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The Antidiabetic Agent Pioglitazone Enhances Adipocyte Differentiation of 3t3-F442a Cells: Regulation of Glucose Transport Activity and Differentiation-Dependent Gene Expression

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THE ANTIDIABETIC AGENT PIOGLITAZONE ENHANCES ADIPOCYTE DIFFERENTIATION OF 3T3-F442A CELLS: REGULATION OF GLUCOSE TRANSPORT ACTIVITY AND DIFFERENTIATION-DEPENDENT GENE EXPRESSION

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CHAPTER I

INTRODUCTION

Adipocytes are highly specialized cells that play a central role in systemic energy homeostasis. These cells have evolved complex biochemical machinery and transport systems that allow them to store energy in the form of triglycerides in times of nutritional abundance, and release this energy in times of nutritional deprivation. Intensive studies of adipocyte biochemistry have revealed considerable information about the hormonal control of carbohydrate and lipid metabolism, particularly the hormone-regulated enzymes involved in these processes (1). Despite that, our understanding of how adipocytes differentiate and the precise role these cells play in maintaining blood glucose homeostasis is still incomplete.

When the function of adipocytes is altered, a number of disease states occur including cachexia, lipodystrophy, obesities, and diabetes (2-4). In the latter state, insufficient disposal of glucose by adipose and muscle leads to elevated blood glucose levels. This hyperglycemic state can be corrected in diabetic patients by treatment with oral hypoglycemic agents which have been mostly shown to increase glucose uptake by stimulating insulin release from the pancreas (5). A more recent and favorable approach is to use insulin-sensitizers, agents that act to enhance sensitivity of target tissues to insulin.

Our preliminary studies with pioglitazone, a novel hypoglycemic agent which presumably acts to enhance insulin sensitivity of target tissues (6), suggested that this agent might act to accelerate adipocyte differentiation.

Adipocyte cells express a program of proteins quite distinctive from other cell types, thus determining the ability of these cells to carry out their specialized function in energy balance (7-10). It is known that the acquisition of specialized phenotype, which characterizes the latter stages of differentiation of cells and tissues, is associated with the coordinate expression of new genes and the concomitant loss of expression of genes specific to less-differentiated cells. We therefore, sought to fully investigate the effect of pioglitazone on cell differentiation to determine how such an effect might contribute to the action of pioglitazone to regulate cellular glucose uptake.

CHAPTER II

REVIEW OF RELATED LITERATURE

Glucose Transport in the Adipocyte

Adipose tissue serves as the primary energy reservoir in mammals, converting excess dietary glucose and fatty acids into triglycerides. When dietary nutrients are lacking, these triglycerides are hydrolyzed to glycerol and fatty acids and released into the systemic circulation. It is estimated that adipose tissue accounts quantitatively for 10-30% of whole-body glucose metabolism (11).

The transport of glucose into muscle and adipose tissue, the rate limiting step of glucose utilization, is mediated by integral membrane carrier proteins belonging to a family of facilitated glucose transporters (12,13). These proteins transport glucose down its concentration gradient by facilitative diffusion, a passive, energy independent process that is tightly regulated in adipocytes by insulin and counterregulatory factors (1).

Stimulatory actions of insulin on glucose transport

The process by which insulin increases glucose transport in peripheral tissues has been studied extensively. Insulin actions are initiated by binding of the hormone to a specific membrane receptor that possesses an intrinsic protein kinase activity localized to its transmembrane ß-subunit (14). Autophosphorylation of the receptor on tyrosine residues results in receptor

activation with subsequent stimulation of glucose transport (15,16). At least two members of the glucose transporter family (GLUT1 and GLUT4) are expressed in both adipose and muscle, tissues where glucose uptake is sensitive to regulation by insulin (17). It was demonstrated that adipocytes contain a large intracellular pool of glucose transporters. Insulin stimulates glucose uptake primarily by induction of a rapid translocation of pre-existing transporters from an inner membrane fraction to the plasma membrane resulting in as much as a 100-fold increase in the V_{max} for transport (18,19). Although both GLUT1 and GLUT4 transporters are capable of translocation, the GLUT1 protein accounts for about 10-fold fewer glucose transporters than GLUT4 protein in primary adipocytes, and these GLUT1 transporters are mostly localized in the plasma membrane. Thus, the effect of insulin appeared to be predominantly on the GLUT4 protein (20,21). Since quantitative comparisons revealed a significant discrepancy between the transport activity and the number of transporters in basal and insulin-stimulated cells, it was suggested that other mechanisms such as changes of the transporter intrinsic activity also contribute to insulin action (19,22-24). Indeed, qualitative alternations of the glucose transporters were reported, including GLUT4 phosphorylation in adipocytes treated with ß-adrenergic agonists (25), or with agents that increased intracellular calcium levels (26). In addition to these acute effects, prolonged insulin treatment further regulated transport activity by a process requiring ongoing protein synthesis that may involve increased glucose transporter gene expression (17,27).

Inhibitory actions of counterregulatory factors on glucose transport

The actions of insulin are opposed by several counterregulatory factors including hormones, neurotransmitters, and metabolic substrates (2,28). Glucagon, the most potent counterregulatory hormone, acts mainly on liver, but it does not affect peripheral glucose metabolism (1). In adipocytes, it was shown that stimulators of lipolysis are inhibitors of insulin-stimulated glucose oxidation and by inference, inhibitors of glucose transport (29). Conversely, antilipolytic agents exert an opposite effect, and produce a moderate augmentation of the insulin-stimulated transport activity (29). In rat adipocyte cells, ligands such as catecholamines stimulate adenylate cyclase leading to elevated cellular cAMP levels and inhibition of insulin-stimulated glucose transport activity (30,31). Similarly, the adenylate cyclase inhibitors such as prostaglandin E_1, nicotinic acid, and non-metabolized adenosine analogues prevent or reverse this inhibition (32). The inhibitory effect of catecholamines in adipocytes reflects both a shift of the concentration-response curve to higher insulin concentrations and a marked reduction of the maximal effect on insulin. The first component may be attributed to inhibition of the insulin receptor kinase, whereas the latter effect appears to reflect a change in intrinsic activity of glucose transporters. In addition, the insulin-induced subcellular translocation of glucose transporters is decreased in cells that have been exposed to ambient levels of cAMP. In contrast to these findings, activation of the lipolytic system in human fat cells at the levels of adrenoreceptors, adenylate cyclase, or protein kinase appears to be associated with an acceleration of the glucose transport rate (29). This indicates that there are important species differences in the regulation of adipocyte glucose transport at

least regarding the effect of lipolytic agents. On the other hand, the action of antilipolytic agents (*i.e.* insulin and adenylate cyclase inhibitors) appear to be similar in human and rat fat cells (29).

Two additional hormones that are known to modulate the regulation of glucose transport by insulin in the rat adipose cells are the glucocorticoids (33) and growth hormone (34). It has been established that glucocorticoids, specifically the analogue dexamethasone, induce a decrease in both basal and insulin-stimulated glucose transport activity (35) either by accelerating degradation of glucose transporters present in the plasma membrane and/or by inducing a redistribution of glucose transporters in favor of the intracellular pool. An excess of circulating growth hormone in both rat and man appears to correlate with insulin resistance and elevated blood glucose levels, while growth hormone-deficient conditions are associated with an increased response to insulin (19); the cellular mechanism underlying this phenomenon has not been established.

Forskolin, an agent known to elevate cellular cAMP levels, is another powerful inhibitor of glucose transport in adipocytes; this agent initiates adenylate cyclase stimulation and inhibit glucose transport at different sites including direct binding to glucose transporters (36). Inhibitors of nucleoside transport (methylxanthines) are also potent inhibitors of glucose transport in rat adipocytes, apparently by direct interaction with the glucose transporters (36).

Finally, metabolic substrates such as glucose and amino acids have profound effects on modulating insulin's stimulatory actions on glucose uptake in primary cultured adipocytes (37). Specifically, circulating levels of glucose and amino acids modulate cellular metabolism through two independent, but integrated,

control systems. The first system is the classical substrate-pancreatic axis, in which glucose and amino acids act as potent secretagogues for the release of insulin. The second control system acts in tandem with the first by enabling insulin target tissues to continuously monitor circulating levels of glucose and amino acids, thereby modulating cellular responsiveness at distinct postreceptor sites within the insulin action pathways. It was recently discovered in the rat primary cultured adipocytes that routing of incoming glucose through the hexosamine biosynthesis pathway leads over several hours to a refractory state in which the ability of insulin to stimulate glucose uptake is severely impaired- a state known as insulin resistance. Glutamine:fructose-6-phosphate amidotransferase was also found to play a central role in the development of insulin resistance, as this enzyme catalyzes the first and limiting step in the formation of hexosamine products (37).

Adipocyte Differentiation

Adipocyte differentiation occurs through a process in which preadipocyte cells, *i.e.* fibroblasts cells populating the future adipose tissues, are reprogrammed to stop dividing and start expressing properties and functions of adipocytes. While the reprogramming process generating committed cells takes place only in embryonic life, the differentiation of these committed cells can take place at any time, even in adult life (38).

Models for studying the adipocyte

Since primary adipocytes isolated from adipose tissue only remain viable a few hours, the ability to study adipocyte differentiation, gene expression, and

morphological and biochemical changes during the process, was greatly facilitated by the isolation of preadipocyte cell lines that could be induced to differentiate into adipocytes in culture. Studies showed that Swiss 3T3 fibroblasts were able to differentiate spontaneously to adipocytes in culture (39). This process took 2-4 weeks, and the percentage of differentiated cells varied from culture to culture. From these established fibroblasts, Green and Kehinde isolated clonal sublines (e.g. 3T3-L1 and 3T3-F442A) that could differentiate to adipocytes at high frequency (40,41). Also established were clonal cell lines ob17 and HG-Fu rising from differentiated adipocytes from the epididymal fat pad of adult ob/ob mouse, PFC6 from the stromal-vascular fraction of epididymal fat pad which contains preadipocyte cells, and the ST13 cell line arising from the parental line ST that was itself established from a mammary carcinoma of the ddN mice (42).

Such differentiation was studied extensively and was found in many respects to resemble the *in vivo* development of adipocytes from stromal vascular cells (the adipose precursor cells). In these cell lines, the preadipocytes are fibroblast-like cells. Once the cell growth was arrested by reaching confluence in culture, the cells exhibited morphological characteristics of adipocytes and accumulated lipid droplets. At the same time, the cells developed enzymatic activities related to fatty acid and triglyceride synthesis (43), and acquired sensitivities to hormones that are important in the regulation of fat and carbohydrate metabolism (44,45).

Under ordinary conditions, only a minority of the cells undergo adipose differentiation. There is therefore a marked heterogeneity in the predisposition for differentiation. This property of preadipose cells ensures that some cells are

retained in developing tissue for later recruitment to formation of more adipose cells (38).

Mitogenic response of differentiating cells and its role in clonal selection

It is known that for preadipocytes, as well as other cell types, growth arrest is a prerequisite for the induction of the differentiation program (46). Paradoxically, among the earliest events taking place in adipocyte differentiation was a mitogenic response in susceptible cells so that they appeared later in clusters (46-48). Studies have shown that after stimulation with adipogenic serum, 3T3-F442A preadipocyte cells underwent a mitogenic response characterized by a burst in DNA replication and cell division before they expressed the differentiated state. In addition, it was demonstrated that DNA synthesis was required for adipose differentiation of cultured cells (48). Growth arrest at the G_1/S boundary triggered the activation of early genes. But the expression of late markers, which led to terminal differentiation and accumulation of neutral lipids, took place after a limited number of mitoses of early-marker-expressing cells. The mitogenic proliferation of those cells responding to the adipogenic factor led to a selective increase in their progeny, all of which formed adipocytes (49). This form of mitogenic response occurred only in the early stages of adipocyte differentiation since fully differentiated cells do not divide (38).

Morphological changes during adipocyte differentiation

Differentiation of preadipocytes into adipocytes is accompanied by changes in their morphological appearance. Preadipocytes appear as extended irregular

fibroblast-like cells. Upon differentiation, such cells enlarge, acquire a spherical shape, and later accumulate lipid droplets in their cytoplasm. Small lipid globules may ultimately fuse into a few or a single fat globule. It was shown that the change in cell shape during differentiation was independent of lipid accumulation. This was demonstrated by culturing cells in biotin-deficient medium wherein the cells were unable to synthesize fatty acids and were therefore unable to accumulate triglycerides (50). Nevertheless, these cells acquired a spherical shape, suggesting that the morphological changes were part of the differentiation program and not simply the result of triglyceride accumulation in their cytoplasm. Measured changes of the synthesis and assembly of cytoskeletal proteins during differentiation were consistent with that concept. Undifferentiated 3T3-L1 cells, for instance, contained actin-like stress fibers and synthesized actin as well as bundles of intermediate filaments (51). Upon differentiation, both structures disappeared and cell content of actin and tubulin decreased several fold. A decrease in the net production of extracellular matrix components, particularly proteoglycans typical of fibrous connective tissue, was also described (52).

Enzymatic changes and triglyceride accumulation during differentiation

During adipocyte differentiation, there is a large increase in the synthesis of more than 50 cellular proteins (7) and a 50- to 100-fold increase in the cellular triglyceride content (40). Triglyceride accumulation can be affected by different factors, which make it an extremely variable feature. Insulin, methyl isobutyl xanthine and dexamethasone were shown to increase, while ß-adrenergic catecholamines decrease, triglyceride accumulation (38,40). A coordinate rise

in most of the enzymes of de novo fatty acid synthesis (53) and of some of the enzymes of the triglyceride synthesis pathway was observed (43,54). This greatly enhanced the ability of cells to synthesize fatty acids, to take up exogenous fatty acids, and to esterify fatty acids obtained from either source (55,56); all of these actions resulted from increased expression of appropriate regulatory enzymes.

Changes in receptor levels and hormonal responses of differentiated cells

Although preadipocyte cells in culture possess cell surface receptors for insulin, no metabolic responses to insulin are observed in these cells. Upon differentiation, there is a dramatic increase in the insulin-binding capacity (57) which is primarily accounted for by a change in receptor number resulting from a parallel rise in the rate of insulin receptor synthesis (44,45). These changes in receptor levels were accompanied by increases in glucose metabolism and insulin responsiveness (58-60), the latter of which was shown to be due to a decreased basal glucose transport rate rather than an increased maximal rate after stimulation by insulin. The coupling between insulin binding and the biological response was greatly enhanced, as was the coupling to adenylate cyclase in response to ACTH and ß-adrenergic catecholamines (7,38,61). In addition, adipocytes differentiated from different cell lines manifest properties similar to adipocytes isolated from adipose tissue, including responsiveness to lipolytic hormones, elevated basal glucose transport activity after prolonged exposure to insulin (62), and resistance to stimulation by insulin after chronic exposure to insulin, glucocorticoids (7), and growth hormone (63).

Factors That Regulate Adipocyte Differentiation

The specific factors that regulate adipocyte differentiation and the mechanisms by which such factors act have not been revealed. Initially, cells have to be committed for differentiation. Evidence of the process whereby committed cells were generated resulted from the experiment in which cells of the nondifferentiating mouse 3T3-C2 line were transfected with DNA from uninduced 3T3-F442A preadipocytes or from human fat tissue (64). Such transfection permitted recovery of 3T3-C2 transfectants that differentiate into adipocytes in the presence of insulin, thus implicating the presence of transregulatory gene or genes in committed 3T3-F442A or human fat cell DNA that was sufficient to commit 3T3-C2 cells to adipocyte differentiation. In order for preadipocytes to subsequently differentiate, the cells must reach a confluent resting state that is followed by a mitogenic response (46). However, growth arrest itself was not sufficient to induce differentiation, since agents such as sodium pyruvate that cause growth arrest only accelerated differentiation in the presence of other adipogenic factors (65). Extracellular and regulatory adipogenic serum factors of different species were thus shown to be required for the process which could be divided into early and late stages. Fetal calf serum is by far the most effective serum used for cellular differentiation (66). In addition, bovine pituitary extracts showed an adipogenic activity that was at least three orders of magnitude higher in pituitary than in fetal calf serum. Only growth hormone was found to promote preadipocyte differentiation, while adipogenic activity was not associated with other pituitary polypeptides (67). A number of studies have implicated both growth hormone (GH) and insulin like growth factor-I (IGF-I) (68-70) during the early events of adipose

differentiation. Growth hormone is suggested to promote preadipocyte differentiation, generating committed precursors sensitive to the proliferative mitogenic action of IGF-I. The late events of the adipose pathway represent the terminal differentiation of the committed cells, and require the presence of modulating signals mainly elicited by physiological concentrations of insulin (43,47). Thus, it seems that a combination of mitogenic-adipogenic signals is required to trigger terminal differentiation of preadipose cells. The requirement for serum, however, is not absolute since 1246 cells were able to differentiate in a serum-free medium containing insulin, transferrin and fibroblast growth factor (7). Another study demonstrated a complete adipose differentiation of 3T3-L1 cells in a chemically defined culture system supplemented with insulin, glucocorticoids, and factors that increase the cAMP content of the cells (71), and a third study showed 3T3-L1 differentiation by IGF-I and insulin in a serum-free culture system (69). Although the expression of the adipocyte phenotype has been shown to be under the control of lipogenic hormones, especially insulin, other factors including indomethasone (60), prostaglandin F2α and 1-methyl-3-isobutylxanthine (72), dexamethasome (65,73), triiodothyronine (74), glucocorticoids (75), and other factors such as RU38486, a potent glucocorticoid and progestin antagonist(76), sodium pyruvate in combination with insulin or dexamethasone (65), the antidiabetic agent AD4743 (77,78), or the expression of transfected *ras* oncogenes (79), have all been variously shown to accelerate adipose differentiation in adipocyte models

On the other hand, adipocyte cells may lose their differentiated function and exhibit properties of less differentiated cells in response to modulation by

environmental influences. Cultured TA1 adipocytes treated with tumor necrosis factor alpha (TNFα) for instance, lose intracytoplasmic lipid and, over a period of days, come to resemble their predifferentiated progenitors (80). Within hours of TNF addition to adipocytes, mRNAs for genes whose expression is augmented during adipogenesis decreased to predifferentiated levels. Transforming growth factor-ß also caused a rapid decrease in expression of adipose genes when added to fully differentiated cells (80,81), while interferon completely inhibited the differentiation of 3T3-L1 mouse fibroblasts into adipocytes (82).

Mechanisms Underlying Activity Changes During Differentiation

The acquisition by adipose precursor cells of the morphological and biochemical characteristics of mature adipocytes is mediated by extensive reprogramming of gene expression resulting in alternations in the abundance of several mRNAs and proteins, and appearance of many new adipocyte-specific mRNAs and proteins. These changes are brought about by both transcriptional regulation at the level of the gene and post-transcriptional regulation between the RNA and the protein product (83-85).

Expression of adipocyte-specific genes

Electrophoretic studies of preadipocyte and adipocyte proteins indicated that there were major quantitative changes in at least 100 protein species and that 40% of the soluble adipocyte proteins represented newly produced material (83,86) including enzymes involved in fatty acid and triglyceride synthesis, such as glycerol phosphate dehydrogenase (GPDH), fatty acid synthetase,

phosphoenol pyruvate carboxykinase, and lipoprotein lipase (7,56). Adipocyte differentiation was later shown to be associated with the coordinate expression of new genes and the concomitant loss of expression of genes specific to less-differentiated cells (8-10,73). Subsequently, new and potentially important gene products of the adipocyte were identified by isolating cDNA clones corresponding to mRNAs that are specifically induced during adipocyte differentiation (84,85). Included are the cDNA clones encoding GPDH, a key enzyme in triglyceride synthesis which provides the glycerolphosphate backbone for acylation and its protein accounts for about 2% of the soluble protein in the adipose 3T3 cells, the adipocyte fatty acid-binding protein, aP2, that accounts for about 6% of soluble protein in the adipose cells, and the adipocyte serine protease homolog, adipsin, that accounts for about 5% of the soluble protein of adipose cells (85). The following section will discuss some of these genes, which are also known to be involved in energy metabolism.

The aP2 gene

The gene encodes an adipocyte homologue of myelin P2. This protein accounts for about 6% of adipocyte soluble proteins and has sequence homology to a class of fatty acid-binding proteins that includes liver fatty acid binding protein, intestinal heart fatty acid-binding protein and cellular retinoic acid-binding protein (87,88). The precise function of this putative lipid carrier protein is unknown, although it was found to be a cellular target for phosphorylation by the insulin receptor tyrosine kinase, and may thus be considered a potential mediator of insulin actions (89-91). The abundance of

aP2 mRNA is increased by a factor of at least 100 during adipocyte differentiation (92).

The adipsin gene

This gene encodes an adipocyte-specific serine protease homolog with complement factor D activity, formerly called 28k, that is synthesized by adipocytes and secreted into the blood stream (93-95). Its protein accounts for about 5% of the adipocyte soluble proteins. The expression of this gene was found to be quantitatively altered in certain models of obesity and diabetes (96-98). Adipsin mRNA abundance is increased in adipose tissue during fasting in normal rats and in diabetes due to streptozotocin-induced insulin deficiency, but its mRNA abundance is decreased during the continuous infusion of glucose, which induces a hyperglycemic, hyperinsulinemic state that is accompanied by an increased adipose mass. Adipsin mRNA expression is also suppressed ($>$ 100-fold) in two strains of genetically obese mice (*db/db* and *ob/ob*), and in obesity chemically induced by injection of monosodium glutamate into newborn mice. Circulating adipsin protein is correspondingly decreased in these animal models of obesity (98).

Adipsin mRNA has also been shown to increase over 100-fold during differentiation of 3T3-preadipocytes (85,99). Studies have shown that insulin markedly increases adipsin secretion and mRNA levels during differentiation, presumably due to the ability of insulin to stimulate differentiation (100). This is in contrast with the finding of another study in which cells were differentiated in serum deprived-medium. These cells already exhibit, at day 1, a maximal amount of mRNA encoding for adipsin, which is tenfold decreased by the

presence of insulin (73). Both studies however, demonstrated negative effects of insulin on the abundance of adipsin mRNA and secretion in mature adipocyte cells. These findings indicate that adipsin appears to be an early marker of adipocyte differentiation, and its gene expression is increased during differentiation, but is dramatically down-regulated by the presence of insulin, especially after differentiation (73,100).

The insulin receptor gene

This gene encodes the insulin receptor which mediates actions of insulin in target cells, presumably through the activation of the receptor tyrosine kinase (15,101). Although insulin receptor is a housekeeping protein that is essential for cell growth and is usually synthesized at low levels in all cells, there are increased numbers of insulin receptors in major target tissues for insulin such as liver, muscle, and adipocytes. In addition, the number of insulin receptors decreases considerably under certain conditions, *i.e.*, chronic exposure of cells to insulin leads to a net loss of receptors from cell surface (down-regulation) by decreasing biosynthesis and accelerating degradation (58). In contrast, differentiation of 3T3-preadipocytes leads to a 10- to 20-fold increase in the number of insulin receptors on the cell surface, and this change is paralleled by a 10-fold increase in insulin receptor mRNA transcripts of two sizes (57,59,102). Prolonged incubation of 3T3-L1 differentiated adipocytes with insulin however, does not induce hormone-mediated down-regulation of the receptor (44).

Glucose transporter genes

Glucose uptake across plasma membrane of insulin-sensitive tissues (heart, skeletal muscle and adipose) is carried out mainly by means of two transporters (20) belonging to a family of at least five tissue-specific facilitated-diffusion proteins (12,13,103-105). GLUT4 is an insulin-responsive glucose transporter expressed exclusively in fat and muscle (106); GLUT1 is a transporter for basal uptake of glucose that is expressed ubiquitously in most tissues. The rate of glucose uptake is subject to both short-term and long-term regulation. It has been shown that insulin acutely, *i.e.* within minutes, increases glucose uptake 10- to 20-fold via the redistribution of glucose transporters from an intracellular pool to the plasma membrane (20,107). After hours of treatment with insulin, there is a further increase in glucose uptake, which requires ongoing protein synthesis and may be mediated by an increase in glucose transporter gene expression (108). Other examples of long-term alternations in the responsiveness of glucose uptake to insulin include the fasted and diabetic states, both of which are associated with a decrease in GLUT4 mRNA and protein (109), and the differentiation of preadipocyte cells. It was demonstrated that undifferentiated 3T3-L1 and 3T3-F442A preadipocytes express only GLUT1 transporter mainly on the cell surface, and are unresponsive to insulin (110). Upon differentiation, a large intracellular pool of glucose transporters is formed by redistribution of GLUT1 and by addition of newly synthesized GLUT1 and GLUT4 transporter into this pool (61,111), contributing to the development of insulin sensitivity in the differentiated cells (61). Once differentiated, 3T3 adipocytes display properties similar to adipocytes from fat tissue with regard to short and long term regulatory effects on glucose transport.

Such cells respond to acute insulin treatment with increases in glucose transport above basal levels (61). Both GLUT1 and GLUT4 appeared to be hormone-sensitive, since in differentiated cells insulin gave rise to transporter translocation from the intracellular compartment to the plasma membrane. Chronic-insulin treatment has been shown to produce an increase in the mRNA and total protein for GLUT1 (108). The expression of GLUT1 is associated with an increase in basal glucose uptake, but not in the insulin-stimulated increase in 2-deoxyglucose uptake rates by 3T3-L1 fibroblasts and adipocytes (112). However, no change was observed in the total cellular mRNA and protein for GLUT4, although availability of GLUT4 at the cell surface was down-regulated to half the level found in the acute treatment (62). Differentiated 3T3 adipocytes, however, differ from those cells isolated from fat tissue with regard to the relative abundance of their glucose transporter isoforms. GLUT4 protein in fat tissue adipocytes is expressed at a considerably higher levels than is GLUT1 protein. This is reversed in 3T3-L1 adipocytes where the relative amount is 4 to 1 for GLUT1 and GLUT4, respectively (21). These observations indicate that the translocation of GLUT4 could largely account for the insulin effect on transport rate, as the case in fat tissue, only if the intrinsic activity of GLUT4 is much higher than that of GLUT1.

Regulation of adipocyte gene expression

The observed coordinate changes in the expression of a group of genes during differentiation has led to intensive investigation of the mechanisms underlying such processes. Run-on transcription activity by isolated nuclei revealed that the large increases in abundance of several mRNAs were

accompanied by marked rises in their nuclear transcription rates. This was particularly shown for highly regulated mRNAs (20- to 100- fold increase in the steady-state mRNA levels) such as those encoding aP2 (8,9), adipsin (9), glycerol phosphate dehydrogenase (9), and phosphoenolpyruvate carboxykinase (56), thus suggesting that an increased rate of specific transcription is primarily responsible for the accumulation of these mRNAs during differentiation. The activation of transcription of those adipocyte-specific genes was not synchronous indicating that the temporal differences in the appearance of different adipocyte mRNAs probably result from differences in the times of activation of transcription (9). However, no changes in the rates of transcription was observed for moderately regulated mRNAs (2- to 4- fold increase or decrease in their steady state mRNA levels) such as fructose-1,6-bisphosphate aldolase, actin and tubulin mRNAs (8). These same studies indicated that post-transcriptional regulatory mechanisms were operative during differentiation of adipose precursor cells. This was inferred from the poor correlation between relative steady-state mRNA levels and transcription rates for some of the genes. Steady-state levels of mRNAs for aP2 and adipsin for instance, were much greater than would be expected from their relative transcription rates, indicating a possible role for regulation of mRNA stability (84).

A search for candidate transcription factor(s) or regulatory sequence element(s) that could be involved in coordinating those observed changes in gene expression revealed several different possible mechanisms. DNase I footprinting for promoters of several adipocyte-specific genes using nuclear extracts from undifferentiated and differentiated 3T3-L1 cells revealed that a differentiation-specific nuclear factor binds to specific regions relative to the

start site of transcription. This transcription factor was later shown to be the CCAAT/enhancer binding protein (C/EBP), the mRNA of which was found at high levels in tissues that metabolize lipids and cholestrol-related compounds, mainly liver and adipocytes (113,114), and its expression was limited to fully differentiated fat and liver cells. Transient cotransfection studies revealed that C/EBP binds to, and trans-activates promoters of several differentiation-dependent genes, including GLUT4 (115), aP2, stearoyl-CoA desaturase 1 (116,117), and insulin receptor (118) gene promoters, as well as the C/EBP promoter itself (119). Hence, it was proposed that C/EBP may play a key role in coordinating transcription of genes involved in energy metabolism during differentiation of adipocytes (120,121). Consistent with this proposed role is the observation that an increase in the rate of C/EBP transcription precedes that of several adipose-specific genes whose promoters are transactivated by C/EBP (122).

On the other hand, a comparison of the DNA sequences 5' to the transcription start site of several differentiation-dependent genes allowed characterization of sequence homologies which might represent core elements involved in differentiation-dependent transcription. Two such elements, designated fat-specific elements FSE 1 and 2, were described (92,123). These elements are FSE1, a 14-base sequence present in multiple nonidentical copies in the 5' flanking region of GPDH, aP2, and adipsin, and FSE2, a 21-base sequence present in a single copy in the 5' flanking regions of only the GPDH and aP2 genes that acts as a negative regulator in preadipocytes (92,123). The gel mobility shift assay indicated that Fos, a nuclear phosphoprotein encoded by the *c-fos* proto-oncogene, is a major component of a protein complex that binds

the FSE2 element in a differentiation-dependent manner (124). This was demonstrated by the ability of antibodies to Fos to disrupt specific binding of factors to the FSE2 sequence but not to factor-binding sequences from several other genes (124). Within the aP2 FSE2 sequence, Fos complexes bind DNA containing the sequence TGACTCA, previously identified as the consensus sequence for the binding of mammalian transcription factor AP-1 and yeast transcription factor GCN4 (125). Consistent with the suggested regulatory role for FSE2 is the observation that Fos complex exerts a negative regulatory effect on aP2 gene expression in undifferentiated 3T3-F442A cells. Another study showed that at least two distinct sequence elements in the aP2 promoter contribute to the expression of chimeric gene in transfection assays (126). An AP-1 site shown to bind to Jun- and Fos-like proteins, serve as a positive regulator of gene expression in adipocytes only, and C/EBP binding sequences upstream to the AP-1 site can stimulate expression in fat cells in the intact aP2 promoter. This indicates that sequences which bind C/EBP and the Fos-Jun complex play major roles in the expression of the aP2 gene during adipocyte differentiation (126). However, recent evidence using a series of transgenic mice containing 5' flanking sequences in the aP2 promoter linked to the bacterial gene chloramphenicol acetyltransferase demonstrated that the proximal-promoter binding site for AP-1 and C/EBP were neither sufficient nor necessary to give adipose-tissue expression *in vivo*. Rather, an upstream adipose specific enhancer was required and sufficient for the expression of the chimeric gene *in vivo* (127).

Also identified are sequences with homology to the triiodothyronine (T3) receptor binding site that are present in multiple copies in the regulatory region

of adipsin, aP2, and GPDH. This is in agreement with the observation that T3 stimulates the differentiation in preadipocyte cell lines and in primary cultures of stromal vascular cells. In addition, GRE core sequence was identified in the 5' flanking region of the aP2 gene which is probably responsible for the induction of the aP2 gene by glucocorticoids (75,84). Although several hormones and transcription factors have been implicated in adipose-specific gene expression, little is known about sequences responsible for transcriptional activation by each hormone. The cellular factors that interact with these hormones to modulate transcription also have not been identified.

Adipocytes and Diabetes

The major depots for whole body glucose disposal are muscle and adipose. Since insulin is known to facilitate glucose disposal, insufficient glucose disposal by these tissues can result either from absolute deficiency of insulin as in insulin-dependent diabetes mellitus (IDDM), or from resistance of target tissues to insulin as in non-insulin-dependent diabetes mellitus (NIDDM). Intensive studies were carried out to characterize mechanisms for cell resistance to insulin. The action of insulin involves many gene products. Target-cell resistance could therefore be due to defects affecting any protein between the receptor and the final insulin-regulated proteins (128). Candidate proteins include the insulin receptor, members of the family of glucose transporter proteins, several enzymes involved in glucose metabolism such as hexokinase, and substrates for insulin-receptor kinase or signaling intermediates (129). In addition, many circulating factors and physiologic states adversely affect the sensitivity of target tissues to insulin. Insulin resistance for instance, is

ubiquitous in obese people and in lean or obese patients with non-insulin-dependent diabetes (NIDDM) (129). Investigation of target-tissue defects in these states indicated a complex etiology (4,130) variously involving decreases in the number of insulin receptors(131) or impaired insulin receptor autophosphorylation and tyrosine kinase activity (132), altered expression of insulin receptor types that are generated by alternative splicing of a primary gene transcript (133), impairment of insulin-stimulated glucose transport, and decreases in the number of GLUT4 glucose transporters as well as impairment of their functional activity in adipocytes of NIDDM and obese subjects (134-138).

Oral Hypoglycemic Agents

Early hypoglycemic agents used for the treatment of NIDDM largely addressed the insulin secretory defects of the disease. *In vitro* studies have shown that first generation-sulfonylureas exert a direct and immediate stimulatory effect on insulin secretion from islet ß cells by increasing sensitivity of islet ß cells to the insulin-releasing effect of glucose (5). Sulfonylureas interact in a specific and saturable manner with a single type of binding site on the cell plasma membrane (5). The more complex substitutions of second-generation sulfonylureas may be responsible for improved anchorage at the binding site, thus conferring greater insulin-releasing potency. In addition, sulfonylureas exert extrapancreatic glucose lowering effects mainly directed towards correcting the defects of glucose metabolism in liver, muscle and fat of NIDDM patients. However, sulfonylureas alone are ineffective in the control of

hyperglycemia if insulin is absent, but they increase the hypoglycemic effect of exogenous insulin (139-142).

A novel class of hypoglycemic agents that are effective in animal models of NIDDM has recently emerged. Thiazolidinediones including pioglitazone, ciglitazone, and englitazone, were all shown to lower plasma glucose levels presumably by enhancing both sensitivity and responsiveness of target tissues to insulin. Epididymal fat pads removed from ob/ob mice treated with ciglitazone were shown to exhibit increased response to insulin in terms of insulin-stimulated glucose oxidation and lipogenesis (143). Such treatment did not change insulin binding, thus suggesting that these agents act to amplify cellular responses to insulin at post-receptor level(s). In agreement with this concept are observations that pioglitazone corrected deficits in glucose transport and GLUT4 mRNA and protein abundance in fat and, to a lesser extent, muscle cells of insulin-resistant animals, and that such actions were dependent on the presence of circulating insulin (6). Pioglitazone was also shown to increase insulin-stimulated autophosphorylation and kinase activity of the insulin receptor in muscle cells (144), further supporting the concept that pioglitazone acts to amplify post-binding steps in the insulin-response cascade.

Proposed studies

Our laboratory has been interested in investigating the mechanism(s) for insulin resistance in non-insulin-dependent diabetes mellitus, *i.e.* how the function of muscle and adipose is altered in insulin resistance and what molecular mechanisms underlie such changes. We are also interested in probing the action of agents that have the potential of reversing this resistance.

One such agent, pioglitazone, is a compound that has been identified empirically for its ability to lower elevated plasma glucose levels in animal models of NIDDM (145).

Hypothesis

I propose to test the hypothesis that the antidiabetic agent pioglitazone acts to enhance cellular glucose utilization by promoting differentiation of preadipocytes to adipocytes, thus inducing expression of genes encoding glucose transporters and other adipocyte-specific proteins.

In this differentiated state, adipocyte cells are known to be fully active in glucose uptake, storage, and metabolism.

Specific Aims

To conduct an *in vivo* animal study of such kind would be complex due to the simultaneous presence of multiple hormonal factors known to affect adipocyte differentiation. There is also difficulty in obtaining pure populations of precursor cells that undergo differentiation under controlled conditions. For these reasons, the *in vitro* cell culture model, the 3T3-F442A preadipocyte/adipocyte cell line will be used. Since our prior *in vivo* studies showed that pioglitazone-enhanced glucose transport activity required the presence of insulin (6), subsequent studies will be carried out in the presence or absence of insulin.

AIM 1

To determine the basis of pioglitazone-mediated acceleration of 3T3 differentiation from fibroblast to adipocytes by examining markers of the adipogenic pathway and the expression of adipocyte-specific genes.

Experimental Design

1- Examine effects of pioglitazone and/or insulin treatments on cell morphology and triglyceride accumulation.

2- Examine effects of pioglitazone and/or insulin treatments during differentiation on glucose transport activity and glucose transporter gene expression (GLUT1 and GLUT4) at mRNA and protein levels.

3- Assess effects of pioglitazone and/or insulin treatments on differentiation-related gene expression of adipocyte-specific genes (aP2, adipsin, insulin receptor) at the mRNA level.

4- Since insulin has been reported to down-regulate some messages after differentiation, mRNA abundance in some cases will be assessed after the removal of the differentiation inducer (insulin or pioglitazone) to rule out such effect.

AIM 2

To probe mechanisms underlying differentiation-dependent changes in mRNA levels of glucose transporters and other highly regulated genes.

Experimental Design

1- Assess whether changes of mRNA levels are due to changes in the transcription rate.

2- If changes in mRNA abundance are not fully explained by changes in transcription rate, I will examine the stability of mRNA for selected genes.

Project Significance

This study of adipocyte cell differentiation induced by a novel hypoglycemic agent will deal with several important areas. First, it will provide useful insight into the regulatory mechanisms involved in the adipocyte differentiation and development, and information on the molecular basis for the development of responsiveness to certain hormones such as insulin. Second, adipocytes are known to play an important role both in normal physiology as a major site for energy storage and release, and in certain pathological states such obesities and obesity-linked diabetes. Therefore, studying the adipocyte gene expression is of great interest because it might provide clues on how disordered gene expression in such disease states would adversely affect the adipocyte function. In addition, this study will expand our understanding of how the hypoglycemic agent pioglitazone acts *in vivo* to enhance insulin sensitivity of target tissues thus correcting elevated blood glucose levels in diabetes. Finally, this combined use of cell culture and pioglitazone treatment should prove to be a useful model system with which to study the molecular mechanisms underlying insulin action on glucose transport.

CHAPTER III

MATERIALS AND METHODS

3T3-F442A cells (39) were kindly provided by Dr. J. Schwartz (University of Michigan, Ann Arbor, MI) and pioglitazone was provided by the Upjohn Co. (Kalamazoo, MI). All tissue culture media and reagents were obtained from Sigma Co. (St. Louis, MO). Culture plates were purchased from Corning glass works (Corning, NY). Guanidine isothiocynate and formamide were purchased from International Biotechnologies, Inc. (New Haven, CT). Nytran nylon membranes were purchased from Schleicher and Schuell (Keene,NH), and Riboprobe preparation kits were obtained from Promega Corp. (Madison, WI). Cytidine 5'-[$^{\alpha-32}$P] triphosphate (>800Ci/mmol), Uridine 5'-[α-^{32}P] triphosphate (800Ci/mmol), and D-[U-^{14}C] Glucose (>230 mCi/mmol) were purchased from Amersham Corp. (Arlington Heights, IL). Immobilon PVDF membranes were obtained from Millipore Corp. (Bedford, MA). Immunoblot assay kit and the alkaline phosphatase-conjugated secondary antibody (GAR-AP) were purchased from Bio-Rad (Richmond, CA). Nonident P40 was purchased from Boehringer Mannheim (W. Germany). All other chemicals were obtained from Sigma Chemical Co. (St.Louis, MO).

Cell Culture and Differentiation

Cells were routinely grown in Dulbecco's Modified Eagle's medium

(DMEM; 4.5 g/liter glucose) in the presence of 10% (vol/vol) calf serum. All formulations of DMEM contained glutamine (2 mM), penicillin (50 U/ml), streptomycin (50 μg/ml), and fungizone (0.25 μg/ml). The cells were inoculated onto 100-mm culture dishes at a density of 500/cm^2, or 35-mm 6-well plates at a density of 1500/cm^2, then were grown to confluence and maintained at 37^{o}C in a humidified 10% CO_2/90% air atmosphere. Medium was changed every 2-3 days. To induce differentiation to adipocytes, confluent fibroblast cultures were switched to differentiation medium, DMEM containing 10% (vol/vol) fetal calf serum and supplemented with insulin (1 μg/ml), pioglitazone (1 μM) unless otherwise indicated. Cell differentiation was assessed judging cell morphology under phase contrast microscopy; cells were considered to be adipocytes if numerous lipid droplets were observed in the cytoplasm. The cell line was used during 10th to 20th passage after clone isolation. To maintain a stock of 3T3-F442A fibroblasts at an early passage, cells were frozen in 15% (vol/vol) Dimethyl Sulfoxide (DMSO)/ culture medium and stored in liquid nitrogen.

Measurement of Triglyceride Accumulation

Triglyceride accumulation was measured by an organic extraction method adapted from J. Schwartz (146). Briefly, monolayers of 3T3-F442A cells on 100-mm dishes were washed once with sterile phosphate buffered saline (PBS, 2.68 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 136.9 mM NaCl) and released from the plate with 3 ml of 0.05% trypsin-EDTA (Gibco Co., Grand Island, NY) in sterile PBS. An aliquot of cells was delivered to a hemocytometer for cell counting and another measured aliquot (0.3 ml) was delivered into 5 ml

Dole's reagent (isopropyl alcohol: heptane: 1 N H_2SO_4; 40: 10: 1), then 3 ml heptane and 2 ml water were added to convert into a 2-phase system. After vigorous mixing, the phases were allowed to separate and the volume of the upper heptane phase was measured. A volume of the upper heptane phase (3 ml) was delivered into a tared glass scintillation vial, dried, and the vial was weighed. The difference in weight indicates triglyceride amount.

Cell counting

Cell monolayers were washed with PBS and released from plate with 3 ml (1 ml for 35-mm plates) 0.05% trypsin-EDTA. A drop of cell suspension was placed in the upper and lower chambers of a hemocytometer, and cells in a total of 10 (1 mm^2 x 0.1 mm) squares were counted. This gives the number of cells in 0.1 mm^3.

Cell/cm^3 = cells counted x 10,000/ 10 (number of squares counted).

Calculation of triglyceride accumulation per cell

mg triglyceride per ml cell suspension:

[mg triglyceride x total vol. heptane phase/3.0 (ml heptane used)] x [1/0.3 (ml of cell suspension used)]

This number divided by the number of cells in a ml yields mg triglyceride per cell.

Measurement of 2-deoxy [^{14}C] Glucose Uptake

Cell monolayers on 35-mm, 6-well plates (Corning brand) were rinsed once with PBS, and incubated in assay medium [DMEM, no-glucose, 5 mM sodium bicarbonate, 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), pH 7.4, 0.1% bovine serum albumin BSA; BIOLOGOS INC. Naperville, IL] 15 min at 22^{o}C. 0.5 μCi of D-[U-^{14}C] glucose in assay medium was added to each well and further incubated 15 min at 22^{o}C. The cells were rinsed three times with PBS + 10 mM glucose, and solubilized by incubation with 0.9 ml of 0.5 N NaOH 30-40 min at 37^{o}C, and transferred to vials. The mixture was neutralized by addition of 105 μl glacial acetic acid, and radioactivity was counted using 10 ml Beckman Ready-Solv CP (147).

Cell counting and calculations

Cells corresponding to each treatment condition were released from 35-mm, 6-well plates with 1 ml of 0.05% trypsin-EDTA and were counted using hemocytometer as described under triglyceride accumulation. Cell-associated radioactivity measured by scintillation counting as Disintegrations per minute (DPM) was divided by the number of cells for each treatment condition to yield DPM per cell.

Northern Blot Analysis of RNA

Total RNA was extracted from cell monolayers by the guanidinium isothiocyanate extraction method adapted from Chomczynski and Sacchi (148). Briefly, cell monolayers were rinsed with sterile PBS and scraped into guanidine thiocyanate solution (4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5%

Sarkosyl and 0.1 M 2-mercaptoethanol; GTS), and extracted with phenol/chloroform in the presence of high salt concentration. The two layers were separated by centrifugation at 7,800 x *g* and RNA in the aqueous layer was precipitated with an equal volume of isopropanol at -70^{o}C. RNA was further resuspended in GTS and reprecipitated, and the final RNA pellet was resuspended in DEPC (Diethyl pyrocarbonate)-treated H_2O and stored at -70^{o}C. RNA yield was calculated using an O.D. 260 reading in spectrophotometer and assuming each 1 O.D. unit = 40 μg/ml RNA. Electrophoretic size-fractionation of RNA (10 μg/lane) through 1% agarose gels and transfer to nylon membranes were performed according to the technique of Fourney *et al* (149). RNA was cross-linked to the membrane using a UV Stratalinker (Stratagene, La Jolla, CA) and hybridized to antisense RNA Riboprobes prepared according to kit protocols (Promega Corp.).

High stringency hybridizations were performed at 65^{o}C in a solution of 10% dextran sulfate, 50% formamide, 5 x SSPE (1 x SSPE = 180 mM NaCl, 10 mM $NaPO_4$, and 1 mM EDTA, pH 7.4), 5 x Denhardt's solution (100 x = 2% Ficoll, 2% BSA, and 2% polyvinylpyrrolidone), 0.1% sodium dodecyl sulfate (SDS), and denatured sheared salmon sperm DNA (100 μg/ml). Dextran sulfate was omitted in some cases from hybridization solution to reduce background. The membranes were washed at 70^{o}C twice in 2 x SSC (1 x = 150 mM NaCl and 15 mM sodium citrate, pH 7.0) with 0.1% SDS and twice in 0.1 x SSC with 0.1% SDS. Membranes were exposed to Kodak XAR-5 film (Eastman Kodak, Rochester, NY) or to Amersham Hyperfilm-MP (manufactured for Amersham by CEA AB, Sweden) at -70^{o}C with an intensifying screen (2-16 hr). Autoradiographic bands were quantitated by two dimensional densitometry on

a Bio-Rad 620 Scanner (Richmond, CA) or on an AMBIS image acquisition analysis system (San Diego, CA). Hybridizations were performed under conditions where autoradiographic detection was in the linear response range. To correct for unequal RNA loading, Northern blots were photographed with Polaroid type55 positive/negative black and white film, and the fluoresence of the ethedium bromide-intercaleted 28S rRNA band on negative films was quantitated by two dimensional densitometry. The system was validated by demonstrating that the signal was linear at least over the range of 1-12.5 μg RNA/lane (150,151).

Description of plasmids

Riboprobes for **GLUT1** were prepared using the entire coding region of rat brain glucose transporter from pSPGT-1 (152) obtained from Dr. Graeme Bell (Chicago, IL). The insert was excised using Bgl II and subcloned into the Bam HI site of pGEM-4Z. The construct was linearized with Eco RI, and T7 RNA polymerase was used for preparation of Riboprobes.

Riboprobes for **GLUT4** were similarly prepared using pSM1D2 obtained from Dr. Morris Birnbaum (Boston, MA). The construct contains a 1.5-kilobase (kb) 5' fragment of cDNA encoding the rat GLUT4 protein (153). This construct was linearized with *EcoR*I, and T7 RNA polymerase was used for transcription.

Riboprobes for **adipsin** were prepared using pGEM-4Z containing a mouse-940bp insert (pAD-20) from *Pst I* site of pBR322 (85) obtained from Dr. Bruce Spiegelman (Boston, MA) and subcloned in our laboratory. The construct was linearized with *Eco RI* and T7 RNA polymerase was use for transcription.

Similarly, Riboprobes for **aP_2** were prepared using a mouse-390bp insert called pAd-5 (85) obtained from Dr. Bruce Spiegelman and subcloned in our laboratory from pBR322 into the *Pst I* site of pGEM-4Z. The construct was linearized with *Hind III*, and SP6 RNA polymerase was used for preparation of Riboprobes.

For the **insulin receptor (IR)**, a 775bp insert for the mouse insulin receptor (original clone provided by Dr. G. Bell and has not been formally described in the literature) was subcloned in our laboratory from pUC into the *EcoRI* site of pGEM-3Z. It was linearized with *Hind III*, and T7 RNA polymerase was used for preparation of Riboprobes.

Plasmid DNA preparation

Plasmid DNA was prepared using Qiagen plasmid preparation kit (Qiagen Inc. Chatworth, CA). Cultures were inoculated from frozen (-70^{o}C) stocks of *E.coli* using 10 μl per 50 ml of 2.5% LB Broth culture medium, and incubated overnight in an environmental shaker at 200-250 rpm. Cells were harvested by centrifugation when the O.D.600 reached 1-1.5, and plasmids were isolated following the kit protocol. This procedure yielded 100 μg (midi-prep kit) to 500 μg (maxi-prep kit) DNA.

Western Immunoblot Analysis

Total particulate membrane proteins were prepared as previously described (61). Briefly, cells were washed with PBS and scraped into homogenization buffer (20 mM Tris, 255 mM Sucrose, 1 mm ethylenediaminetetraacetic acid EDTA, 1 mm phenylmethyl sulfonyl fluoride PMSF, 10 units/ml Trasylol) and

were homogenized with ten strokes of Tekmar tissumizer (Tekmar Inc, Cincinnati, OH). Total membranes were prepared by centrifugation of the homogenate at 200,000 x *g* 4°C 75 min. Protein concentrations were determined by the Bradford assay (154) using Bio-Rad/Bradford Protein Assay Kit and bovine serum albumin as a standard. Initial Western blot experiments revealed diffuse bands of GLUT1 and GLUT4 proteins, possibly due to heterogeneous glycosylation of the transporter proteins. Therefore, protein samples were routinely treated with peptide N-glycosidase F (1 unit per 100 μg protein; Boehringer Mannheim) in a buffer containing 20 mM sodium phosphate, pH 7.5, 10 mM EDTA, 1.7% Triton x-100, 1 mm PMSF for 48-72 hr at 37°C (109) to remove sugar residues, and then mixed with 1/4 vol of 4 x electrophoretic sample buffer (200 mM Tris-HCl, pH 6.8, 400 mM dithiothreitol, 8% SDS, 40% glycerol, 0.4% bromophenol blue) and stored at -20°C. Samples were thawed and loaded in parallel onto two discontinuous 12% polyacrylamide gels, and size-fractionated using a Mini Protean II Dual Slab Cell (Bio-Rad) according to the method of Laemmeli (155). The amount of protein loaded (10 μg/lane for GLUT1 and 60 μg/lane for GLUT4) was empirically determined to be within the linear response range of the detection system used. Separated proteins from both gels were electrophoretically transferred to Immobilon PVDF membranes (Millipore Corp., Bedford MA) using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). One membrane was stained with Coomassie R-250 (0.1% Coomassie R-250, 40% methanol, 10% acetic acid) for total protein. The second membrane was used for the colorimetric detection of GLUT1 or GLUT4 using a double antibody system and the Immuno-Blot Assay Kit (Bio-Rad). Manufacturer's instruction were followed, except 5% BSA was

used for membrane blocking. The primary antibody (RaIRGT, against GLUT4 and RaGLUTRANS against GLUT1, East Acres Biologicals, Southbridge, MA) were diluted to 1:2000 and 1:3000 respectively and the alkaline phosphatase-conjugated second antibody (GAR-AP, Bio-Rad) was diluted 1:3000 before use. Resulting signals were quantitated with the reflective mode of a model 620 Video Densitometer (Bio-Rad). Sample loading corrections were made based on densitometry data from the Coomassie-stained membrane.

Run-on Transcription Activity

Nuclei were isolated from 3T3-F442A cells by a method adapted from Konieczny *et al* (156). Cell monolayers were washed twice with resuspension buffer RSB (10 mM Tris, pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$); scraped from plates in RSB^+ buffer (10 mM Tris, pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonident P40, 2.75 mM DTT), and disrupted by 10 strokes in a Dounce glass homogenizer. The nuclei were concentrated by centrifugation at 1000 x *g* and suspended in nuclei suspension buffer NSB (50 mM HEPES, pH 8.0, 5 mM $MgCl_2$, 0.1 mM EDTA, pH 8.0, 2 mM DTT, 40% glycerol) stored at -70^{o}C. Nuclear transcription assays were adapted from Sasaki *et al* (157) with some modifications. Briefly, after thawing on ice, equal amounts of nuclei (0.5-1 mg DNA) - assessed by fluorometric quantitation of DNA (158)- were used in assays carried out at 26^{o}C for 20 min in a solution of 75 mM Hepes, pH 8.0, 100 mM KCl, 0.1 mM EDTA, 0.1 mM PMSF, 0.5 mM CTP, 0.5 mM ATP, 0.5 mM GTP, 0.25 U/ml RNasin, 1 μCi/μl αP^{32}-UTP -800 Ci/mM) in a total reaction vol of 140 μl. The assays were terminated by incubation with 2 x stopping buffer (40 mM Tris, pH 7.5, 20 mM $CaCl_2$, 200 μg/ml proteinase K, 250 μg/ml tRNA,

5 U/ml RNase-free DNase) for 20 min at 37^{o}C. Nuclei were digested with EDTA/SDS (100 mM EDTA, pH 8.0, 8% SDS) for 10 min at 37^{o}C and RNA was isolated by chloroform/phenol extraction (1:1), and precipitation with 7.5 M ammonium acetate-100% ethanol. RNA transcripts were resuspended in 1 x stopping buffer (without tRNA) and incubated for 20 min at 37^{o}C, followed by another EDTA/SDS digestion, phenol/chloroform extraction and precipitation as above. The transcripts were suspended in T.IE suspension buffer (0.1 mM EDTA, pH 8.0, 10 mM Tris, pH 7.5) and reprecipitated. RNA transcripts were resuspended in 200 μl hybridization solution (50% formamide, 5 x SSPE, 5 x Denhardt's solution, 0.1% SDS, 200 μg/ml denatured sheared salmon sperm DNA solution), the amount of RNA-associated ^{32}P-radioactivity determined by liquid scintillation counting. Samples were adjusted to contain equal amounts of radioactivity using hybridization solution (1-2 x 10^{7} cpm). Hybridizations were carried out in a total volume of 500 μl at 65^{o}C for 18-24 hr using nylon membranes to which 1 μg of each cRNA was applied using a dot-blot apparatus (Mini-fold, Schleicher and Schuell, Keene, NH) and attached by UV cross-linking. 1 μg was empirically found to be appropriate for signal detection. Each cRNA was prepared from its vector plasmid as described for Northern Blotting using RNA Riboprobe Kit according to kit protocol (Promega Corp.), and confirmation of full length transcripts was made by electrophoresis with molecular markers on 1% agarose gel. cRNA Riboprobes for the plasmid vector control (pGEM-4Z) and appropriate genes were denatured in a solution containing 50% formamide, 16.2% formaldehyde for 15 min at 65^{o}C and applied to the membrane in 10 x SSC buffer. Membranes were first prehybridized for 2 hr at 65^{o}C in hybridization solution. After hybridization,

filters were washed at 70°C twice in 2 x SSC with 0.1% SDS and twice with 0.1 x SSC with 0.1% SDS. Membranes were then exposed to Kodak XAR-5 films at -70°C for 2-4 days. Filters were then cut and ^{32}P-labeled radioactivity hybridized to each cRNA Riboprobe was released from filters with 0.04 N NaOH and neutralized with 0.1 N acetic acid. Five ml of Beckman Ready-Solv CP scintillation fluid were added and radioactivity was quantitated by scintillation counting.

DNA quantitation by fluorescence

To determine the amount of nuclei needed for each transcription reaction, the amount of DNA was measured according to the method of Labarca and Paigen (158). Briefly, an aliquot of nuclei was delivered into phosphate-buffered saline with EDTA (PBSE = 0.05 M Na_2HPO_4, 2 M NaCl, 2 mM EDTA, pH 7.4) and sonicated for 10 seconds. The compound Hoechst 33258, (2-[2-(4-hydroxy phenyl)-6-benzimidazolyl]-6-(1-methyl-piperazyl)-benzimidazol.3HCl) (Sigma Co. St.Louis, MO), was added to each sample and the fluorescence enhancement was measured in fluorometer with excitation set at 356 nm and emission set at 458 nm. Calf thymus DNA was used to generate a standard curve.

Measurement of mRNA Stability

Confluent monolayers of 3T3-F442A cells were differentiated with insulin, pioglitazone or both agents as described under cell culture and differentiation. On day 7 of differentiation, mRNA synthesis was inhibited by addition to the medium of actinomycin D-mannitol (Sigma Co. St.Louis, MO) to a final

concentration of 5 μg/ml. Total RNA was extracted from separate monolayer cultures at 0, 1, 2, 4, 6, and 24 hr after the addition of transcription inhibitor. Messenger RNA transcript abundance was determined by Northern blot analysis and hybridization and densitometry of resultant autoradiographic bands as described above.

Calculations

Data were expressed as percent of mRNA remaining after actinomycin D treatment relative to their levels before the treatment (time 0). The half life of each message was calculated using the Enzfitter program (Elsevier Biosoft, Elsevier Science Publishers, Amesterdam). mRNA levels were fitted to a single exponential decay curve according to the equation y = Exp (-k.t) by non linear least square regression analysis. In this equation, y, is the concentration at time 't', and k is the first order rate constant of the exponential decay plot. The half-life of mRNA was then calculated by dividing ln2 with k.

Statistical Analysis

Statistical analysis was performed using SAS version 6 (SAS institute, Inc., Cary, NC). All hypothesis tests were two-sided and were considered significant if p-value was less than or equal to .05

Dose-response experiments were analyzed using one-factor analysis of variance (ANOVA). In one set of analyses, the factor was dose of insulin, and in the second set of analyses, the factor was dose of pioglitazone. If there was a significant dose response, then Tukey's pairwise comparison procedure was used to locate the differences among dose levels.

Time-course experiments were analyzed using a three-factor analysis of variance (ANOVA) with interactions procedure. The factors used were day of the experiment, insulin or control, pioglitazone or control. Beginning with the higher order interaction terms, successive models were run deleting one non-significant factor or interaction at a time until the final model only contained terms which were significant at $p \leq 0.05$.

Protein experiments and time-course experiments, in some cases, were analyzed using a two-factor analysis of variance with interaction procedure.

CHAPTER IV

RESULTS

Effects of insulin and pioglitazone on 3T3-F442A preadipocyte differentiation

Treatment of confluent 3T3-F442A cells with insulin (1 μg/ml), pioglitazone (1 μM), or both agents in the presence of 10% fetal calf serum resulted in conversion of cells into lipid-accumulating adipocytes as assessed by phase contrast microscopy (Table I, Figure 1). While only limited numbers of cells differentiated into adipocytes by either treatment alone, both agents had approximately additive effects shown most clearly on day 3 (I, 8 $\pm$ 3; P, 15 $\pm$ 0; I+P, 30 $\pm$ 5) and day 5 (I, 42 $\pm$ 8; P, 45 $\pm$ 4; I+P, 84 $\pm$ 5) and reaching maximal levels on day 7 (I+P, 95 $\pm$ 0) of differentiation. These morphological changes were accompanied by time-dependent changes in cellular triglyceride content (Figure 2), assessed by organic extraction of triglyceride. This effect was measurable on days 3, 5, and 7. There were marked changes on day 7 of differentiation (C, 0.39 $\pm$ 0.05; I, 0.599 $\pm$ 0.07; P, 1.34 $\pm$ 0.19; I+P, 2.2 $\pm$ 0.27). These results indicate that both insulin and pioglitazone act as accelerators of adipocyte differentiation and that their effects are additive or synergistic.

Effects of insulin and pioglitazone on glucose transport activity and glucose transporter gene expression

To determine whether the observed cell differentiation was accompanied

Table I. Pioglitazone and insulin-dependent differentiation of 3T3-F442A expressed as % differentiated cells.

Day	CS	INS	PIO	INS+PIO
0	0	-	-	-
1	0	2	2	3
3	2	8	15	30
5	2	42	45	84
7	2	60	80	95

Cells were plated, grown to confluence and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum. To induce differentiation, cells were switched to DMEM medium supplemented with 10% fetal calf serum, insulin (INS), pioglitazone (PIO) or both (I+P) as described under materials and methods. Untreated age-matched fibroblasts maintained in growth medium with only calf serum (CS) were used as control. Cell differentiation was assessed by phase contrast microscopy. Data represent mean values for n = 2-6 determinations. One-way analysis of variance (ANOVA) on day 7 for CS vs INS, CS vs PIO, CS vs INS+PIO, $p < 0.0001$.

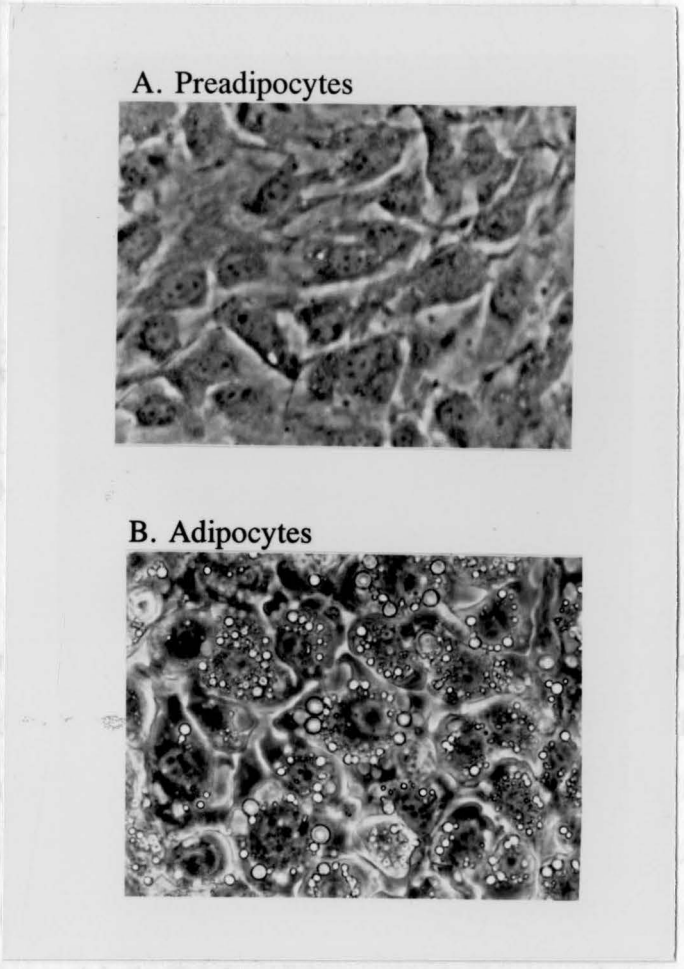


Figure 1. Phase contrast microscopy showing the change in morphology upon differentiation from preadipocytes (A) to adipocytes (B) induced by treatment with a combination of insulin and pioglitazone. Magnification x300.

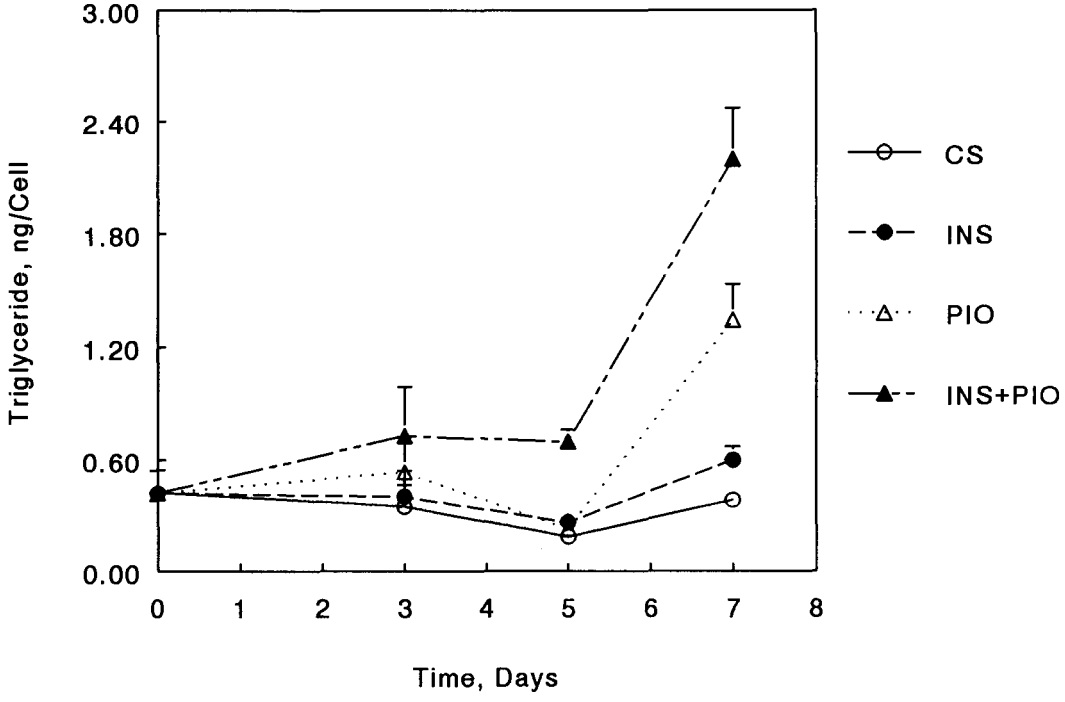


Figure 2. Time-course showing increasing abundance of triglyceride during differentiation of 3T3-F442A fibroblasts into adipocytes. 3T3-F442A cells were plated, grown to confluence and differentiated with INS (1 μg/ml), PIO (1 μM), or both (INS+PIO) as described under materials and methods. At indicated time points, triglycerides were extracted from cells with Dole's reagent and the organic layer was dried. Triglyceride accumulation was determined by weighing the dried residue. Untreated age-matched fibroblasts maintained in growth medium (CS) were used as control.

Each data point represents mean $\pm$ S.E.M for n = 9 determinations. A three factor analysis of variance (ANOVA; insulin, pioglitazone, time) with interactions showed significant time-dependent effects on triglyceride abundance by INS ($p = 0.017$), PIO ($p < 0.0001$), INS+PIO ($p = 0.02$).

with changes in cellular metabolic activities, we examined the transport level of 2-deoxy [^{14}C] glucose into cells differentiated with insulin and/or pioglitazone as compared to control undifferentiated cells. Differentiation of 3T3-F442A cells with insulin, pioglitazone, or both agents, resulted in enhanced basal 2-deoxy glucose transport in a time-dependent manner (Figure 3). While either insulin or pioglitazone alone showed respectively 22- and 29-fold enhancement above control of glucose transport on day 7 of differentiation, both agents acted synergistically to increase glucose transport activity on each of the days examined, reaching maximal levels of 96-fold on day 5 and seemingly started to decline to lower levels (46-fold) on day 7 of differentiation. Age-matched undifferentiated cells kept in growth medium showed no increase glucose transport during the same period of time, and even showed a 40% decline in their rate of glucose transport by day 7, possibly an indication of the cells becoming more quiescent.

To assess whether such enhanced glucose transport activity could be explained by changes in adipocyte-glucose transporter expression, we analyzed the levels of GLUT1 and GLUT4 mRNA and protein abundance. GLUT1 mRNA abundance increased in a dose-dependent manner reaching maximal levels of 2.5-fold and 2-fold after 7 days of treatment with 10 μg/ml insulin (Figure 4A) and 10 μM pioglitazone (Figure 5A), respectively, as compared to undifferentiated control cells (day 0). Age-matched undifferentiated cells kept in growth medium showed $<10\%$ increase in GLUT1 mRNA abundance at the same time. In contrast to this moderate elevation of GLUT1 mRNA, GLUT4 mRNA abundance was dramatically increased in a dose-dependent manner as much as 20-fold above its levels in undifferentiated cells by either insulin

(Figure 4B) or pioglitazone (Figure 5B). Age-matched cells kept in growth medium showed <2.5-fold increase in GLUT4 mRNA abundance. Furthermore, GLUT4 mRNA abundance was significantly increased by the lowest tested dose of each of insulin and pioglitazone, with maximal effects at 5 μg/ml insulin (Figure 4B) and 1 μM pioglitazone (Figure 5B).

In addition, both mRNA transcripts analyzed by Northern blotting, increased in a time-dependent manner during differentiation. While either insulin (1 μg/ml) or pioglitazone (1 μM) treatment increased GLUT1 mRNA abundance by 2.3- and 1.5-fold, respectively, above undifferentiated cells (day 0), insulin and pioglitazone together acted synergistically to increase this message by almost 6-fold by day 7 (Figure 6). GLUT1 mRNA levels did not change during the same period in undifferentiated age-matched cells kept in growth medium. In contrast to the observed synergism on GLUT1 mRNA, GLUT4 mRNA abundance increased to similar levels above undifferentiated cells (day 0), 3.8-, 4.6, and 5.2-fold by insulin, pioglitazone, or both, respectively (Figure 7).

These changes in GLUT1 and GLUT4 mRNA levels were accompanied by changes in their respective protein products as assessed by Western blotting. While either insulin or pioglitazone treatment increased GLUT1 protein levels on day 7 of differentiation by 2.3-and 3.5-fold, respectively, both agents acted synergistically to increase GLUT1 protein levels by almost 10-fold (Figure 8) above those in their age-matched undifferentiated cells. In contrast to the observed synergistic effect on GLUT1 protein, GLUT4 protein levels increased by 2.2-, 6.6-, and 4-fold by insulin, pioglitazone, or both, respectively (Figure 9).

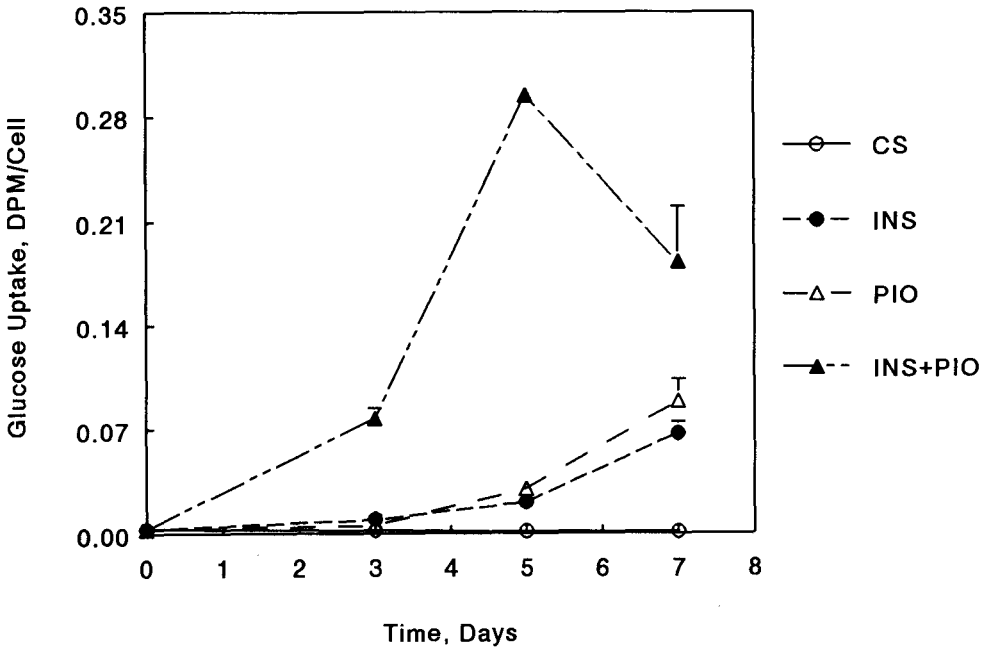


Figure 3. Time-course showing increasing glucose transport activity during differentiation. Confluent 3T3-F442A cells on 35 mm, 6-well plates were induced to differentiate into adipocytes by insulin (INS), pioglitazone (PIO) or both (INS+PIO). On indicated time points, glucose transport activity was determined by measuring cell-associated radioactivity following the uptake of 2-deoxy-D-[U-^{14}C] Glucose as described under materials and methods. Untreated, age-matched fibroblasts maintained in calf serum growing medium (CS) were used as control. Each data point represents mean $\pm$ S.E.M for n = 3 determinations. A two way analysis of covariance (ANCOVA) showed significant effects on glucose uptake by INS ($p = 0.0006$), PIO ($p < 0.0001$), INS+PIO ($p < 0.0001$).

Figure 4. Insulin dose effect on GLUT1 and GLUT4 mRNA abundance. 3T3-F442A cells were plated and grown in DMEM containing 10% calf serum. To induce differentiation, confluent fibroblasts were maintained in DMEM containing 10% fetal calf serum and supplemented with insulin at 0, 0.1, 1, 5, 10 μg/ml. Total RNA was isolated from cells on day 7. Samples of total RNA (10 μg/lane) were electrophoretically size-fractionated on agarose gels, and Northern blots were hybridized to Riboprobes specific for the rat GLUT1 mRNA (A), or the rat GLUT4 mRNA (B). Autoradiographic bands were quantitated by densitometry and normalized for loading based on the abundance of the 28S ribosomal RNA. Each data point represents mean $\pm$ S.E.M for n = 6 determinations. One way ANOVA showed significant dose-dependent effect of insulin treatment on mRNA abundance for GLUT1 ($p < 0.0001$) and GLUT4 ($p = 0.006$).

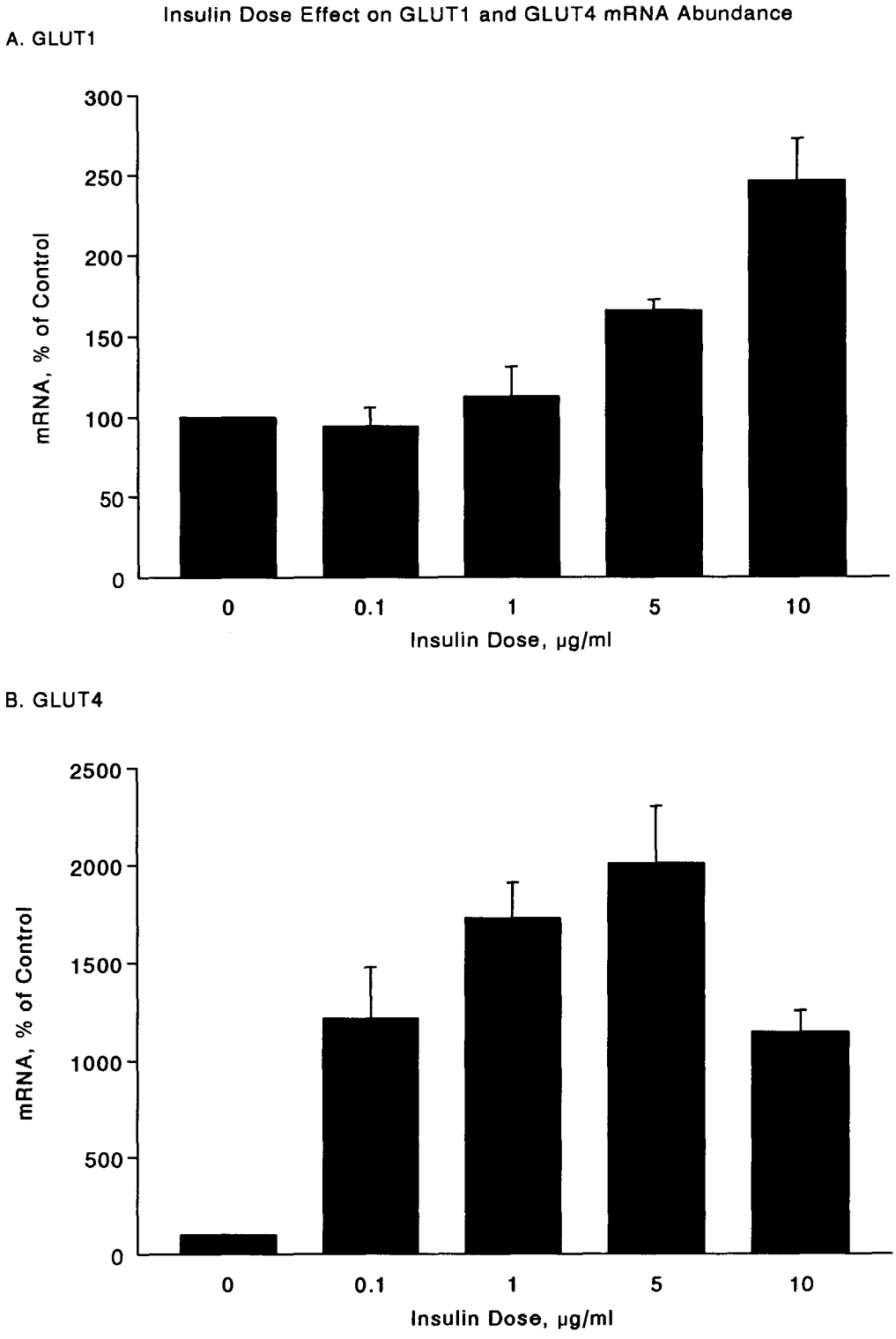

Insulin Dose Effect on GLUT1 and GLUT4 mRNA Abundance
A. GLUT1
mRNA, % of Control
300
250
200
150
100
50
0
0
0.1
1
5
10
Insulin Dose, µg/ml
B. GLUT4
mRNA, % of Control
2500
2000
1500
1000
500
0
0
0.1
1
5
10
Insulin Dose, µg/ml

Figure 4.

Figure 5. Pioglitazone dose effect on GLUT1 and GLUT4 mRNA abundance. 3T3-F442A cells were plated and grown to confluence as described under materials and methods. Differentiation was induced by maintaining cells in DMEM containing 10% fetal calf serum and supplemented with 0, 0.1, 1, 5, 10 μM pioglitazone. Total RNA was isolated from cells on day 7, and samples of 10 μg/lane were electrophoretically size-fractionated on agarose gels. Northern blots were hybridized to Riboprobes specific for the rat GLUT1 mRNA (A), or the rat GLUT4 mRNA (B). Autoradiographic bands were quantitated by densitometry and normalized for correct loading base on the 28S ribosomal RNA abundance. Each data point represents mean $\pm$ S.E.M for n = 6 determinations. One way ANOVA demonstrated significant dose-dependent effect of pioglitazone on mRNA abundance for GLUT1 ($p < 0.0001$) and GLUT4 ($p = 0.0001$)

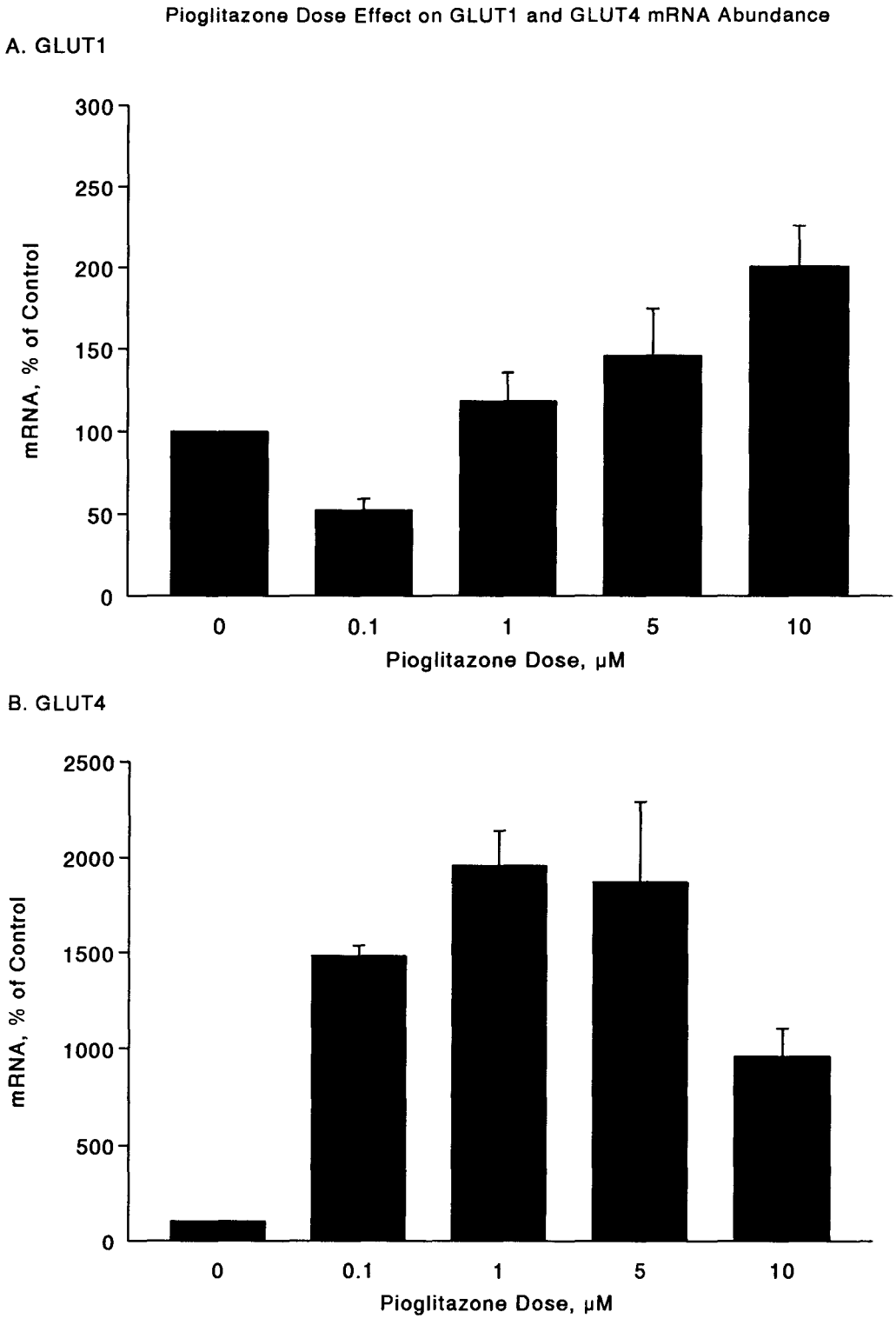
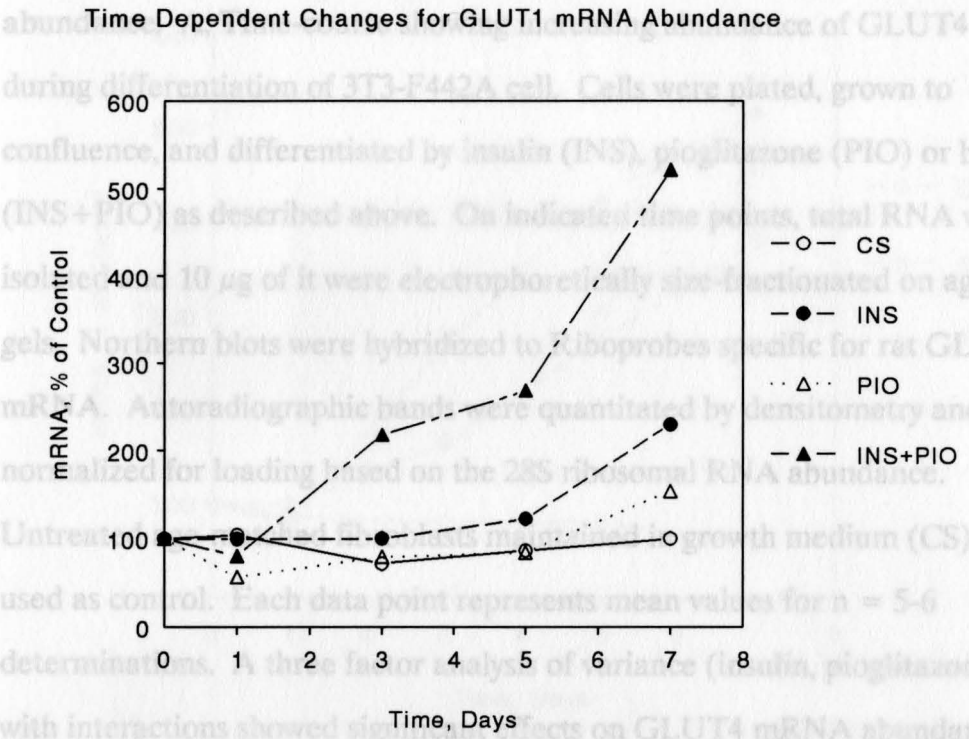

Pioglitazone Dose Effect on GLUT1 and GLUT4 mRNA Abundance
A. GLUT1
mRNA, % of Control
300
250
200
150
100
50
0
0
0.1
1
5
10
Pioglitazone Dose, μM
B. GLUT4
mRNA, % of Control
2500
2000
1500
1000
500
0
0
0.1
1
5
10
Pioglitazone Dose, μM

Figure 5.

Figure 6. Effect of insulin and pioglitazone treatments on GLUT1 mRNA abundance. A, Time-course showing increasing abundance of GLUT1 mRNA during differentiation of 3T3-F442A cells. Cells were plated, grown to confluence and differentiated with insulin (INS), pioglitazone (PIO), or both (INS+PIO) as described above. Total RNA was isolated on indicated time points (days 1, 3, 5, 7). 10 μg of total RNA were electrophoretically size-fractionated on agarose gels and Northern blots were hybridized to Riboprobes specific for rat GLUT1 mRNA. Autoradiographic bands were quantitated by densitometry and normalized for loading based on 28S ribosomal RNA abundance. Untreated age-matched fibroblasts maintained in growth medium (CS) were used as control.

Data represent mean values for n = 5-6 determinations. A three factor analysis of variance (insulin, pioglitazone, time) with interactions showed significant effect on GLUT1 mRNA abundance from combined treatment with insulin and pioglitazone ($p = 0.0001$). B, Representative blot showing GLUT1 mRNA abundance in control CS cells and in cells treated with INS, PIO, INS+PIO on day 7 of differentiation.

A.


Time Dependent Changes for GLUT1 mRNA Abundance
mRNA, % of Control
600
500
400
300
200
100
0
0
1
2
3
4
5
6
7
8
Time, Days
CS
INS
PIO
INS+PIO

B.

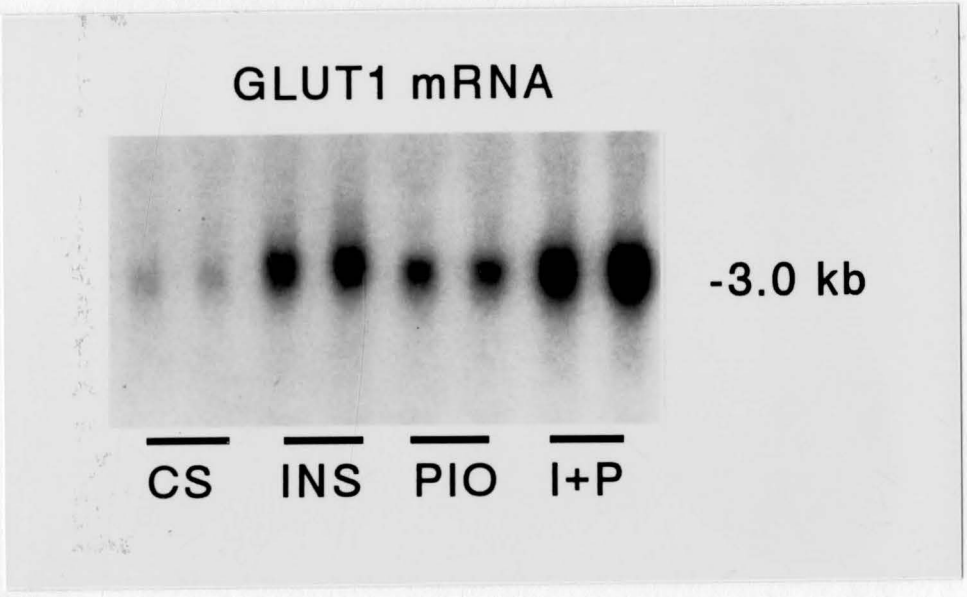
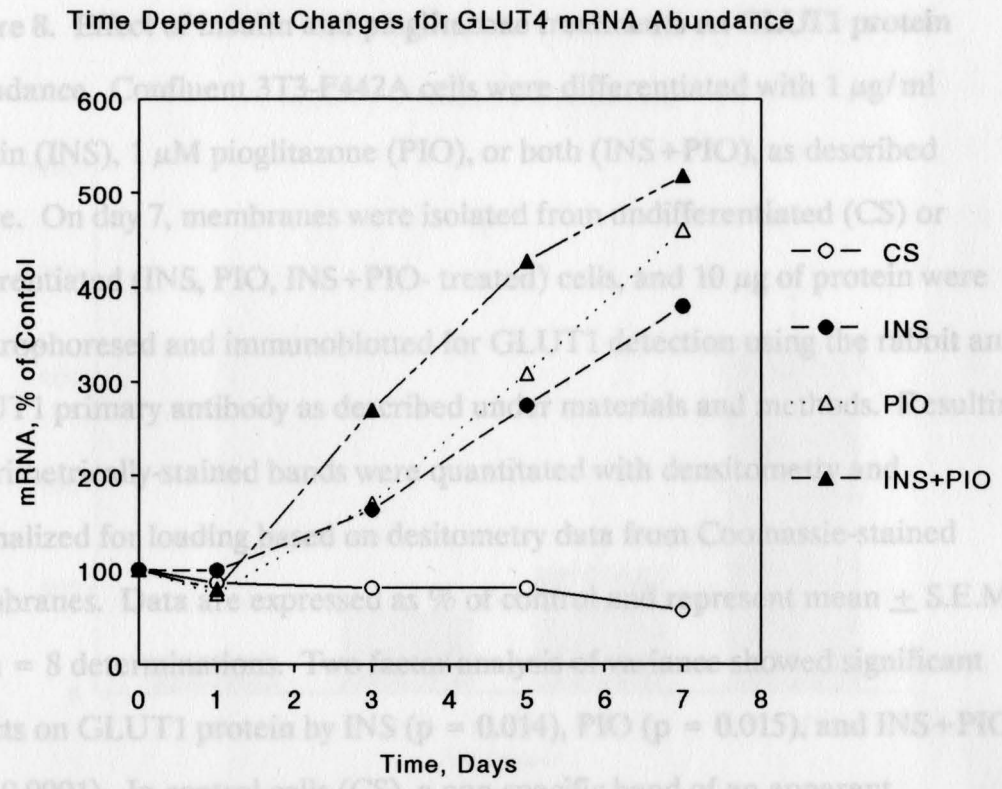

GLUT1 mRNA
-3.0 kb
CS
INS
PIO
I+P

Figure 6.

Figure 7. Effect of insulin and pioglitazone treatments on GLUT4 mRNA abundance. A, Time-course showing increasing abundance of GLUT4 mRNA during differentiation of 3T3-F442A cell. Cells were plated, grown to confluence, and differentiated by insulin (INS), pioglitazone (PIO) or both (INS+PIO) as described above. On indicated time points, total RNA was isolated and 10 μg of it were electrophoretically size-fractionated on agarose gels. Northern blots were hybridized to Riboprobes specific for rat GLUT4 mRNA. Autoradiographic bands were quantitated by densitometry and normalized for loading based on the 28S ribosomal RNA abundance. Untreated age-matched fibroblasts maintained in growth medium (CS) were used as control. Each data point represents mean values for n = 5-6 determinations. A three factor analysis of variance (insulin, pioglitazone, time) with interactions showed significant effects on GLUT4 mRNA abundance by INS ($p = 0.0001$) or PIO ($p < 0.0001$). B, Representative blot showing GLUT4 mRNA abundance in control (CS) and treated (INS, PIO, INS+PIO) cells on day 7 of differentiation.

A.


Time Dependent Changes for GLUT4 mRNA Abundance
mRNA, % of Control
600
500
400
300
200
100
0
0
1
2
3
4
5
6
7
8
Time, Days
CS
INS
PIO
INS+PIO

B.

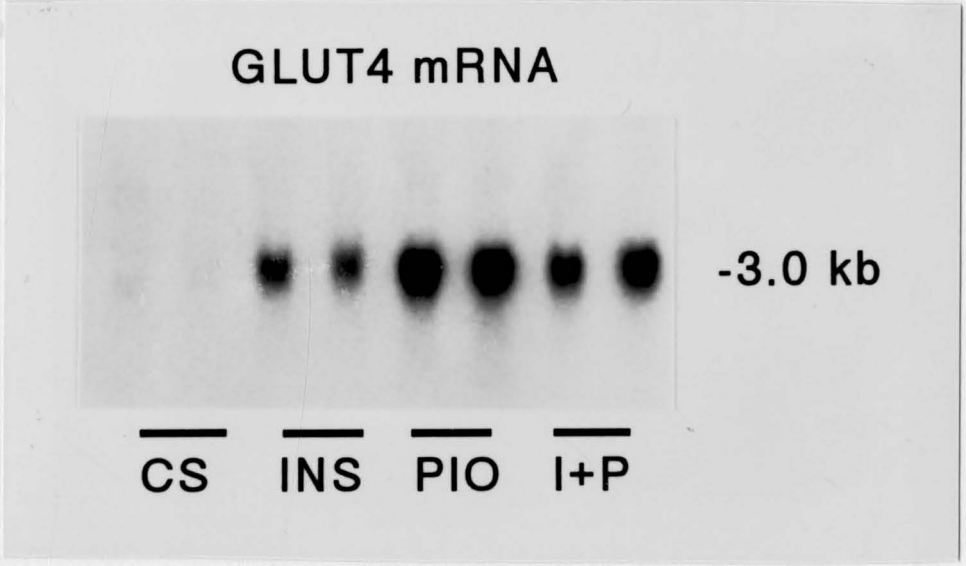
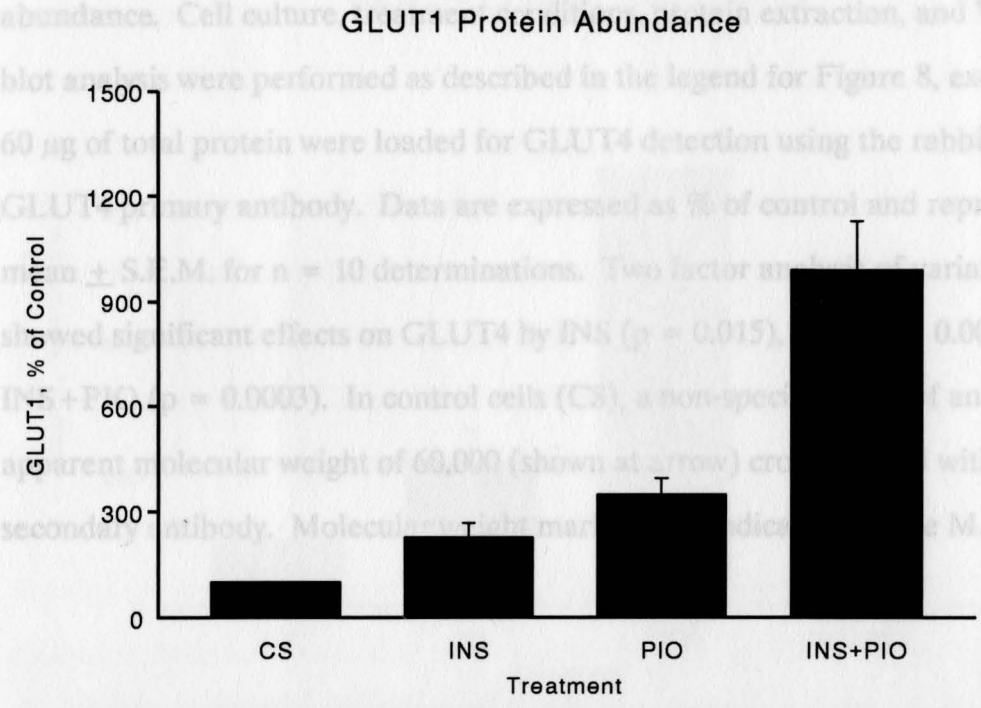

GLUT4 mRNA
-3.0 kb
CS
INS
PIO
I+P

Figure 7.

Figure 8. Effect of insulin and pioglitazone treatments on GLUT1 protein abundance. Confluent 3T3-F442A cells were differentiated with 1 μg/ml insulin (INS), 1 μM pioglitazone (PIO), or both (INS+PIO), as described above. On day 7, membranes were isolated from undifferentiated (CS) or differentiated (INS, PIO, INS+PIO- treated) cells, and 10 μg of protein were electrophoresed and immunoblotted for GLUT1 detection using the rabbit anti-GLUT1 primary antibody as described under materials and methods. Resulting colorimetrically-stained bands were quantitated with densitometry and normalized for loading based on desitometry data from Coomassie-stained membranes. Data are expressed as % of control and represent mean $\pm$ S.E.M for n = 8 determinations. Two factor analysis of variance showed significant effects on GLUT1 protein by INS ($p = 0.014$), PIO ($p = 0.015$), and INS+PIO ($p < 0.0001$). In control cells (CS), a non-specific band of an apparent molecular weight of 60,000 (shown at arrow) cross reacted with secondary antibody. Molecular weight markers are indicated in lane M.

A.


GLUT1 Protein Abundance
GLUT1, % of Control
1500
1200
900
600
300
0
CS
INS
PIO
INS+PIO
Treatment

B.

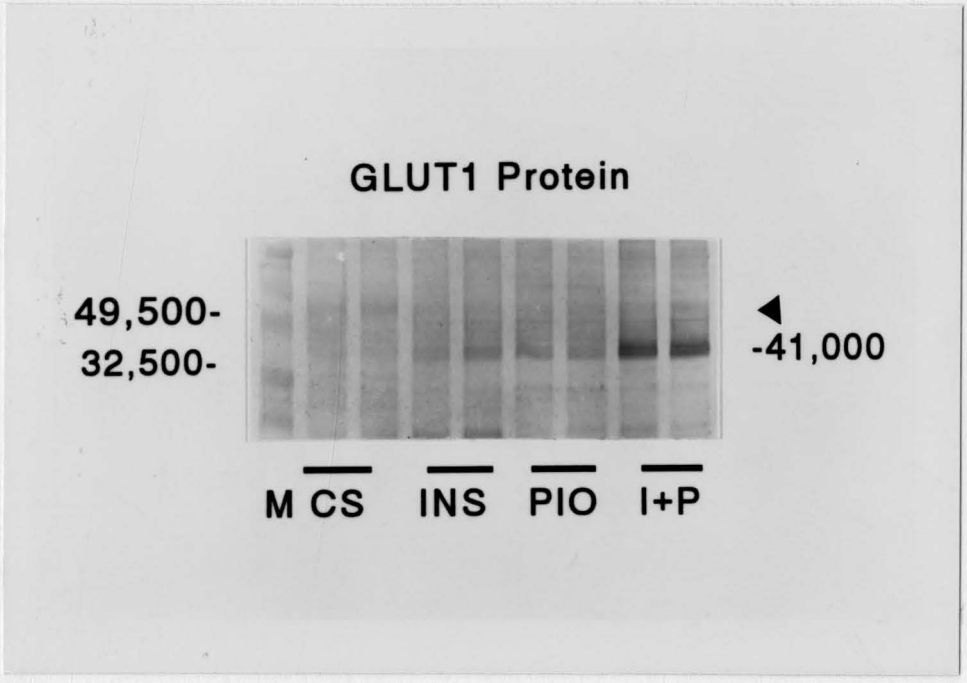
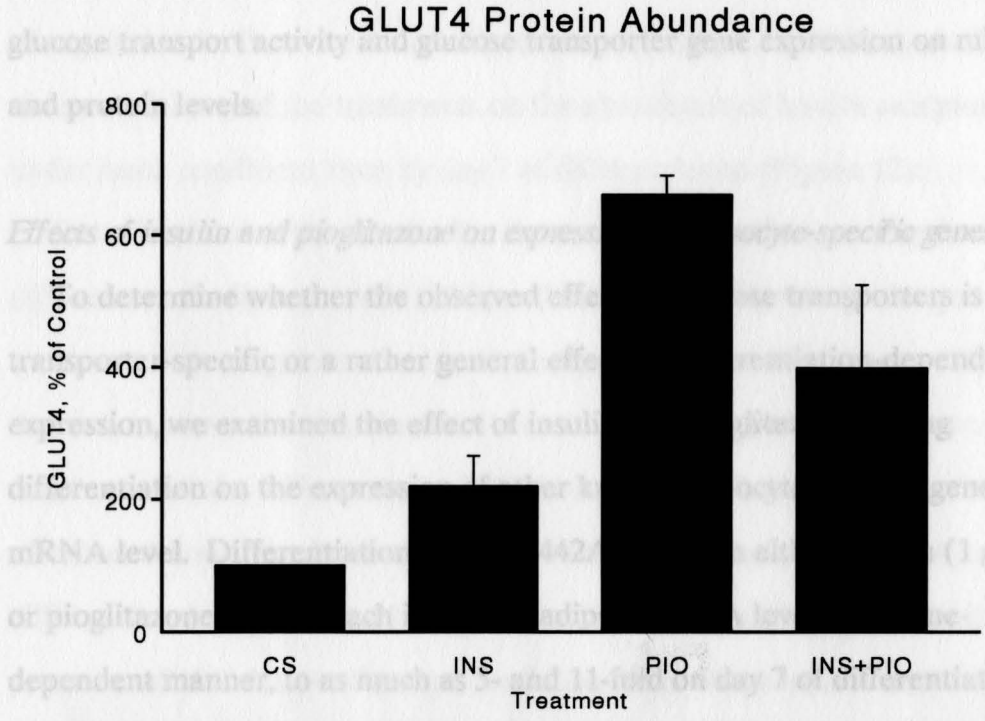

GLUT1 Protein
49,500-
32,500-
-41,000
M
CS
INS
PIO
I+P

Figure 8.

Figure 9. Effect of insulin and pioglitazone treatments on GLUT4 protein abundance. Cell culture, treatment conditions, protein extraction, and Western blot analysis were performed as described in the legend for Figure 8, except that 60 μg of total protein were loaded for GLUT4 detection using the rabbit anti-GLUT4 primary antibody. Data are expressed as % of control and represent mean $\pm$ S.E.M. for n = 10 determinations. Two factor analysis of variance showed significant effects on GLUT4 by INS ($p = 0.015$), PIO ($p = 0.007$), INS+PIO ($p = 0.0003$). In control cells (CS), a non-specific band of an apparent molecular weight of 60,000 (shown at arrow) cross reacted with secondary antibody. Molecular weight markers are indicated in lane M.

A.


GLUT4 Protein Abundance
GLUT4, % of Control
800
600
400
200
0
CS
INS
PIO
INS+PIO
Treatment

B.

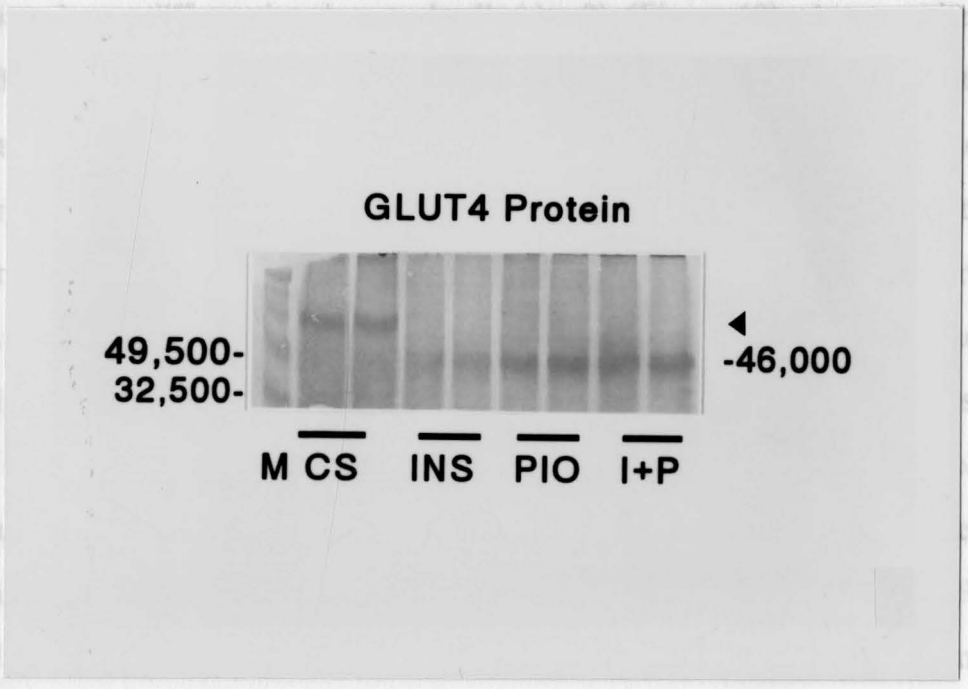

GLUT4 Protein
49,500-
32,500-
-46,000
M
CS
INS
PIO
I+P

Figure 9.

Taken together, these results indicate that differentiation of 3T3-F442A cells with insulin and pioglitazone is accompanied by concomitant increases in glucose transport activity and glucose transporter gene expression on mRNA and protein levels.

Effects of insulin and pioglitazone on expression of adipocyte-specific genes

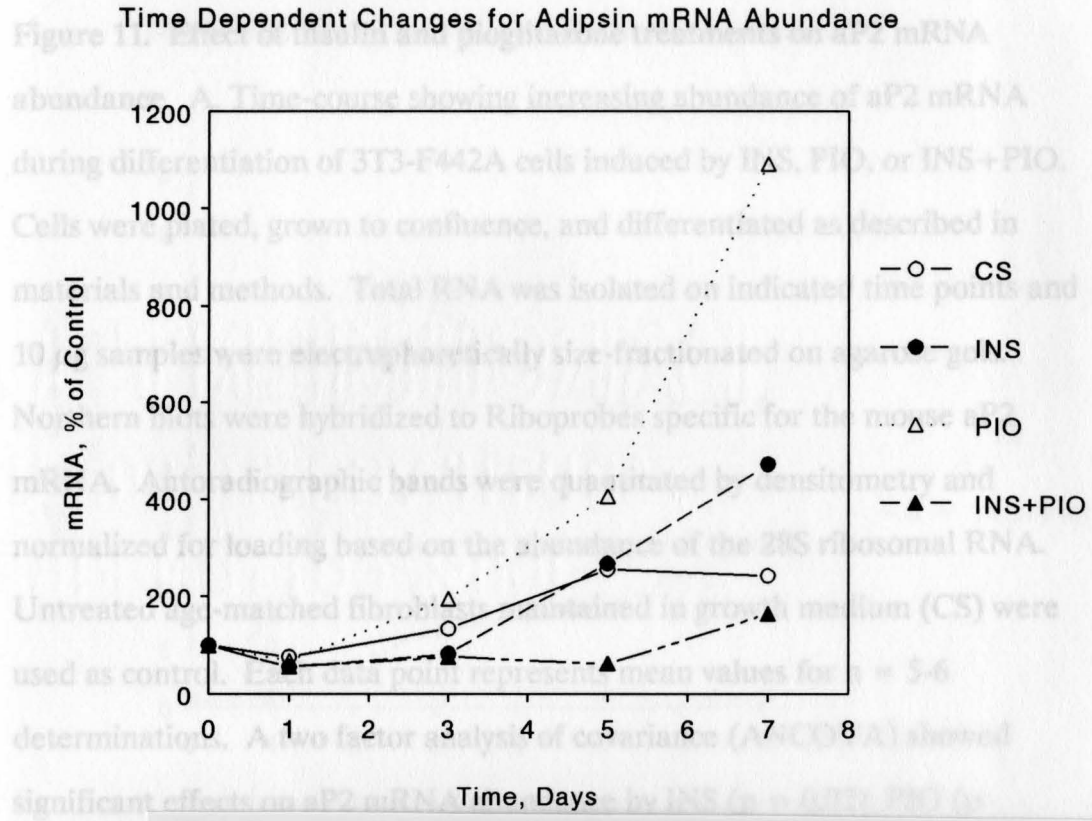
To determine whether the observed effect on glucose transporters is a transporter-specific or a rather general effect on differentiation-dependent gene expression, we examined the effect of insulin and pioglitazone during differentiation on the expression of other known adipocyte-specific genes on mRNA level. Differentiation of 3T3-F442A cells with either insulin (1 μg/ml) or pioglitazone (1 μM) each increased adipsin mRNA levels in a time-dependent manner, to as much as 5- and 11-fold on day 7 of differentiation, as compared to undifferentiated control cells (day 0), (Figure 10). Age matched undifferentiated cells kept in growth medium showed <2.5-fold increase in this message during the same period of time. The combined insulin/pioglitazone treatment significantly inhibited expression of adipsin mRNA induced by either treatment alone. On the other hand, aP2 mRNA abundance assessed under the same conditions increased dramatically in a time-dependent manner by either insulin, pioglitazone, or the combined treatment. These effects were seen as early as day 1 of differentiation and reached maximal levels of 93- and 77-fold on day 3 of differentiation by pioglitazone or insulin/pioglitazone respectively, and after that the message levels start to decline (Figure 11). In contrast, aP2 mRNA abundance continues to increase with insulin treatment, though to much lesser levels, reaching only 27-fold on day 7 of differentiation. Undifferentiated

age-matched control cells kept in growth medium showed <3-fold increase in aP2 mRNA abundance during the same period. In contrast to these high levels of regulation on the abundance of both adipsin and aP2 mRNAs, no effect was observed for any of the treatments on the abundance of insulin receptor mRNA under same conditions even by day 7 of differentiation (Figure 12).

Since it is known that insulin has down-regulating effect on adipsin mRNA in differentiated adipocyte cells (100), and it is also known that prolonged insulin treatment down-regulates the insulin receptor itself in many tissues (58), we decided to assess the abundance of these two messages after treatment and withdrawal of the differentiation inducer in order to sort out differentiation effects and down-regulation effects. For this experiment, 3T3-F442A cells were differentiated with insulin (1 μg/ml), pioglitazone (1 μM), or both, in the presence of 10% fetal calf serum, for 7 days, after which insulin and pioglitazone were removed from the feeding medium and cells were kept in medium containing only 10% fetal calf serum for another six days (day 13). Total RNA was extracted from cells on day 7 and day 13, and the abundance of adipsin and insulin receptor mRNAs was assessed on Northern blots. Treatment of cells with either insulin or pioglitazone resulted in 5.6- and 6-fold increase of adipsin mRNA while the combined treatment resulted in about 40% decrease in the message on day 7 of differentiation when compared to undifferentiated control cells - day 0 - (Figure 13). After insulin and pioglitazone withdrawal, there is further increase in adipsin mRNA of about 6- and 5.5-fold on day 13 above corresponding levels on day 7. Furthermore, such withdrawal from cells differentiated with the combined insulin/pioglitazone

Figure 10. Effect of insulin and pioglitazone treatments on adipsin mRNA abundance. A, Time-course showing increasing adipsin mRNA abundance during differentiation 3T3-F442A cells induced by treatment with either insulin (INS) or pioglitazone (PIO) alone and inhibitory effect by the combined treatment (INS+PIO). Cells were plated, grown to confluence and differentiated with INS, PIO, or both (INS+PIO) as described under materials and methods. Total RNA was isolated on indicated time points, and 10 μg samples were electrophoretically size-fractionated on agarose gels. Northern blots were hybridized to Riboprobes specific for the mouse adipsin mRNA. Autoradiographic bands were quantitated by densitometry and normalized for loading based on the abundance of the 28S ribosomal RNA. Untreated age-matched fibroblasts maintained in growth medium (CS) were used as control. Each data point represents mean values for n = 5-6 determinations. A three factor ANOVA (insulin, pioglitazone, time) with interactions showed significant time-dependent effects on adipsin mRNA abundance by INS ($p < 0.0001$) or PIO ($p < 0.0001$). The inhibitory effect of the combined treatment (I+P) was also significant ($p < 0.0001$). B, Representative blot showing adipsin mRNA abundance in control (CS) and treated (INS, PIO, INS+PIO) cells on day 7 of differentiation.

A.


Time Dependent Changes for Adipsin mRNA Abundance
mRNA, % of Control
1200
1000
800
600
400
200
0
0
1
2
3
4
5
6
7
8
Time, Days
CS
INS
PIO
INS+PIO

B.

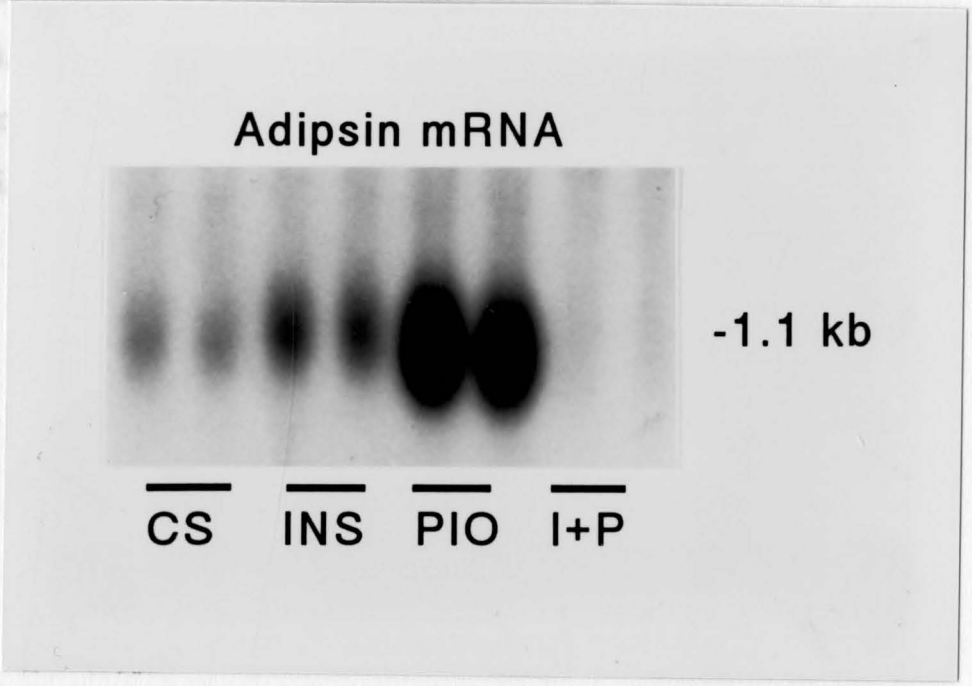
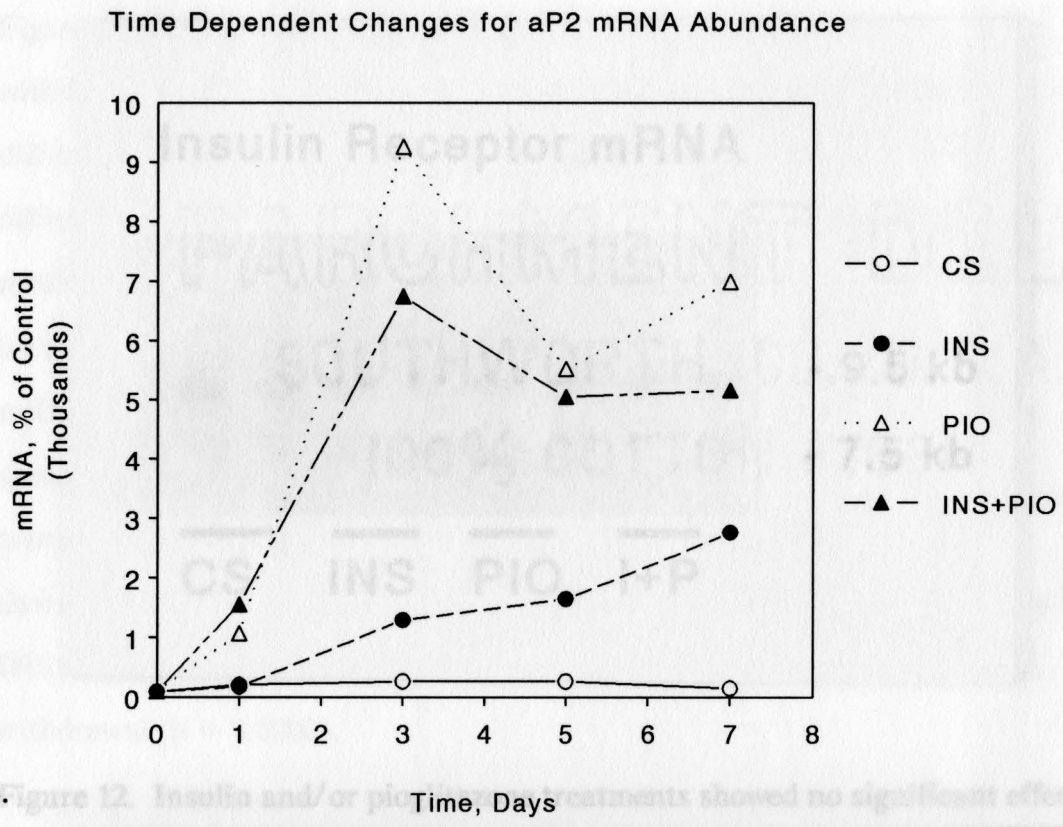

Adipsin mRNA
-1.1 kb
CS
INS
PIO
I+P

Figure 10.

Figure 11. Effect of insulin and pioglitazone treatments on aP2 mRNA abundance. A, Time-course showing increasing abundance of aP2 mRNA during differentiation of 3T3-F442A cells induced by INS, PIO, or INS+PIO. Cells were plated, grown to confluence, and differentiated as described in materials and methods. Total RNA was isolated on indicated time points and 10 μg samples were electrophoretically size-fractionated on agarose gels. Northern blots were hybridized to Riboprobes specific for the mouse aP2 mRNA. Autoradiographic bands were quantitated by densitometry and normalized for loading based on the abundance of the 28S ribosomal RNA. Untreated age-matched fibroblasts maintained in growth medium (CS) were used as control. Each data point represents mean values for n = 5-6 determinations. A two factor analysis of covariance (ANCOVA) showed significant effects on aP2 mRNA abundance by INS ($p = 0.02$), PIO ($p < 0.0001$), INS+PIO ($p = 0.0002$). B, Representative blot showing aP2 mRNA abundance in control (CS) and treated (INS, PIO, INS+PIO) cells on day 7 of differentiation.

A.


Time Dependent Changes for aP2 mRNA Abundance
mRNA, % of Control (Thousands)
Time, Days
CS
INS
PIO
INS+PIO
0
1
2
3
4
5
6
7
8
9
10

B.

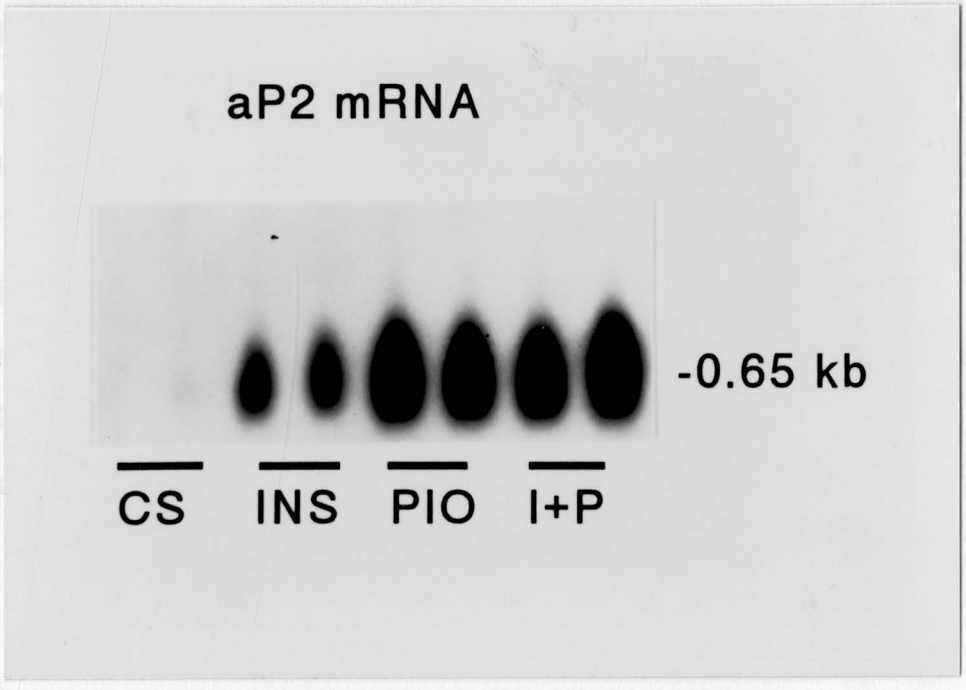

aP2 mRNA
-0.65 kb
CS
INS
PIO
I+P

Figure 11.

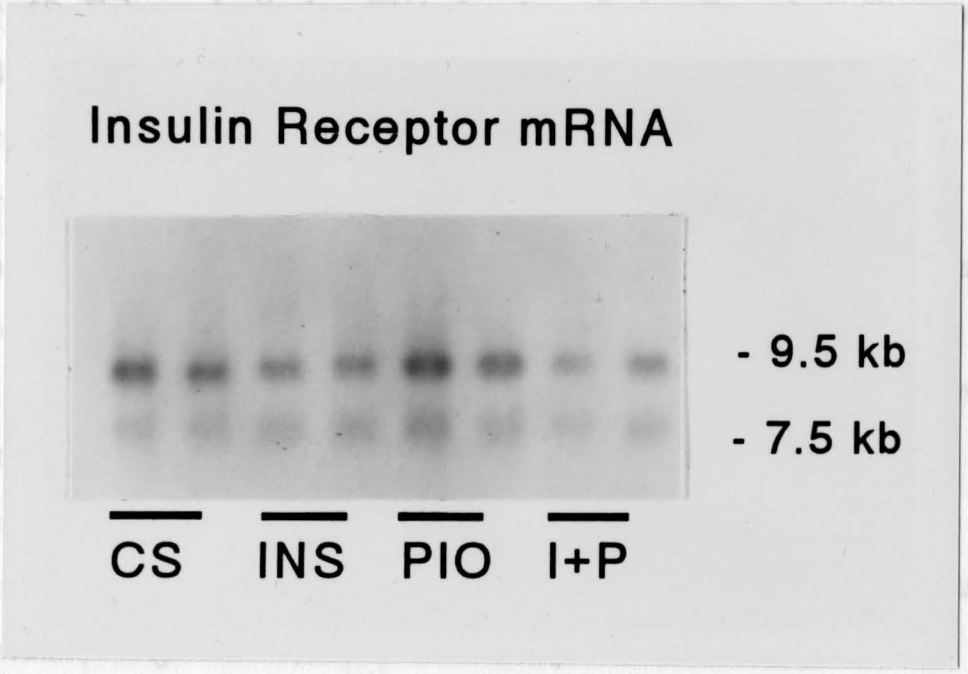


Figure 12. Insulin and/or pioglitazone treatments showed no significant effect on insulin receptor mRNA abundance. 3T3-F442A cells were plated, grown to confluence, and differentiated with INS, PIO, or INS+PIO as described under materials and methods. Untreated age-matched fibroblasts (CS) were used as control. Total RNA was isolated on selected time points (day 1, 3, 5, 7) of differentiation. Samples of 10 g of total RNA were electrophoretically size-fractionated on agarose gels and Northern blots were hybridized to Riboprobes specific for the mouse insulin receptor mRNA. The blot represents typical results on day 7 of differentiation.

Figure 13. Enhanced adipsin mRNA abundance by insulin and pioglitazone withdrawal after differentiation. 3T3-F442A cells were plated and differentiated as described under materials and methods. On day 7 of differentiation, insulin and pioglitazone were removed from differentiation medium and cells were maintained in DMEM containing 10% fetal calf serum for 6 days after which total RNA was isolated and the abundance of adipsin mRNA was assessed using Northern blotting analysis as described earlier. A quantitation of autoradiographic bands corresponding to adipsin mRNA transcripts is shown. Each data point represents mean $\pm$ S.E.M for n = 4 determinations. Student's test for unpaired groups showed significant effects for INS withdrawal ($p = 0.007$), PIO withdrawal ($p = 0.002$), and INS+PIO withdrawal ($p = 0.0002$).

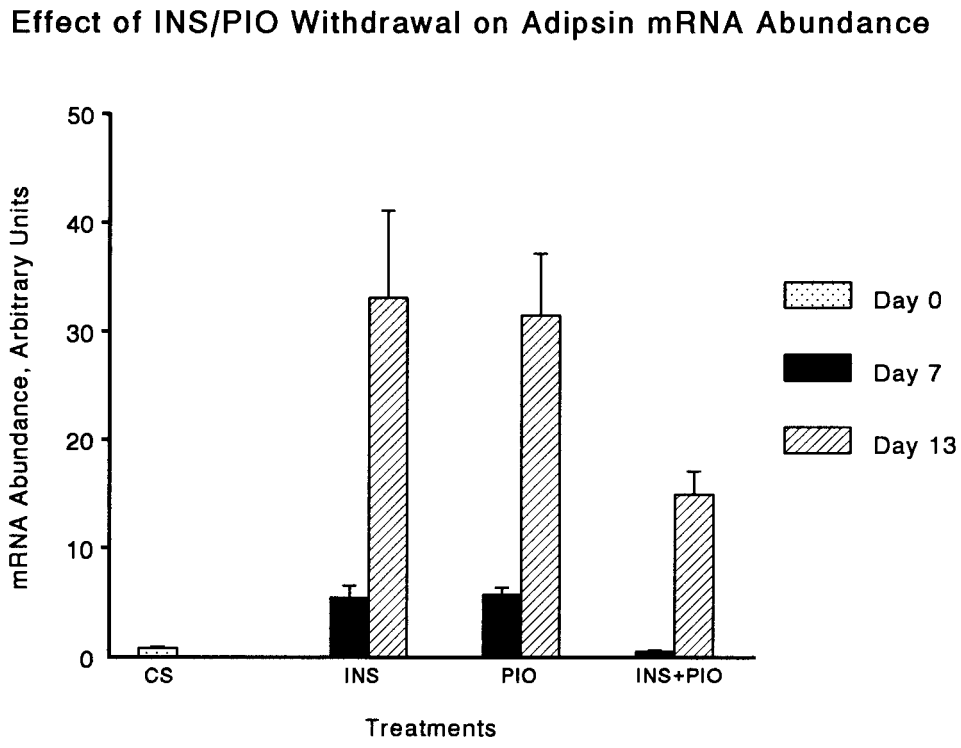
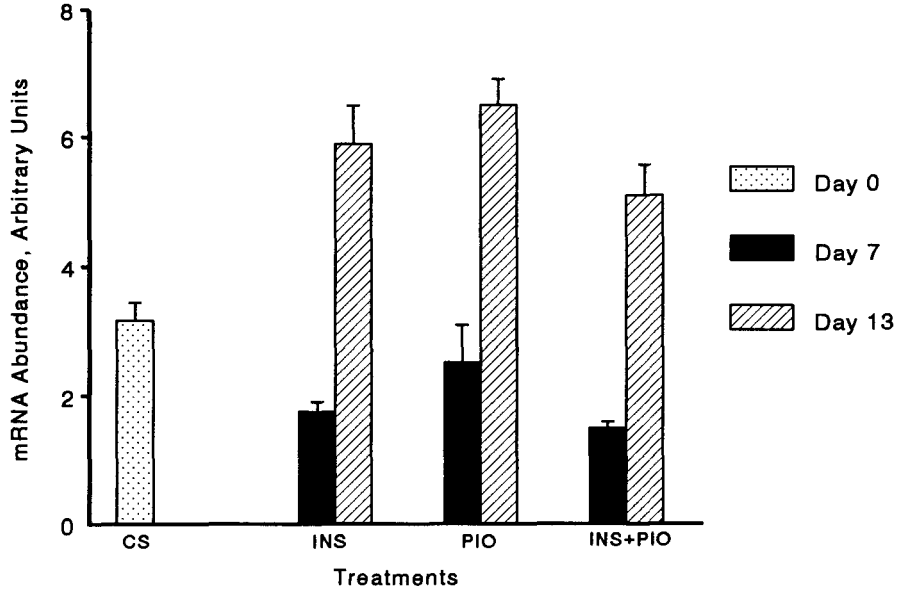

Effect of INS/PIO Withdrawal on Adipsin mRNA Abundance
mRNA Abundance, Arbitrary Units
50
40
30
20
10
0
CS
INS
PIO
INS+PIO
Treatments
Day 0
Day 7
Day 13

Figure 13.

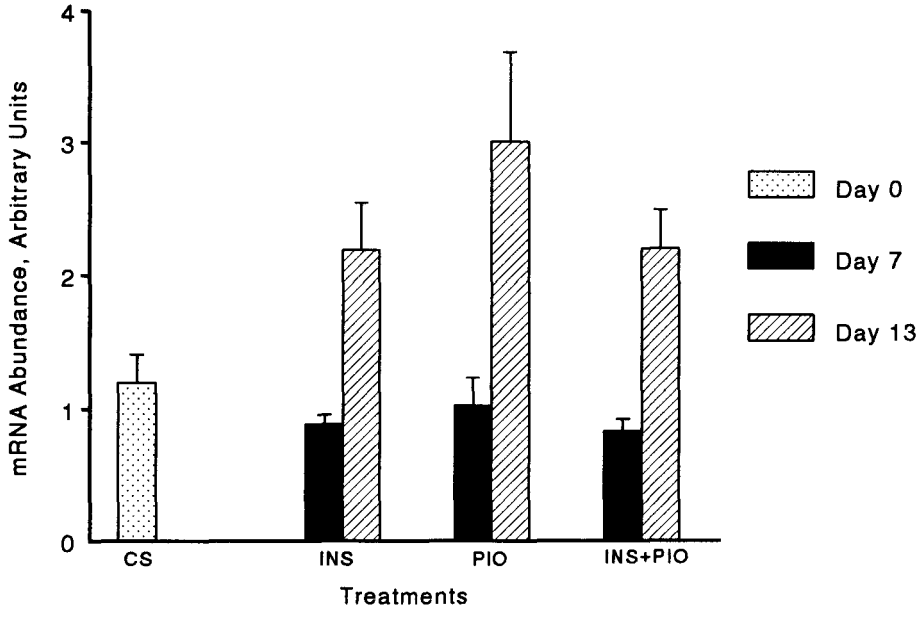
Figure 14. Enhanced insulin receptor mRNA abundance by insulin and pioglitazone withdrawal after differentiation. Cell culture, differentiation, treatments, and message abundance analysis as described for figure 13. A, Quantitation of autoradiographic bands corresponding to the 9.5 kb insulin receptor mRNA transcript. Each data point represents mean $\pm$ S.E.M for n = 4 determinations. Student's test for unpaired groups showed significant effects on the abundance of the 9.5 kb insulin receptor message by INS ($p = 0.0002$), PIO ($p = 0.0007$), and INS+PIO withdrawal ($p < 0.0001$). B, Quantitation of autoradiographic bands corresponding to the 7.5 kb insulin receptor mRNA. Each data point represents mean $\pm$ S.E.M for n = 4 determinations. Student's test showed significant effects on the 7.5 kb insulin receptor message abundance for INS withdrawal ($p = 0.005$), PIO withdrawal ($p = 0.0016$), and INS+PIO withdrawal ($p < 0.0024$). C, Representative blot showing enhanced expression of insulin receptor message of two sizes after insulin and pioglitazone withdrawal.

Effect of INS/PIO Withdrawal on Insulin Receptor mRNA Abundance

A.



B.



C.

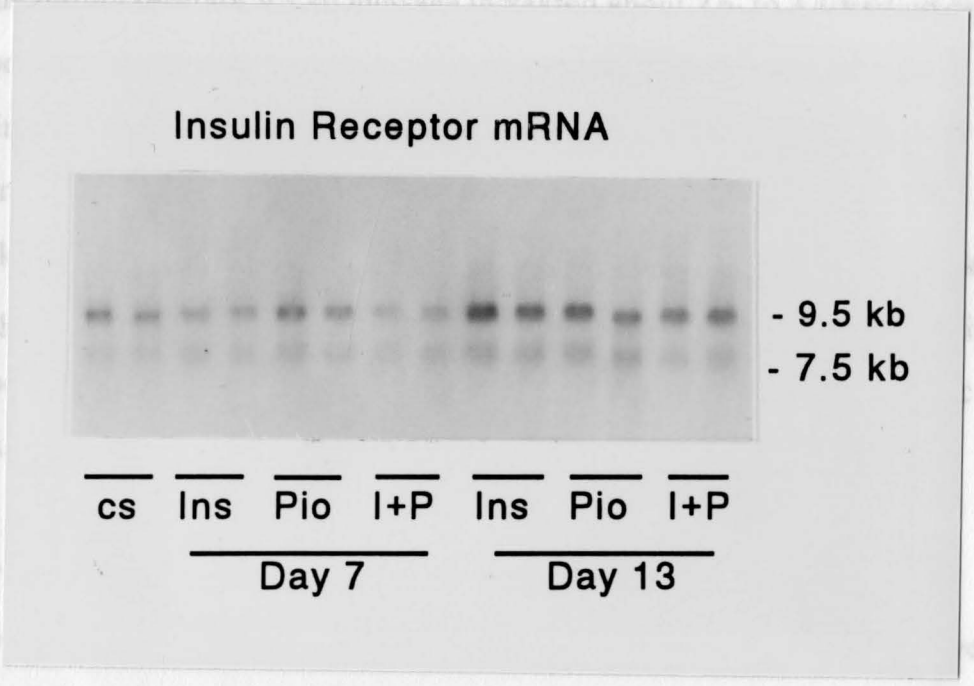

Insulin Receptor mRNA
- 9.5 kb
- 7.5 kb
cs
Ins
Pio
I+P
Ins
Pio
I+P
Day 7
Day 13

Figure 14.

treatment showed 29-fold increase of adipsin mRNA levels on day 13 above their corresponding levels on day 7.

The insulin receptor 9.5 kb message increased about 2.6- to 3.4-fold on day 13 above corresponding levels on day 7 after insulin, pioglitazone, or insulin/pioglitazone withdrawal (Figure 14A, 14C). Similar results were obtained for the insulin receptor 7.5 kb message (Figure 14B, 14C).

Taken together, all these results indicate that both insulin and pioglitazone have general differentiation effect, causing 3T3-F442A cells to differentiate into adipocytes with concomitant increases in the expression of adipocyte specific genes.

Effect of insulin and pioglitazone on transcription activity

To assess whether observed changes in abundance of different mRNAs were due to changes in corresponding gene transcription rate, we performed the nuclear transcription run-on assay. 3T3-F442A cells were differentiated with insulin (1 μg/ml), pioglitazone (1 μM), or both, and nuclei were isolated from differentiated cells on day 7 and from undifferentiated control cells on day 0. Transcription activity was measured by allowing the incorporation of [^{32}P]UTP into elongating nascent transcripts in isolated nuclei, and hybridizing the resultant labeled transcripts into immobilized cRNA Riboprobes of interest. As shown in Figure 15 and in Table II, transcription activity was below detection for GLUT1, GLUT4, and insulin receptor in control cells and no change could be detected with differentiated cells. Unexpectedly high levels of transcription activity were detected for adipsin and aP2 in undifferentiated control cells. There are detectable increases in transcription activity for aP2 and detectable

decreases for adipsin in differentiated cells. In all cases, changes in mRNA abundance for all studied genes could not be explained by corresponding changes in transcription activity.

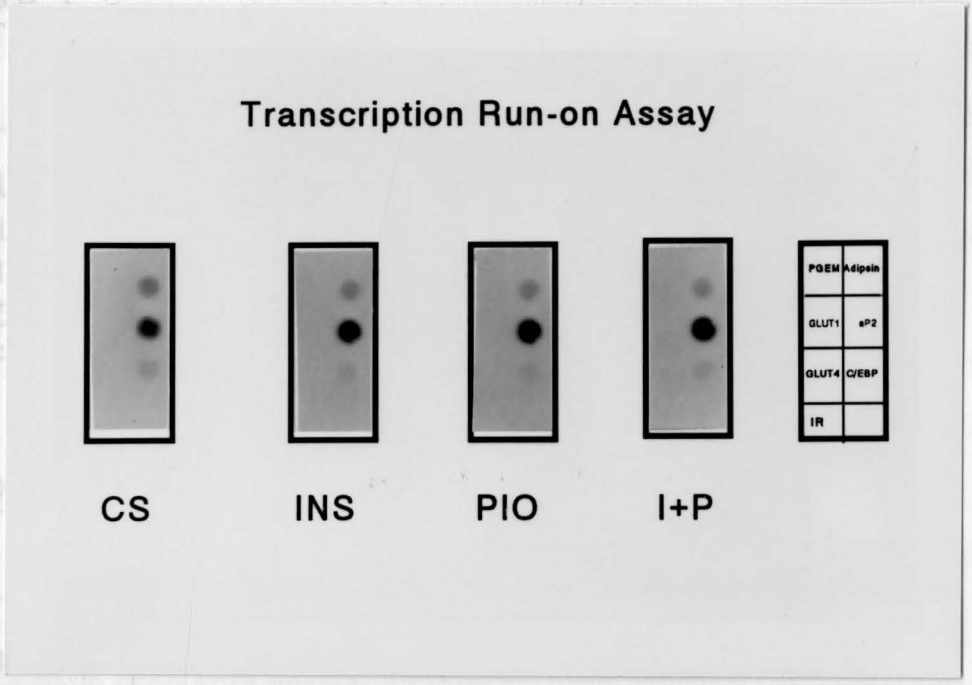


Figure 15. Regulation of gene transcription activity by insulin and pioglitazone-induced differentiation of 3T3-F442A cells. Nuclei were isolated from cells differentiated with insulin (INS), pioglitazone (PIO), or both (INS+PIO) on day 7 of differentiation, and from preconfluent undifferentiated cells (CS). Transcription run-on assay by isolated nuclei was carried out as described under material and methods. Resultant labeled transcripts were hybridized to indicated cRNAs (1 μg each), and to plasmid vector cRNA (pGEM-4Z). A representative autoradiogram is presented from an experiment performed twice each with a different preparation of nuclei.

Table II. Regulation of gene transcription activity by insulin and pioglitazone-induced differentiation of 3T3-F442A cells.

EXP. 1

	CS	INS	PIO	INS+PIO
GLUT1	N.D.	N.D.	10	N.D.
GLUT4	N.D.	11	15	N.D.
INS.REC.	N.D.	N.D.	27	N.D.
ADIPSIN	208	152	255	142
aP2	452	454	896	595

EXP. 2

	CS	INS	PIO	INS+PIO
GLUT1	N.D.	N.D.	0.6	N.D.
GLUT4	N.D.	57	N.D.	N.D.
INS.REC.	N.D.	N.D.	N.D.	N.D.
ADIPSIN	181	125	131	108
aP2	395	524	569	586

Transcription run-on assay was carried out as described under figure 15. After hybridization and autoradiography, filters were cut and 32 P-labeled radioactivity hybridized to each cRNA was quantitated by scintillation counting. Data are expressed as count per minute (cpm) after subtracting counts corresponding to vector-associatd radioactivity as background. N.D. indicates that signal was not detected.

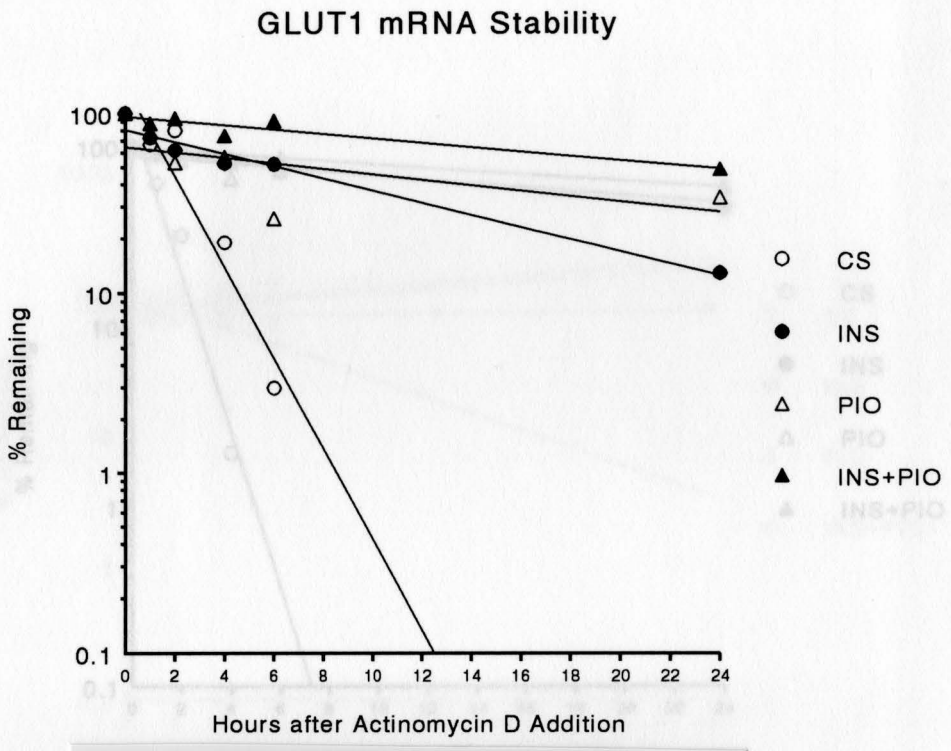
Effect of insulin and pioglitazone on mRNA stability

Since observed increases in specific mRNA levels could not be explained by corresponding increases in their transcription rates, we investigated possible effects of insulin and pioglitazone on the stability of these mRNAs. 3T3-F442A cells were differentiated with insulin (1 μg/ml), pioglitazone (1 μM), or both for 7 days. The transcription inhibitor agent, actinomycin D, was added at a final concentration of 5 μg/ml to differentiated cells on day 7 or to undifferentiated control cells used just before reaching confluence (day 0), and total RNA was extracted from cells at indicated times after addition of the transcription inhibitor (0, 1, 2, 4, 6, 24 hr). The abundance of remaining mRNA transcripts for each gene was assessed on Northern blots. Differentiation of 3T3-F442A cells increased the GLUT1 mRNA half life from about 2.2 hr in control undifferentiated cells to about 6.3, 4.1, and 24 hr in cells differentiated with insulin, pioglitazone, or both, respectively (Figure 16, Table III). The magnitude of GLUT1 mRNA stabilization likely accounts for the previously described corresponding increases in GLUT1 mRNA abundance. Similarly, GLUT4 mRNA half life increased from about 1.2 hr in undifferentiated control cells to about 20.2, 24, and >24 hr in cells differentiated with insulin, pioglitazone, or both, respectively (Figure 17, Table III). Again, the magnitude of GLUT4 mRNA stabilization most likely accounts for described increases in mRNA abundance. Adipsin mRNA half life also increased from about 5.9 hr in control undifferentiated cells to more than 24 hr in cells differentiated with either insulin or pioglitazone or both (Figure 18, Table III), and this increase in half life could account for observed changes in mRNA abundance, except in the case of the combined treatment were the observed decrease in mRNA steady

state level could be due to transcriptional inhibition. On the other hand, there was no measurable change in mRNA stability for aP2 which showed half lives of more than 24 hr in control undifferentiated as well as differentiated cells (Figure 19, Table III).

Figure 16. Enhanced GLUT1 mRNA stability by treatment with insulin, pioglitazone, or both. Confluent 3T3-F442A cells were differentiated with INS, PIO, or INS+PIO for 7 days as described under materials and methods. On day 7, the transcription inhibitor agent actinomycin D (Act D) was added to differentiated and undifferentiated (CS) control cells to a final concentration of 5 μg/ml and total RNA was extracted from cells at indicated time points (0, 1, 2, 4, 6, 24 hr). Abundance of mRNA was assessed by Northern blotting analysis as described earlier. A, Quantitation of autoradiographic bands corresponding to the remaining GLUT1 mRNA after Act D addition in undifferentiated and differentiated cells. Data are expressed as percent of mRNA remaining after Act D treatment relative to their levels before the treatment (time 0). Each data point represents mean values for n = 2 determinations. B, Blot showing remaining GLUT1 mRNA after transcription inhibition in an experiment done in duplicate, except for the time "0" point of INS, where only a single sample is shown. Hours of film exposure were 40, 18, 24, and 8 hr for CS, INS, PIO, I+P respectively.

A.


GLUT1 mRNA Stability
% Remaining
100
10
1
0.1
0
2
4
6
8
10
12
14
16
18
20
22
24
Hours after Actinomycin D Addition
CS
INS
PIO
INS+PIO

B.

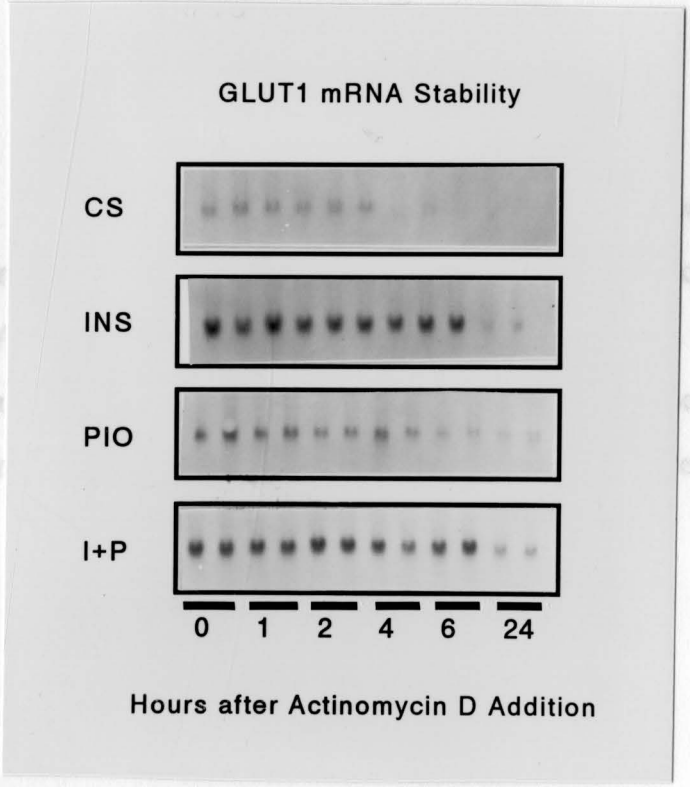

GLUT1 mRNA Stability
CS
INS
PIO
I+P
0
1
2
4
6
24
Hours after Actinomycin D Addition

Figure 16.

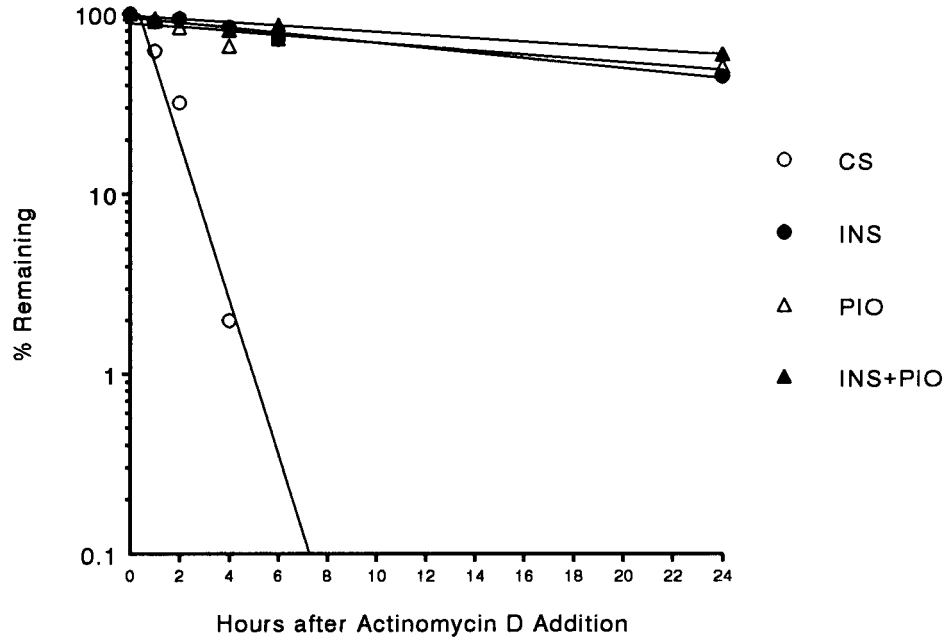


Figure 17. Enhanced GLUT4 mRNA stability by treatment with insulin, pioglitazone, or both. Cell culture, differentiation, actinomycin D treatment, and mRNA analysis were done as described under figure 16. Data, which represent mean values for n = 2 determinations, are expressed as percent of mRNA remaining after Act D treatment relative to their levels at time 0.

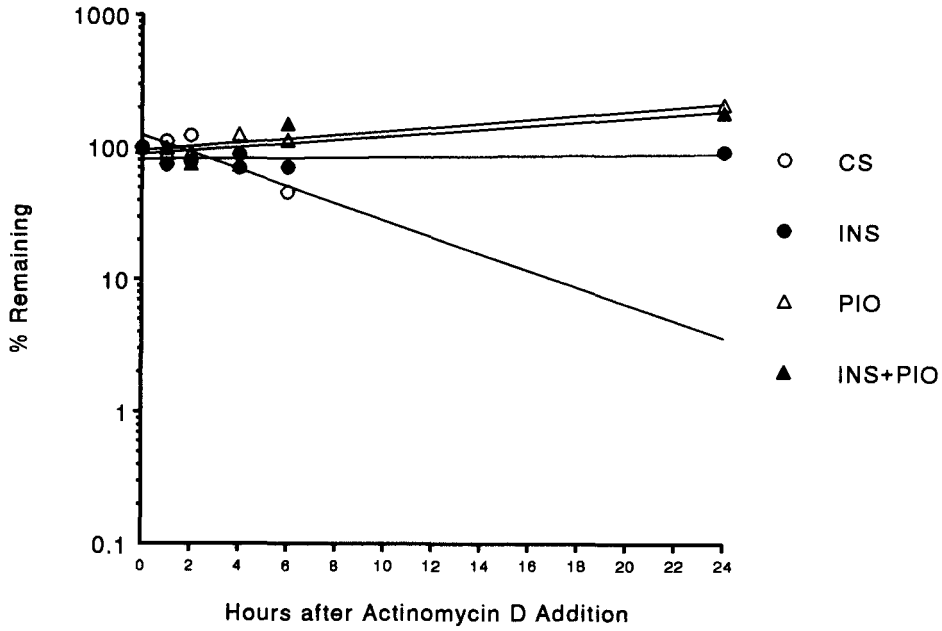


Figure 18. Enhanced adipsin mRNA stability by treatment with insulin, pioglitazone, or both. Cell culture and differentiation, actinomycin D treatment, and mRNA analysis as described under figure 16. Data, which represent mean values for n = 2 determinations, are expressed as percent mRNA remaining after Act D treatment relative to their levels at time 0.

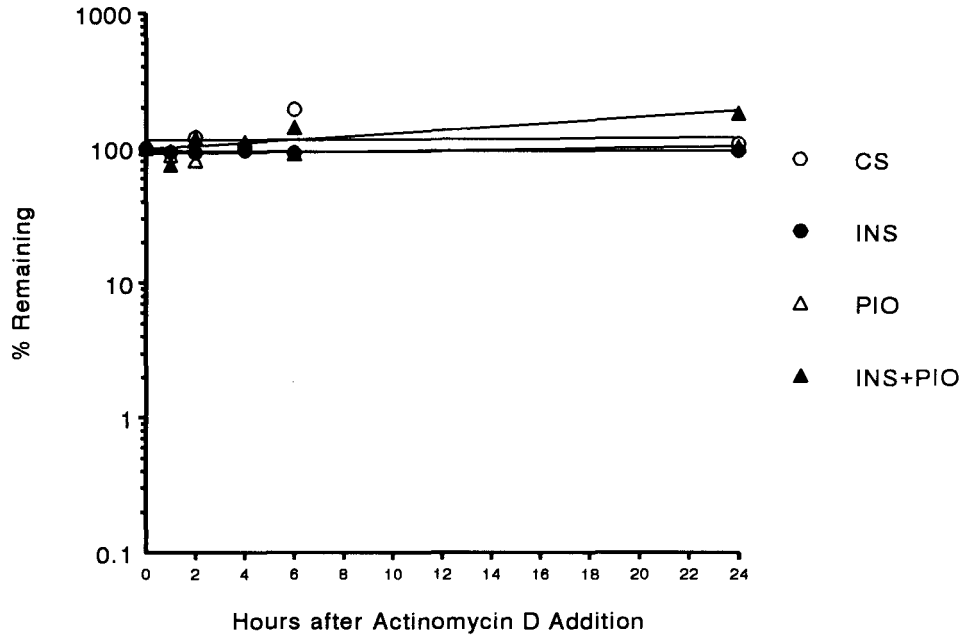


Figure 19. aP2 mRNA stability in undifferentiated and differentiated 3T3-F442A cells. Cell culture, differentiation, actinomycin D treatment and mRNA analysis as described for figure 16. Data, which represent mean values for n = 2 determinations, are expressed as percent of mRNA remaining after Act D treatment relative to their levels at time 0.

Table III. Calculated mRNA half lives in hours for undifferentiated and differentiated 3T3-F442A cells. Data from actinomycin D (Act D) treatment experiment (Fig. 16, 17, 18, 19) were fitted to a single exponential decay curve by non linear least square regression analysis. The estimated first-order rate constant was used to calculate the mRNA half life.

	CS	INS	PIO	INS+PIO
GLUT1	2.2	6.3	4.1	> 24
GLUT4	1.2	20.2	24	>24
Adipsin	5.9	>24	>24	>24
aP2	>24	>24	>24	>24

CHAPTER V

DISCUSSION

Effect of insulin and pioglitazone on 3T3-F442A preadipocyte differentiation

Pioglitazone, 5-[4-(2-(5-ethyl-pyridyl)ethoxy)-benzoyl]-2,4-thiazolidinedione, is an antidiabetic agent that has been shown to ameliorate hyperglycemia in animal models of non-insulin-dependent diabetes mellitus (6,145,159). Although a clear cut mode of action for pioglitazone has not been elucidated, this agent appears to potentiate insulin action both *in vivo* and *in vitro* as measured by enhanced insulin-stimulated glucose transport, suppressed hypertriglyceridemia and hyperinsulinemia, but without increasing insulin secretion (145,159). However, pioglitazone required the presence of insulin for these effects and did not change insulin binding, thus suggesting that it might act to amplify cellular responses to insulin. In fact, pioglitazone treatment was shown to increase insulin-stimulated autophosphorylation of insulin receptors in Wistar fatty rats (144), and to correct deficits in GLUT4 mRNA and protein in fat and muscle of insulin deficient rats and KKA^{Y} obese mice (6).

The purpose of my work was to further probe action mechanisms for pioglitazone. Since adipose tissue functions as an energy reservoir wherein glucose is used to synthesize triglycerides, treatment with agents such as pioglitazone, which enhance glucose uptake, would be expected to be associated

with changes in adipose tissue function and development. I thus decided to investigate the effect of pioglitazone treatment on the differentiation of preadipocytes to adipocytes as an indicator of adipose tissue development. Such differentiated adipocytes are known to be fully active in glucose uptake, storage, and metabolism. The results of this study clearly showed that the antidiabetic agent pioglitazone acts as a potent accelerator of adipocyte differentiation of 3T3-F442A cells. This was evidenced by the demonstration that treatment of fibroblast-like cells with pioglitazone led to acquisition of the morphological appearance of lipid-accumulating adipocytes with concomitant increases in triglyceride accumulation. Both insulin and pioglitazone acted synergistically on these parameters further supporting the notion that pioglitazone acts to amplify cellular responses to insulin.

Although previous *in vivo* studies have shown the hypoglycemic effect of pioglitazone to be dependent upon the presence of circulating insulin, my study demonstrated effects of pioglitazone on cell differentiation without added insulin. These two observations however, would become consistent when we consider the fact that in my study differentiation was carried out in serum-containing medium which is known to contain physiologically significant concentrations of insulin. Therefore, under my experimental conditions, pioglitazone might act to enhance cellular responses to low concentrations of insulin.

Effect of insulin and pioglitazone on glucose transport activity and glucose transporter gene expression

In the present study, I showed that the differentiation of 3T3-F442A cells

with insulin and pioglitazone was accompanied by concomitant increases in adipocyte basal glucose transport activity. Recent studies have established that facilitated diffusion of glucose across the plasma membrane of adipose cells is mediated by two glucose transporter proteins (105,106). Multiple mechanisms may exist by which hormones and other factors control the rate of glucose uptake across the plasma membrane. These include the rapid translocation of pre-existing transporters from intracellular pool to the plasma membrane, modulation of the intrinsic activity of pre-existing plasma membrane glucose transporters, and stimulation of the synthesis of new transporters (12). My results demonstrated increases in the expression of both GLUT1 and GLUT4 transporters on mRNA and protein levels. These results are in agreement with previous studies in which GLUT1 and GLUT4 mRNA and protein levels were shown to increase during differentiation of 3T3 preadipose cells (61,108). The increase in glucose transport activity in my study however, was at least an order of magnitude higher than the combined increase in both transporter proteins indicating that another mechanism such as increased transporter intrinsic activity may contribute to the observed effect. This is not an unusual observation since regulation of intrinsic transporter activity in 3T3-L1 adipocytes has been previously reported (160,161). Inhibition of protein synthesis in 3T3-L1 adipocytes by anisomycin, for instance, appeared to stimulate glucose transport primarily by enhancing the intrinsic catalytic activity of cell surface GLUT1 and to a lesser extent, GLUT4 proteins (161), while tumor necrosis factor-α treatment reportedly increased glucose transport and GLUT1 transporter intrinsic activity in 3T3-L1 preadipose cells (160).

Although adipocyte differentiation by insulin or pioglitazone increased the expression of two transporter proteins and basal glucose uptake, no significant increase in insulin-responsiveness of differentiated cells was observed in the present study (data not shown). This could possibly be due to the chronic exposure of cells to low concentrations of insulin in the serum during differentiation, thus causing continuous translocation of existing, as well as newly synthesized transporters, into the plasma membrane further contributing to the high level of basal glucose uptake. Consequently, acute insulin exposure does not induce further translocation normally known to be responsible for enhanced acute insulin- responsiveness. Consistent with that mechanism are two observations established by previous studies. First, chronic exposure of differentiated 3T3-L1 adipocytes to insulin has been shown to result in persistent state of enhanced basal glucose transport activity which was resistant to further stimulation by insulin during hexose uptake assays (45), and second, chronic insulin treatment of 3T3-F442A (108) and 3T3-L1 (62) adipocytes induced a redistribution of glucose transporters in these cells from plasma membrane to low density microsomes which was associated with marked resistance of cells to restimulate glucose transport and particularly to recruit GLUT4 to the cell surface following an additional insulin treatment (62). The first study showed that chronic insulin treatment increased total cellular GLUT1 but did not change total cellular GLUT4 (45), while the latter study showed that insulin resistance was associated with selective down-regulation of cell-surface GLUT4 glucose transporter and particularly with a marked resistance of cells to further recruit GLUT4 to the cell-surface following an additional insulin treatment; while levels of GLUT1 at the surface were

markedly increased (62). These observations are in contrast with results reported in another study in which insulin sensitivity was shown to increase during differentiation of 3T3-L1 cells (61). In that study however, adipocyte differentiation was induced by insulin, dexamethasone, and isobutylmethylxanthine (IBMX), all of which are independently known to regulate glucose transport and glucose transporter activity. Indeed, a similar study showed that basal glucose uptake in 3T3-L1 adipocytes was 50% lower than that in fibroblasts which was due to more than 90% inhibition of the intrinsic catalytic activity of GLUT1 on the surface of mouse 3T3-L1 adipocytes (162). Therefore, a mechanism that markedly suppressed basal glucose transport catalyzed by GLUT1 was implicated as the major contributor to the dramatic insulin sensitivity of glucose uptake in 3T3-L1 adipocytes in these studies. Thus, elevated basal glucose transport activity in my study would be consistent with continuous translocation of existing, as well as newly synthesized, GLUT1 transporter into the cell-surface. In addition, changes in intrinsic activity of cell-surface transporters would be speculated to account for any discrepancy between increased GLUT1 expression and increased basal glucose uptake activity. Although there is an apparent contradiction between increased GLUT4 expression and cell unresponsiveness, these two observations are consistent with increased GLUT4 expression due to the differentiation of preadipocytes to adipocytes, and with cell inability to recruit GLUT4 to the cell-surface due to the continuous exposure to insulin during differentiation.

Effects of insulin and pioglitazone on adipocyte-specific gene expression

The present study indicated that the effect of insulin and pioglitazone on

gene expression was not glucose transporter-specific since the abundance of mRNAs encoding other adipocyte-specific genes were increased at the same time, further confirming that pioglitazone has a general differentiation effect. Adipsin and aP2 mRNAs for instance, were both increased by insulin or pioglitazone treatments, in agreement with earlier studies in which these two mRNA transcripts were shown to increase during adipocyte differentiation (8,10,85,92,99). No significant effect on insulin receptor gene expression was detectable in my experiments under the same condition despite the fact that its message and protein product have previously been reported to increase during 3T3-L1 adipocyte differentiation (44,57,59,60,102). The disparity between my present results and those reported in previous studies could be due to the use of different cell lines and/or different differentiation conditions. In all these studies, the 3T3-L1 preadipocyte/adipocyte cell line was used as a model system. In addition, their differentiation medium variously contained dexamethasone, IBMX, insulin, and indomethasone, all of which are known to regulate adipocyte differentiation (44,57,59,60). My results also showed that cell differentiation induced by a combination of insulin and pioglitazone completely inhibited adipsin mRNA expression. Such results implicate some negative mechanism contributing to the observed down-regulation effect on adipsin and insulin receptor genes. In fact, prolonged insulin treatment has previously been shown to downregulate insulin receptor protein and mRNA in various tissues by accelerating its degradation and decreasing its biosynthesis (58). Insulin was also shown to exert negative effect on the abundance of adipsin mRNA in differentiating, as well as in fully differentiated 3T3-F442A adipocytes via a mechanism which involved primarily a rapid inhibition of the

transcriptional rate (73,100,163). Thus, it appears that insulin has both positive effects due to its ability to accelerate differentiation, and negative effects due to direct down-regulation at different pre-translational and/or post-translational levels, on insulin receptor and adipsin mRNAs. Indeed, my results demonstrated that insulin and pioglitazone withdrawal after differentiation relieved the negative down-regulating effect, thus revealing the differentiation-dependent increases for these two mRNAs. The fact that insulin completely down-regulated adipsin mRNA only in the presence of pioglitazone provides additional evidence that pioglitazone acts to amplify cellular responses to insulin.

Effect of insulin and pioglitazone on transcription activity and mRNA stability

It has been previously established that alternations in the steady state level of several mRNAs during differentiation were accompanied by activation of specific gene transcription (8-10,75). This was particularly shown for mRNAs whose abundance is dramatically increased (20- to 100-fold) during differentiation, including the aP2 and glycerolphosphate dehydrogenase genes. These same studies showed no significant change in the rates of transcription of mRNAs whose abundance was only moderately altered (2- to 4-fold) during differentiation, such as those encoding fructose-1,6-bisphosphate, ß-actin, α-actin, and ß-tubulin (8). While transcriptional activation of adipsin gene expression during 3T3-F442A differentiation was reported in one study (9), another study failed to show such activation for this gene under similar conditions (10). In these studies however, aP2 and adipsin mRNAs were found to be far more abundant than would be predicted by their nuclear transcription

alone, suggesting that other levels of control may contribute to the relative abundance of these mRNAs. In my present study, run-on transcription by nuclei isolated from undifferentiated 3T3-F442A cells or from cells differentiated with insulin and/or pioglitazone revealed little or no change in the transcription activity for all studied genes. No transcription signal could be detected for GLUT1 and GLUT4 mRNAs in either undifferentiated or differentiated cells, although both mRNAs increased in abundance moderately (2- to 6-fold) during differentiation. Transcription of adipsin and aP2 was strongly detectable in undifferentiated cells, but no significant change in their transcription activity could be detected upon differentiation despite the dramatic increase (20- to 70-fold) in their steady state levels. There were two exceptions. Insulin, in combination with pioglitazone, decreased the transcription of adipsin mRNA, while pioglitazone moderately increased (< 2-fold) the transcription of aP2 mRNA. In all cases, the increase in the steady state level of all studied mRNAs could not be explained by changes in their corresponding transcription activities suggesting that other levels of control, possibly mRNA stability, may contribute to the relative abundance of certain mRNAs during adipocyte differentiation. To further investigate such a possibility, I compared mRNA half lives in undifferentiated and differentiated cells. Differentiation of 3T3-F442A cells by insulin and/or pioglitazone increased the mRNA half lives for GLUT1, GLUT4, and adipsin above their values in undifferentiated cells. Furthermore, the stabilization of these mRNAs caused by differentiation appears to fully account for the increase in their steady state levels under most of the treatment conditions. There has been no correlation, however, between the increase in adipsin mRNA half life and its

steady state levels in the combined insulin and pioglitazone treatment. In this case, the observed inhibition of gene transcription activity may account for the extremely low abundance of adipsin mRNA. My results also failed to explain the dramatic induction of aP2 gene expression upon differentiation, since its mRNA was apparently very stable (half-life > 24 hr) in both undifferentiated and differentiated cells. This could be due to several reasons. First, RNA extracted from undifferentiated cells may has been contaminated with RNA from a small number of spontaneously-differentiating cells in the same culture. Thus, if aP2 gene expression is greatly enhanced very early in the process, My results would falsely indicate very stable mRNA in undifferentiated cells. Although I purposely used undifferentiated cultures before they reached confluence, such spontaneous cell differentiation is still possible. Thus, further *in situ* hybridization experiments would be required to confirm whether their is an expression of aP2 gene in a minor population of spontaneously differentiating fibroblast cells. An alternate possibility is that another post-transcriptional step, such as RNA processing contributes to the accumulation of aP2 mRNA during differentiation. Since Northern blots showed only a single aP2 mRNA species of the same size in both undifferentiated and differentiated cells, such RNA processing would have to occur very early and extremely fast during differentiation to explain our ability to detect a presumably processed aP2 mRNA in undifferentiated cells. Another possibility is that aP2 mRNA is stable in undifferentiated cells (half life >24 hr) and it becomes more stable with differentiation, but my results showed no measurable change in half life because we carried out the stability study for only 24 hours. Measurements of the aP2 mRNA half life, or that of any other adipocyte-specific mRNA, in

undifferentiated preadipocytes have not been reported before because of the extremely low abundance of the RNA in these cells. One study however, reported aP2 mRNA half life in differentiated adipocytes and found it to be far more stable in the adipocyte cytoplasm than other mRNAs examined, with an estimated half life of 12 hours (10). Since that study did not show aP2 mRNA half life in preadipocytes, it failed to indicate whether the apparent stability of the aP2 is dependent on differentiation or whether it is an intrinsic property of the RNA molecule. In either case, my results showed far more stable mRNA (>24 hr) than was reported which leads to another possibility. Actinomycin D treatment has been shown specifically to stabilize certain mRNAs. For instance, actinomycin D has been reported to have stabilizing effect on mRNAs for all protein kinase A subunits (164). Since aP2 mRNA half life in adipocytes was reported to be 12 hours using 5,6-dichloro-1-ß-D-ribofuranosylbenzimidazole (DRB) as a transcription inhibitor, the apparent stability of aP2 mRNA in undifferentiated and differentiated cells in my study could be due to a direct stabilizing effect on the aP2 gene by actinomycin D treatment. This issue would be resolved in future studies by using a different transcription inhibitor such as DRB, or thiolutin, or using a different method such as [^{3}H]uridine-chase method to assess message stability.

Taken together, my results indicate that the mechanism underlying differentiation-dependent changes in mRNA abundance induced by insulin or pioglitazone appears to be mostly on the level of mRNA stability rather than transcriptional activation. Although this is the first study that directly shows increased mRNA abundance during differentiation by increasing message stability, such mechanism of regulation at mRNA stability level has been

reported under various conditions. Treatment of 3T3-F442A mature adipocytes with retinoic acid for instance, specifically decreased the adipsin mRNA level (165). The rate of adipsin gene transcription remained unchanged, while the half life of adipsin mRNA was greatly shortened in retinoic acid-treated adipocytes (37.6 to 7.3 hr) as compared with untreated cells. Another example is the induction of GLUT1, as well as other immediate early growth related protooncogenes, in 3T3-L1 fibroblasts by treatment with tumor necrosis factor-α (160,166) and 8-bromo-cAMP (167). While transcriptional activation of immediate-early genes correlated well with subsequent accumulation of their respective mRNAs, increased GLUT1 mRNA was due to apparent increase in the stability of this message (45 min to several hours) and not to increased transcription. Another study showed that increased GLUT1 mRNA abundance by chronic exposure of L6 myocytes to insulin was due to increased transcription as well as prolonged half life (2-2.5 hr to 5 hr) (168).

Significance and future studies

My findings demonstrated that pioglitazone, like insulin, has the ability to accelerate the *in vitro* adipocyte differentiation of 3T3-F442A cells with concomitant increases in the expression of two glucose transporter genes. This finding is consistent with a recent report of pioglitazone having adipogenic activity in 3T3-L1 cell line (169). This is the second example of an antidiabetic agent with adipogenic activity. A recent study demonstrated that another antidiabetic agent with similar thiazolidinedione derived structure, AD4743, enhanced adipocyte differentiation of 3T3 T mesenchymal stem cells (77). Although not established *in vivo*, this would constitute an interesting mechanism

of action for hypoglycemic agents by enhancing insulin sensitivity of target tissues, thus correcting blood glucose levels in diabetes. Therefore, the effect of pioglitazone on adipocyte differentiation from stromal vascular cells (the *in vivo* adipocyte progenitor cell) should be investigated and characterized in future experiments to establish the pertinence of such an effect to its *in vivo* hypoglycemic activity. The observations that pioglitazone acted synergistically with insulin on adipocyte differentiation and on glucose transport activity provide additional support to the concept that pioglitazone acts to amplify cellular responses to insulin, but do not prove it. Since cell differentiation was carried out in the presence of serum, I was not able to ascertain whether pioglitazone induced effects on cell differentiation and glucose transport activity were dependent on the presence of insulin in the serum or not. Thus, it is necessary to develop a defined serum-free differentiation medium in order to determine the role of each of insulin and pioglitazone individually and how they interact with each other to regulate adipocyte differentiation and glucose transport activity.

Pioglitazone also showed direct transcriptional activation effect on the aP2 gene. Considering the fact that aP2 mRNA is expressed at high levels in adipocytes (87), and that it has a postulated role as a mediator of insulin actions (89), aP2 presents an interesting candidate gene for further exploration of insulin action mechanisms, their perturbations in diabetes, and possible corrective effects of pioglitazone treatment.

My findings have interesting implications for better understanding the processes involved in cellular differentiation and development. This is the first report directly showing increased abundance of adipocyte-specific mRNA

during differentiation as result of increasing message stability, although the concept was previously inferred (8-10). The stabilization or destabilization of mRNAs in response to some biological or pharmacological stimuli has been recognized as an important post-transcriptional step in the regulation of gene expression (170-173). Despite that, the mechanism underlying such processes, including the signals that trigger mRNA degradation, the structural elements of an individual mRNA recognized by the degradative enzymes, as well as the enzymes and other *trans*-acting factors that are involved in mRNA degradation, remain largely unknown (170,173-176).

Recent studies demonstrated that some mRNA sequences may play important role in determining degradation rates. Of particular importance are the 3' terminal untranslated region (UTR) and poly(A) sequences (174). The rate of mRNA degradation was shown to be dependent on the presence or absence of the 3' terminal poly(A), and that the de-adenylated mRNA apparently had a faster rate of turnover (170,177,178).

Although not studied within the context of differentiation, there is some evidence that adipsin and GLUT1 mRNAs are regulated through effects on their 3'-UTR sequences. A recent study has shown that within a physiological range of concentrations, insulin exerts a negative effect on adipsin mRNA abundance in 3T3-F442A adipocytes, which was found to involve a rapid inhibition of the transcriptional rate (163). At the same time, insulin showed a specific effect on the length of the poly(A) tract of the adipsin mRNA. The study did not show the consequence of decreasing the adipsin poly(A) tract length, but speculated that it could affect translational efficiency or the stability of mRNA. Indeed, upon examining my Northern blots carefully, both GLUT1

and adipsin mRNAs appeared as broad signals in differentiated cells. Such heterogeneity in mRNA length could be speculated to be the result of variation of the poly(A) tract length which would be consistent with the previously cited study (163). Further experiments using RNase H digestion of mRNA would be required to prove such a possibility.

Another study showed that tumor necrosis factor-α stimulated hexose transport in quiescent 3T3-L1 preadipocytes by stabilizing the relatively labile mRNA coding for the basal glucose transporter, GLUT1 (166). The 3'-UTR of GLUT1 mRNA contains a single copy of the destabilizing AUUUA motif in the context of an AU-rich region. The stability of GLUT1 mRNA was found to be partially controlled by its interaction with sequence-specific mRNA binding protein, the adenosine-uridine binding factor (AUBF), which was speculated to mediate mRNA stabilization by blocking the AU-destabilizing motifs. Further sequence analysis of the 3'-UTR of other adipocyte-specific genes is required to identify putative *cis*-elements through which such regulation might occur.

Thus, increasing mRNA abundance during differentiation by increasing message stability presents an interesting phenomenon awaiting further probing with regard to the differentiation and development fields. Future efforts should be particularly directed towards identifying common mRNA sequences that may function as stabilizing elements in the differentiation-induced mRNAs, and the potential regulatory mRNA binding proteins that are upregulated in response to various differentiation-inducing factors.

Overall, this study of pioglitazone effects in a cell culture system should prove to be a useful model with which to study molecular mechanisms underlying cell differentiation as well as insulin action on glucose transport.

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The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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