line is one of the preferred models for the examination of mesenchymal cell differentiation into adipocytes (adipogenesis). Unfortunately, transfection of 3T3-L1 cells is inefficient; therefore, Orlicky et al (236) generated a stable 3T3-L1 line that expressed a gene encoding the coxsackie and adenovirus receptor (CAR). Thus, 3T3-L1 CARΔ exhibit improved adenovirus infection efficiency relative to 3T3-L1. Both cell lines were grown in DMEM (Dulbecco's modified Eagle's medium). For 3T3-L1, 10% calf serum was added to the medium in the growth stage and 10% FBS (fetal bovine serum) during differentiation. In contrast, 10% HI-FBS (heat-inactivated fetal bovine serum) was used for 3T3-L1 CARΔ in both growing and differentiating stages. The media are also supplemented with 20 mM HEPES, 100 units/ml penicillin, and 100 μg/ml streptomycin. The cells were maintained in a humidified atmosphere with 95% air/5% CO_2. To induce differentiation, the cells were allowed to reach confluence and after two days, cells were placed into DMEM (10% FBS or 10% HI-FBS supplemented) with 0.5 mM 1-methyl 3-isobutylxanthine (MIX), 0.25 μM dexamethasone, and 10 μg/ml insulin. This was considered day 0. After two days, the media were refreshed, but only 10 μg/ml insulin was added. The media were refreshed every 48 hours until the end of the experiment. Cells started differentiating on day 4; cells could be cultured up to day 12 although most experiments were terminated by day 8.

4.2 Cell treatments

For experiments investigating modulators of PKC and PKA on adipogenesis and Cx43 phosphorylation, the cells were treated with adipogenic induction medium after addition of PKC β_I inhibitor, bisindolylmaleimide I, KT5720, and forskolin. (See Table 1). Cells were lysed on day 2 and then at intervals until the morphologic features of differentiation were established (Day 8); the medium was changed every 2 days and treatments added concurrently. CLA treatment was achieved by adding 60 μM t10-c12, 60 μM c9-t11, and 60μM of both isomers directly to the media. The specific concentration of CLA was based on the results of Kristin Streuber, who showed 60 μM of CLA was the optimal dose capable of affecting adipocyte differentiation and lipid accumulation (237). The frequency of treatment and experiment termination were like other experiments.

4.3 Adipocyte size

Photographs of 3T3-L1 cells were taken before the cells were lysed. Digital images were captured with an Olympus digital camera (Model No.C-5050) and cell area was measured with the open-source image analysis program ImageJ v1.34s (Rasband, WS, ImageJ, US National Institutes of Health, Bethesda, MD, http://rsb.inf.nih.gov/ij/, 1997-2006) as previously described by Noto et al (238).

4.4 Adenovirus Infection

Constitutive Cx43 expression was achieved with adenovirus infection. Adenovirus expressing Cx43 (Q Bio gene) was titred using the tissue culture infectious dose (TCID) method. In this method HEK-293 cells were infected with different

concentrations of adenovirus in two separate sets of 96 well plates. The cells were maintained in 2% DMEM for 10 days. At the end of the titring period, the number of wells with dead cells were counted and this value was used to calculate the PFU (plaque formation unit) of the adenoviral stock (PFU is a measure of the adenovirus concentration). The Cx43-expressing adenovirus was applied to the cells on day 0 (growth-arrested stage) at the same time as the adipogenic cocktail. The negative control (not exposed to adenovirus) was processed in exactly the same way as treated cells. Expression of Cx43 was used to determine the efficiency of infection. The adenovirus was applied at 0, 50, 100 and 150 multiples of infectivity (MOI). One hundred MOI was the most effective dose so it was employed in all the experiments. The experiment was terminated by addition of lysis buffer and differentiation endpoints were quantified by imunoblotting for adipocyte markers such as A-FABP, perilipin, adiponectin and adipsin.

4.5 Immunoblotting

Protein was extracted from growing, growth-arrested and differentiating 3T3-L1 and 3T3-L1 CARΔ cells by lysing with 2×sample buffer (20% glycerol, 0.05 M Tris and 10% SDS). After sonicating the samples, the Bicinchoninic Acid (BCA) protein assay from Pierce (Rockford, IL) was used to determined the protein concentration of all cell lysates to enable equal protein loading on to the SDS-polyacrylamide gels.

Ten μg of lysate protein was separated on SDS-polyacrylamide gels (% depended on the size of the desired protein, the protein smaller than 30 kDa run on 15% gel, range of 30-100 kDa protein run on 7.5% or 10% gel and for protein greater

than 100 kDa 5% gel was used) at 20 mA constant current per gel for 70-90 minutes and transferred electrophoretically to PVDF (polyvinylidene difluoride) membranes at 100 volts for 60 minutes for 1.0 mm thick gels. For large proteins (5% gel), the amount of methanol used for the protein transfer was halved. To determine the molecular mass, Bench Mark™ Prestained Protein Ladder (Bio–Rad) was used. The membranes were blocked with 3% BSA-TBST (bovine serum albumin in Tris-buffer saline with Tween-20) then exposed to primary antibody for 1 hour. Subsequently, the primary antibody was removed and the membrane washed for a minimum of 20 minutes in 1× TBST, with the TBST refreshed at least four times. The membrane was then incubated with horseradish peroxidase-conjugated secondary antibody for 1 hour and the wash repeated as previously described. All secondary antibodies were used at a dilution of 1:10000 in PBS containing 1% BSA except rabbit HRP, which was used at 1:15000 (only for Cx43 polyclonal). The antibodies are listed in Table 2. The membrane was dipped in ECL plus Western blotting detection system (GE Health Care) and the relative intensity was captured by exposing the membrane to film (Kodak Scientific Imaging). The band of interest on the film was quantified by densitometry with a model GS-800 Imaging Densitometer (Bio-Rad Laboratories, Hercules, CA).

4.6 Adipose tissue sections

To investigate the presents of Cx43 in adipose tissue by immunofluorescence staining, 5 μm sections of rat adipose tissue frozen in OCT (Optimal Cutting Temperature) embedding medium were used. The working temperature on the Cryostat was set to -50°C. The sectiones were kept at -80°C until they were used for immunofluorescence staining.

4.7 Immunofluorescence

To study the distribution pattern and relative amounts of Cx43 and FAS, a marker of mature adipocytes, immunofluorescence staining was performed on 3T3-L1 or 3T3-L1 CARΔ cells grown on coverslips. The same growth conditions as those used for the immunoblotting experiments were used. Immunofluorescence was conducted as previously described by Doble et al (239). Briefly, cells were fixed with fresh 4% paraformaldehyde. The coverslips were then washed in PBS, and treated with 3% BSA for 1 hour to prevent nonspecific antibody binding. The cells were incubated overnight at 4°C in a moist chamber with the desired primary antibody. After three PBS washes, the cells were incubated for 1 hour with fluorescein donkey anti-rabbit Alexa IgG (H+L) (Molecular Probe) or fluorescein donkey anti-mouse Texas Red (Jackson Lab.) containing 1% BSA in PBS. After two PBS washes, the coverslips were stained with Hoechst 33342 (nuclei dye) for 2-3 minute after diluting to 1:40000 in PBS. After three PBS washes, the coverslips were mounted (biømeda corp.) and allowed to dry overnight. Negative controls were processed in the same manner as those in the experimental groups except that the primary antibody was omitted. The cells were viewed and photographed using a Zeiss Axiovert 3.0 microscope.

4.8 Subcellular fractionation

To investigate the localization of Cx43 during differentiation and confirm the immunofluorescence results, subcellular fractionation was employed. The subcellular Proteome Extraction kit from Calbiochem (Cat #539791, Lot #B77036) was used on 3T3-L1 cells on day -3 (growing), day 0 (growth arrested), day 2 (mitotic clonal

expansion), day 4 (mid-differentiation) and day 8 (differentiated) to seprate cytosol, membrane, nuleus and sytosckeleton fractions. The same procedure was applied to undifferentiated cells. Whole cell lysates were also collected at each stage. The experiment was performed in 4°C. The protein content of each fraction was assayed as described previously and 5 μg protein was loaded on to a 10% SDS-polyacrylamide gel. Western blotting was used to detect total Cx43 and specific markers of each fraction.

4.9 Animal study

Seven week old male fa/fa and lean Zucker rats were fed t10,c12 and/or c9-t11CLA for a period of 8 weeks (0.4% of diet was from CLA in free fatty acid form). Zucker rats on 0% CLA in semi-purified control diet (CTL) were used as controls. Animals were terminated when 15 weeks old and the adipose tissues collected for histology and protein analysis purposes were frozen immediately.

4.10 Data analysis

Data are presented as mean ± SEM (standard error of the mean). Statistical analysis was performed using one way or two-way Analysis of Variance (ANOVA) using SAS program, as indicated in the figure legends. Significant differences among treatment group means were determined with Duncan's Multiple Range test. Differences were considered statistically significant at $P<0.05$.

Table 1. Chemicals and functions

Chemical	Functions	Working Conc.	Vehicle	Company	Catalog #
Bisindolylmaleimide I	PKC α, β_I,β_{II}, γ, δ and ε inhibitors	10^{-5} M	DMSO	Calbiochem	203290
cAMP-CPT	Active cAMP (PKA activator)	4×10^{-3}M	Water	Calbiochem	
Dexamethasone	synthetic glucocorticoid agonist	0.25 μM	Water	Sigma	D4902
Forskolin	PKA Activator	10^{-5} M	DMSO	Sigma	F-6886
Insulin	Activate Insulin receptor	10^{-6} M	Water	Calbiochem	407694
KT5720	PKA selective inhibitor	10^{-6} M	DMSO	Biomol	EI-199
methylisobutylxanthine	cAMP phosphodiesterase inhibitor	0.5 mM	DMSO	Sigma	I5879
PKC β1 inhibitor	PKC β_I inhibitor	10^{-6} M	DMSO	Calbiochem	539654
Ponceau S	Protein stain	N/A	Water	Sigma	P7170-1L
c9-t11 CLA	*Cis*9-*trans*11 CLA isomer	60 μM	Ethanol	Cayman Chemical Co.	90140
t10-c12 CLA	*Trans*10-*cis*12 CLA isomer	60 μM	Ethanol	Cayman Chemical Co.	90145

Table 2. Antibodies and applications

Antibody	Secondary	Application	Dilution	Size (kDa)	Catalog #	Company	Function
ACRP30 (Adiponectin)	Rabbit	WB	1:1000	30	107920	Calbiochem	Adipokine
Actin	Mouse	WB IF	1:1000 1:400	42	A-2547	Sigma	Cytoskeleton protein
Adipsin	Goat	WB	1:1000	45,28	SC-12402	Santa Cruz	Adipokine
A-FABP	Goat	WB	1:2000	16	SC-18661	Santa Cruz	Cytoplasmic protein that mediates fatty acid uptake and its transportation
Calnexin	Rabbit	IF	1:150	90	2433	Cell Signaling	ER protein
Calreticulin	Goat	IF	1:400	47	Custom	Dr. Mesaeli Lab.	ER protein
Chemerin	Goat	WB	1:1000	16	AF2325	R&D System	Adipokine
Cx43 (total)	Rabbit	WB IF	1:20,000 1:2000	43	Custom	Dr. Kardami Lab.	Gap junction protein
Cx43 (total)	Mouse	IF	1:100	43	610062	Transduction laboratory	Gap junction protein
Cx43 (UnPhospho)	Mouse	IF	1:200	43	13-8300	Zymed	Gap junction protein
eEF2	Rabbit	WB	1:1000	100	2332	Cell Signaling	Loading control
FAS	Mouse	WB IF	1:1000 1:250	265	610962	BD Trans Labs	Enzyme involved in fatty acid synthesis

Table 2. (cont'd)

Antibody	Secondary	Application	Dilution	Size (kDa)	Catalog #	Company	Function
GAPDH	Mouse	WB	1:5000	36	Ab1484	Abcam	Loading control
GM130	Mouse	IF	1:150	130	SC-55590	Santa Cruz	Cis Golgi matrix protein
IGF-1 receptor β	Rabbit	WB	1:1000	95	3027	Cell Signaling	Membrane protein
Leptin	Rabbit	WB	1:1000	16	SC-842	Santa Cruz	Adipokine
PPAR γ	Mouse	WB	1:750	67	SC-7273	Santa Cruz	Adipocyte specific transcription factor
Perilipin	Guinea Pig	WB	1:4000	50	RDI-PROGP29	Fitzgerald	Adipocyte lipid droplet coated protein

5 Results

5.1 Adipogenesis and expression of Cx43

To examine the expression of total Cx43 during adipogenesis, 3T3-L1 cells were induced to differentiate into adipocytes by being subjected to adipogenic cocktail containing INS, DEX and MIX two days post-confluence. This was designated day 0. After two days, the media were refreshed and only insulin was added. The media were refreshed every 48 hours until the end of the experiment on day 8. Cx43 was analyzed by Western blotting with an antibody that detects both phosphorylated and unphosphorylated Cx43 (this Ab is designated Total Cx43). Representative results are shown in Figure 3a. Preadipocytes expressed low levels of both phosphorylated and non-phosphorylated Cx43. Placing the cells into adipogenic medium triggered an increase in the amount of Cx43 on days 1 and 2. In parallel, Cx43 phosphorylation was elevated. Thereafter, both Cx43 protein levels and phosphorylation decreased. By day 4, phosphorylated Cx43 was no longer detectable. Levels of total (non-phosphorylated) Cx43 in day 6 adipocytes were decreased compared to those in preadipocytes. Quantification of these data (both phosphorylated and non-phosphorylated forms) showed there was a significant increase ($P=0.001$, $n=5$) in the level of total Cx43 on day 2 as compared to day 0. Also, levels of total Cx43 on days 6 and 8 were significantly lower ($P=0.001$, $n=5$) compared to day 2 (Figure 3b). In these experiments, adiponectin (ACRP30) was used as a marker of adipocyte maturation. Adiponectin expression was first detected in 3T3-L1 cells on day 2 and was maximally

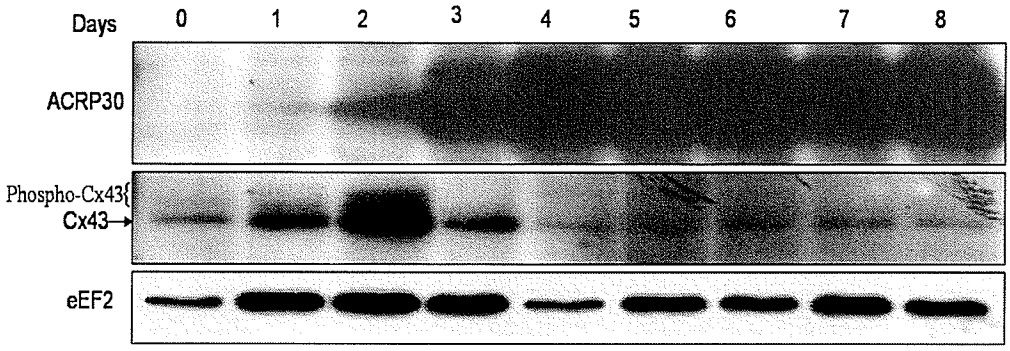
expressed from day 4 until the experiment was terminated (Figure 3a, data not quantified). GAPDH or eEF2 were employed as internal loading controls.

Figure 3. Expression of Cx43 and its phosphorylated form during 3T3-L1 cells differentiation

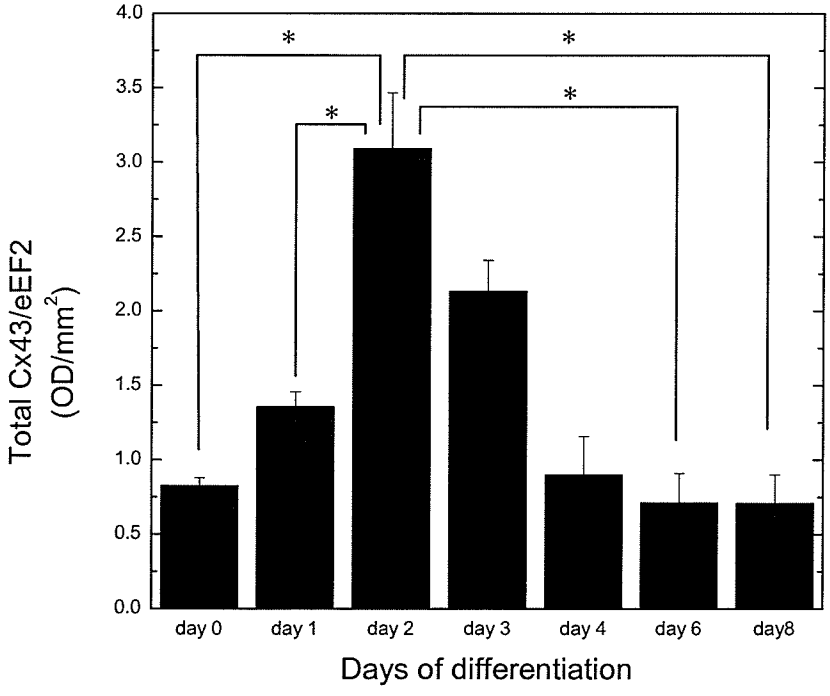
(a) Adipocyte lysates were prepared from cells at different stages of adipogenesis; day 0 (growth arrested), day 2 (mitotic clonal expansion), day 4 (mid-differentiation) and day 8 (differentiated). Western blotting was done with Rabbit Abs for total Cx43 and ACRP30 (mature adipocyte marker). eEF2 was used as internal loading control. Ten μg protein was loaded on each lane.

(b) Densitometric assessment was used to quantify total Cx43 band intensity. Data were adjusted according to the loading control (eEF2). Data are shown as mean ± SEM. Statistical analysis was conducted using one way ANOVA (P=0.001) and Duncan's Multiple Range Test. Differences among means are indicated by *, n=4.

(a)


Days
0
1
2
3
4
5
6
7
8
ACRP30
Phospho-Cx43
Cx43
eEF2

(b)


Total Cx43/eEF2
(OD/mm^2)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*
*
day 0
day 1
day 2
day 3
day 4
day 6
day8
Days of differentiation

5.2 Cx43 expression in adipose tissue

To confirm the results of the in vitro experiments, levels of total Cx43 were measured in adipose tissue, using Western blotting and immunofluorescence methods. Protein extracts prepared from the perirenal adipose tissue of 15 week old male lean Zucker rats (kindly provided by Vanessa DeClercq) were loaded on a 10% SDS-polyacrylamide gel and the amount of total Cx43 was determined. A lysate of day 4 differentiated 3T3-L1 cells was used as a positive control and GAPDH served as an internal loading control (Figure 4). In addition, immunocytochemistry was used to examine sections of adipose tissue. Total Cx43 antibody was used in these experiments. Negative controls were processed in the same manner as those in the experimental groups except that the primary antibody was omitted.

We were unable to detect Cx43 in adipose tissue by Western blotting. Immunofluorescence showed no difference between negative control and the experimental groups, which indicates Cx43 is absent in adipose tissue (Figure 5).

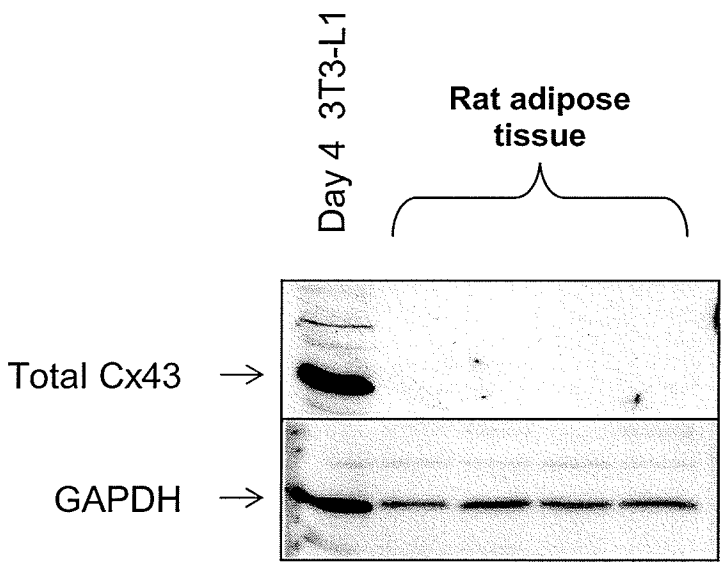


Figure 4. Total Cx43 expression in adipose tissue

Western blot of adipose tissue from four different animals probed for total Cx43. Cell lysate of 4 day differentiated 3T3-L1 cells was used as a positive control. GAPDH served as loading control.

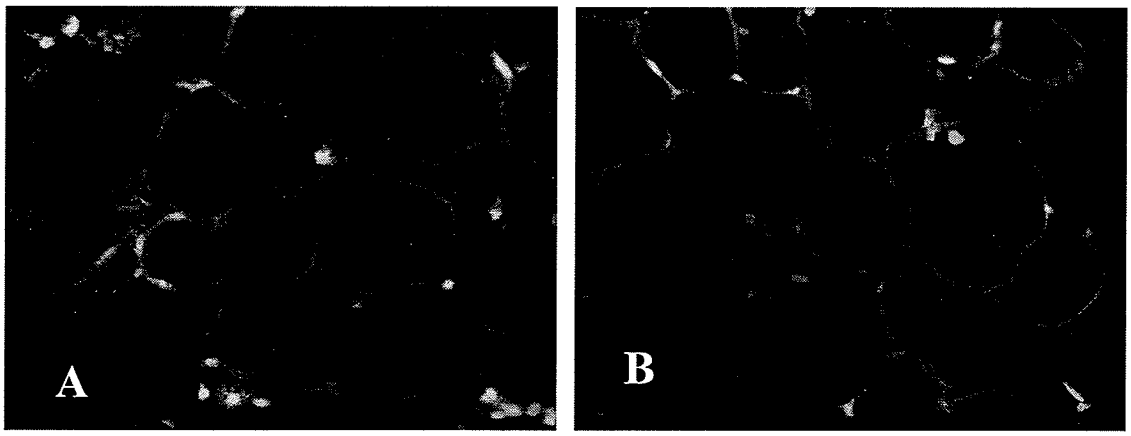


Figure 5. Total Cx43 expression in adipose tissue

Immunofluorescence staining of 5 μm sections of rat adipose tissue for total Cx43. No difference was observed between control (A) (without primary antibody) and the actual experiment (B) (with primary antibody). Total Cx43 is indicated in green and nuclei are stained blue.

5.3 Expression and localization of total Cx43 during adipogenesis

Immunofluorescence staining was performed on 3T3-L1 cells grown on coverslips. Cells were differentiated as previously described. Cells were fixed with fresh 4% paraformaldehyde at different stages of adipogenesis, including day -2 (growing pre-adipocytes), day 0 (growth arrested), day 2 (mitotic clonal expansion), day 4 (start of differentiation), day 6 (mid-differentiation) and day 10 (fully differentiated). Fixed cells were maintained at 4°C in PBS until differentiation was completed (day 10). Cells were then stained with antibodies to total Cx43, with the latter serving as a marker for mature adipocytes (Figure 6).

In preadipocytes (day -2 & 0), Cx43 was located in the cytoplasm, whereas during the early stages of differentiation (day 2) it was present mostly in the cell membrane. After day 6, the pattern of Cx43 staining resembled the pre-adipocyte stage, with no Cx43 in the membrane (Figure 6).

To examine the phosphorylation status of Cx43 during adipogenesis, immunofluorescence staining was performed on fixed cells at various differentiation stages. Cells were co-stained with total Cx43 and unphosphorylated Cx43 antibodies and images of these individual antibodies were merged to identify phosphorylated Cx43.

This experiment showed that pre-adipocytes contain both phosphorylated and unphosphorylated Cx43 (Figure 7). However, Cx43 phosphorylation is increased as cells become confluent (day 0) and during the hormonal induction stage (as observed in Figure 3).

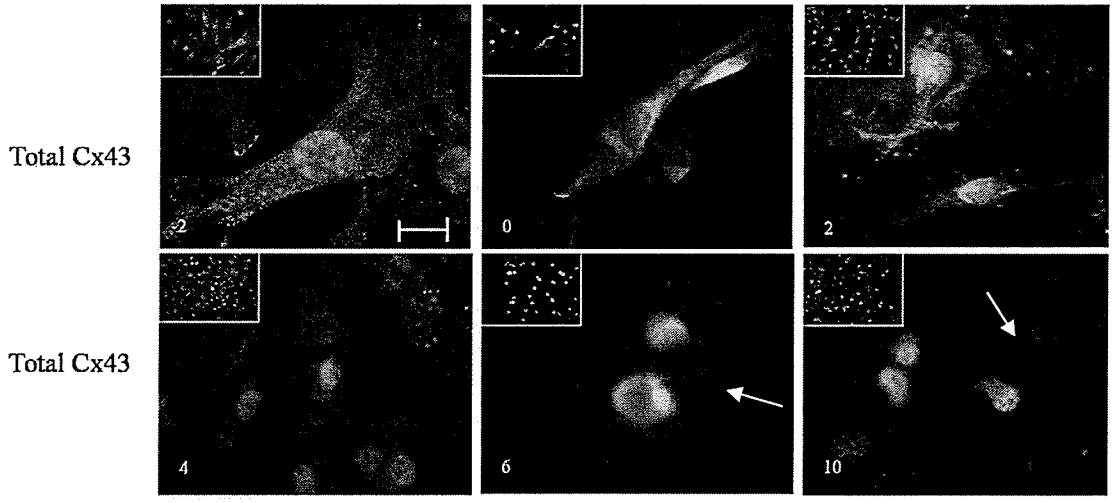
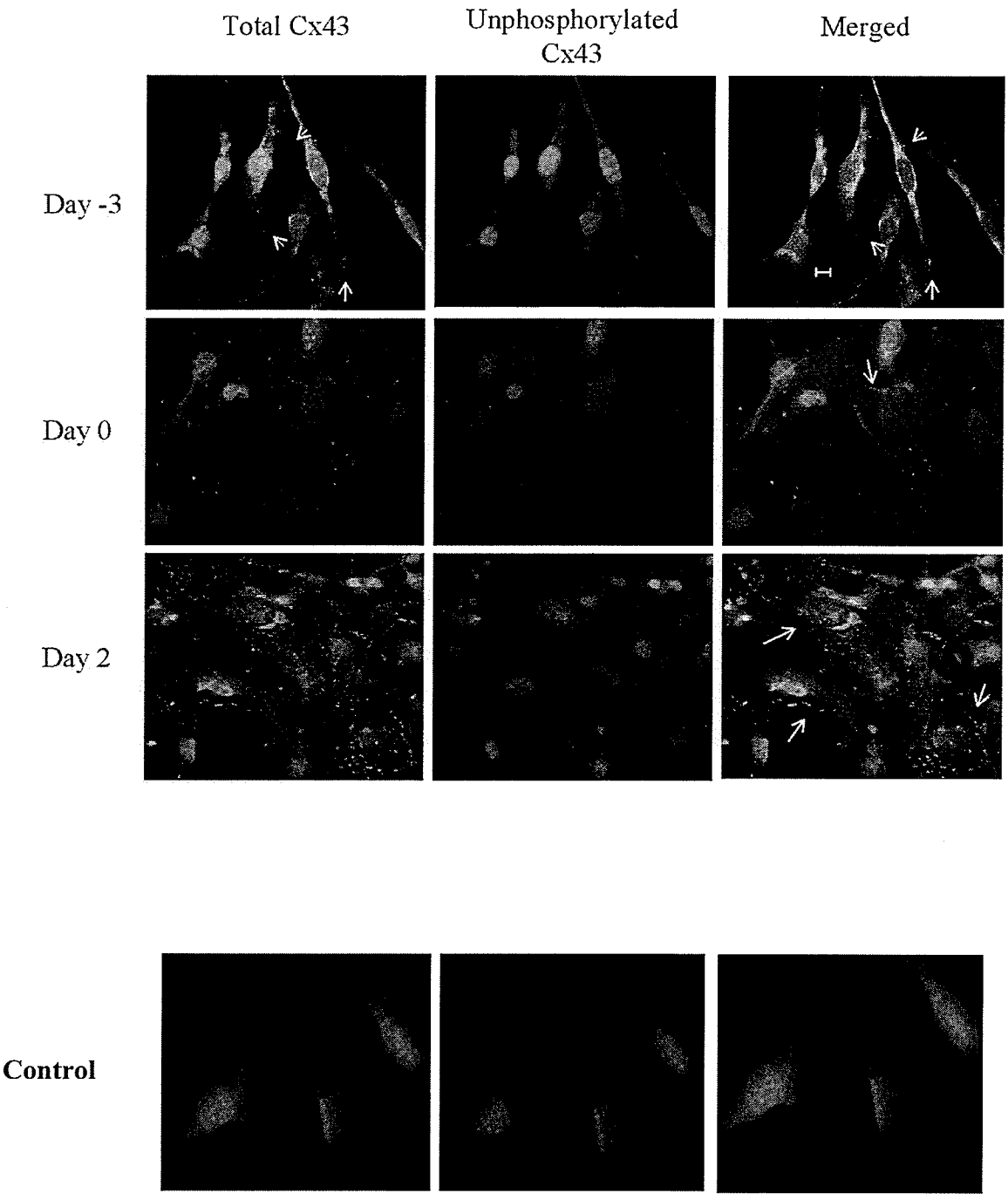


Figure 6. Localization of Cx43 during adipogenesis

Immunofluorescence staining was performed on fixed cells at different stages of differentiation as indicated in bottem left corner of each picture. The photos depict cells on day -2 (growing), day 0 (growth arrest), day 2 (mitotic clonal expansion), day 4 (start of differentiation), day 6 (mid-differentiation) and day 10 (fully differentiated). Total Cx43 is indicated in green and the nuclei are blue. The arrows show the lipid droplets in differentiated cells on days 6 and 10. The inset shows the general population of 3T3-L1 cells on the indicated differentiation days. The pictures are representative of 3 independent experiments. Bar represents 2 μm (indicated on image of day -2) and is applicable to all other panels.

Figure 7. Phosphorylation and localization of Cx43 in the early stages of adipocyte differentiation

3T3-L1 pre-adipocytes were fixed on day -3 (growing stage, top), day 0 (growth arrested, middle) and day 2 (mitotic clonal expansion, bottom), and examined by immunocytochemistry as described previously. The cells were co-stained with total Cx43 and unphosphorylated Cx43 antibodies and images of these individual antibodies were merged to identify phosphorylated Cx43. Green is total Cx43 and red is unphosphorylated Cx43. The arrows show the green dots which represent phosphorylated Cx43. The phosphorylated Cx43 mainly appears around the cells in a position that likely represents the plasma membrane. The pictures are representative of 3 independent experiments. Control panels show 3T3-L1 cells stained in the absence of primary antibody. Bar represents 2 μm (indicated on merged image of day -3) and is applicable to all other panels.


Total Cx43
Unphosphorylated Cx43
Merged
Day -3
Day 0
Day 2
Control

5.4 Role of adipogenic medium on expression and phosphorylation state of total Cx43 during adipogenesis

The amount of Cx43 can be influenced by two factors, confluency of the cells and adipogenic medium. As cells became confluent, the amount of total as well as phosphorylated (>41 kDa) Cx43 increased. Addition of adipogenic cocktail also led to up-regulation of phosphorylated (>41 kDa) and unphosphorylated (~ 41 kDa) Cx43 after two days (see Figure 3). Since phosphorylation of Cx43 is important for its functions, the role of the three components of the adipogenic cocktail in these events was investigated.

Two day post-confluent 3T3-L1 cells were treated with INS, DEX and MIX individually and in combination for 48 hours. Cells exposed to the three ingredients and without any treatment served as positive and negative controls, respectively. At the end of the experiment, cells were lysed using 2×sample buffer and the level of total Cx43 measured by Western blotting.

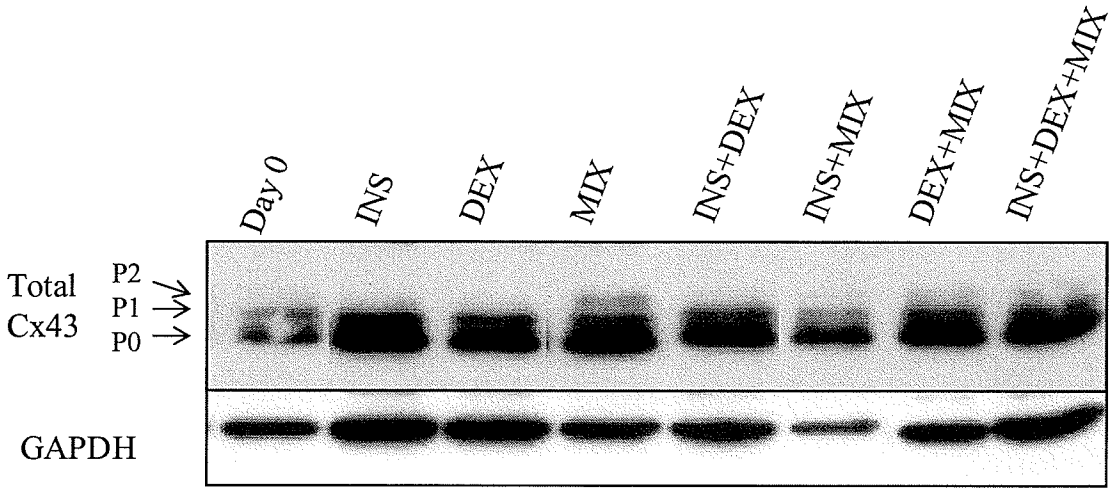
The treatment with MIX led to the highest degree of Cx43 phosphorylation (only treatment exhibiting P2 band), followed by DEX and INS (Figure 8a). Although statistical analysis indicated no difference on total Cx43 expression between the latter two treatments (Figure 8b), MIX treated cells had one extra phosphorylated band (P2) compared to DEX in all of the experiments. This suggests that MIX is the component that stimulates complete Cx43 phosphorylation.

Figure 8. Effect of INS, DEX and MIX separately on expression of total Cx43 on day 2

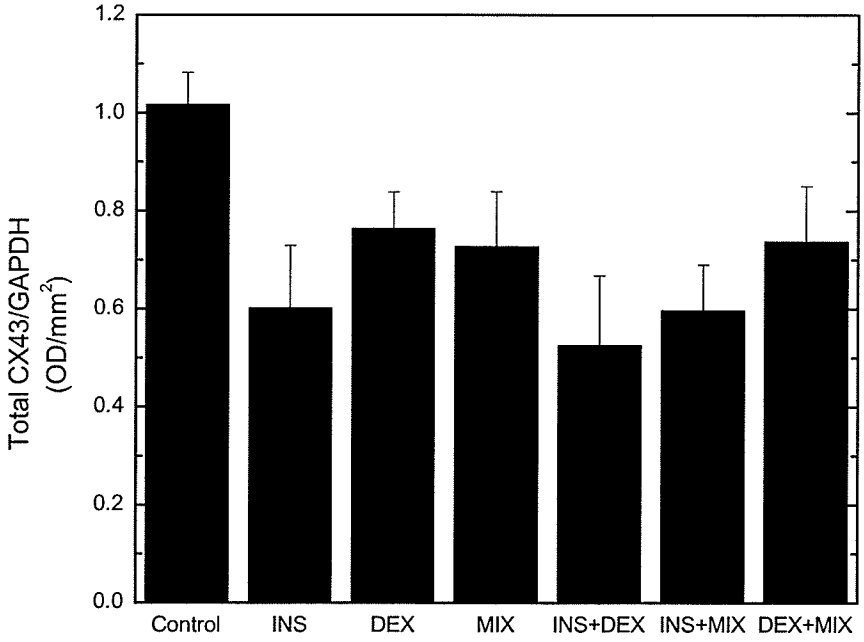
(a) Western blot of two day post-confluent 3T3-L1 cells treated with INS, DEX and MIX individually and in combination for 48 hours. Cells exposed to the three ingredients served as positive controls. Three Cx43 bands with different levels of phosphorylation (unphosphorylated and minimally phosphorylated = P0, highly phosphorylated = P1 and P2) are indicated. GAPDH was used as the loading control. The data are representative of the results obtained in three independent experiments.

(b) Densitometric assessment of total Cx43 levels in 3T3-L1 adipocyte lysates treated with INS, DEX and MIX separately or in combination for 48 hours. Cells treated with all ingredients were used as control. Data are adjusted for the loading control. Data are presented as mean ± SEM (n=3). Statistical analyses were performed using one way ANOVA and Duncan's Multiple Range Test. No significant differences were detected.

(a)


Day 0
INS
DEX
MIX
INS+DEX
INS+MIX
DEX+MIX
INS+DEX+MIX
Total Cx43
P2
P1
P0
GAPDH

(b)


Total CX43/GAPDH (OD/mm²)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
INS
DEX
MIX
INS+DEX
INS+MIX
DEX+MIX

5.5 Effect of different protein kinases on Cx43 phosphorylation during adipogenesis

Cx43 can be phosphorylated at various sites by many different kinases including PKA and PKC (223). Since, PKC and PKA are important in adipogenesis, we looked at the effect of PKC and PKA inhibitors and/or activators on Cx43 phosphorylation in relation to adipogenic state.

Bisindolylmaleimide I (classical PKC inhibitor), PKC β1 inhibitor, KT5720 (PKA inhibitor), forskolin and CPT-cAMP (cAMP analog that activates PKA) were added to the cells on day 0 of differentiation, concurrent with the adipogenic cocktail. Cells were lysed on day 2 and day 8, when the morphologic features of differentiation were established; the medium was changed every 2 days and the treatments refreshed. No differences between the treatments and control were observed on day 2, (data not shown). On day 8, when control cells (differentiated adipocytes with no treatment) showed very low expression of Cx43, cells treated with bisindolylmaleimide I exhibited very high Cx43 expression. It was evident that these cells were not differentiated, since adiponectin was not expressed (Figure 9a). These data suggest that bisindolylmaleimide I both inhibits adipocyte differentiation and prevents down regulation of Cx43 after day 2. In contrast, the cells differentiated (indicated by presence of adiponectin) in the presence of the PKC β1 inhibitor.

Interestingly, inhibition of PKA with KT5720 prevented adipogenesis, although it did not alter down regulation of Cx43. Thus, PKA affects adipocyte differentiation but has no effect on Cx43.

Treatment of 3T3-L1 cells with forskolin alone reduced adiponectin productions (Figure 9b). Western blotting also showed higher expression of Cx43 in forskolin treated cells in comparison to control cells, although CPT-cAMP treatment had no effect on adipokine production (as indicated by adiponectin (ACRP30) expression) and/or Cx43 expression (Figure 9b). Since both PKA and PKC inhibitors block adipogenesis (Figure 9a), but differentially affect Cx43, it was considered important to know if activation of PKA can rescue the inhibition of adipogenesis that occurs in the presence of PKC inhibitors. Cells treated with a combination of bisindolylmaleimide I and forskolin and cAMP showed lower Cx43 expression but there was no effect on adipokine production (Figure 9c). This effect of bisindolylmaleimide I on cell differentiation was not reversed when PKA was activated with CPT-cAMP (Figure 9c). This finding suggests that PKC and PKA possibly regulate adipogenesis through different pathways. Since the presence or absence of adiponectin (ACRP30) was used to indicate differentiated and undifferentiated status respectively, no quantification of adiponectin levels was performed.

In addition, a significant enlargement in the size of bisindolylmaleimide I treated cells was observed, as indicated by the increase in the number of cells with larger area relative to the untreated controls (Figure 10a & b).

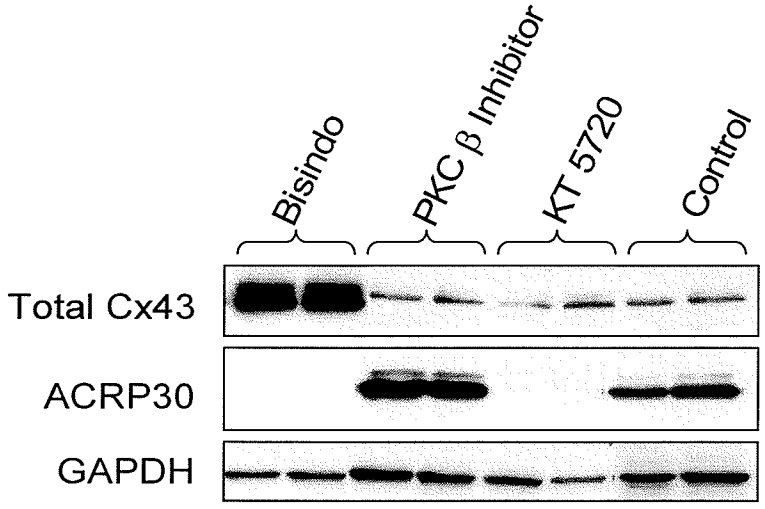
Figure 9. Effect of PKA and PKC inhibitors on Cx43 expression and phosphorylation in relation to adipocyte differentiation

(a) Western blot of protein lysates from 3T3-L1 cells treated with bisindolylmaleimide I (Bisindo) (10^{-5} M), KT5720 (10^{-6} M) and PKC β1 inhibitor (10^{-6} M) until day 8 of differentiation. The inhibitors concentration is according to the product sheets from the company. The membrane was probed for total Cx43, adiponectin (ACRP30) and GAPDH, which served as a loading control. The figure provides a representative picture of 3 independent experiments.

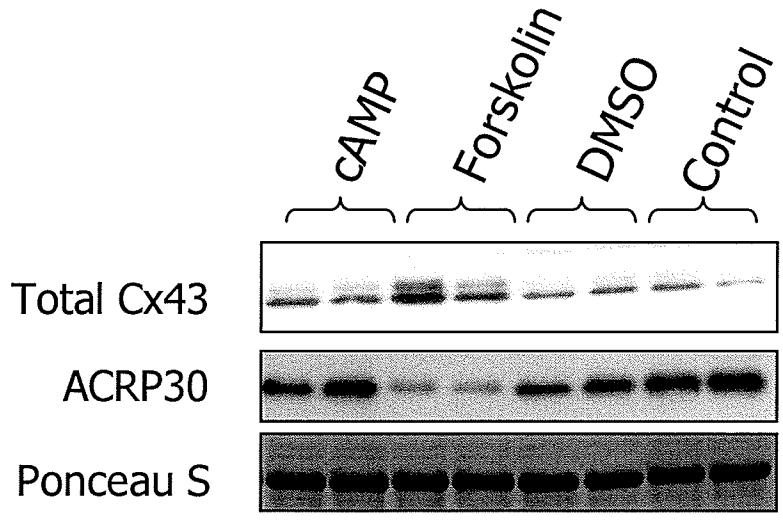
(b) Western blot of protein lysates from 3T3-L1 cells treated with PKA activators (forskolin (10^{-5} M) and CPT-cAMP (4×10^{-3} M)) and DMSO as vehicle until day 8 of differentiation, probed for total Cx43 and adiponectin (ACRP30) and stained with ponceau S, which served as a loading control. In this figure, CPT-cAMP is abbreviated as cAMP. The inhibitor concentrations were selected on the basis of their effectiveness for other lab members. The figure provides a representative picture of 3 independent experiments.

(c) Western blot of protein lysates from 3T3-L1 cells treated with a combination of bisindolylmaleimide I (Bisindo) (10^{-5} M), forskolin (10^{-5} M), CPT-cAMP (4×10^{-3} M) and DMSO as vehicle until day 8 of differentiation, probed for total Cx43 and adiponectin (ACRP30) and stained with ponceau S, which served as a loading control. The inhibitors and activators concentrations are according to the product sheets from the company. The figure provides a representative picture of 3 independent experiments.

(a)


Bisindo
PKC β Inhibitor
KT 5720
Control
Total Cx43
ACRP30
GAPDH

(b)


cAMP
Forskolin
DMSO
Control
Total Cx43
ACRP30
Ponceau S

(c)

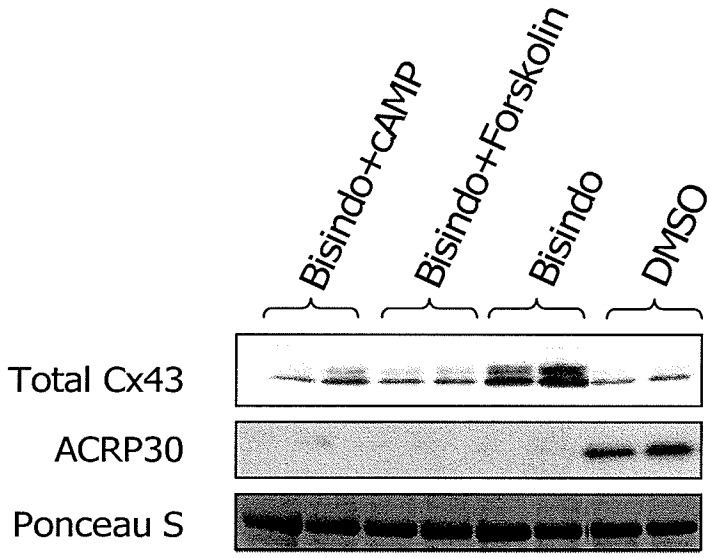
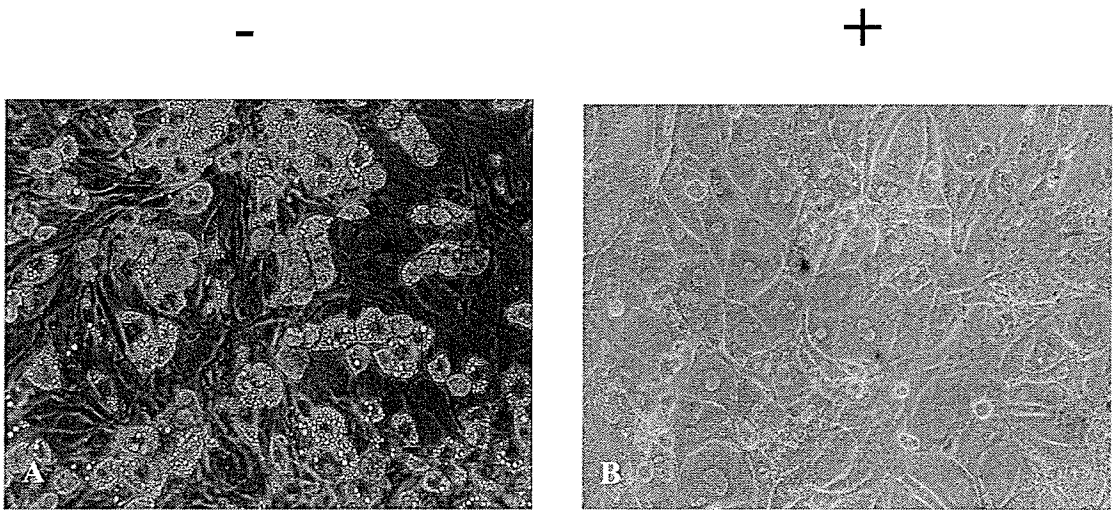

Bisindo+cAMP
Bisindo+Forskolin
Bisindo
DMSO
Total Cx43
ACRP30
Ponceau S

Figure 10 Effect of bisindolylmaleimide I on 3T3-L1 cell size on day 8 of differentiation

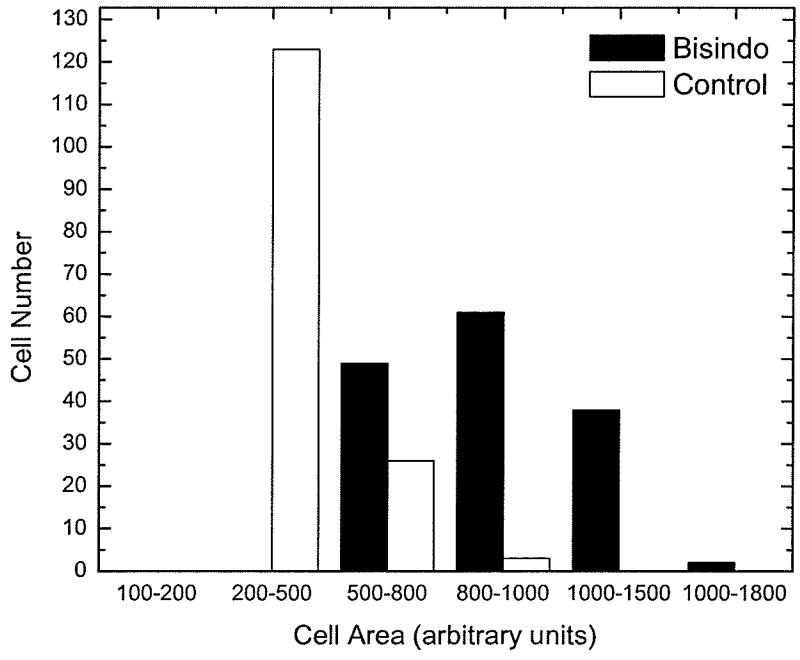
(a) Photomicrographs of 3T3-L1 cells treated for 8 day with 10^{-5} M bisindolylmaleimide I. A=untreated control, B= treated. Bisindolylmaleimide I caused major changes in cell morphology. Pictures are captured using a 25× objective.

(b) Cell size distribution comparing bisindolylmaleimide I (Bisindo) treated cells and control. The data were derived as described in Methods following analysis of 150 cells for each treatment, n=4.

(a)


-
+
A
B

(b)


Bisindo
Control
Cell Number
130
120
110
100
90
80
70
60
50
40
30
20
10
0
100-200
200-500
500-800
800-1000
1000-1500
1000-1800
Cell Area (arbitrary units)

5.6 Subcellular localization of Cx43 during adipogenesis

Since immunofluorescence staining (Figure 6) suggests that Cx43 localization changes during adipocyte differentiation, cells were fractionated to determine which cellular compartment contained Cx43. Fractions of 3T3-L1 cells were prepared on day -3 (growing), 0 (growth arrested), day 2 (mitotic clonal expansion), day 4 (mid-differentiation) and day 8 (differentiated) as described in Methods. The same procedure was applied to undifferentiated cells, the 3T3-L1 cells which are not triggered to differentiate by stimulation cocktail were lysed either 2 or 8 days after being confluent for two days. Whole cell lysates were also collected at each stage. Actin, IGF-1 receptor β, GAPDH and Oct-1 were used as markers for cytoskeleton, membrane, cytosol and nucleus, respectively.

Western blotting showed that Cx43 localization did not change during adipocyte differentiation. Cx43 was found in the cytoskeleton/membrane fraction and not in others (Figure 11a). Based on the distribution of the IGF-1 receptor β, considered a membrane marker (Figure 11b), it was concluded that the kit did not effectively separate the membrane and cytoskeletal components and so it was not possible to determine whether Cx43 is present in the membrane, the cytoskeleton or both. However, subcellular fractionation did confirm that Cx43 was down regulated during adipogenesis (Figure 11a).

Likewise, Western blotting of undifferentiated 3T3-L1 fractions showed that Cx43 was located in the same fractions (cytoskeleton/membrane) (Figure 11c).

To investigate the issue of membrane versus cytoskeletal localization, co-staining for total Cx43 and actin (cytoskeleton marker) was employed. The merged

microscopic image showed no overlap, which indicates Cx43 was not present in the cytoskeleton (Figure 12).

Subcellular fractionation of 3T3-L1 cells showed that Cx43 remained in the membrane fraction during differentiation (Figure 11a), whereas immunofluorescence showed Cx43 was located in the cytosol before stimulation (day -3) and after clonal expansion (day 4) (Figure 6). These contrasting observations suggested that Cx43 was present on one of the membranous organelles present in the cytosol, such as the Golgi apparatus or the endoplasmic reticulum (ER).

3T3-L1 cells were co-stained on day 0 (growth arrested), day 2 (mitotic clonal expansion) and day 4 (mid-differentiation) with Golgi or ER specific markers in addition to total Cx43. GM130 was used as the Golgi marker and calnexin and calreticulin were used as markers for ER. On day 0, cytosolic Cx43 was found in the ER but not in the Golgi (Figure 13a). However, two days after adding adipogenic medium, Cx43 was mostly phosphorylated (Figure 7) and predominantly localized in the plasma membrane. Cx43 staining was very weak in either the Golgi or the ER (Figure 13b). On day 4 (beginning of differentiation), phosphorylation of Cx43 was reduced as previously shown by Western blotting (Figure 3) and the protein was found in both Golgi and ER compartments (Figure 13c).

Figure 11. Subcellular fractionation of 3T3-L1 cells during adipogenesis

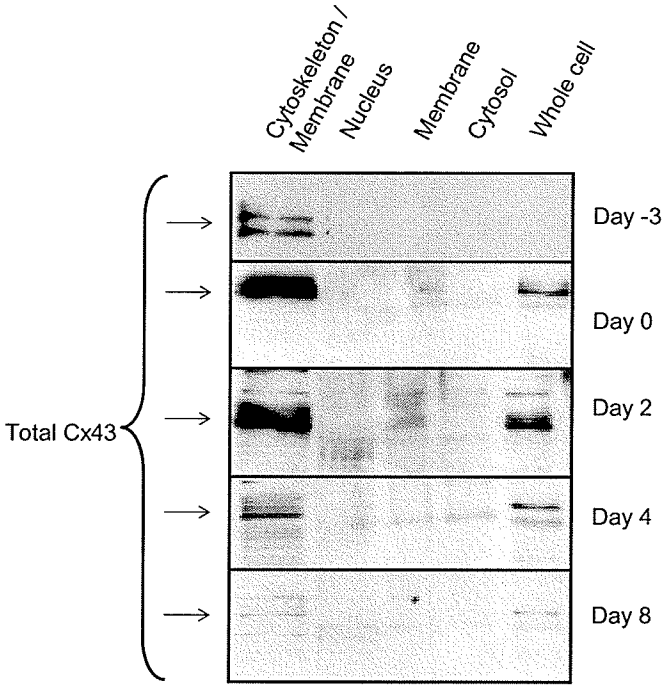
(a) Western blot of subcellular fractions obtained from 3T3-L1 cells on day -3 (growing), day 0 (growth arrest), day 2 (mitotic clonal expansion), day 4 (start of differentiation) and day 8 (fully differentiated) showing the presence of total Cx43.

(b) Western blot of 3T3-L1 cell fractions, probed with different markers for each fraction. Actin = cytoskeleton, IGF-1 receptor β = membrane, GAPDH = cytosol and OCT-1 = nucleus

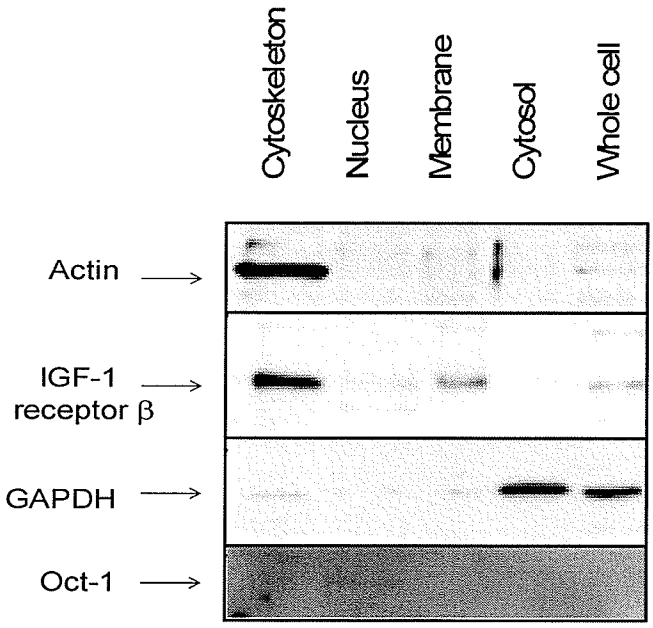
(c) Western blot of undifferentiated 3T3-L1 fractions on day 2 and 8, probed for total Cx43.

The blots presented in this figure are representative of the results obtained from three independent experiments.

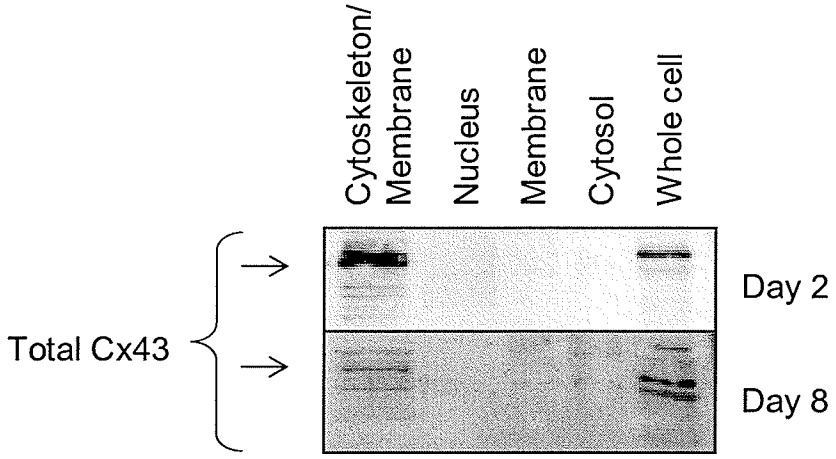
(a)


Cytoskeleton / Membrane
Nucleus
Membrane
Cytosol
Whole cell
Total Cx43
Day -3
Day 0
Day 2
Day 4
Day 8

(b)


Cytoskeleton
Nucleus
Membrane
Cytosol
Whole cell
Actin
IGF-1 receptor β
GAPDH
Oct-1

(c)


Cytoskeleton/ Membrane
Nucleus
Membrane
Cytosol
Whole cell
Total Cx43
Day 2
Day 8

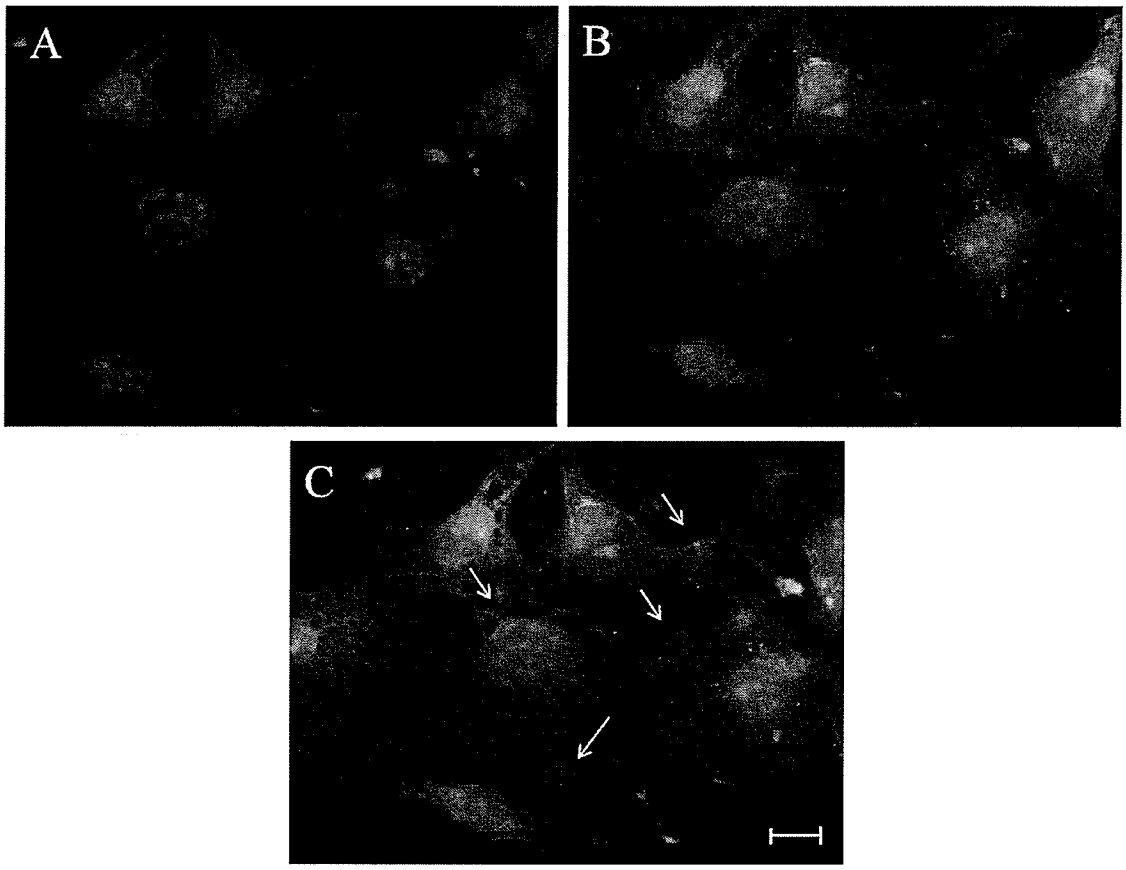


Figure 12. Immunofluorescence staining of the cytoskeleton marker actin and total Cx43 on day 2

3T3-L1 cells were fixed in day 0 and subsequently examined by immunofluorescence microscopy. Total Cx43 is indicated in green (B) and actin in red (A). The merged picture (C) shows the absence of overlap between actin and Cx43, as indicated by the arrows which point to regions of actin staining. Bar represents 2 μm (indicated on merged image of day -3) and is applicable to all other panels.

Figure 13. Immunofluorescence microscopy to determine the relative presence of Cx43 in the plasma membrane, Golgi apparatus and Endoplasmic Reticulum

(a) 3T3-L1 cells were fixed on day 0 (growth arrested) and co-stained with calnexin (ER marker, green, panel B) and total Cx43 (red, panel A), or co-stained with GM130 (Golgi marker, red, panel D) and total Cx43 (red, panel E). Panels C and F show the results of merging calnexin + Cx43 and GM130 + Cx43, respectively. The arrows show areas of overlap between Cx43 and GM130 or calnexin.

(b) 3T3-L1 cells were fixed on day 2 (mitotic clonal expansion) and co-stained with GM130 (Golgi marker, red, panel A) and total Cx43 (green, panel B), or co-stained with calnexin (ER marker, green, panel D) and total Cx43 (red, panel E). Panels C and F show the results of merging GM130 + Cx43 and calnexin + Cx43, respectively. The arrows show areas of overlap between Cx43 and GM130 or calnexin.

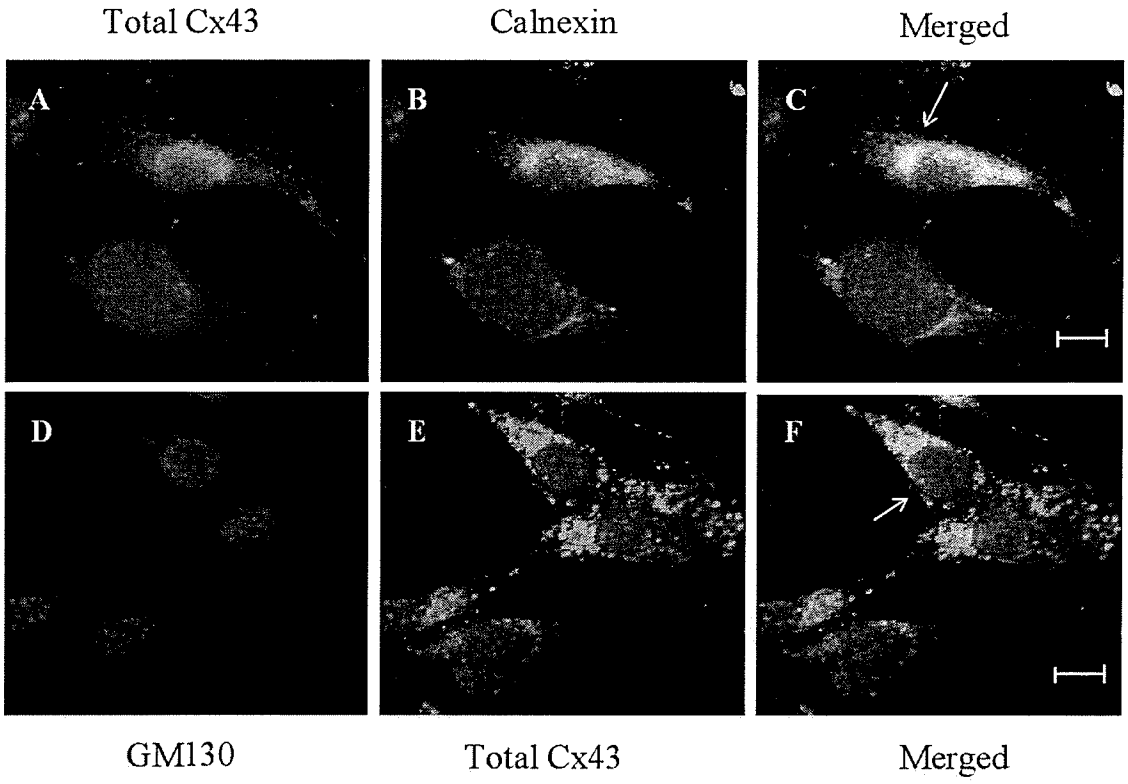
(c) 3T3-L1 cells were fixed on day 4 (beginning of differentiation) and co-stained with calreticulin (ER marker, red, panel A) and total Cx43 (green, panel B).GM130 (Golgi marker, red, panel D) and total Cx43 (green, panel E), or co-stained with Panels C and F show the results of merging calreticulin + Cx43 and GM130 + Cx43, respectively. The arrows show areas of overlap between Cx43 and GM130 or calnexin.

(d) The control shows the 3T3-L1 (day -3) with no primary antibody.

Panels a-d in this figure are representative of the results obtained from two independent experiments. Likewise, all images were captured using a 40× objective. Bar represents 2 μm (indicated on merged images) and is applicable to all other panels.

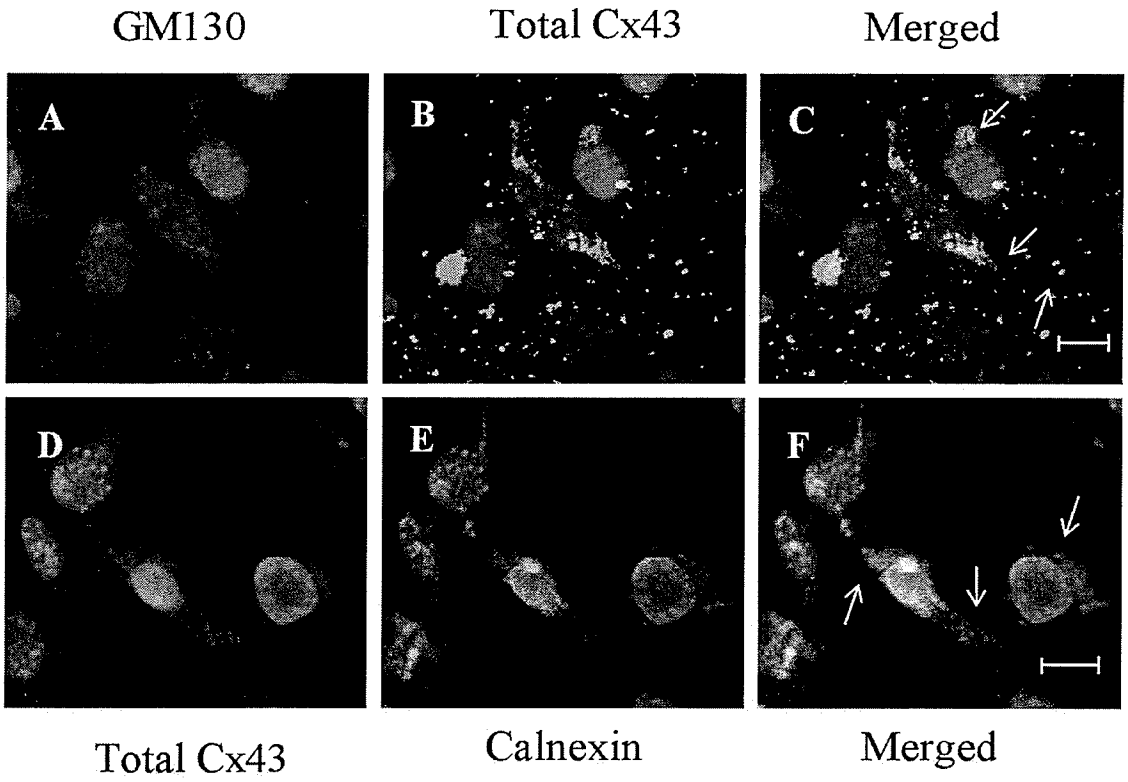
(a)

Day 0



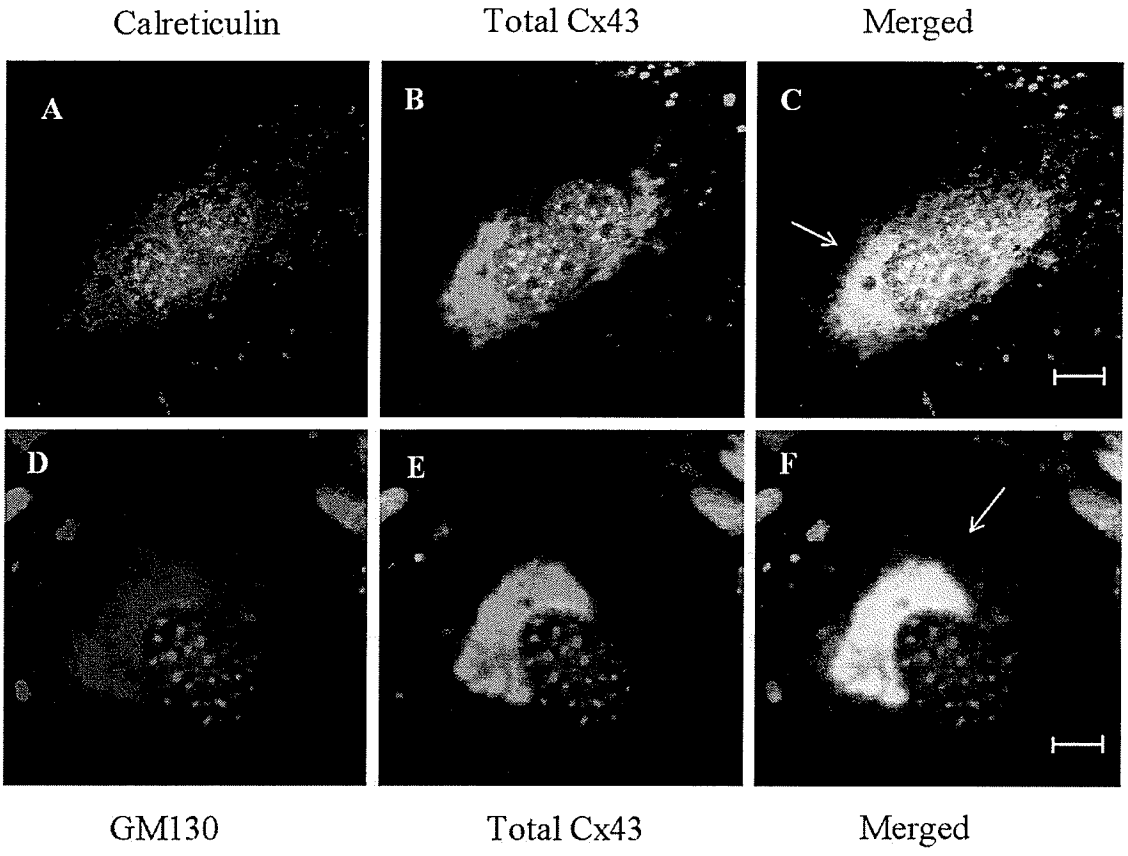
(b)

Day 2



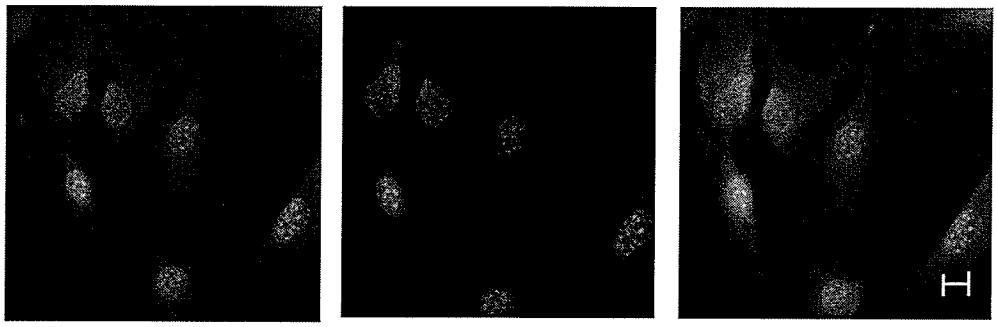
(c)

Day 4


Calreticulin
Total Cx43
Merged
A
B
C
D
E
F
GM130
Total Cx43
Merged

(d)

Day -3



5.7 Effect of constitutive expression of Cx43 during adipogenesis

Alterations in the level of Cx43 during adipogenesis were reported previously by others (233, 234) and confirmed in this study (Figure 3). Yanagiya et al (235) showed that Cx43 is necessary for adipogenesis, but after clonal expansion Cx43 decreased. We investigated if this down regulation is necessary for adipocyte differentiation.

Two day post-confluent 3T3-L1CARΔ were treated with adipogenic medium and concurrently were infected with adenovirus carrying Cx43. Controls were treated with the same cocktail but not infected with adenovirus. Cells were lysed on days 0 and 1 (growth arrested), days 2 and 3 (mitotic clonal expansion), day 4 (mid-differentiation) and days 6 and 8 (differentiated). Perilipin was used as a marker for lipid droplet formation and adiponectin as an adipokine production marker. Adenoviral delivery of Cx43 was very effective, with increased amounts detectable at 24 hours (Figure 14, upper panel). Furthermore, Cx43 levels remained elevated for the 8-day duration of the experiment (Figure 14, upper panel).

However, as can be seen from the Western blots, constitutive expression of Cx43 had no effect on adipokine production, since adiponectin did not change following treatment. Likewise, Cx43 expression had no effect on lipid droplet formation (shown by perilipin) (Figure 14) and fatty acid metabolism (A-FABP expression (Figure 27)) (see also Appendix 1).

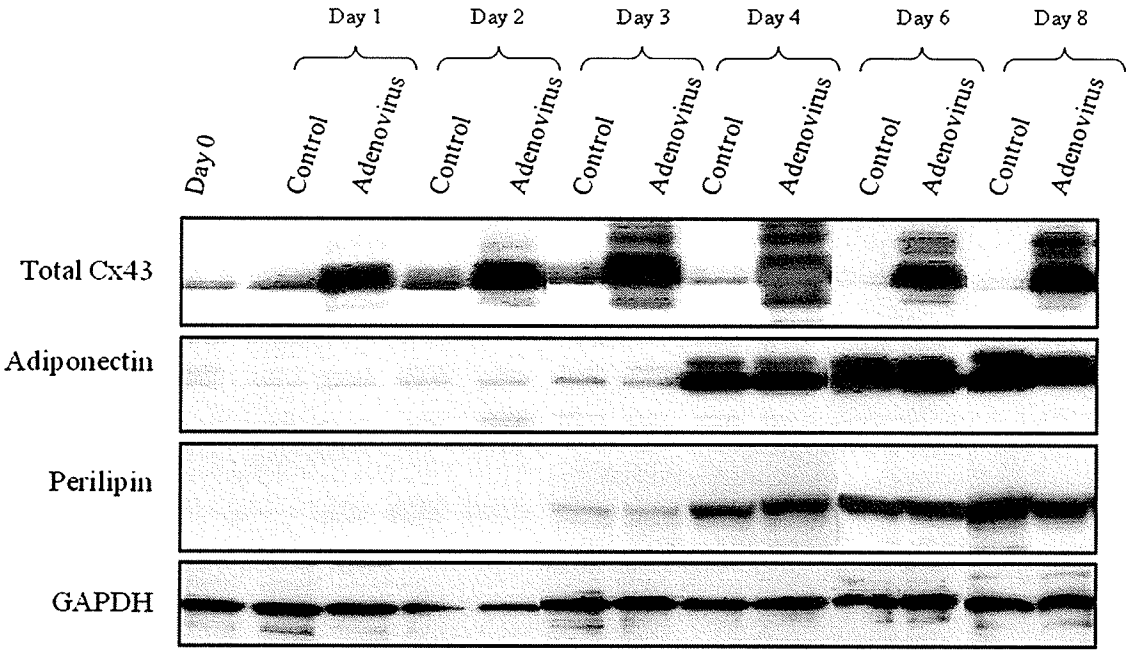


Figure 14. Effect of constitutive expression of Cx43 on 3T3-L1-CARΔ differentiation

Constitutive expression of Cx43 in 3T3-L1-CARΔ cells on day 0 (growth arrested), day 2 (mitotic clonal expansion), day 4 (mid-differentiation) and day 8 (differentiated) was achieved by infecting the cells with an adenovirus (Ad) carrying Cx43. Top panel shows the expression of total Cx43 in infected and uninfected (control) 3T3-L1-CARΔ. Adipokine production or lipid droplet formation was determined by measuring the protein expression of ACRP30 and perilipin, respectively. GAPDH was used as the loading control. Data were not quantified. The figure provides a representative blot of 3 independent experiments.

5.8 Effect of CLA on adipogenic markers during 3T3-L1 differentiation

CLA has been reported to affect the differentiation of adipocytes (178, 179, 185). This was confirmed by treating 3T3-L1 cells with 60 μM c9-t11 CLA and t10-c12 CLA separately and then monitoring lipid droplet formation 4 days after addition of the stimulation cocktail.

In comparison to control, CLA isomer treated cells had increased numbers of lipid droplets in cells (Figure 15a). Quantification showed that in CLA treated cultures, the number of cells with lipid droplets went from 48% in the control to 78% with c9-t11 and 85% with t10-c12 (Figure 15b), the total number of cells did not change. These data suggest CLA treatment accelerates the differentiation of adipocytes.

To investigate the role of CLA on adipogenic markers, two-day post-confluent (day 0) 3T3-L1 cells were treated with 60 μM of c9-t11and t10-c12, individually and together, at the same time cell differentiation was triggered by addition of the stimulation cocktail. CLA treatments were added whenever the media was changed (every 48 h). Cells were lysed on day 0 (growth arrested), day 2 (mitotic clonal expansion), day 4 (mid-differentiation) and day 8 (differentiated). Markers for lipid droplet formation and adipokine production were subsequently investigated. The results for days 0, 2 and 4 were not quantified. Data from day 8 were quantified because cells are fully differentiated at this time point and any trends seen earlier should be at their greatest. Likewise, the effect of time (days of differentiation) on adipogenesis markers was not investigated in this study (with the exception of total Cx43) because the 3T3-L1 cells were not in their mature state until day 8.

We found that A-FABP expression showed a trend (P=0.058, n=4) with t10-c12 CLA compared to control on day 8 of differentiations. (Figure 16a & b).

Western blotting showed that perilipin expression was lower in t10-c12 CLA treated cells in comparison with c9-t11, but the differences did not reach a significant level (n=4) (Figure 17a & b).

In addition to markers of lipid droplet formation, adipokine production was also altered by CLA isomers. In agreement with Brown et al (190), we found that adiponectin levels were lower with t10-c12 CLA treatment on day 8 compared to c9-t11 treated cells, but the difference did not reach a significance level (Figure 18a & b). Adiponectin expression increased during 3T3-L1 differentiation as expected.

In addition to the adipokines studied earlier, we extended the CLA studies to also look at adipsin and chemerin protein level, because of their important role in obesity (68, 240).

Both glycosylated (44-37 kDa) and non glycosylated (26 kDa) forms of adipsin was detectable only on day 8. Expression of adipsin (both glycosylated and non-glycosylated) increased in c9-t11 CLA treated cells and the control over time, but not in t10-c12 CLA treated cells. Its expression was very low in t10-c12+c9-t11 CLA treated cells. Adipsin levels in t10-c12 CLA treated cells were significantly lower in comparison to the control and c9-t11 CLA treatments on day 8 ($P<0.01$, n=4). The same effect was observed when the cells were treated with a mixture of the isomers (t10-c12+ c9-t11). No difference was observed between c9-t11 CLA compared to control and t10-c12 compared to the isomer mixture (t10-c12+ c9-t11) at that stage (Figure 19a & b).

Chemerin is a recently recognized pro-inflammatory adipokine, but the effect of CLA on this adipokine has not been previously studied. Our experiment demonstrated that chemerin is detectable at day 4 and its expression was affected by CLA treatments. On day 8, chemerin expression showed a tendency to remain low in t10-c12 CLA isomer treated cells compared to control ($P=0.09$, $n=4$). (Figure 20a & b).

PPARγ, leptin and FAS expression were likewise examined in CLA treated 3T3-L1 during the 8 days of differentiation. There were no significant differences seen with CLA isomer treatment in this study (representative Western blots are shown in Appendix 2).

5.9 Modulation of Cx43 with CLA during adipogenesis

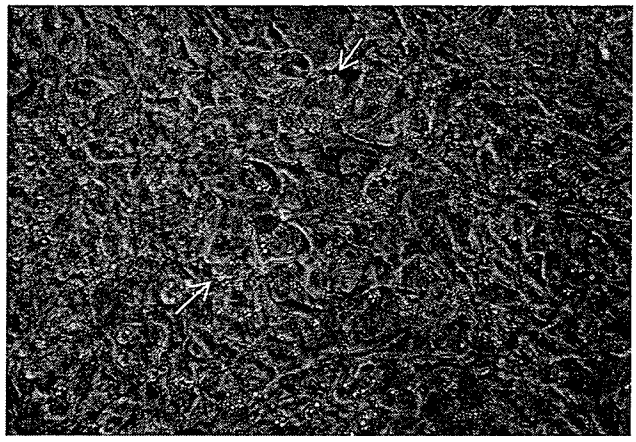
After proposing that Cx43 may serve as a new adipogenic marker, it was important to investigate the effect of nutraceuticals such as CLA on the level of Cx43 and its phosphorylation state during adipogenesis. The results show that total Cx43 expression was not altered by any of the treatments (CLA isomers) over the time course, although there were significant changes in total Cx43 during differentiation (days 2, 4 and 8) ($P<0.0001$, $n=4$) (Figure 21a & b).

In general, CLA did not change Cx43 expression and/or its phosphorylation state during adipogenesis (Figure 21a & b). This result may be linked to the fact that changes in Cx43 occur early in differentiation, while CLA affects middle and late differentiation events.

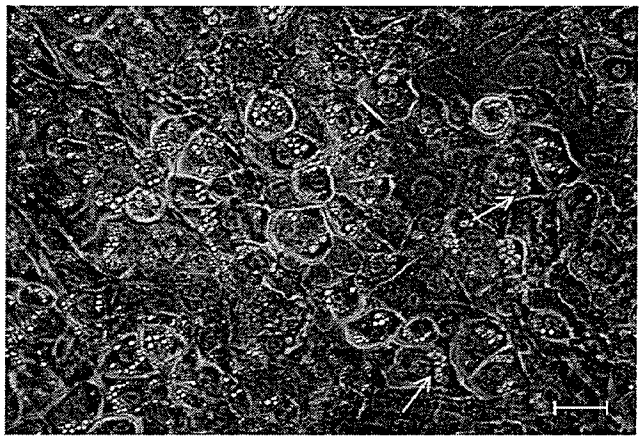
Figure 15. Stimulation of adipocyte differentiation by CLA isomers in early stage

(a) Pictures of 3T3-L1 cells were taken 4 days after addition of adipogenic cocktail. The panels show control and CLA treated (60 μM c9-t11 and 60 μM t10-c12 isomer) cells. Arrows indicate the lipid droplets. Pictures were captured using a 10× objective. Bar represents 10 μm (indicated on c9-t11 image) and is applicable to all other panels. **(b)** Cells were treated with 60 μM c9-t11 and t10-c12 CLA for 4 days. The number of cells with droplets were quantified and divided to the total number of counted cells (100 cells) and plotted as means ± SEM. Statistical analysis was performed using one-way ANOVA(P=0.01) and Duncan's Multiple Range Test. Differences among means are indicated by #, n=3. There was significant interaction among the treatments and control. The experiment was repeated three times and 100 cells were counted in each experiment.

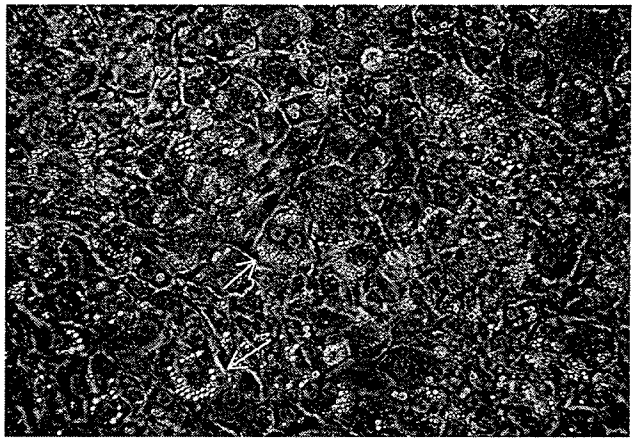
(a)



Control



9-11



10-12

(b)

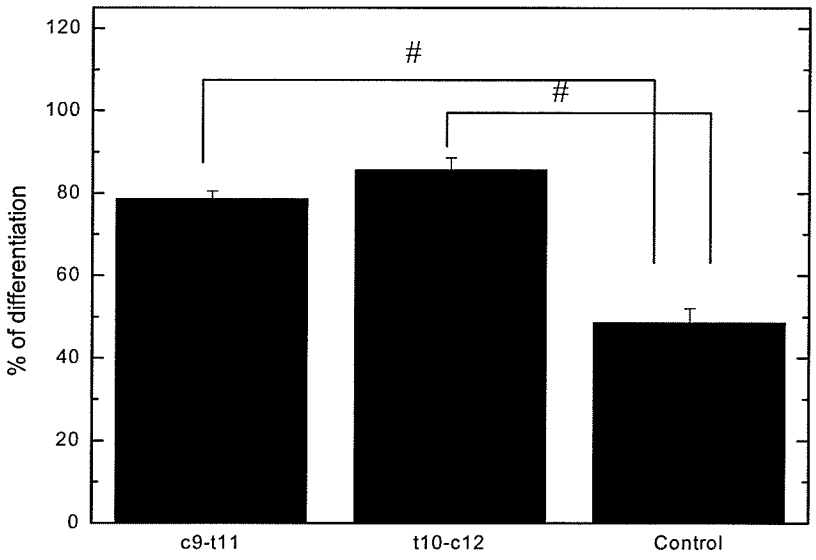
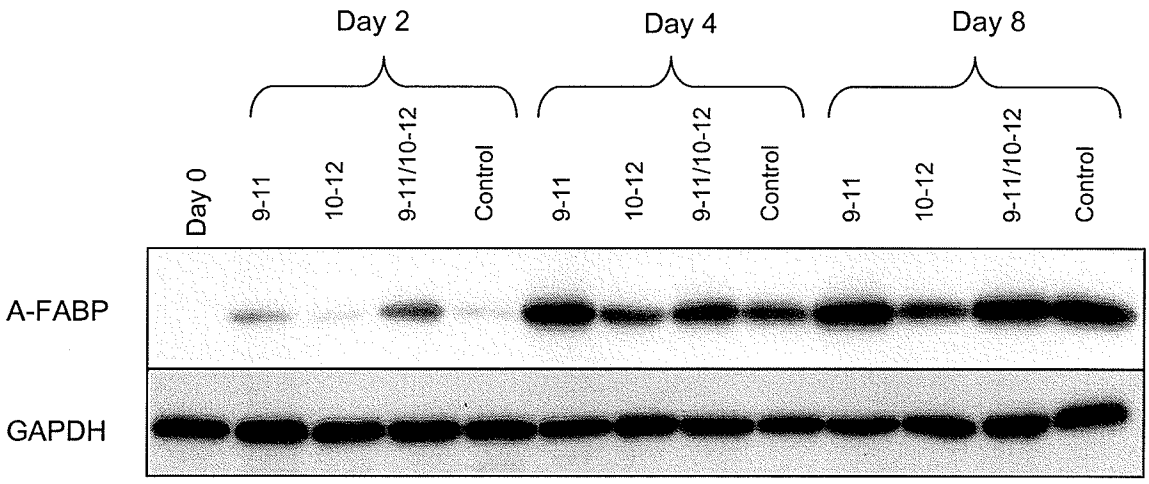

120
100
80
60
40
20
0
% of differentiation
#
#
c9-t11
t10-c12
Control

Figure 16. Effect of different CLA isomers on expression of A-FABP during adipogenesis

(a) Western blot of 3T3-L1 cells lysed after treatment with 60 μM c9-t11, 60 μM t10-c12 CLA isomers or the combination of both (defined as c9t11t10c12 on the figure) on days 0, 2, 4 and 8 of differentiation. The blots were probed for A-FABP. GAPDH was used as a loading control. The figure provides a representative picture of 4 independent experiments.

(b) Densitometric assessment was used to quantify A-FABP band intensity shown in the previous panel on day 8. Data were adjusted with the loading control (GAPDH). Statistical analysis was performed using one-way ANOVA (P=0.058) and Duncan's Multiple Range Test. Trends among means (P=0.058) are indicated by †, n=4. Interactions among the treatments were examined only on day 8.

(a)


Day 2
Day 4
Day 8
Day 0
9-11
10-12
9-11/10-12
Control
9-11
10-12
9-11/10-12
Control
9-11
10-12
9-11/10-12
Control
A-FABP
GAPDH

(b)

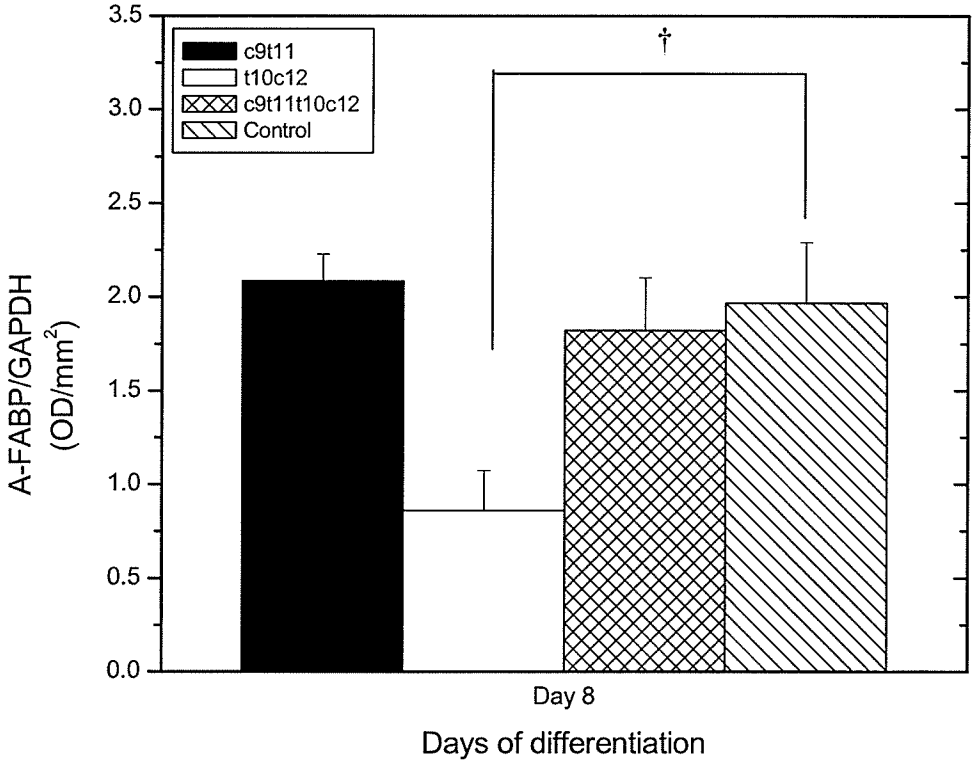
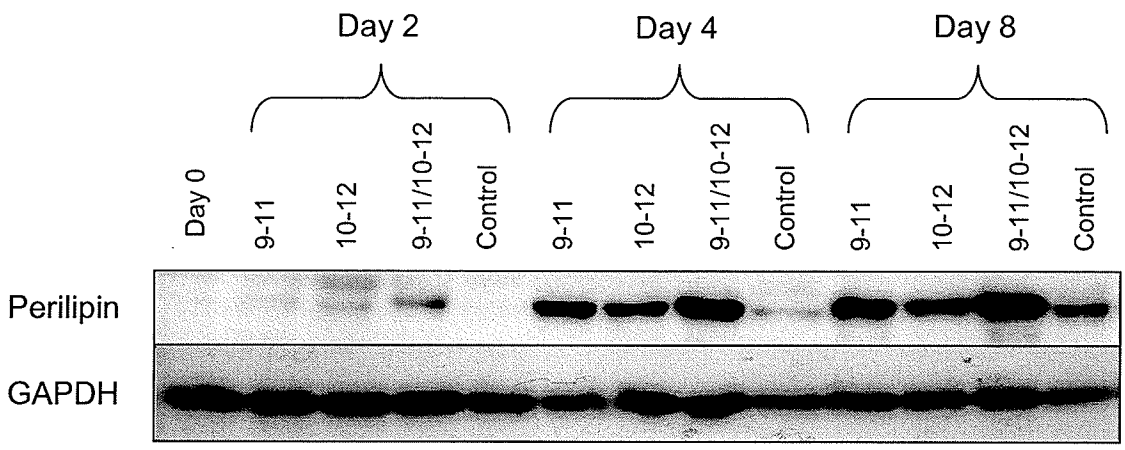

c9t11
t10c12
c9t11t10c12
Control
†
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
A-FABP/GAPDH
(OD/mm²)
Day 8
Days of differentiation

Figure 17. Effect of different CLA isomers on expression of perilipin during adipogenesis

(a) Western blot of 3T3-L1 cells lysed after treatment with 60 μM c9-t11, 60 μM t10-c12 CLA isomers or the combination of both (defined as c9t11t10c12 on the figure) on days 0, 2, 4 and 8 of differentiation. The blots were probed for perilipin. GAPDH was used as a loading control. The figure provides a representative picture of 4 independent experiments.

(b) Densitometric assessment was used to quantify perilipin band intensity shown in the previous panel on day 8. Data were adjusted with the loading control (GAPDH). Statistical analysis was performed using one-way ANOVA and Duncan's Multiple Range Test. Trends among means (P=0.105) are indicated by †, n=4. Interactions among the treatments were examined only on day 8.

(a)


Day 2
Day 4
Day 8
Day 0
9-11
10-12
9-11/10-12
Control
9-11
10-12
9-11/10-12
Control
9-11
10-12
9-11/10-12
Control
Perilipin
GAPDH

(b)

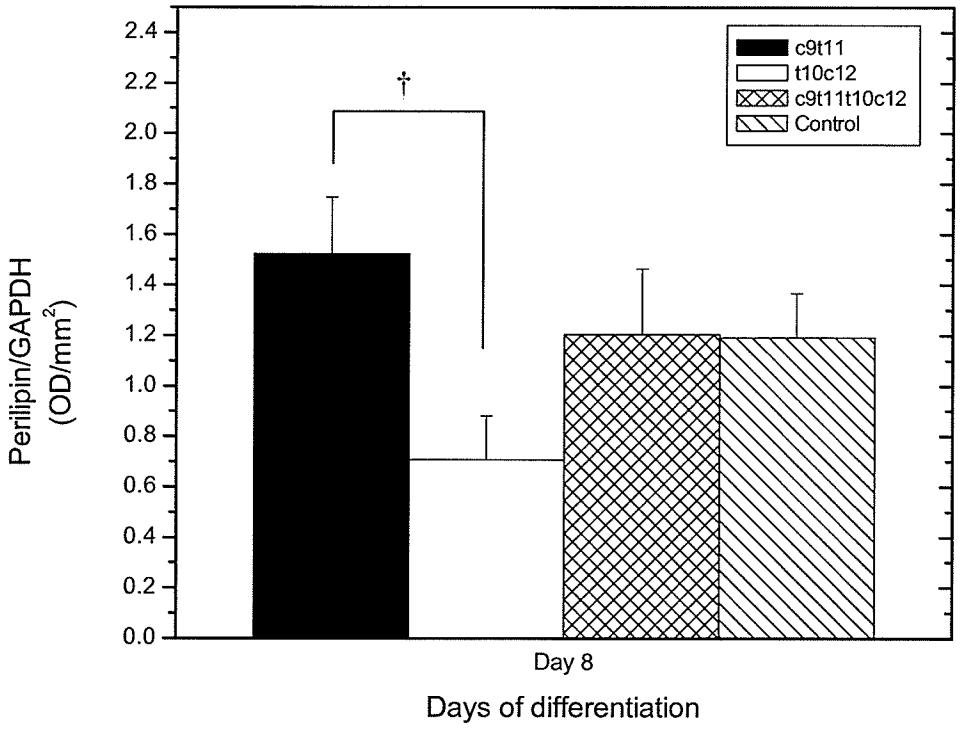
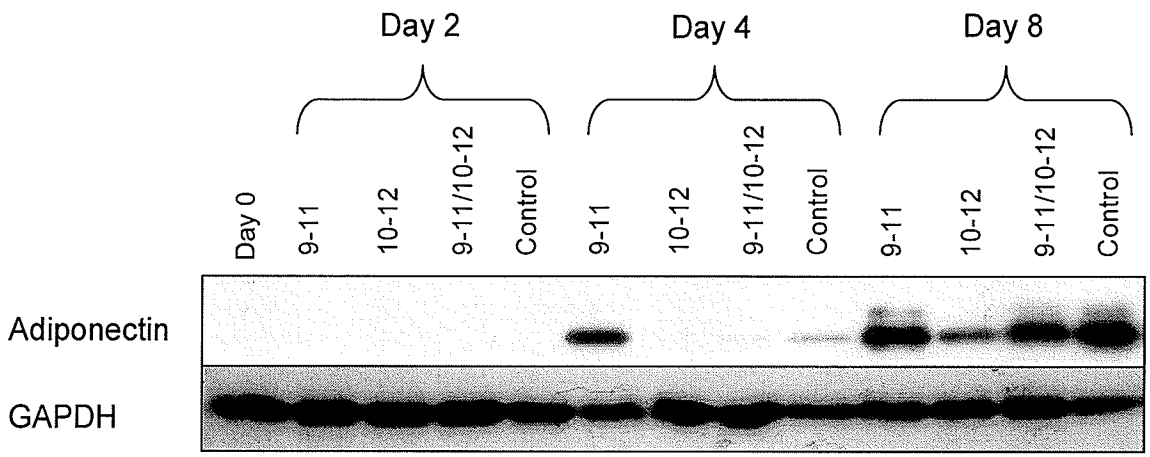

c9t11
t10c12
c9t11t10c12
Control
†
Perilipin/GAPDH (OD/mm²)
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Day 8
Days of differentiation

Figure 18. Effect of different CLA isomers on expression of adiponectin during adipogenesis

(a) 3T3-L1 cells were treated with 60 μM c9-t11, 60 μM t10-c12 CLA isomer or the combination of both (defined as c9t11t10c12 on the figure) on days 0, 2, 4 and 8 after adding adipogenic cocktail. Adiponectin was detected in cell lysates by Western blotting and compared to GAPDH, loading control. The figure provides a representative picture of 4 independent experiments.

(b) Densitometric assessment was used to quantify adiponectin band intensity shown in the previous panel on day 8. Data were adjusted with the loading control (GAPDH). Statistical analysis was performed using one-way ANOVA and Duncan's Multiple Range Test. Trends among means (P=0.06) are indicated by †, n=4. Interactions among the treatments were examined only on day 8.

(a)


Day 2
Day 4
Day 8
Day 0
9-11
10-12
9-11/10-12
Control
9-11
10-12
9-11/10-12
Control
9-11
10-12
9-11/10-12
Control
Adiponectin
GAPDH

(b)

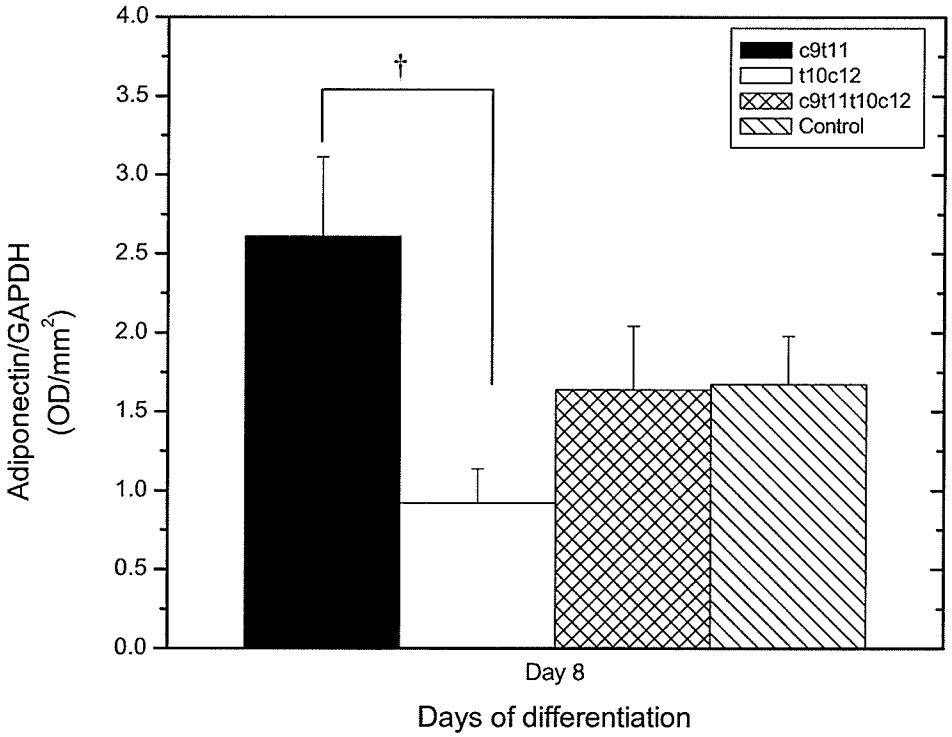

4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
†
c9t11
t10c12
c9t11t10c12
Control
Adiponectin/GAPDH
(OD/mm^2)
Day 8
Days of differentiation

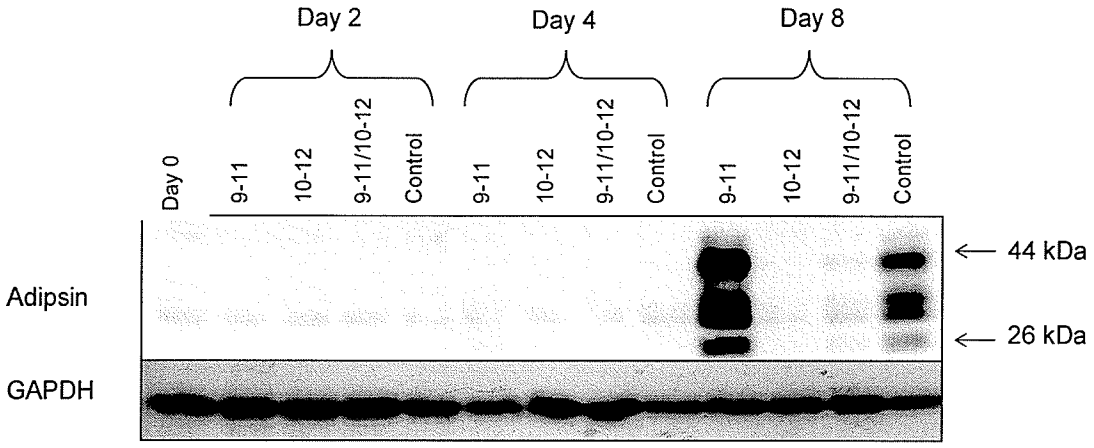
Figure 19. Effect of different CLA isomers on expression of adipsin during adipogenesis

(a) Western blot of 3T3-L1 cells lysed after treatment with 60 μM c9-t11, 60 μM t10-c12 CLA isomers or the combination of both (defined as c9t11t10c12 on the figure) on days 0, 2, 4 and 8 of differentiation. The blots were probed for glycosylated (44-37 kDa) and non-glycosylated (26 kDa) forms of adipsin. GAPDH was used as a loading control. The figure provides a representative picture of 4 independent experiments.

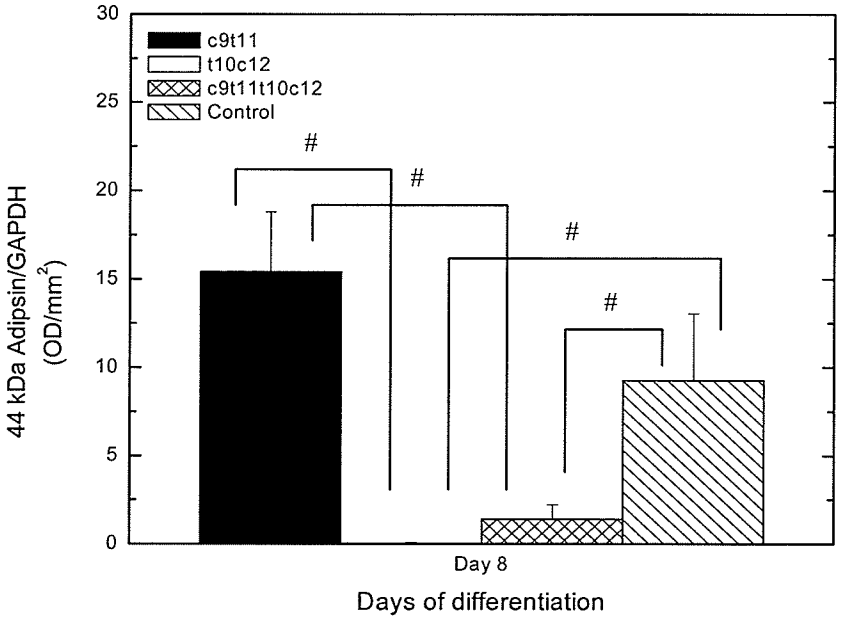
(b) Densitometric assessment was used to quantify the glycosylated (44-37 kDa) adipsin band intensity shown in the previous panel on day 8. Data are adjusted by loading control (GAPDH). Statistical analysis was performed using one-way ANOVA (P=0.004) and Duncan's Multiple Range Test. Differences among means are indicated by #, n=4. Interactions among the treatments were examined only on day 8.

(c) Densitometric assessment was used to quantify the non-glycosylated (26 kDa) adipsin band intensity shown in the panel (a) on day 8. Data are adjusted by loading control (GAPDH). Statistical analysis was performed using one-way ANOVA (P=0.004) and Duncan's Multiple Range Test. Differences among means are indicated by #, n=4. Interactions among the treatments were examined only on day 8.

(a)


Day 2
Day 4
Day 8
Day 0
9-11
10-12
9-11/10-12
Control
9-11
10-12
9-11/10-12
Control
9-11
10-12
9-11/10-12
Control
Adipsin
GAPDH
44 kDa
26 kDa

(b)


c9t11
t10c12
c9t11t10c12
Control
#
#
#
#
44 kDa Adipsin/GAPDH
(OD/mm2)
30
25
20
15
10
5
0
Day 8
Days of differentiation

(c)

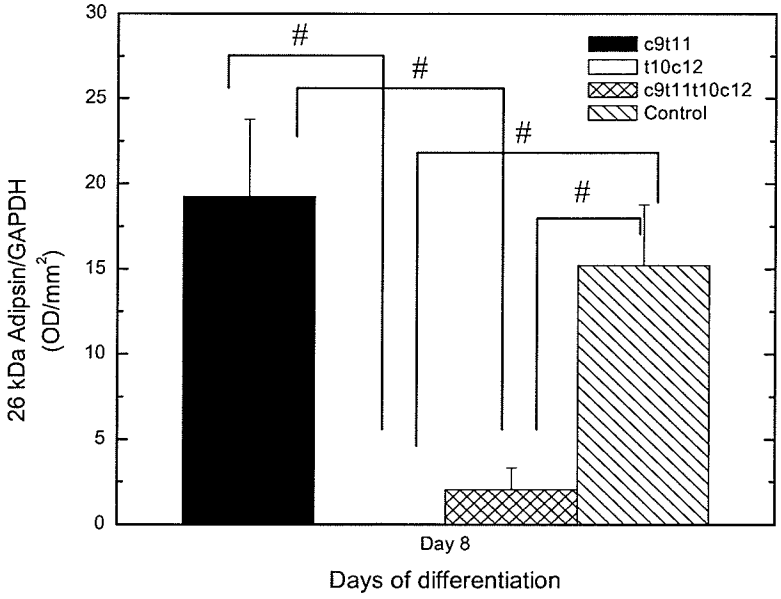
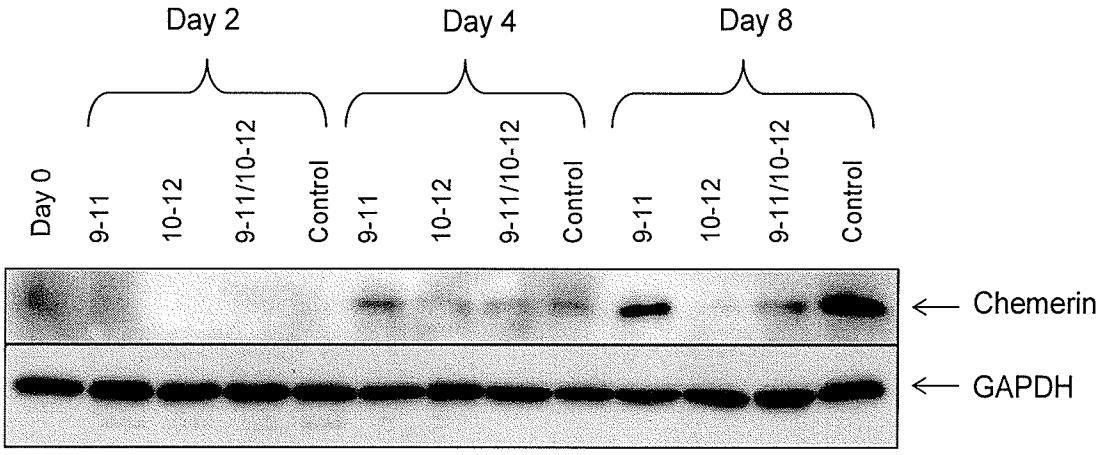

30
25
20
15
10
5
0
26 kDa Adipsin/GAPDH
(OD/mm^2)
#
#
#
#
c9t11
t10c12
c9t11t10c12
Control
Day 8
Days of differentiation

Figure 20. Effect of CLA isomers on chemerin expression during adipogenesis

(a) Western blot of 3T3-L1 cells lysed after treatment with 60 μM c9-t11, 60 μM t10-c12 CLA isomers or the combination of both (defined as c9t11t10c12 on the figure) on days 0, 2, 4 and 8 of differentiation. The blots were probed for chemerin. GAPDH was used as a loading control. The figure provides a representative picture of 4 independent experiments.

(b) Densitometric assessment was used to quantify chemerin band intensity shown in the previous panel on day 8. Data were adjusted according to the loading control (GAPDH). Statistical analysis was performed using one-way ANOVA and Duncan's Multiple Range Test. Trends among means (P=0.09) are indicated by †, n=4. Interactions among the treatments were examined only on day 8.

(a)


Day 2
Day 4
Day 8
Day 0
9-11
10-12
9-11/10-12
Control
9-11
10-12
9-11/10-12
Control
9-11
10-12
9-11/10-12
Control
Chemerin
GAPDH

(b)

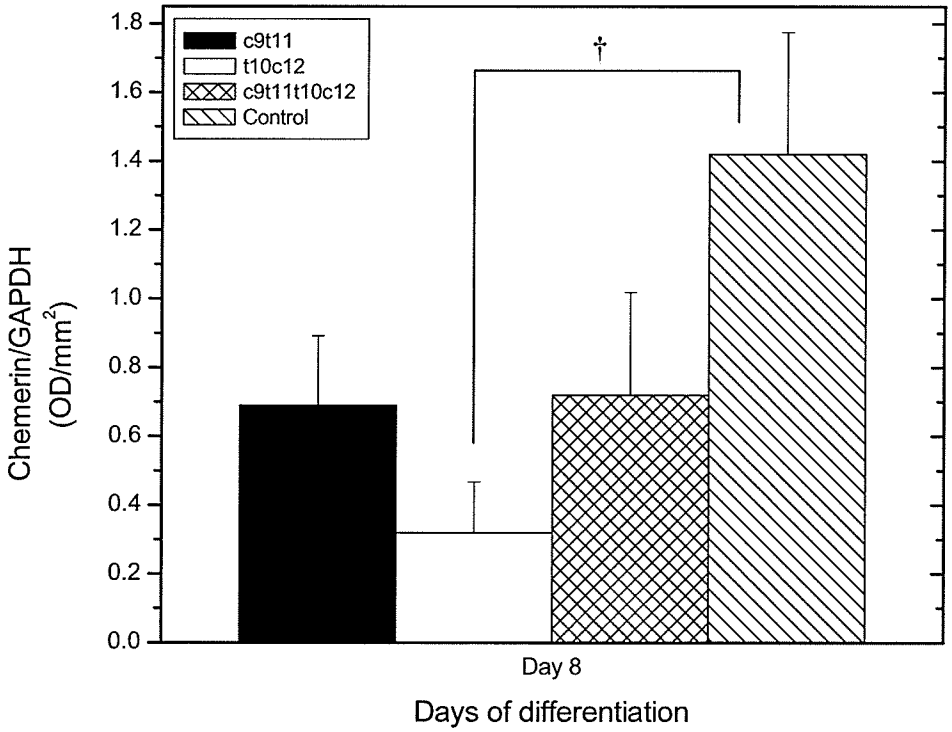
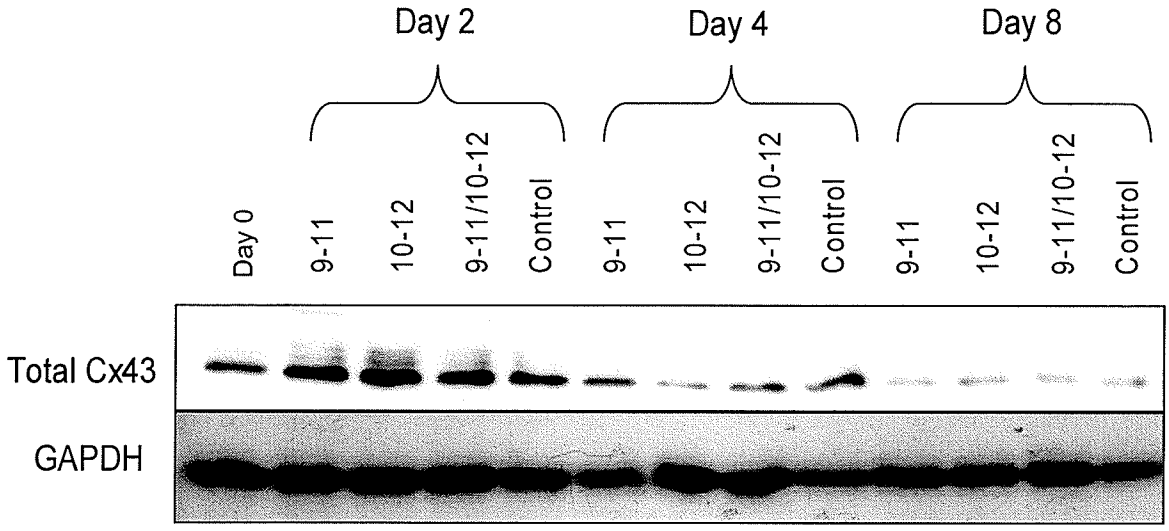

c9t11
t10c12
c9t11t10c12
Control
†
Chemerin/GAPDH
(OD/mm²)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Day 8
Days of differentiation

Figure 21. Effect of different CLA isomers on expression of Cx43 during adipogenesis

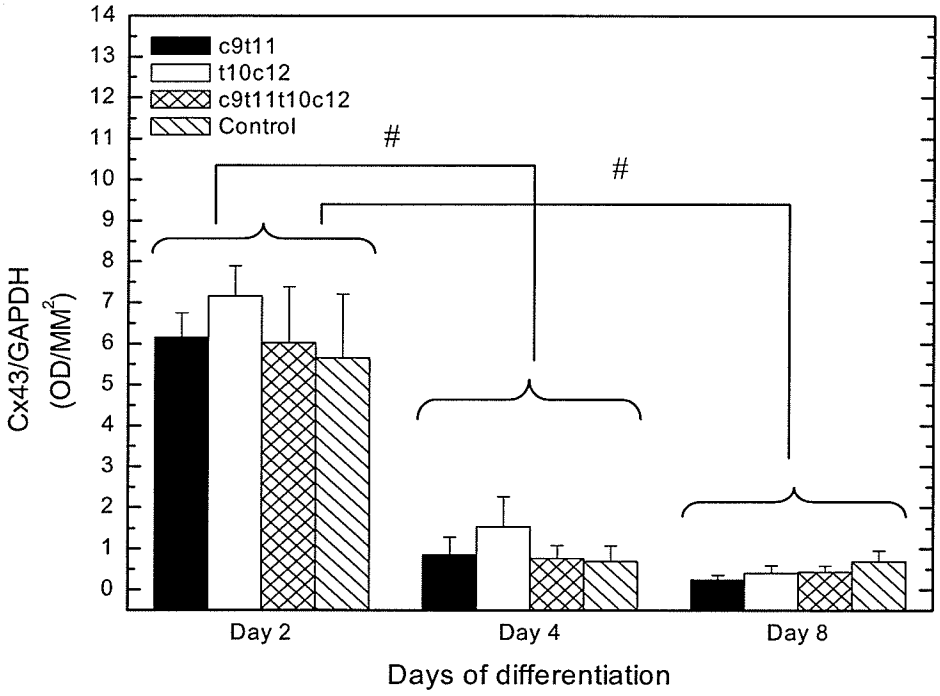
(a) Western blot of 3T3-L1 cells lysed after treatment with 60 μM c9-t11, 60 μM t10-c12 CLA isomers or the combination of both (defined as c9t11t10c12 on the figure) on days 0, 2, 4 and 8 of differentiation. The blots were probed for total Cx43. GAPDH was used as a loading control. The figure provides a representative picture of four independent experiments.

(b) Densitometric assessment was used to quantify total Cx43 band intensity shown in the previous panel. Data were adjusted according to the loading control (GAPDH). Statistical analysis was performed using two-way ANOVA to compare the effect of different days of differentiation and treatments (CLA isomers). No interactions were found among treatments. The figure shows a main effect of time (days of differentiation), #P=0.0001, n=4.

(a)


Day 2
Day 4
Day 8
Day 0
9-11
10-12
9-11/10-12
Control
9-11
10-12
9-11/10-12
Control
9-11
10-12
9-11/10-12
Control
Total Cx43
GAPDH

(b)


c9t11
t10c12
c9t11t10c12
Control
#
#
Cx43/GAPDH
(OD/MM^2)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Day 2
Day 4
Day 8
Days of differentiation

5.10 Effect of different CLA isomers on Cx43 expression in rat adipose tissue

To confirm the results of the *in vitro* experiments, the level of Cx43 in adipose tissue was measured using Western blotting. For this purpose, protein was extracted from the perirenal fat of fa/fa Zucker rats (7 week old, male) fed c9-t11 and t10-c12 CLA for 8 weeks (0.4% of diet was from CLA in free fatty acid form) and the perirenal fat of lean Zucker rats was used as a control. Protein extracts from adipose tissue were kindly provided by Vanessa DeClercq. Protein was loaded on 10% SDS-polyacrylamide gels and the levels of total Cx43 determined. The protein lysate prepared from 4 day differentiated 3T3-L1 cells was used as a positive control and GAPDH served as an internal loading control.

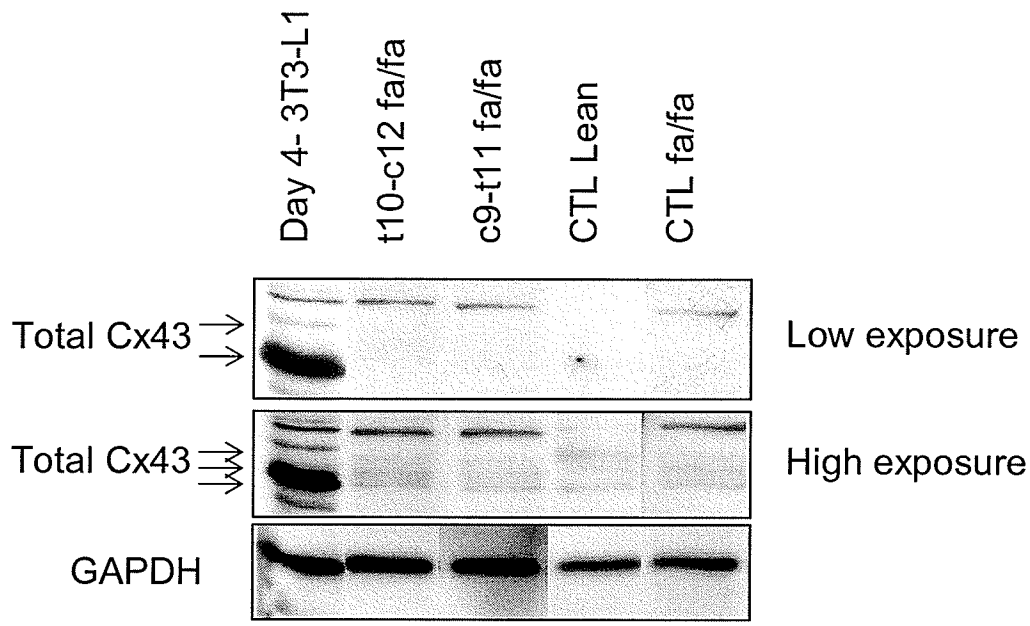
The results showed that Cx43 is at very low levels in this tissue, since a very high exposure was required to detect Cx43. Nevertheless, no significant changes in either Cx43 amount or phosphorylation were observed among the groups (Figure 22).

Figure 22 Effect of different CLA isomers on Cx43 expression in rat adipose tissue

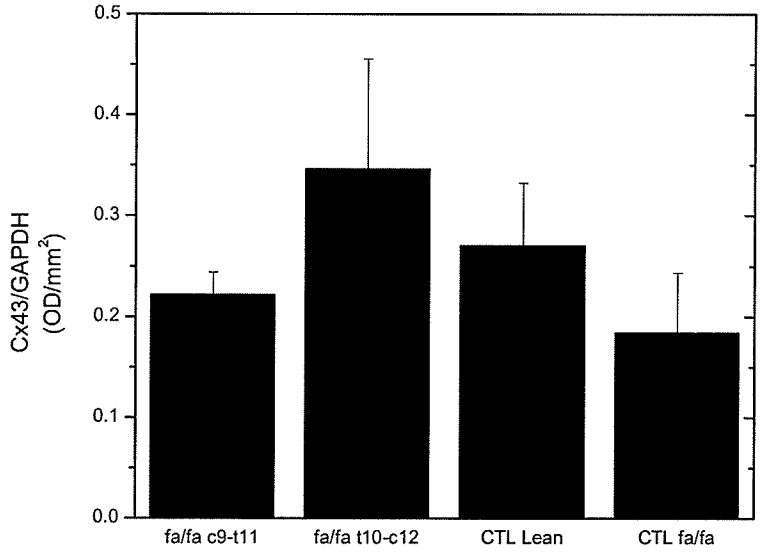
(a) Western blot for total Cx43 of protein extracted from adipose tissue of fa/fa Zucker rats, fed with c9-t11 and/or t10-c12 CLA isomers. Protein extracts of adipose tissue from fa/fa and lean Zucker rats fed 0% CLA semi-purified control diet (CTL) were used as controls. The Cx43 bands are shown at two different exposures. GAPDH was used as loading control, n=4.

(b) Densitometric assessment was used to quantify the total Cx43 band intensity shown in the previous panel (higher exposure was used). Data were adjusted according to the loading control (GAPDH). Statistical analysis was performed using one-way ANOVA. There were no significant differences.

(a)


Day 4- 3T3-L1
t10-c12 fa/fa
c9-t11 fa/fa
CTL Lean
CTL fa/fa
Total Cx43
Low exposure
Total Cx43
High exposure
GAPDH

(b)


Cx43/GAPDH (OD/mm²)
0.5
0.4
0.3
0.2
0.1
0.0
fa/fa c9-t11
fa/fa t10-c12
CTL Lean
CTL fa/fa

6 Discussion

6.1 Modulation of Cx43 during adipogenesis

Gap junctions are aqueous intracellular channels that allow molecules with molecular weights of less than 1 kDa, such as cAMP, ions and second messengers, to pass directly from the inside of one of cell to that of another (215). Cx43 and its phosphorylation play an important role in gap junction formation and function. An association between gap junction communication and differentiation of myoblasts and osteoblasts were reported by several groups (241-244). Balogh et al (241) showed that a decrease in Cx43 expression was correlated with rat L6 myoblast differentiation. Likewise, Lecanda et al (243) demonstrated that Cx43 modulates the expression of specific osteoblastic gene products by regulating the transcriptional activity of their promoters. Also, it has been known that adipocytes, myoblasts, and osteoblasts differentiate from a common mesenchymal progenitor cell (245). Therefore, there is a possibility that gap junction communication via Cx43 expression and/or phosphorylation, have a vital role during adipocyte differentiation. We demonstrated that Cx43 expression was robustly increased two days after differentiation during clonal induction. At this stage, Cx43 is in a highly phosphorylated state based on the presence of both P1 and P2 bands (Figure 3a), and according to the literature this will lead to an increase in gap junction communication. As adipocytes start to differentiate, a process that can be recognized by morphological changes and lipid droplet accumulation, Cx43 expression is down regulated significantly (Figure 1 & 4). This

result is in agreement with Umezawa et al (234) who found that Cx43 was down regulated at the gene level during marrow stromal cell differentiation.

We also showed that, two days after induction of differentiation with stimulation cocktail, Cx43 was highly phosphorylated (see Figure 3a) and possibly localized in the cell membrane (Figure 7). At this stage, Cx43 phosphorylation could promote activation of gap junctions, thus resulting in the transport of second messengers and cAMP which are required for adipocyte differentiation.

In support of our finding that changes in expression and phosphorylation of Cx43 occur during the adipogenesis, Yanagiya et al (235) in 2007 reported that an increase in Cx43 and gap junction communication is required for mitotic clonal expansion during adipogenesis. This group showed that 3T3-L1 pre-adipocytes failed to differentiate in the presence of the gap junction inhibitor 18-α glycyrrhetinic acid (AGA). However, they did not examine the phosphorylation state of Cx43 which is necessary for gap junction function.

We also investigated the cellular localization of Cx43 during 3T3-L1 pre-adipocyte differentiation since it has an important role in the function of gap junctions. Musil et al (219) in 1990 demonstrated that in communication deficient S180 and L929 cells Cx43 is absent from their membranes, but considerable intracellular staining was detected. These results show the pivotal importance of Cx43 localization on gap junction communication. The subcellular fractionation of 3T3-L1 in different stages of adipogenesis, day -3 (growing), day 0 (growth arrested), day 2 (mitotic clonal expansion), day 4 (mid-differentiation) and day 8 (differentiated), showed that Cx43

was localized in the membrane fraction, which also included membrane of various cytosolic organelles. Immunofluorescence staining demonstrated that Cx43 was localized mostly in the ER and less in the Golgi apparatus on day 0, but two days after addition of stimulus (day 2 of differentiation) Cx43 was mostly found on the cell periphery, possibly in the plasma membrane. It can be argued that this is the phosphorylated form of Cx43 due to the high level of gap junction communication in this phase, as well as the staining pattern that was observed (Figure 13a & b). As the morphological appearance of the cells changed during differentiation (day 4), Cx43 expression decreased and became localized in cytosolic organelles such as the ER and Golgi. At this point, Cx43 is in the cytosol, which indicates it cannot participate in cell-cell communication by gap junctions. This finding is in agreement with Azarnia et al (233) who showed that adipocytes do not communicate via gap junctions. However, it has been established that Cx43 can influence gene expression via a gap junction-independent process (246). Thus, Cx43 could have an effect adipogenesis even when present only in the cytosol.

Yanagiya et al (235) showed that Cx43 expression is required for initiating differentiation, but whether down regulation of Cx43 is also a necessary event has not been tested. Constitutive expression of Cx43 during 3T3-L1 CARΔ differentiation by adenovirus carrying Cx43 showed high expression of Cx43 on days 4 to 8, a period during which the levels of Cx43 normally decreased. Constitutive Cx43 expression did not have any effect on production of adipokines as indicated by adiponectin expression (Figure 14). Likewise, there was no effect on the formation of lipid droplets as shown

by perilipin (Figure 14) and A-FABP (Figure 23), or the expression of transcription factors such as PPARγ (data not shown) that mediate adipogenesis. Based on these results it was concluded that the loss of Cx43 is not required for progression through adipogenesis. Thus the decrease in Cx43 is a consequence of the differentiation process, likely through changes in the relevant transcription factor(s), rather than a causal event.

Regardless, Cx43 expression is pivotal for the clonal expansion phase at the start of differentiation, but apparently not afterwards. On the other hand, the relation between Cx43 phosphorylation and gap junction functionality has not been evaluated within the context of adipogenesis. As was mentioned previously, elevated expression of Cx43 does not lead to the formation of gap junctions if this protein is present in the cytosol (219). Phosphorylation of Cx43 may occur before Cx43 associates with the plasma membrane (230). Consequently it is important to investigate if constitutive expression of Cx43 leads to its phosphorylation, and if it does, where it becomes localized. Although phosphorylation of Cx43 in nonjunctional plasma membrane regions is not necessary, it has been reported that Cx43 in gap junctions is phosphorylated (primarily in the P2 form) and resistant to solubilization by Triton X-100 (230). In contrast, phosphorylated Cx43 in nonjunctional plasma membranes is soluble in 1% Triton X-100 (230). Although the Cx43 constitutively expressed in 3T3-L1 CARΔ was in both phosphorylated and unphosphorylated forms, neither Cx43 subcellular localization nor gap junction activity were investigated. Thus, it is a possibility that the phosphorylated Cx43 seen with Western blotting was not present in

the membrane. As a result, the gap junctions may not be functional and these were not required for adipogenesis.

The protein kinase inhibitor experiments supported the idea that different kinases such as PKC and PKA modulate Cx43 phosphorylation in different ways, Cx43 stayed phosphorylated in presence of bisindolylmaleimide I (PKC inhibitor) on day 8, but PKA inhibitors did not prevent the down regulation of Cx43 on day 8 although both inhibit adipogenesis (Figure 9a & b). Furthermore, these results suggest the possibility that phosphorylation of Cx43 during adipocyte differentiation has an important role. Therefore, further investigation is required to determine the role of Cx43 during adipocyte differentiation. In addition, since bisindolylmaleimide I treatments leads to cell enlargement but not lipid droplet accumulation and adipokine production, it is likely that PKC inhibition may play a pivotal role in the induction of obesity (247). Further analysis with respect to the role of PKC, PKA and other kinases is therefore recommended.

6.2 Modulation of adipogenesis by CLA

All stages of adipogenesis, from the very early stages when mesenchymal stem cells convert to pre-adipocytes to the final stage of terminal differentiation, are subject to environmental factors, particularly food, which can operate by modulating specific signaling pathways involved in regulating the differentiation process. A number of recent articles have examined the effect of CLA on factors associated with adipogenesis. House et al (189) reported a down-regulation of genes such as perilipin, caveolin, glycerol-3-phosphate acyltransferase, adiponectin, adipsin and PPARγ in the adipose tissue of mice fed t10-c12 CLA for 14 days. In addition, Brown et al (190) showed with cultures of human SV cells containing newly differentiated adipocytes that chronic (18 days) t10-c12 CLA but not c9-t11 CLA treatment decreases the expression of markers of adipocyte differentiation, including A-FABP, adiponectin, aP2, LPL, perilipin, GLUT4, PPAR γ 1/2 and C/EBP-α.

In this study, the effect of CLA on adipokine production and lipid droplet formation was examined from a different point of view. Our observation that t10-c12 CLA treatment decreased expression of adipokines such as adiponectin, (Figure 17) confirmed previous reports (190, 248). Likewise, adipocyte lipid droplet proteins were altered by t10-c12 CLA treatments (Figure 15 & 16), in agreement with an earlier report (191). As well, neither of these changes were obtained with c9-t11 CLA. While some of these results have already been described by other investigators (189, 190), this is the first time the effect of CLA on chemerin and adipsin has been studied. Although CLA had the same effect on production of these two adipokines as the others

examined previously (189, 190), the data establish that chemerin and adipsin expression occurs in conjunction with the other adipokines. Adipsin was the only adipokine that had its production (non-glycosylated form) and secretion (glycosylated form) blocked by t10-c12 CLA treatment. This was also evident with cells treated with the isomer mixture. In experiments with other adipokines, the effect of the isomer mixture most closely resembled c9-t11 CLA treatment. These data show that t10-c12 CLA has the dominant effect with respect to adipsin expression, whereas the opposite is true for other adipokines.

Interestingly, the effect of CLA on adipokine production was dissociated from its effect on lipid droplet formation. Changes in adiponectin expression as well as adipsin and chemerin expression were noticeable after 4 days with t10-c12 CLA treatment and this divergence was statistically significant by day 8.

On the other hand, changes in expression of adipocyte lipid droplet markers such perilipin and A-FABP could be seen in early differentiation (day 2). This finding was confirmed by our observation which showed CLA treatments resulted in significantly higher numbers of droplets in CLA treated cells versus control. These findings suggest that lipid droplet formation is altered by short term treatment with CLA, whereas the effect on adipokine production is a late event.

Thus, we have shown that t10-c12 CLA down regulates adipokine production and lipid droplet formation, but these processes are likely modulated through different pathways during adipogenesis or the same pathways that are activated at different times.

The present study also suggests that both CLA can stimulate adipocyte differentiation (Figure 15a & b) as indicated by the number of lipid droplets the mixture of isomer had the same effect (date not shown). Furthermore, the changes in lipid droplet numbers were paralleled by changes in lipid droplet protein (perilipin) and mediators of lipid metabolism (A-FABP). This finding is novel given that other studies have reported that t10-c12 CLA has anti-adipogenic effects at the end of the differentiation (191, 192). Interestingly, we did see an anti-adipogenic effect of t10-c12 CLA in the late stages of differentiation (reduction in adipokine production and lipid droplet protein expression) which implies CLA differentially affects early and late adipogenesis.

Our data regarding perilipin and A-FABP expression in the fully differentiated cells (day 8) demonstrate a significant decrease in these two lipid droplet markers with t10-c12 CLA treatment, although we did not observe any association between the number of differentiated cells (number of cells containing lipid droplets) and t10-c12 CLA treatment at that phase. As a result, we can conclude that perilipin and A-FABP are not accurate markers for lipid droplet formation. An alternative explanation is that t10-c12 treatment may cause changes on lipid droplet size but this was not noticeable on day 8 of differentiation. In support of this view, Chungs et al (191) showed that t10-c12 CLA treated cultures had smaller lipid droplets compared to c9-t11 treated cultures. Furthermore, the small lipid droplets created by t10-c12 isomer treatment contained higher levels of adipose-differentiation related protein (ADRP). In contrast, while t10-c12 CLA treatment also caused an increase in cytosolic perilipin, this protein was more abundant in cells with larger droplets. However, Chungs et al (191) showed that

t10-c12 CLA decreases the perilipin level in a whole cell lysate of SV after 8 days of treatment. These findings may explain the decrease in perilipin we observed with t10-c12 CLA, although the number of differentiated cells (indicated by lipid droplets) remained the same. Our results therefore suggest that t10-c12 CLA has a greater effect on lipid droplet formation than on adipokine production. The size of lipid droplets in early and late differentiation was not measured in our studies, but is planned for the future.

The mechanism by which t10-c12 and c9-t11 have different effects on 3T3-L1 cells is not known at this time. However, since these two isomers differentially regulate adipogenesis, there is the possibility that they have different affinities for transcription factors that regulate adipogenesis, such as PPARγ.

In our study we could not detect any significant change in PPARγ, leptin and FAS expression with t10-c12 and/or c9-t11CLA treatment during adipogenesis. The lack of change may be a function of the study length. Most of the published studies had durations of 12 to 16 days (190, 198), but in our case we terminated the study at 8 days. The decision was based on the fact that we have very high expression of Cx43 from day 0-4 and by day 6-8 Cx43 expression was decreased significantly. If the experimental period was increased, then Cx43 expression may not be detectable. Overall, our model is most similar to diet-induced obesity, since adipogenesis leads to an increase in the number of adipocytes. Our results suggest that CLA can stimulate obesity by promoting adipogenesis, but t10-c12 CLA decreases the expression of adipokines when added to mature adipocytes.

Since we are the first group to propose that Cx43 may serve as an adipogenic marker, we decided to examine the effect of t10-c12 and c9-t11CLA isomers on Cx43 expression during adipogenesis, based on evidence that these molecules can affect adipocyte differentiation (192). We did not find any association between CLA treatment and Cx43 expression in either early (day 2) or late (day 8) differentiation stages (Figure 21). On the other hand, there is evidence for a rapid (less than 24 hours) effect of CLA on lipid droplet markers such as perilipin and adipophilin (191). Likewise CLA may also influence Cx43 phosphorylation prior to the day 2 time point examined in this study. Investigations centered on the day 0 to day 2 time period are therefore warranted, specifically because different cellular processes are activate relative to later in the differentiation process.

Our examination of adipose tissue from rats fed t10-c12 and c9-t11 CLA also confirmed that CLA did not have an effect on the expression and/or phosphorylation of Cx43 in adipose tissue. At the time of this analysis, no adipose tissue from CLA-fed mice was available, but an investigation examining the effect of CLA on Cx43 expression in mouse adipose tissue is recommended.

7 Conclusion

Our study has confirmed that Cx43 is down-regulated in adipogenesis. In extending this work, we have shown that an increase in relative levels of phosphorylated Cx43 occurs two days after differentiation, and the modified protein is primarily located on the cell membrane. This study further established that increased levels of phosphorylated Cx43 can serves as marker for early-stage adipocyte

differentiation, prior to expression of adipokines and formation of lipid droplets. As well, the subcellular localization of Cx43 did not change when examined by Western blotting after fractionation, however, microscopy showed that subcellular localization of Cx43 varies during differentiation, with Cx43 moving from the cytosol (cell membrane compartment, Golgi and ER) on day 0 to the cell periphery on day 2 and back into the cytosol (cell membrane compartment, Golgi and ER) on day 4.

We also investigated the effect of constitutive expression of Cx43 during adipogenesis. Our results showed that over-expression of Cx43 during adipocyte differentiation have no apparent effect on adipokine production or lipid droplet formation. On the other hand, inhibition of PKC but not PKA prevents down-regulation of Cx43, although both treatments inhibit adipogenesis. Inhibition of PKC by bisindolylmaleimide I also had a major effect on cell morphology. These results suggest the possibility that phosphorylation of Cx43, an event that is required for its function, may play an important role in adipogenesis. Another kinase that is involved in Cx43 phosphorylation and has important role on adipogenesis is MAPK (ERK1/2), which was discussed previously. Further analysis will be necessary to determine which phosphorylation sites are critical for adipocyte differentiation.

We have shown that the t10-c12 CLA isomer suppresses the expression of adipokines such as adiponectin, adipsin and chemerin, and it has same effect on lipid droplet markers such as perilipin and A-FABP. On the other hand, the t10-c12 and c9-t11 CLA isomers did not affect expression and/or phosphorylation of Cx43 during adipocyte differentiation. In addition, the t10-c12 and c9-t11 CLA isomers

individually stimulate differentiation at an early stage. We conclude that CLA regulates both adipocyte production and lipid droplet formation, but these actions are mediated through distinct mechanisms or similar mechanisms that are temporally distinct.

7.1 Future directions

1. Identification of the phosphorylated amino acids on Cx43 by mass spectrometry during clonal induction phase would be important for understanding their function in the context of adipogenesis.
2. The effect of CLA on the expression and/or phosphorylation of Cx43 over short (less than 24 hours) and long (14 days) time periods needs to be investigated.
3. Further investigation is required to identify the mechanism by which CLA regulates adipocyte production and lipid droplet formation.
4. Tissue specific over expression of Cx43 should be used to further investigate the effect of Cx43 on adipocyte differentiation. This study should include mutants of Cx43 phosphorylation.
5. Scrape loading assay at different stages of differentiation will confirm the results regarding the subcellular localization of Cx43 and its relationship with gap junction function in adipogenesis.

7.2 Strengths and limitation of the research

One of the greatest strengths of this study was the use of an *in vitro* model. By using 3T3-L1 cells as the experimental model we were able to investigate the mechanism of action in relation to specific stages of adipocyte differentiation from pre-adipocytes to adipocytes. Our experiments with various inhibitors could not be performed with animal models. Also studying constitutive expression of Cx43 *in vivo* was not possible without employing a very expensive procedure.

Working with an *in vitro* model is always a weakness, since findings made during the course of experiments should be confirmed *in vivo* too. On the other hand, the 3T3-L1 cells are already pre-adipocytes which prevent us from studying the first stages of adipogenesis, specifically the commitment of stem cells to the adipocyte linage (pre-adipocyte). Another limitation in this study was in regards to localization of Cx43 in infected cells. It is not known whether the high expression of Cx43 resulting from adenovirus infection affected its localization in the plasma membrane or other cell membrane compartments. Also, if Cx43 is present in the plasma membrane, do the infected cells have active gap junctions?

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9 Appendix

9.1 Appendix 1: the effect of constitutive expression of Cx43 on A-FABP expression

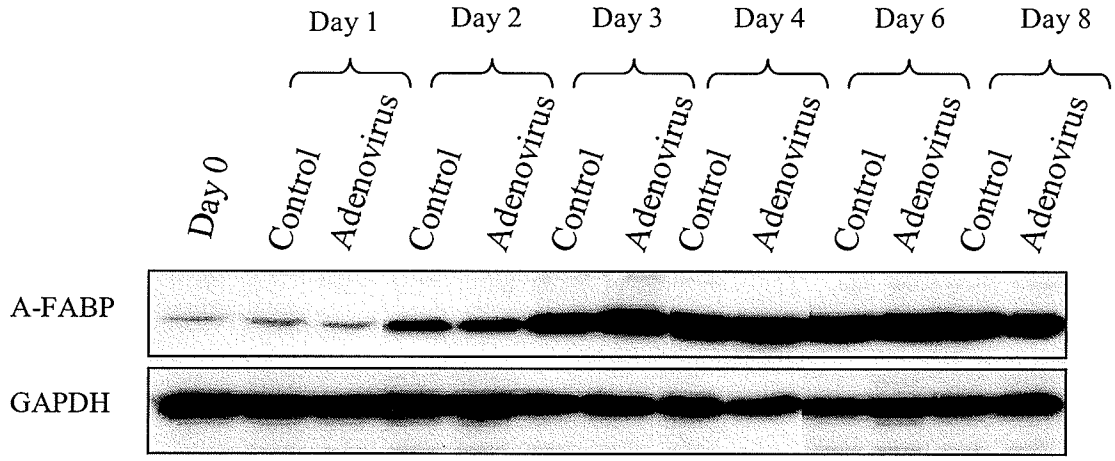

Figure 23. Effect of constitutive expression of Cx43 in 3T3-L1-CARΔ on A-FABP expression

Constitutive expression of Cx43 in 3T3-L1-CARΔ cells on day 0 (growth arrested), day 2 (mitotic clonal expansion), day 4 (mid-differentiation) and day 8 (differentiated) was achieved by infecting the cells with an adenovirus carrying Cx43. Western blotting was used to monitor expression of A-FABP over this 8 day period. GAPDH served as the loading control. n=4. Data not quantified.

9.2 Appendix 2: The effect of CLA isomers on PPARγ, leptin and FAS during adipogenesis

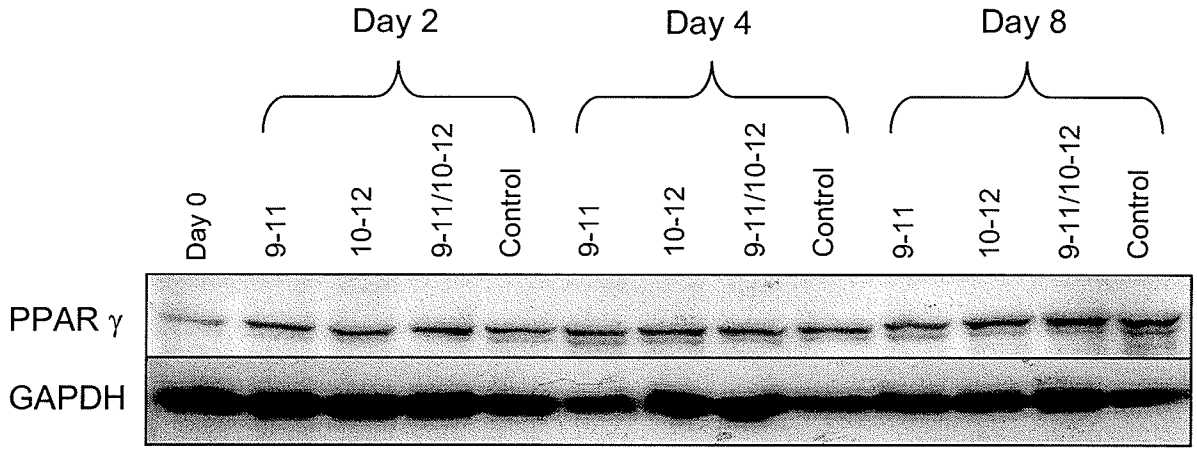

Figure 24. Effect of different isomers of CLA on expression of PPARγ during adipogenesis

Western blot of 3T3-L1 cells lysed after treatment with 60 μM c9-t11 and/or 60 μM t10-c12 CLA isomers on days 0, 2, 4 and 8 of differentiation. The blots were probed for PPARγ. GAPDH was used as a loading control. The figure provides a representative picture of 4 independent experiments. Data not quantified.

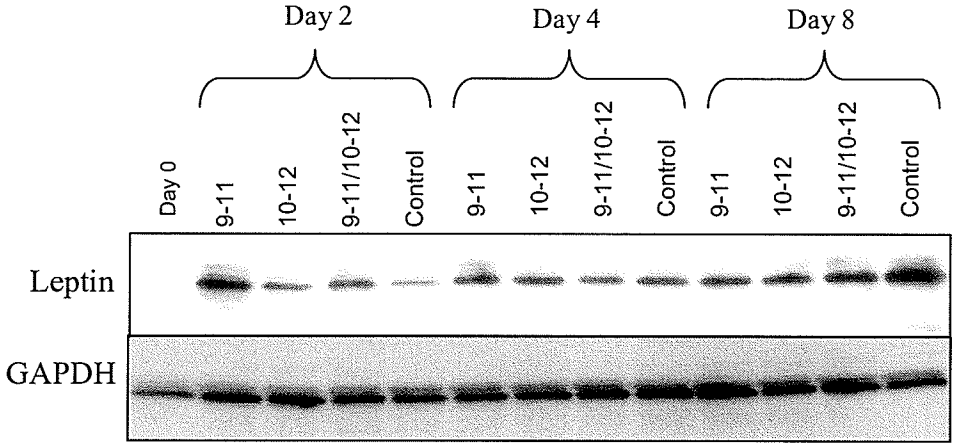

Figure 25. Effect of different isomers of CLA on expression of Leptin during adipogenesis

Western blot of 3T3-L1 cells lysed after treatment with 60 μM c9-t11 and/or 60 μM t10-c12 CLA isomers on days 0, 2, 4 and 8 of differentiation. The blots were probed for leptin. GAPDH was used as a loading control. The figure provides a representative picture of 4 independent experiments. Data not quantified.

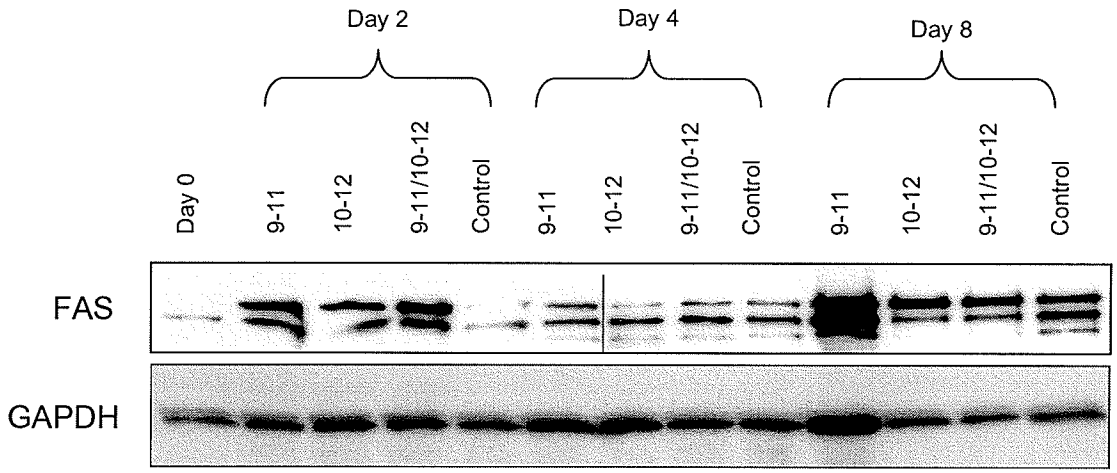

Figure 26. Effect of different isomers of CLA on expression of FAS during adipogenesis

Western blot of 3T3-L1 cells lysed after treatment with 60 μM c9-t11 and/or 60 μM t10-c12 CLA isomers on days 0, 2, 4 and 8 of differentiation. The blots were probed for FAS. eEF2 was used as a loading control. The figure provides a representative picture of 4 independent experiments. Data not quantified.