AN ABSTRACT OF THE DISSERTATION OF

Terry Brandebourg for the degree of Doctor of Philosophy in Animal Science presented on September 4, 2003.
Title: Nutritional Regulation of Adipocyte Differentiation in Animals.

Abstract Approved: Redacted for privacy
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The incidence of obesity has increased in the U.S. until an obesity epidemic now exists. American consumers are acutely aware of the consequences of obesity and are increasingly associating the consumption of high-fat foods with having a negative impact upon their health. This has in turn forced producers to grow leaner animals. Since excess fat is an undesired commodity, the need to understand mechanisms that regulate adipose tissue differentiation has never been more important. Understanding the underlying regulation of adipogenesis is a vital first step toward developing strategies that limit fat accretion in growing animals. The resulting increase in consumer demand and improved productive efficiency associated with such technologies should ensure that the animal industry will remain solvent in the years to come. However, despite a great deal of effort directed at elucidating the regulation of adipogenesis during the last decade, our understanding of adipose tissue growth and development in agriculturally important animals remains very limited.

This study used a porcine stromal-vascular cell primary culture system to study the nutritional regulation of adipogenesis. Specifically, the underlying molecular mechanism governing the anti-adipogenic actions of retinoic acid and conjugated linoleic acids was investigated. Retinoic acid inhibited the differentiation of pig preadipocytes through activating the RARα receptor and inhibiting the expression of

the key adipogenic transcription factors PPARγ and ADD1 while simultaneously increasing the expression of COUP-TF. Conjugated linoleic acids inhibited the differentiation of pig preadipocytes in an isomer-specific fashion via a mechanism that also involved reduced expression of PPARγ and ADD1 and the increase of COUP-TF message abundance. Importantly, C/EBPα and β expression was unaffected in either study.

Finally, feeding retinoic acid to growing broilers at levels 32 times greater than the minimum NRC requirements tested the hypothesis that retinoic acid is a potent inhibitor of adipogenesis. Feeding retinoic acid decreased total carcass lipid by 14% and abdominal fat mass by 10%. However, abdominal fat retinoic acid levels were unaltered by the diet suggesting that a physiological mechanism exists in the chicken which prevented retinoic acid from accumulating in adipose tissue and may have limited the response to supplementing the diet with retinoic acid.

Nutritional Regulation of Adipocyte Differentiation in Animals

By

Terry Brandebourg

A DISSERTATION

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Presented September 4, 2003

Commencement June 2004

Doctor of Philosophy dissertation of Terry Brandebourg presented on September 4, 2003.

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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Terry Brandebourg, Author

ACKNOWLEDGEMENTS

A very special thank you to my advisor, Dr. Ching Yuan Hu, who guided me with one hand and turned me loose with the other. I thank you for allowing me to learn from my mistakes while ensuring they would grow fewer in number. Thank you also for correcting my faults with gentle encouragement. You have shown me that a true scientist must also be a gentleman- I will aspire to the behavior you've modeled during my program. I gratefully and humbly recognize that anything I achieve in my scientific career will have your inspiration indelibly written upon it.

I also thank my committee members, Dr. Gita Cherian, Dr. Alfred Menino, Dr. Daniel Rockey, and Dr. Alvin Smith for their guidance and support during my graduate studies. Special appreciation is extended to Dr. Cherian whose guidance and support were particularly above the call of duty. Also, thank you Dr. Menino- your contribution to my success far exceeds reagents and PCR machines. My family and I will always treasure your friendship.

Appreciation is also extended to Dr. David Froman and Allen Feltman. Both were always willing to help in my time of need and I greatly value their friendships. Allen's input was essential to the success of my program and his friendship was a beacon during the rough seas that often occur during graduate school. Special thanks are also expressed to Irene Pilgrim and Jim Morrison for help they extended at the poultry farm. This dissertation simply could not have been completed without the generous assistance of Irene. Appreciation is also extended to Dr. Ann Brodie and Viola Manning who took me under their wing during my first months in Corvallis and to Dr. Agus Suryawan who taught me primary culture. Thanks also to Tim Langford

whose friendship and help in the laboratory are much appreciated. Thanks also to Dr. Jennifer Duringer who patiently helped with HPLC and for Dr. Buhler for allowing access to his HPLC machine.

Grateful thanks are extended to my colleagues in the basement. The help and cooperation received from these people and other members of the Department of Animal Sciences have made both my education and my family's stay in Corvallis enjoyable and successful.

I also wish to thank my parents, William and Jane Brandebourg for supporting me in all of my academic endeavors and for teaching me how to be a father and a husband. Thanks also to Elmer and Jane Muhlenkamp for their support throughout my education. Finally, I would like to thank my wife, Catherine, for her love, patience, and sacrifice throughout my education and for her dedication to providing such a wonderful family and home.

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CHAPTER 1
INTRODUCTION

The incidence of obesity has increased during the last few decades in the U.S. until an obesity epidemic now exists as an estimated one-third of all Americans are currently considered to be obese and greater than one-half of all Americans are overweight. Obesity is largely considered a pathological state characterized by a loss of equilibrium between energy intake and energy expenditure that is accompanied by both an increase in the number and size of adipocytes. Unfortunately an obese individual has dramatically increased risks for developing several disorders including type II diabetes, hypertension, heart disease, cancer and even reproductive dysfunction. American consumers are acutely aware of the consequences of obesity and have come to associate the consumption of foods high in fat with having a negative impact on their health. This has in turn forced producers to grow animals that are leaner. Since the production of excess fat is an undesired commodity and the main production cost is feed, the animal industry has recognized that improving production efficiency must be a top priority.

The need to understand mechanisms that regulate adipose tissue differentiation has never been more important. Understanding the underlying regulation of adipogenesis may help identify potential targets that are critical for differentiation. Such discoveries may lead to strategies which limit fat accretion in growing animals.

This, in turn, could have an immediate and dramatic impact upon society as the ability to produce healthier food could directly improve human health. An increased ability of the producer to meet consumer demands while simultaneously improving productive efficiency could likewise ensure that the animal industry will remain solvent in the years to come.

Despite a great deal of effort directed at elucidating the regulation of adipogenesis during the last decade, our understanding of adipose tissue growth and development in agriculturally important animals remains very limited. Work using cell culture systems has revealed that the adipocyte differentiation program is regulated by several transcription factors. In the current model, the expression of C/EBP-β during early differentiation induces expression of PPAR-γ. Ligand-activated PPAR-γ, cooperatively with C/EBP-α, transactivates a group of adipocyte-specific genes leading to terminal differentiation and the emergence of the adipocyte phenotype. PPAR-γ is now considered the "master regulator" of adipogenesis while C/EBP-α potentiates the process by up-regulating the expression of proteins which confer insulin sensitivity upon the differentiating adipocytes. While this central regulatory cascade has been well characterized in the literature, a large black box in our understanding exists between the early events initiated by the administration of either positive or negative regulators of differentiation and the effects these compounds have on the regulation of key proteins in the adipogenic cascade.

Importantly, it is not clear that observations concerning the regulation of adipogenesis in clonal cell lines can be reliably extrapolated to the regulation of

adipogenesis in meat producing animals in vivo. Clearly species-specific differences in the regulation of adipogenesis must surely exist. Furthermore, clonal cell lines suffer from artifactual changes in their behavior arising from their clonal nature. Thus, primary cultures of pig preadipocytes were used in this study to directly investigate the nutritional regulation of adipocytes differentiation in order to provide a more faithful model of the regulation of adipogenesis in production animals.

Conjugated linoleic acids (CLAs) are naturally occurring derivatives of linoleic acid which have been shown to limit fat accretion in growing animals. However, controversy exists concerning the mechanism of action for CLAs in adipose tissue. This study directly investigated the possibility that CLAs can inhibit the differentiation of pig preadipocytes and probed the underlying mechanism for such action. It is suggested that CLAs decrease fat accretion in pigs in part through negative effects upon adipocyte hyperplasia. Importantly, a potential novel negative regulator of adipogenesis, COUP-TF, was identified in this work.

Previous work in this laboratory had indicated that all-trans retinoic acid (RA) potently inhibited the differentiation of pig preadipocytes. This study builds upon that work by further investigating the molecular mechanism for the antiadipogenic action of RA. Specifically, the underlying molecular mechanism governing the anti-adipogenic action of retinoic acid was investigated. Finally this study tested the hypothesis that retinoic acid is a potent inhibitor of adipogenesis in vivo by feeding RA-supplemented rations to growing broilers and measuring the affect upon carcass adiposity and growth performance.

This study will help elucidate the nutritional regulation of adipogenesis in vitro. Using primary cultures of pig preadipocytes, we have identified a novel transcription factor, COUP-TF, which appears to represent a negative regulatory protein common to the anti-adipogenic actions of both RA and CLAs. This research could lead to a novel use of vitamin A that the animal industry could adopt immediately. Furthermore, this combined work proposed here should bring us closer to developing a genetic approach that could be utilized to limit fat accretion in livestock.

CHAPTER 2
LITERATURE REVIEW

Overview of Fat Metabolism.

Excessive fat accretion in livestock is undesirable from a production standpoint. Since fat is an undesired commodity and feed represents a considerable productive cost, adipose tissue accretion represents an energy sink which decreases the producer's return on investment.

Fat accretion in vertebrates is the synergistic result of the processes of hyperplasia (increase in adipocyte number) and hypertrophy (increase in adipocyte size) and is thus regulated by a complex interaction of genetic, endocrine, and nutritional factors. Hyperplasia functions as a major factor determining the propensity of the adult animal to fatten by determining the total number of fat cells. While it is currently thought that fat cell number can increase throughout the lifespan of most domestic animals-especially when the animal is in a positive energy balance- the extent to which the adult animal fattens is ultimately dictated by the hypertrophy of existing fat cells (associated with energy being consumed in excess of the animal's requirements for growth and maintenance) during the "fattening" stage of growth.

Adipose tissue functions primarily as an energy reserve. Excess energy consumed by the animal is stored in the form of triacylglyceride (TAG) in the mature adipocyte. Thus the extent of hypertrophy in adipose tissue is largely a function of the relative rates of TAG synthesis and degradation. This equilibrium is in turn controlled

by the relative contributions of de novo lipogenesis, lipolysis, absorption of dietary fat and fatty acid oxidation (Allen et al., 1976).

Adipocyte differentiation gives rise to adipose tissue and provides the animal with the necessary metabolic machinery for fat metabolism and consequently, fat accretion. Thus one plausible strategy to limit fat accretion would be to inhibit adipocyte differentiation thus limiting adipose tissue formation. Any decrease in adipose tissue mass would ultimately be expected to reduce the potential of an animal to fatten. Thus an understanding of the basic mechanisms which regulate adipose tissue development may lead to exciting and novel technologies that prove useful in preventing the excessive accretion of fat in livestock species.

Development of Porcine Adipose Tissue

Fat tissue contains primarily lipid, water, and collagen and is found ubiquitously throughout the animal carcass to varying degrees but is generally concentrated in predictable anatomical locations of the body termed depots. In pigs, the major depots of the carcass are subcutaneous (under the skin and overlying superficial muscles; i.e. backfat), intermuscular (between muscles; i.e. seam fat), and intramuscular (within muscles; i.e. marbling) (Wood, 1990). At market weight, the subcutaneous depot represents 75% of the total extractable lipid in the pork carcass and contains roughly 45% of the adipose cells found in the porcine body (Lee et al., 1973 a, b). Moody and Zobrisky (1966) first characterized three distinct layers within backfat and these individual layers are measurable both in the live animal (via real-time ultrasound) and the carcass. Furthermore, each layer is metabolically distinct (Allen et al., 1976).

Backfat layer thickness is typically measured as the ¾ fat depth (3/4 distance across the loin from the midline) at the 10^{th}-11^{th} rib interface. Back fat and intramuscular fat represent the two most economically important depots in the pork carcass since backfat is the largest depot but is also undesirable and often trimmed and discarded while marbling is considered a primary determinant of pork quality by consumers at the point of sale.

Adipose tissue develops in a unique pattern as the pig grows and approaches maturity. Although pigs represent a relatively low fetal load to the dam, newborn pigs are born with extremely low lipid stores (Reed et al., 1993). At birth, the amount of intramuscular fat is greater than the subcutaneous depot and represents greater than 50% of the extractable lipid of the carcass (Kauffman et al., 1986). However, piglets ingest a high fat diet during while suckling and there is a resultant rapid increase in fat stores during the immediate postnatal period of growth. This slows temporarily in association with weaning and accelerates once again as the pig approaches sexual maturity (Reeds et al., 1993). This latter acceleration occurs presumably because the animal reaches its potential for lean gain and the slower rate of muscle growth allows dietary energy to support the growth of adipose tissue.

At market weight, the majority of carcass fat is contained in the subcutaneous depot but as the pig approaches sexual maturity, the rate of intramuscular fat deposition surpasses that of the subcutaneous depot (Carr et al., 1978; Gu et al., 1992). At birth all adipocytes are multilocular (contain multiple fat droplets) but by as early as day 3 postpartum, many have become unilocular (containing one central lipid

droplet). Marked increase in adipocytes size is accompanied by an increase in the size of the central droplet with age (Mersmann et al., 1975). It is important to note that small cells are observed at all ages yielding biphasic cell size distributions. Fortin (1986) monitored the development of backfat in pigs from 14.5-137 kg live weight using a serial slaughter procedure. It was observed that the middle subcutaneous layer developed more rapidly than the outer layer with the rate of development of the inner subcutaneous fat layer being intermediate. Compared to subcutaneous fat, adipocytes of the latter depots are physiologically less mature and exhibit a delayed growth pattern characteristic of late maturing tissues (Lee and Kauffman, 1974). These authors concluded that intramuscular fat behaves differently from subcutaneous fat both in terms of cellularity and metabolic activity. The depot sequence of adipose tissue development in pigs occurs as follows: subcutaneous fat matures earliest (middle>inner>outer layer) followed by intermuscular fat and finally intramuscular fat develops the latest (Allen et al., 1976).

The swine industry is faced with the problem of adopting a strategy to limit subcutaneous fat without negatively affecting the amount of marbling that is present. Currently it is not understood how the temporal development of adipose tissue is regulated in growing animals but differences in the development of fat depots suggests that the growth of adipose tissue is regulated in a depot specific manner. Interestingly, a number of studies have reported moderate to low phenotypic and genotypic correlations between percent intramuscular fat and subcutaneous fat depth (Wood et al., 1986; Cameron, 1990; Lo et al., 1992). The lack of a correlation between these

traits suggests that selection to increase intramuscular fat without increasing subcutaneous fat may be possible. Thus, it is possible that technologies may be developed which could effectively target adipose tissue development in a depot specific manner allowing producers to finely manage the carcass composition of their pigs.

It is possible that limiting adipocyte differentiation in growing animals could significantly limit adipose tissue accretion. In the pig, adipocytes hyperplasia appears to contribute to adipose tissue growth up until the fifth month postpartum. This period may be extended in pigs with lower genetic potential for lean gain. When compared to other depots, adipocyte hyperplasia in intramuscular fat occurs latter in the growth period and adipocyte number in this depot appears to contribute greater to overall depot size while adipocyte volume is the primary contributor to size in other fat depots (Allen et al., 1976). This suggests that therapies aimed at inhibiting adipogenesis might be administered during early stages of growth to maximally limit subcutaneous fat without effecting marbling.

Adipocyte Differentiation

Insulin stimulates lipogenesis and fat storage during periods of caloric excess that are characteristic of production scenarios in the pork and poultry industries (Walton and Etherton, 1986, Dunshea et al., 1992c). Adipose tissue becomes sensitive to insulin and acquires the necessary proteins for energy metabolism as fibroblast-like adipocyte precursor cells (preadipocytes) terminally differentiate into adipocytes. This conversion is induced by extracellular hormones, growth factors, and nutrients

which presumably bind to receptors present in preadipocytes and trigger various signaling pathways. These events result in the activation of specific trans-acting factors that initiate the cell differentiation program. Consequently elucidating the processes of adipocyte differentiation will provide a basis for understanding how hormones and metabolites influence fat accretion in growing animals as these signals are apparently integrated through a common adipogenic transcription control pathway (Gregoire et al., 1998).

Differentiation Program

Adipocytes are derived from fibroblast-like precursor cells whose differentiation is characterized by a timed cascade of transcriptional events. This process has been well studied using 3T3-L1 preadipocyte cell culture models and has been the subject of several excellent reviews (Cognet and Lane, 1993, Sul et al., 1993, Cornelius et al., 1994, Klaus, 1997, Loftus and Lane, 1997, Gregoire et al., 1998).

The differentiation process is summarized in figure 1. Mature adipocytes are derived from stem cells that can give rise to three mesodermal cell types (adipoblasts, chondroblasts, and myoblasts). These ultimately differentiate into adipose tissue, bone and muscle. MyoD has been implicated as the principal protein that directs multipotent cells to commit to the muscle lineage (Muscat et al., 1995). It is very likely that an analogous as yet uncharacterized protein directs commitment of multipotent cells to the adipocyte lineage.

During adipocyte differentiation, stem cells undergo determination to unipotential adipoblasts in response to specific DNA methylation events. Adipoblasts

then become committed to the adipocyte lineage and form preadipocytes. Commitment corresponds with a brief period of growth arrest, and the appearance of early adipogenic markers (i.e. β-adrenergic receptors). Adipoblasts can form aggregates *in vivo* that release growth factors such as FGF and PDGF which stimulate blood vessel development in neighboring regions (Ailhaud et al., 1992). This leads to increased adipocyte cluster size as increased blood flow facilitates greater exposure to circulating nutrients and hormones.

Preadipocytes proliferate in culture until confluence signals the arrest of growth due to contact inhibition. Exposure to mitogenic agents such as growth hormone, glucocorticoids and IGF-1 allow the arrested cells to overcome contact inhibition. This stimulates a brief period of clonal expansion consisting of several rounds of mitosis. Presumably, DNA replication and the accompanying changes in chromatin structure increase the accessibility of cis-DNA elements to the trans-acting factors which activate the transcription of genes important for the expression of the adipocyte phenotype. These events lead to the coordinate transcriptional activation of a large group of genes that confer the adipocyte phenotype and increased insulin sensitivity as illustrated by the expression of such proteins as the fatty acid binding protein (FABP), lipoprotein lipase (LPL), glucose transporter 4 (GLUT4), the insulin receptor, lipogenic enzymes (i.e. ACC and FAS) and leptin. These events result in increased lipid filling and the preadipocyte terminally differentiates into a mature adipocyte.

Transacting Factors Involved in Differentiation

The differentiation of adipoblasts into mature adipocytes results from the coordinate regulation of proteins caused by corresponding changes in gene expression. Of particular interest have been trans-acting proteins that control the promoters of genes whose expression is altered during adipocyte differentiation. The characterization of transcription factor binding sites has led to the identification of the CCAAT/ enhancer binding protein (C/EBP) and peroxisome proliferator-activated receptor (PPAR) protein families. The members of these families comprise a control pathway that directs the timed cascade of transcriptional events governing the adipocyte differentiation program.

C/EBP Proteins.

C/EBP-α is a member of the CCAAT/ enhancer binding protein family of basic leucine zipper transcription factors which includes the isoforms C/EBP-β, -δ ,-γ,-ε and -ζ (CHOP) (Cornelius et al.,1994). The CEBP family of proteins regulate the expression of genes involved in a diverse array of processes including the acute-phase response, glucose homeostasis, cytokine action and adipocyte differentiation. CEBP proteins are highly modular, sequence-specific DNA binding proteins which display a preference for binding to CCAAT boxes in promoter regions of target genes (Johnson et al., 1987) and which possess separate domains for activation, DNA-binding, and dimerization (Hanson, 1998). The dimerization domain ("leucine zipper") by which CEBP proteins form homo- and heterodimers with other family members is both highly conserved and required for DNA binding (Landschulz et al., 1989). However

the activation domain is much less conserved thus as a result C/EBP proteins range from very strong transcriptional activators (i.e. C/EBP-α) to dominant negative repressors (i.e. C/EBP-ζ). Though not adipose-specific, C/EBP-α is only expressed in tissues that have a high lipogenic capacity including adipose tissue, liver, intestine and lung (Birkenmieir et al., 1989). Consistent with this, C/EBP-α orchestrates the coordinate transcriptional activation of several adipose-specific genes including ACC, GLUT4, the insulin receptor, leptin, aP2, and stearol CoA desaturase 1 (SCD-1) (Tea et al., 1994, Kaestner, 1990, McKeon and Pham, 1991, Cognet and Lane, 1993, Delabrousse et al., 1996, Hwang et al., 1996).

Evidence of function. Several lines of evidence indicate C/EBP proteins are involved in the regulation of adipocyte differentiation. First the expression of C/EBP-α is dramatically induced as clonal expansion ceases just prior to the coordinate expression of a group of genes during differentiation of 3T3-L1 preadipocytes (Cognet and Lane, 1993). Binding sites for C/EBP exist within the promoters of several differentiation-induced genes such as aP2 and GLUT4, and the mutagenesis of these binding sites blocks the transactivation of these genes (Christy et al., 1989, Kaestner et al., 1990, Cheneval et al., 1991). Further, the overexpression of C/EBP-α can induce adipocyte differentiation in fibroblasts independent of hormonal stimuli (Freytag et al., 1994, Lin and Lane, 1994). Conversely, Lin and Lane (1992) have used C/EBP antisense mRNA to block terminal differentiation of preadipocytes *in vitro*. In this study, the expression of C/EBP-α antisense mRNA blocked the transcription of aP2, GLUT4, and SCD-1 and the resulting 3T3-L1 cells failed to accumulate triacylglyceride. Wang et al (1995)

extended these findings to investigate the role of C/EBP *in vivo* using a C/EBP knockout mouse model. The knockout mice in this study failed to accumulate fat. Taken together, these data indicate that C/EBP-α plays a vital role in the coordinate transcriptional activation of several adipocyte-specific genes during differentiation and thus plays an important role in the induction of the adipocyte phenotype.

Regulation of C/EBP-α gene expression. The C/EBP-α promoter itself contains several potential binding sites for transcription factors including USF, NF-1, Sp1, CUP (AP2α), and the C/EBP proteins (Christy et al., 1991, Legraverend et al., 1993,Cognet and Lane, 1993). Several of these transacting factors have been implicated in the positive or negative regulation of C/EBP-α gene expression.

The C/EBP proteins can bind their own promoters leading to autoactivation, consequently, the expression of C/EBP-α is potentially regulated in differentiated cells by itself. This model is supported by the demonstration that C/EBP-α can transactivate its own promoter and expression of C/EBP-α antisense mRNA can block the transcription of C/EBP-α in 3T3-L1 cells (Lin and Lane, 1992, Lin et al., 1993). Timchenko et al. (1995) also demonstrated C/EBP-α promotes the binding of USF to an ebox in the C/EBP-α promoter. These data suggest that C/EBP-α can regulate its own expression through autoactivation as well as indirect mechanisms.

Another trans-acting factor, Sp1, has a negative effect on C/EBP-α expression through its ability to block C/EBP protein binding to the C/EBP-α promoter (Cognet and Lane, 1993a). Footprinting data has located an Sp1 binding site adjacent to the consensus C/EBP site in the C/EBP-α promoter (Christy et al., 1991). Sp1 is

constitutively expressed in preadipocytes. However cAMP stimulates the phosphorylation of Sp1 which leads to Sp1 dissociating from the promoter and becoming rapidly proteolysed (Cornelius et al., 1994). If the Sp1 site functionality is abolished through targeted mutation, C/EBP proteins can transactivate C/EBP-α expression without cAMP stimulation of adipocytes (Cognet and Lane, 1993b). Thus, it appears that Sp1 represses C/EBP-α gene transcription by competing with C/EBP binding to the C/EBP-α promoter and this repression can be overcome with transient increases in cAMP.

Several lines of evidence indicate that another trans-acting factor, CUP (C/EBP- undifferentiated protein), also represses C/EBP-α gene expression. CUP is only expressed in preadipocytes and its down regulation coincides with the expression of C/EBP-α during the later stages of differentiation (Cornelius et al., 1994). DNase I footprinting analysis of the 5' flanking region of the mouse C/EBP-α gene has revealed two CUP binding sites within the C/EBP-α promoter (Cognet and Lane, 1993a). Mutation of these sites leads to the upregulation of C/EBP-α expression. Conversely, overexpression of CUP in 3T3-L1 cells inhibits C/EBP-CAT expression (Cognet and Lane, 1993b). Thus CUP appears to play an important role in the timing of differentiation-related events by repressing C/EBP-α gene expression until CUP expression itself ceases.

Translational Regulation of C/EBP homologs. It has recently become apparent that the use of alternative translational start sites represents another mode of regulation within the C/EBP protein family. Three variants of 42, 40 and 30 kDa in size are

known to exist for the C/EBP-α isoform with the products of the first and third in frame AUG sites being most abundant (Lin et al., 1993). The 42 kDa variant of C/EBP-α has three transactivation domains that mediate the cooperative binding of C/EBP-α to TBP (TATA box-binding protein) and TFIIB which are two components of the RNA polymerase basal transcription apparatus (Nervo and Ziff, 1994, Nirvo and Ziff, 1995). The p30 variant retains dimerization and DNA-binding domains but has an altered transactivation potential compared to the p42 variant. Thus while both p42CEBPα and p30C/EBPα transactivate C/EBP-α responsive promoters, the p42 form is a more potent transcription factor (Hendricks and Darlington, 1995). Further suggesting the importance of alternative translation as a regulatory mechanism, the ratio of the p42 to p30 variants changes over the course of adipocyte differentiation (Lin et al., 1993, Bachmier and Loffler, 1997).

At least two variants of C/EBP-β are translated from different in frame AUG sites as well. The full length 32 kDaCEBPβ (LAP) encodes the activation, dimerization and DNA-binding domains characteristic of the modular C/EBP protein family (Williams et al., 1995). However a truncated C/EBP-β protein (LIP) is translated from the third in frame AUG site and possesses only DNA binding and a dimerization domains (Matsuno et al., 1996). Consequently LIP acts as a dominant negative inhibitor of transcription when forming heterodimers with LAP LIP does not have an effective activation domain (Descombes and Schebler, 1997).

Post-translational Regulation of C/EBP homologs. While it is evident that C/EBP-α is essential for preadipocyte differentiation, the importance of C/EBP-α in mature

adipocytes is still largely uninvestigated. However recent evidence suggests that in fully differentiated adipocytes, the post-translational regulation of C/EBP activity may be an important mechanism for mediating the effects of hormones upon gene expression. C/EBP-α is phosphorylated on at least six sites in fully differentiated adipocytes (Hemati et al., 1997). At least two of these sites have been implicated as important targets for insulin. MacDougald et al. (1994) demonstrated that insulin stimulated the rapid dephosphorylation of C/EBP-α and repressed expression of the C/EBP-α gene. The authors proposed the downregulation of GLUT4 gene expression in response to insulin may be due to the corresponding downregulation and dephosphorylation of C/EBP-α. In a subsequent experiment Hemati et al. (1997) observed that when insulin-induced dephosphorylation of C/EBP-α was blocked by Wortmanin (a PI-3 kinase inhibitor), the insulin-induced decline in GLUT4 mRNA was greatly reduced. However blocking the insulin-induced repression of C/EBP-α gene expression with a MAP kinase inhibitor failed to attenuate the ability of insulin to repress GLUT4 expression. These data suggest post-translational regulation may be an important regulatory mechanism of C/EBP proteins in differentiated adipocytes. Further, the apparent involvement of C/EBP-α in the regulation of GLUT4 gene expression by insulin in terminally differentiated adipocytes suggests C/EBP-α may play an important role in the hormonal control of gene expression in the adipose tissue of mature animals.

Regulation of C/EBP homologs by ST. Using 3T3-F442A preadipocytes as a model of ST-induced differentiation, Clarkson et al. (1995) has demonstrated differential

regulation of C/EBP-β and δ by ST. These investigators determined that ST induced translation of C/EBP-β and the transcription of the C/EBP-δ gene. The effects of ST were concomitant with increased c-fos and c-jun expression and could be blocked by inhibitors of Janus kinase 2 and protein kinase C. These data suggest that ST-induced phosphorylation stimulates increased C/EBP-β and -δ levels which together may initiate adipocyte differentiation (Clarkson et al 1995).

However recent work using hypophysectomized porcine fetuses as a model to study adipocyte differentiation suggests ST modulates lipogenesis rather than hyperplasia in the fetus. It is clear that hypophysectomy results in increased lipogenesis and decreased hyperplasia in fetal adipose tissue (Ramsay et al., 1987, Hausman et al., 1993). Smith et al. (1988) determined that IGF-1 rather than insulin (which interacts with the IGF-1 receptor at high concentrations) is the factor required for the induction of adipocyte differentiation. Consistent with a decrease in hyperplasia of adipocytes, serum IGF-1 is decreased in the fetus by hypophysectomy (Hausman and Wright, 1996). However ST treatment does not influence IGF-1 levels in these animals while ST does abolish the hypophysectomy-induced stimulation of lipogenesis (Hausman et al., 1998). These data contradict a role for ST in the adipocyte differentiation of the fetus.

PPARγ Proteins.

PPAR-γ (peroxisome proliferator-activated receptor-gamma) is a member of a nuclear hormone receptor family that was originally identified based upon the activation of its members by agents that stimulate peroxisomal β-oxidation of fatty

acids. Originally identified as a component of ARF6 (adipocyte regulatory factor), PPAR-γ is a ligand-activated transcription factor that is expressed in a primarily adipose-specific, differentiation-dependent fashion (Tontonoz et al., 1994a). Activated PPARs form heterodimers with the retinoic X receptor (RXR) and then bind response elements (DR-1) in target genes consisting of a direct repeat of PuGGTCA spaced by one nucleotide (Schoonjans et al., 1996). Such activation promotes adipocyte differentiation in several cell lines (Chawla et al., 1994, Brun et al., 1996).

Alternate promoter usage. The PPAR-γ gene encodes two proteins, PPARγ1 and PPARγ2, that differ in their amino termini and result from alternative promoter usage (Zhu et al., 1995). PPARγ1 is expressed ubiquitously in low levels while PPARγ2 is expressed abundantly in an adipose-specific manner (Braissant et al., 1996). Further, PPARγ2 expression follows a differentiation-induced pattern with its expression being dramatically upregulated preceding the induction of C/EBP-α and a host of other adipose-specific genes (Tontonoz et al., 1994a). The expression pattern of PPAR-γ2 is consistent with studies in which the expression of PPARγ2 in fibroblastic cell lines led to stimulation of adipocyte differentiation (Tontonoz et al., 1994b, Hu et al., 1995).

Ligand-dependent regulation. Ligand binding activates receptors in the PPAR family leading to increased DNA binding by the activated receptor and the subsequent induction of transcription of the target gene. Ligands for PPAR-γ have recently been discovered. Several members of a new class of drugs which can increase insulin sensitivity, the thiazolidinediones, bind PPARγ directly and have been shown to stimulate adipocyte differentiation (Kletzien et al.,1992, Sandock et al., 1993,

Ibrahimi et al., 1994, Forman et al., 1995, Lehmann et al., 1995). Further, derivatives of prostaglandins D2 and J2 are natural ligands and strong activators of PPARγ with 15-d-PGJ2 being the most potent activator characterized to date (Kliewer et al., 1994, Forman et al., 1995, Kliewer et al., 1995). However, it is currently unclear if 15-d-PGJ2 acts as an endogenous ligand. Further, a number of fatty acids and their metabolites are known to activate PPARs but have not been demonstrated to be true ligands (Loftus and Lane, 1997). Certainly the possibility exists that PPAR-γ2 could mediate the effects of a wide range of fatty acids and their derivatives in the adipocyte as the search for endogenous ligands for the PPAR proteins is in its infancy.

Ligand-independent regulation. Several non-ligand agents can also modulate the activity of PPARγ2 in cell culture. Retinoic acid (RA) represses PPAR binding to target DNA presumably by competing with PPAR for dimerization with RXR (Kawanda et al., 1996). However a recent study has shown that liganded RAR can block the ability of C/EBP-β to activate transcription which may explain the inhibitory effects of RA (Schwarz et al., 1997). Another transcription factor, COUP-TF (Chicken Ovalbumin Upstream Promoter-Transcription Factor) inhibits PPAR-γ action as well. COUP-TF, a member of the steroid/thyroid hormone receptor superfamily, inhibits the transcription of PPAR-regulated genes by binding to DR-1 sites in their promoters (Power et al., 1992). This prevents PPAR-γ binding to the site. PPAR action is also controlled at the level of transcription of the PPAR gene itself as several studies have shown that administration of high levels of TNF-α induce a rapid decrease in PPAR-γ protein and mRNA in cultured adipocytes and that fasting can

decrease PPARγ mRNA levels in both rodents and pigs (Zhang et al., 1996, Vidal-Plug et al., 1996, Xing et al., 1997, Houseknecht et al., 1998). Finally PPAR-γ action is also modulated by phosphorylation. Growth factors such as EGF and PDGF inhibit adipocyte differentiation. Several studies have associated this inhibition with decreased transcriptional activation of PPAR-γ and an increased phosphorylation of a consensus MAP kinase site on PPAR-γ. Further, the mutation of this site blocks the inhibitory effects of growth factors on PPAR-γ and differentiation (Hu et al., 1996, Zhang et al., 1996, Camp et al., 1997).

Model of transcription factor interaction during adipocyte differentiation

Figure 2.2 illustrates the current model of the control pathway that directs the transcriptional events governing the adipocyte differentiation program. In this model, the ST-induced expression of C/EBP-β above a threshold level early during differentiation induces expression of PPAR-γ. Activated PPAR-γ, cooperatively with induced C/EBP-α, transactivate a group of adipocyte-specific genes leading to terminal differentiation and the emergence of the adipocyte phenotype.

The model depicted in Figure 2.2 is widely supported throughout the literature. Several studies have demonstrated that C/EBP-β and -δ are maximally expressed during clonal expansion preceding the induction of PPAR-γ and C/EBP-α and their expression wanes during the terminal phase of differentiation (Cao et al., 1991, Yeh, 1995). Furthermore, the ectopic expression of a truncated C/EBP-α isoform prevents differentiation (Yeh, 1995). Consistent with these findings, the work of Wu et. al. (1995) clearly demonstrate that C/EBP-β induces PPAR-γ as the ectopic expression of

C/EBP-β in NIH-3T3 cells induced both PPAR-γ and adipogenesis. Further, the co-expression of both C/EBP-β and -δ induced PPAR-γ expression to a greater magnitude (Wu et al., 1996). Zhu et al has identified at least two consensus C/EBP binding sites within the promoter of the mouse PPAR-γ gene (Zhu et al., 1995). Thus these data strongly suggest the induction of C/EBP-β and -δ result in expression of PPAR-γ. Furthermore, C/EBP-α and PPAR-γ promote adipocyte differentiation in a synergistic fashion. While either overexpression of C/EBP-α or PPAR-γ is sufficient to stimulate differentiation, the co-expression of C/EBP-α and PPAR-γ promoted greater accumulation of triacylglycerol (Tontonoz et al., 1994). Consequently the expression of C/EBP-α likely plays a role with PPAR-γ2 in maintenance of differentiation.

There is very little data concerning the in vivo expression of transcription factors during the development of adipocytes. However the existing data suggest the pattern of expression observed in cell lines may not parallel the pattern of in vivo expression. For instance, Lee et al. (1998) examined the fetal expression of C/EBP proteins in pigs and observed that C/EBP-α was expressed throughout fetal development but found no evidence that C/EBP-β and -δ expression preceded the expression of C/EBP-α.

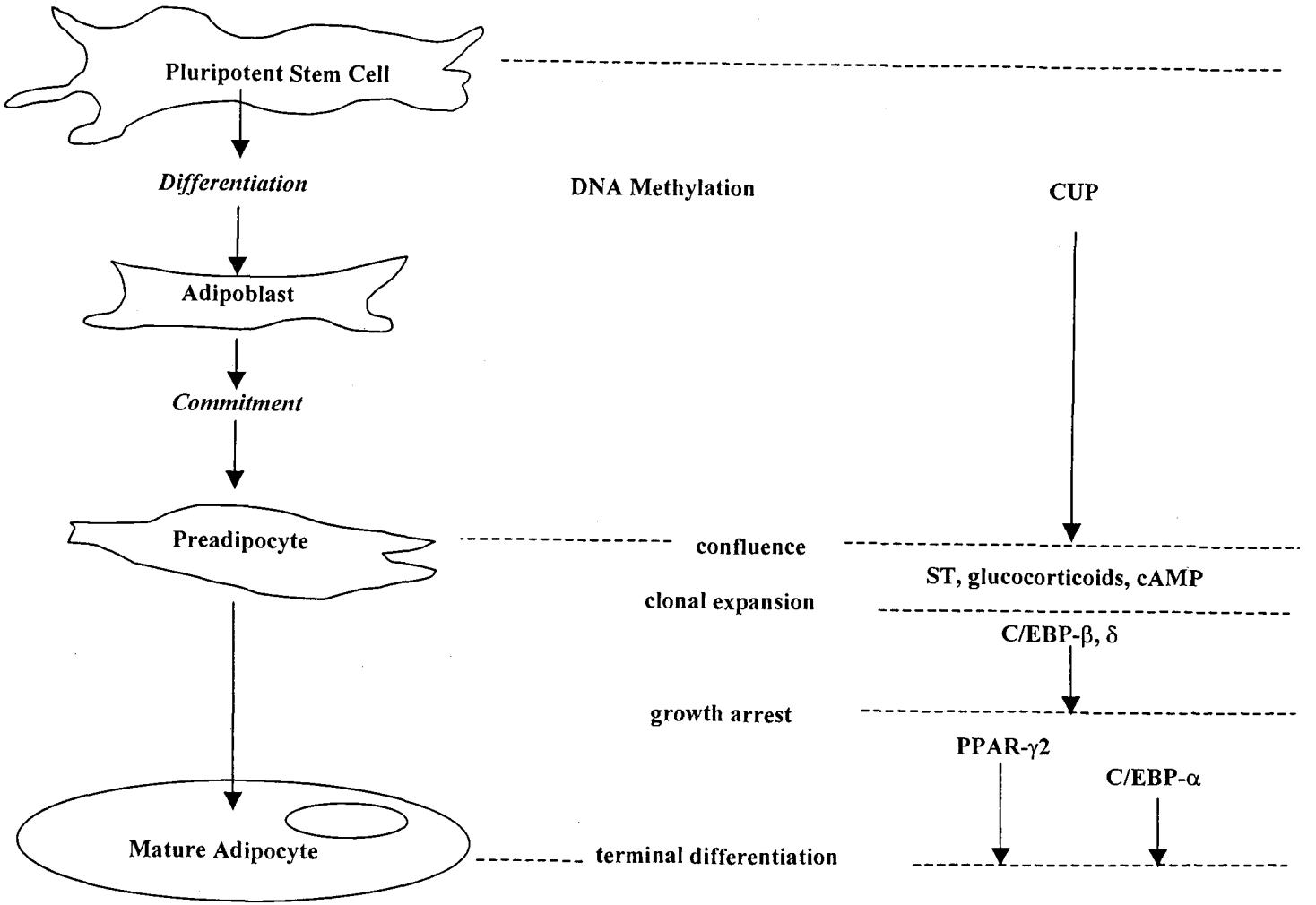


Figure 2.1. The Adipogenic Differentiation Program

Metabolism of Dietary Fats

The poultry industry has recently tended toward the formulation of high-energy rations for broilers that have necessitated rations that contain up to 10% dietary fat and which effectively inhibit lipogenesis in the liver. Conversely, the standard diet fed in the pork industry is rich in carbohydrate and relatively low in fat content, thus fat accretion in the pig is primarily achieved though *de novo* synthesis of fat from starch (Whittemore, 1992). However several classic studies confirm that in swine like in poultry, feeding a high-fat diet dramatically inhibits the *de novo* synthesis of fat (O'Hea et al., 1970, Allee et al., 1971 a,b,c). Under such conditions, adipose tissue generally reflects the fatty acid composition of the diet as monogastric animals can quantitatively transfer dietary fatty acids to fat depots (Allee, 1997). For instance several studies have demonstrated a direct relationship between dietary linoleic acid and carcass linoleic acid levels in growing pigs (Madsen et al., 1992). However it should be noted that considerable modification (i.e. desaturation and chain elongation) of ingested fats may occur. This illustrates the importance of understanding lipid metabolism to both better maximize lean growth and continue to provide a quality product to the consumer.

Digestibility of Dietary Fat Sources

It is generally agreed that fat included up to ten percent in a ration will not significantly decrease the efficiency of utilization in the pig or chicken (Whittemore, 1992). However the digestibility of fatty acids from different sources varies greatly thus altering the animal's ability to utilize them. Fat sources high in unsaturated fatty

acids are generally better utilized than sources high in saturated fats. (McDonald et al., 1995). Further, fat sources such as soya- or palm acid oils, which have greater than thirty- percent free fatty acids (FFA), tend to be poorly digested (NRC, 1998). Thus the ratio of saturated to unsaturated fat in the dietary source is important for both the dietary value of the foodstuff for the pig and the subsequent human value of the pork. Fatty acid chain length, the extent of unsaturation and the nature of esterification all influence the intestinal absorption of dietary fats in chickens as well (Moran, 1989). The majority of fat in the typical diet exists as neutral lipid in the form in TAG which must be hydrolyzed in the intestine before absorption.

Digestion and Deposition of Dietary Fat in Monogastrics

Fat digestion in monogastrics occurs primarily in the small intestine. The stomach contributes to the process by creating an emulsion through the churning action of smooth muscle contractions in the stomach wall (Berne and Levy, 1996). When the digesta enters the small intestine, bile is released further emulsifying the fat. Lipases released from the pancreas act on the emulsion to catalyze the hydrolysis of FFA from positions 1 and 3 of the TAG to yield 2-monoglycerols (Tso et al, 1985). Less than ten percent of the original Tag will remain unhydrolyzed. These are then gradually absorbed by enterocytes of the intestinal wall. In general, unsaturated fatty acids are absorbed more quickly than saturated fatty acids. Fatty acids that are less than 14 carbons in length subsequently enter the portal vein and are directly transported to the liver. Fatty acids greater than 14 carbons in length are reesterified

into TAG and subsequently enter the circulation via the lymphatic system as part of nascent chylomicrons.

Chylomicrons are a specialized type of lipoprotein that is essentially a lipid droplet enveloped by a protein-rich skin. Thus chylomicrons represent an important mechanism that functions to solubilize fatty acids, TAG, and cholesterol of dietary origin thus allowing these key components of dietary fat to be transported from the intestine through the lymph and into the blood supply where they are transported to peripheral tissues.

Uptake of Dietary Fatty Acids by Adipocytes.

TAG are delivered to adipose tissue by chylomicrons which carry fats of dietary origin or by VLDL which carry TAG that were synthesized de novo in the liver. Adipose tissue can then accumulate long chain fatty acids from the blood plasma through the action of LPL on either the above mentioned circulating lipoproteins or via albumin rich in free fatty acids (Murray et al., 1996). Initially synthesized in adipocytes, LPL migrates to the surface of capillary endothelial cells where it hydrolyzes fatty acids from the circulating pool of TAG (Nilsson-Ehle et al., 1976). The LPL mechanism is important for allowing fat storage during the feeding of high fat diets. However LPL likely plays a limited role in fat accretion in pigs fed the standard low fat diet (Mersmann, 1990).

Uptake and storage.

Once liberated by the action of LPL, free fatty acids diffuse across the plasma membrane into the cytosol of nearby adipocytes. Intracellular lipid binding proteins

bind the fatty acids so that the concentration of intracellular free fatty acids is essentially negligible (Bernlohr and Simpson, 1996). These free fatty acids can then be directly esterified into TAG. The long chain fatty acid tails in TAG associate into a large droplet that gradually fills the whole of the adipocyte thus crowding the nucleus into a narrow ring around the cell exterior (Salati and Goodridge, 1996). Developing adipocytes are identified because of the presence of several such lipid droplets in the cytosol. In the pig, only these multilocular adipocytes are present at birth (Anderson and Kaufmann, 1973). As the adipocyte matures and continues to fill with TAG, the lipid droplets coalesce into one large unilocular droplet characteristic of the hypertrophied adipocyte.

Regulation of hypertrophy.

Dietary fats are efficiently incorporated into TG droplets under lipogenic conditions and are rapidly mobilized during lipolytic states (Bernlohr and Simpson 1996). TAG synthesis is stimulated by insulin and induction of the LPL system while TAG degradation is achieved through the actions of the β-adrenergic pathway and hormone-sensitive lipase (HSL) (Mersmann, 1990). Thus the flux and magnitude of the FFA pool in adipocytes is constantly changing in response to hormonal, metabolic and genetic determinants. This has been starkly demonstrated in a recent study by Bernlohr et al. (1997). The targeted disruption of the aP2 gene, which codes for the lipid binding protein in adipocytes, resulted in the compensatory up-regulation of the keratinocyte lipid binding protein gene in adipose tissue and the expansion of the fatty acid pool size (Bernlohr et al 1997).

Regulation of Gene Expression by Dietary Fatty Acids

It has been demonstrated that feeding a high-fat diet results in a marked depression of the lipogenic capacity of the pig (O'Hea et al., 1970, Allee et al., 1971 a,b,c). These early studies by Allee et al. established that fatty acid synthesis and total activities of lipogenic enzymes were reduced in pig adipose tissue by dietary fat. Further this reduction was dependent on the amount of fat in the diet. Since the tissue concentration of lipogenic enzymes is a major determinant of the capacity of a tissue to synthesize fatty acids via the *de novo* pathway, dietary fats may suppress lipogenesis by down regulating lipogenic genes in porcine adipose tissue (Clarke, 1993).

Effects of Fatty Acids

Both *in vivo* and cell culture studies demonstrate that specific fatty acids can alter gene expression leading to changes in cell metabolism, and differentiation (Jump et al., 1997). Specifically, an increase in cellular fatty acids has been associated with increased peroxisomal and mitochondrial fatty acid oxidation, and decreases in glucose uptake, glycolysis, and lipogenesis (Malasanos et al., 1991, Pan et al., 1994). Studies in rat liver indicate that PUFA suppress lipogenesis by inhibiting transcription of genes for ACC, FAS and malic enzyme. These effects are very rapid, occurring within hours, and persist as long as the PUFA remain in the diet (Clarke and Jump, 1994).

Chemistry

Suppression of the lipogenic rate is dependent upon the type of fat ingested (Clarke, 1994). Sources rich in saturated fats such as tallow, lard, and olive oil have little effect on hepatic lipogenesis. Conversely sources rich in PUFA such as corn, safflower, or menhaden oil can effectively inhibit lipogenesis. Inhibition of lipogenic gene expression by PUFA requires a fatty acid with at least 18 carbons and double bonds located at the 9 and 12 positions and the fatty acid must apparently be a substrate for Δ6 desaturase activity (Clarke, 1994). Further, highly unsaturated fatty acids found in fish oils such as eicosapenaenoic (20:5,n-3) and docosahexaenoic (22-6, n-6) acids are particularly potent inhibitors of hepatic fatty acid synthesis (Harris, 1986).

Mechanism.

The molecular basis for PUFA-mediated inhibition has been examined using the S14 gene as a model which responds to PUFA control in a similar manner as fatty acid synthase (FAS) (Clarke et al., 1990, Jump et al., 1994). Since members of the peroxisome proliferator activated receptor (PPAR) family of transcription factors are regulated by fatty acids, they are potential mediators of PUFA action on gene expression (Jump et al., 1995). WY14,643, a strong peroxisome proliferator, suppresses the mRNA for both S14 and FAS (Jump et al., 1995). Deletion studies have mapped the site of PUFA action to a region in the promoter which binds factors that mediate the T3 transactivation of S14 gene transcription (Jump et al., 1997). However, deletion studies indicate that WY14,643 targets a different region of the S14

promoter than the PUFA site in the liver (Ren et al., 1996). This suggests that PPAR may mediate the effects of PUFA several ways. PPAR may act to sequester RXR thus interfering with thyroid hormone activation of transcription. PPAR may also mediate the effect of PUFA by competing with other transcription factors (Jump et al., 1997). Alternatively, PUFA may direct gene transcription via an as of yet uncharacterized trans-acting factor.

Conjugated Linoleic Acid

Conjugated linoleic acid is a mixture of fatty acid isomers that are produced by ruminants during fermentation. Thus, CLA naturally occurs in dairy and meat products derived from ruminants. The cis-9, trans-11 isomer is the predominant isomer present in foodstuffs while both the cis-9, trans-11 and trans-10, cis-12 isomers are present in significant quantities in commercial preparations of crude CLA mixtures. Emerging data has begun to indicate that various CLA isomers may have specific and differing biological activities. Brown et al. (2001) reported that the antiadipogenic action of CLA was entirely due to the trans-10, cis-12 isomer in cultures of 3T3-L1 preadipocytes. A recent report by Kang et al. (2003) further supports a role for trans-10, cis-12 isomer as the active anti-adipogenic isomer as these authors reported that trans-10, cis-12 CLA inhibited the differentiation of 3T3-L1 preadipocytes and significantly inhibited the expression of PPARγ and LPL mRNA.

The effect of CLAs upon preadipocyte differentiation has not been resolved. Several studies indicate that CLAs inhibits the differentiation of murine-derived 3T3-L1 preadipocytes using either GPDH or SCD activity, expression of the PPARγ,

CEBPα, aP2 or SCD genes, or TG content as indicators to measure preadipocyte conversion (Brodie et al., 1999; Choi et al., 2000; Evans et al., 2000). However using glucose incorporation into lipid as an indicator, Satory and Smith (1999) observed that CLAs promoted lipid filling in 3T3-L1 cells, which indirectly suggests that differentiation was not inhibited. There is a paucity of data that addresses the effect of CLA on differentiation of porcine preadipocytes. However, one study suggests that CLAs can increase the differentiation of porcine S-V cells. Ding et al. (2000) observed that treating S-V cells for 24 h with 50-300 μM cis-9, trans-11 resulted in increased ORO staining compared to linoleic acid. In that study, neither 50-300 μM of cis-9, trans-11 nor trans-10, cis-12 had an effect on the mRNA abundance of the PPARγ, CEBPα, or aP2 genes after 24 hrs treatment. It is not surprising that an effect of CLAs was not observed using ORO as a marker of differentiation in that study since little lipid accumulation is observed in primary cultures of S-V cells 24 hrs post-induction. Furthermore, 24 hours may not be sufficient to allow significant changes in the expression of key adipogenic transcription factors. Evans et al., (2001) failed to see a decrease in PPARγ protein levels following 2 days treatment of 3T3-L1 preadipocytes with either cis-9, trans-11 or trans-10, cis-12 CLA, but did report a significant decrease in PPARγ protein expression following 6 days treatment with trans-10, cis-12. In that study, cis-9, trans-11 CLA failed to change PPARγ protein levels even after 6 days.

Interestingly, there exists controversy concerning the effect of CLA and its isomers upon the expression of adipogenic transcription factors. Using 3T3-L1

preadipocytes, Brodie et al. (1999) observed that a crude CLA mix decreased GPDH activity concomitant with decreases in the mRNA abundances of C/EBP-α, PPARγ and aP2. In agreement with these results, Evans et al. (2001) reported that 50 μM trans-10, cis-12 CLA decreased protein levels for both PPARγ and aP2 in 3T3-L1 preadipocytes following 6 days of treatment suggesting that trans-10, cis-12 CLA is the active isomer. However, Choi et al. (2000) reported that a crude CLA mix decreased both PPARγ2 and aP2 mRNA abundance in a dose dependent fashion following six days of treatment but in their study, 10-100 μM trans-10, cis-12 CLA failed to decrease the mRNA abundances for either C/EBP-α or PPARγ. However most recently, Kang et al. (2003) reported that 100 μM trans-10, cis-12 CLA decreased both the mRNA and protein levels for aP2 and PPARγ in 3T3-L1 preadipocytes but, in their study, C/EBP-α and not C/EBP-β protein was decreased. Studies using adipose-tissue-derived S-V cells have likewise produced contradictory results. Ding et al. (2000) reported that cis-9, trans-11 CLA increased the differentiation of porcine S-V cells after 24 hrs treatment in a serum-free primary culture system but they did not observe an effect upon either PPARγ, C/EBP-α, or aP2 gene expression. More recently, McNeel and Mersmann (2003) used a serum-containing primary culture system to investigate the chronic effect of CLAs on porcine preadipocytes and reported that while a crude mix of CLA decreased PPARγ and aP2 mRNA versus control levels, neither the cis-9, trans-11 nor the trans-10, cis-12 CLA isomer individually effected the expression of these genes. However using S-V cells derived from human adipose tissue, Brown et al. (2003) recently reported that trans-

10, cis-12 CLA decreased PPARγ, CEBP-α, and aP2 mRNA abundance. Finally feeding trials have been more consistent. Kang and Pariza, (2001) fed .5% trans-10, cis-12 CLA to mature mice for four weeks and observed a 40% decrease in PPARγ mRNA versus controls though the decrease was not statistically significant. Takahashi et al. (2002) fed C57BL/6J mice 2% CLA for 21 days and reported these mice had decreased WAT and BAT which correlated with decreased PPARγ mRNA abundances in these adipose tissue depots. Thus while contradictory effects of CLA upon adipogenic transcription factors exists in the literature, taken together there is a consensus that CLA decreases the expression of PPARγ mRNA abundance.

Vitamin A and Retinoids

Vitamin A and its derivatives perform essential roles in a myriad of biological processes including vision, reproduction and the growth and differentiation of various tissues (Mangelsdorf et al., 1994). The importance of vitamin A as a chromophore vital to vision has been recognized for many years. However, research during the last decade has provided a wealth of information regarding the function of this essential vitamin relative to many other important biological processes in vertebrates.

The term "vitamin A" is a rather generic term which describes any compound that possesses the biological activity of retinol (Blomhoff, 1994). The term "retinoid" has evolved from a rigid, chemically defined definition in the 1970s to one which now includes any compound that is either a naturally occurring form of vitamin A or is any synthetic analog of retinol which may or may not even display biological activity (IUOAC-IUB, 1982). Clearly the definition of a retinoid has expanded as increasing

appreciation of the importance these compounds in the normal physiology of organisms have placed retinoid biology center stage in medical research.

Retinoic acid and possibly some of its metabolites (i.e. 9-cis-retinoic acid) are the active forms of vitamin A responsible for mediating the majority of nonvisual functions of retinoids (Blaner and Olson, 1994). The mechanism of action of vitamin A was largely a mystery until the discovery of nuclear receptors such as RAR-α that were specific for retinoic acid and its metabolites (Petkovich et al. 1987; Giguere et al., 1987). These important advances in the understanding of retinoid action led to the suggestion that the definition of retinoid should be further modified to reflect these new insights into retinoid action. Now retinoids include any substance that elicits a biological response by binding to and activating a specific retinoic acid receptor with the program for the biological response of the target cell residing in the retinoid receptor rather than in the retinoid ligand itself (Sporn and Roberts, 1994). Currently there are six well-defined retinoid receptors (RAR-α, -β, and -γ as well as RXR-α, -β, and -γ). All-trans-retinoic acid or its metabolite, 9-cis-retinoic acid, bind to and activate retinoic acid receptors. The activated receptors then bind to specific retinoic acid-response elements (RAREs) on DNA and subsequently direct the transcription of target genes. Interestingly, RXR can form heterodimers with a variety of hormone and orphan receptors suggesting that vitamin A might influence more processes than is currently appreciated.

It is now clear that retinoic acid mainly acts through regulating gene expression in a process mediated by intracellular receptors. Thus it is of vital

importance that retinoic acid be present in the target cell. It is clear that retinoic acid activity in a certain tissue is essentially regulated by controlling its availability. One or all of the following mechanisms can control the activity of retinoic acid in a given tissue: 1. Availability and delivery of dietary vitamin A to target tissues, 2. Tight control of retinoic acid synthesis and catabolism, 3. Control of the balance between the all- trans and 9-cis isomers of retinoic acid, 4. Presence and regulation of retinoic acid receptors. Thus knowledge of how retinoic acid is absorbed, metabolized, and distributed among tissues and how its levels are regulated in cells is essential to understanding retinoic acid action. The following review will discuss the various systems that contribute to the overall control of retinoic acid in a specific tissue with specific consideration of adipose tissue.

Dietary Source and Chemical Properties

Vitamin A is a nearly colorless, fat-soluble, long-chain, unsaturated alcohol consisting of isoprene units with alternating double bonds on a side chain that is conjugated to a β-ionone ring (vitamin chapter 2). Retinol is the alcohol form of the vitamin. Replacement of the alcohol goup by an acid yields retinoic acid. The replacement of the alcohol group by an aldehyde results in retinal. The principal form of vitamin A in animal products is the storage form, retinyl palmitate (long-chain fatty acid esters of retinol).

Vitamin A itself does not occur in plants. However, carotenoid pigments, the most active of which is beta-carotene, are abundant in green and yellow vegetables and fruits. The carotenoids are readily converted to retinol in the intestine of

vertebrates and are hence collectively termed provitamin A. Further, vitamin A can also be obtained by consuming foods of animal origin that are rich in retinyl palmitate (i.e. eggs, whole milk, meat products). Vitamin A requirements are commonly stated in units because different forms of vitamin A have different biological activities (NRC, 1994). Requirements for vitamin A are most commonly expressed in International units (IU) with an IU being defined as the biological activity of .3 μg of vitamin A alcohol (retinol), .344 μg vitamin A acetate, or .55 μg vitamin A palmitate (NRC, 1994). Furthermore, 1 IU of vitamin A activity is equal to .6 μg of beta-carotene (NRC, 1994).

The chemical properties of vitamin A dictate that special consideration should be taken when planning experiments. Retinol and its derivatives are hydrophobic compounds that are highly unstable in the presence of oxygen and yield a mixture of dehydrated and double bonded rearrangement products in acids. Light catalyzes double-bond isomerization of most retinoids. These properties require retinoids to be handled experimentally in an inert atmosphere, avoiding contact with acids and under dim illumination.

Absorption of Vitamin A: First Critical Step in Controlling Availability

Vitamin A is fat soluble and provided that fat absorption is normal in the individual animal, oral doses of either vitamin A or carotenoids that do not greatly exceed the physiological requirement should be efficiently absorbed (AHFS, 2001). As discussed in the previous section on fat absorption, it is not surprising that the main site of absorption of vitamin A is the proximal jejunum of the small intestine since bile

salts, pancreatic lipase and dietary fat all contribute to the efficient absorption of fat-soluble compounds.

The primary dietary sources of vitamin A are carotenoid-rich fruits and vegetables and animal products rich in preformed retinyl esters. Briefly, pepsin in the stomach and proteolytic enzymes in the small intestine sequentially act on proteins in vitamin A-rich diets to release retinoids (Ong, 1993; Ross, 1993). Bile salts, released from the liver, subsequently emulsify the carotenoids and retinyl esters thus allowing pancreatic lipase and retinyl ester hydrolase to then efficiently digest the emulsions. Essentially all of the retinyl esters consumed in the diet are enzymatically converted to retinol in the intestinal lumen prior to absorption by enterocytes. Ringtrup and Ong (1992) identified a retinyl ester hydrolase activity in the brush-border membrane of the rat intestine that was potent enough to hydrolyze all of the retinyl ester estimated to be in the experimental diet. Conversely, carotenoids are primarily converted to vitamin A in the intestinal mucosa as enterocytes absorb the bulk of dietary carotenoid which then undergoes oxidative cleavage to retinal and subsequent reduction to retinol (Blaner and Olson, 1994).

Cellular retinoid binding protein type II (CRBP-II) is highly expressed in the rat intestinal mucosa and plays a central role in the uptake and processing of retinoids in the intestine (Quick and Ong, 1990; Ong et al., 1991). CRBP-II proteins in the enterocyte rapidly bind retinal and retinol. Once bound by CRBP-II, retinal is rapidly converted to retinol by the action of retinaldehyde reductase (Ong et al., 1991). Then another important enzyme, lecithin:retinol acyltransferase (LRAT), converts the

CRBP-bound retinol to retinyl esters which are then packaged into nascent chylomicrons (Quick and Ong, 1991). Thus exemplifying a universal theme in retinoid metabolism, binding proteins (CRBP-II) carry lipid-soluble retinol through the aqueous environment of the intestinal mucosa and then delivers the retinol to the proper enzymes thus assuring the proper metabolism of retinol during its absorption. The end result of the absorption process is that dietary retinoids and carotenoids are ulitmately absorbed as the free alchohol, retinol, and packaged into nascent chylomicrons. It is noteworthy however, that a small portion of retinol may be oxidized to retinal and then to retinoic acid which may be delivered to the portal system and then ultimately to the circulation bound to serum albumin (Wolf 1995). A large portion of the retinoic acid of dietary origin appears to be removed from circulation by peripheral tissues (Blaner and Olson, 1994).

Factors affecting the absorption of vitamin A.

In addition to the normal physiological processes of fat absorption, there are several factors which may affect the absorption of vitamin A. Carotenoids are absorbed with differing efficiencies apparently depending upon their chemical nature (Johnson et al., 1997; Erdman et al., 1998; Van den Berg, 1999). Stahl et al. (1995) concluded that carotenoids in the trans-form are more efficiently absorbed. Not surprisingly, dietary fat is important in the absorption of vitamin A and dietary antioxidants also seem to positively effect absorption (Takyi, 1999, vitamin chapter 2000). Importantly for the design of feed trials, the dietary level of vitamin A also apparently affects absorption. In this regards, Donoghue et al., (1983) reported that in

lambs, absorption decreased dramatically as the step-wise increase in dietary vitamin A approached levels predicted to be toxic.

Hepatic metabolism and storage of retinol

After secretion into the lymphatic system, nascent chylomicrons undergo extensive lipolysis to become chylomicron remnants which are largely removed from the circulation by the liver (Mahley and Hussain, 1991). Thus a majority of the body's retinoids are stored in the liver as retinyl-esters.

The liver is composed primarily of parenchymal cells. There are three other major types of non-parenchymal cells in the liver (kupffer cells, endothelial cells and stellate cells). Only parenchymal and stellate cells play a role in the hepatic storage and metabolism of retinoids (Blaner and Olson, 1994). Mounting biochemical evidence has established stellate cells as the major storage site for retinyl esters in the liver as it is clear that these cells are highly specialized for the metabolism of retinoids (Blaner et al., 1995). Their cytoplasm is abundantly filled with large lipid droplets and they are enriched with CRBP proteins as well as multiple enzymes, which hydrolyze and synthesize retinyl esters (Blaner and Olson, 1994). Further, the level of vitamin A in the diet has been shown to markedly influence both the size and number of lipid droplets in the cytoplasm of stellate cells (Goodman and Blaner, 1984). Additionally, both thin layer chromatography and HPLC have been used to determine that the lipid content of stellate cells is comprised of approximately 65% retinyl esters and 20% triglyceride (Hendricks et al., 1987; Yamada et al., 1987; Yumoto et al., 1988). In contrast, parenchymal cells have minimal amounts of retinoids in their cytoplasm.

Finally, Moriwaki et al., (1988) clearly demonstrated that dietary retinol but not triglyceride levels can affect the lipid composition of stellate cells in rats.

The uptake of retinoid by the liver is now well characterized. Parenchymal cells first take up retinyl esters from circulating chylomicron remnants and the retinyl esters are subsequently transferred to stellate cells for storage. The mechanism by which retinyl ester is transferred between parenchymal cells, stellate cells and the plasma is still unclear however, it is commonly accepted that retinyl ester must be hydrolyzed to retinol before its transfer from parenchymal to stellate cells (Blaner and Olson, 1994). Several studies have indicated that newly absorbed retinyl ester is rapidly hydrolyzed inside the parenchymal cell (Blomhoff et al., 1985; Blaner et al., 1987). Once hydrolyzed inside the parenchymal cell, the majority of retinol is transferred to stellate cells while the remainder of retinol can by directly secreted into the circulation bound to RBP. Parenchymal cells are the major site of synthesis and secretion of RBP in the body (Yamada et al., 1987). Thus while stellate cells function to store retinyl ester, one function of parenchymal cells is to regulate the storage of retinyl ester by controlling the absorption of dietary retinoid and the secretion of stored retinyl ester.

Uptake, storage and metabolism of retinol by extrahepatic tissues

Peripheral tissues play an important role in vitamin A homeostasis as many extrahepatic tissues can absorb and store retinol. Tissues such as adipose tissue, testes and bone marrow all contain significant amounts of vitamin A as well as the enzymatic machinery necessary to metabolize retinyl ester (Blaner and Olson, 1994).

Retinol is transported through the circulation bound to RBP and the bulk of circulating retinol is hepatic in origin. It is currently thought that the vitamin A status of the animal dictates the amount of retinol secreted by parenchymal cells as secretion of stored retinol increases during times of low vitamin A intake (Moriwaki et al., 1988). Thus poorly characterized feedback mechanisms exist to ensure the retinoid needs of peripheral tissues are met.

The uptake of retinol by peripheral tissues is thought to occur by receptor-mediated endocytosis initiated by the binding of RBP to specific cell-surface receptors (Gjoen et al., 1987). However evidence also exists for the spontaneous disassociation of retinol from RBP in the plasma and the rapid diffusion of the free retinol across the plasma membrane of target cells (Noy and Xu, 1990). Whatever the mode of uptake, once inside the cell, free retinol is rapidly bound by CRBP proteins. Noy and Blaner (1991) have demonstrated that cellular uptake is actually dictated by the intracellular content of apo-CRBP (CRBP which is free of retinol). In their model, retinol uptake would increase in response to anything that increased the amount of apo-CRBP content. Such stimuli could include increased synthesis of CRBP or either the formation of retinyl ester from retinol or the oxidation of retinol to retinoic acid (neither retinyl ester nor retinoic acid can bind CRBPs).

Adipose Tissue

Adipose tissue contributes significantly to total body retinol stores. Using HPLC, Tsutsumi et al., (1992) reported that retinoid levels in six different adipose tissue depots of chow fed rats were similar and averaged 20-nmol vitamin A per gram

of adipose tissue. It was determined that 66% of the retinoid in these depots was retinol and the remaining amount was retinyl ester. Tsutsumi further determined that the retinoid was stored only in mature adipocytes and was not quantitatively found in stromal-vascular cells. Based upon this study both rat adipocytes and parenchymal cells contained similar levels of vitamin A. This suggests that the adipocyte is likely an important storage site for retinoids.

Retinoic acid formation and metabolism

Since the discovery of nuclear receptors specific for retinoic acid, the processes that generate and maintain cellular levels of retinoic acid have come under greater scrutiny. It is now apparent that the control of retinoic acid content in a tissue is largely dependent upon the balance between the activity of retinoic acid synthesizing and retinoic acid catabolizing enzymes. The conversion of retinol to the active forms of retinoic acid can occur in most tissues although the exact mechanisms responsible are unknown (Blaner and Olson, 1994). It is currently thought that retinol is first oxidized to retinaldehyde which is then irreversibly oxidized to retinoic acid (Luu et al., 2001). Once formed, retinoic acid has one of two fates. It can either bind to intracellular retinoic acid receptors or it can be metabolized by the cytochrome P-450 system.

Though the uptake of retinol and its subsequent oxidation appears to be the major mechanism by which retinoic acid becomes available to tissues, the delivery of retinoic acid in the plasma represents another potentially important mechanism. As noted earlier, a small but significant amount of retinoic acid is ever present in the

circulation as it can be derived from both the diet and from the oxidation of retinol in the liver. Noy et al., (1992) determined that serum albumin has a distinct binding site for retinoic acid suggesting that plasma pools of retinoic acid have some physiological significance. Furthermore, retinoic acid can rapidly diffuse across membranes. Thus it is clear that albumin-delivered retinoic acid could easily enter a target cell.

Geison and Johnson (1970) demonstrated that retinoic acid appears in the circulation after radiolabeled retinoic acid was orally administered to rats. Furthermore, Smith et al., (1973) showed that in the rat, retinoic acid is absorbed in the intestine and roughly two-thirds of the absorbed dose is subsequently distributed as retinoic acid throughout the body. More recently several investigators have determined that circulating levels of all-trans-retinoic acid range between 4 to 14 nmol/L in humans and 7 to 9 nmol/L in rats (De Leedheer et al., 1982; Napoli et al., 1985; Eckhoff and Nau, 1990). These studies taken together suggest that circulating levels of retinoic acid may have significant physiological importance and that it may be possible to enrich tissues by administering exogenous retinoic acid to experimental animals.

While it is clear that the liver and adipose tissue represent significant sites for retinoid storage, it is equally clear that neither tissue stores retinoic acid for any appreciable length of time. The cytochrome P-450 system actively metabolizes retinoic acid. Furthermore, it is clear that oral administration of excessive doses of retinoids can induce the cytochrome P-450 system (Leo et al., 1984; Wouters et al., 1992; Luu et al., 2001).

Metabolites of all-trans-retinoic acid that have been measured in vivo include 13-cis-RA, 9-cis-RA, and retinoyl β-glucuronide (Tang and Russell, 1990; Barua et al., 1991; Eckhoff, et al., 1991; Heyman et al., 1992; Levin et al., 1992). The existence of 9-cis-RA was first reported in 1992 (Heyman et al., 1992; Levin et al., 1992). Mangelsdorf et al., (1992) quickly demonstrated that 9-cis-RA can activate RXR receptors forty-fold more potently than all-trans-retinoic acid could. The importance of such activation was demonstrated in the earlier discussion which detailed the regulation of adipocyte differentiation. Little is known about the formation and catabolism of the 9-cis and 13-cis isomers. Furthermore, very little is known about the biological activity of other retinoic acid metabolites such as retinoyl B-glucuronide.

CHAPTER 3
ISOMER-SPECIFIC REGULATION OF DIFFERENTIATNG PIG PREADIPOCYTES BY CONJUGATED LINOLEIC ACID

Abstract

Conjugated linoleic acids (CLAs) decrease body fat in growing animals by a poorly understood mechanism. The possibility that CLAs decrease adipocyte hyperplasia was investigated by examining the isomer-specific effect of CLAs on the proliferation and differentiation of pig preadipocytes in primary culture. Both a crude CLA preparation and the pure trans-10, cis-12 CLA isomer inhibited sn-glycerol-3-phosphate dehydrogenase (GPDH; EC 1.1.1.8) activity while cis-9, trans-11 CLA failed to decrease GPDH activity. This suggests that CLAs inhibit the differentiation of pig preadipocytes and the inhibitory effect is isomer-specific with trans-10, cis-12 CLA being an active isomer in crude CLA mixtures. Interestingly, increasing concentrations of cis-9, trans-11 CLA blunted the inhibitory effect of trans-10, cis-12 CLA on GPDH activity suggesting the presence of cis-9, trans-11 isomer in crude CLA preparations may limit the anti-adipogenic action of CLA. Next, the effect of CLAs on preadipocyte proliferation was determined using WST-1 cleavage as a marker for proliferation. Preadipocyte number was decreased only by 25 μmol/L trans-12, cis-10. Finally, we investigated the isomer-specific impact of CLAs on the expression of genes known to encode proteins that regulate adipogenesis. Trans-10, cis-12 CLA decreased expression of PPARγ and ADD1 mRNA and increased the expression of COUP-TF mRNA. The expression of COUP-TF antisense mRNA in 3T3-L1 preadipocytes blocked the ability of CLAs to inhibit adipogenesis.

Collectively, trans-10, cis-12 CLA inhibits the differentiation of pig preadipocytes by a mechanism that appears to involve both the down-regulation of PPARγ and ADD1 and the up-regulation of COUP-TF gene expression.

KEY WORDS: CLA, Adipogenesis, Pig, PPARγ

Introduction

Conjugated linoleic acids (CLAs) are naturally occurring isomers of linoleic acid that have been associated with antiatherogenic, antidiabetic and antitumorogenic action in mammals (reviewed by Brown and McIntosh, 2003; Pariza et al., 2000). Several growth trials have suggested that CLAs may also regulate body fat accumulation in growing pigs. Dugan et al. (1997) was the first to demonstrate that pigs fed CLAs deposit less fat and have improved feed efficiency. Subsequent studies have confirmed that feeding CLAs to growing pigs can limit fat deposition (Ostrowska et al., 1999; Bassaganya-Rieva et al., 2001; Thiel-Cooper et al., 2001; Wiegand et al., 2001; Ostrowska et al., 2003). Although the effect of CLAs was less dramatic in some studies (O'Quinn et al., 2000; Eggert et al., 2001; Ramsay et al., 2001; Weber et al., 2001), all growth trials to date have shown some positive effect either upon growth performance, pork quality, or pig health. Consequently, CLAs hold great promise as feed additives in swine rations.

While CLAs can limit adipose tissue accretion in growing pigs, the underlying mechanisms are poorly understood. Fat deposition in swine results from the additive

effects of hyperplasia (increase in fat cell number via the proliferation and differentiation of preadipocytes) and hypertrophy (increase in size of the mature fat cell). In vitro studies utilizing murine-derived 3T3-L1 preadipocytes suggest that CLAs may inhibit adipocyte hyperplasia through inhibitory actions on both preadipocyte proliferation and differentiation (Brodie et al., 1999; Choi et al., 2000; Evans et al, 2001; Kang et al, 2003). Satory and Smith (1999), however, observed that CLAs promoted de novo lipogenesis in 3T3-L1 adipocytes suggesting that CLAs do not inhibit preadipocyte differentiation. Furthermore, there is some disagreement as to whether CLAs decrease triglyceride accumulation in cultured preadipocytes by inhibiting differentiation or by preventing differentiated fat cells from filling with lipid (Choi et al., 2000; Evans et al., 2002).

Studies using primary cultures of preadipocytes have also yielded conflicting results. Using stromal-vascular (S-V) cells originating from human adipose tissue, Brown et al. (2003) reported that tran-10, cis-12 CLA inhibited the differentiation of human preadipocytes. However, McNeel and Mersmann (2003) reported that CLAs failed to inhibit either the proliferation or differentiation of pig preadipocytes. Ding et al (2000) suggested that CLAs actually stimulated the differentiation of pig preadipocytes. Given that a stimulatory effect of CLAs on adipogenesis seems at odds with the ability of CLAs to decrease carcass adiposity and the results of *in vitro* studies as a whole have been conflicting, the effect of CLAs on the hyperplasia of adipocytes in pigs remains controversial.

Better understanding the mechanism underlying the ability of CLAs to limit adiposity and determining which CLA isomers possess this activity may allow formulation of designer CLA mixtures which, when fed to growing animals, results in more efficient growth and a more ideal body composition. Our objective was to better characterize the isomer-specific effect of CLAs on the proliferation and differentiation of pig preadipocytes in primary culture.

Materials and Methods

Materials. Linoleic acid was purchased from Sigma-Aldrich (St. Louis, MO). The 95% CLA mixture (40% cis-9, trans-11 CLA; 44% trans-10, cis-12 CLA; 11% cis-10, cis-12 CLA) was obtained from NU-CHEK Prep Inc (Elysian, MN). The individual trans-10, cis-12 CLA and cis-9, trans-11 CLA isomers were purchased from Matreya, Inc (Pleasant Gap, PA). Dulbecco's modified Eagle's medium, nutrient mixture F-12, dexamethasome, dihydroxyacetone phosphate (DHAP), isobutylmethylxanthine, reduced nicotinamide adenine dinucleotide (NADH), gentamicin sulfate, HEPES buffer, hydrocortisone, insulin, transferrin were purchased from Sigma Chemical (St. Louis, MO). Collagenase (type I) was purchased from Worthington Biochemical (Freehold, NJ), fetal calf serum from Intergen (Purchase, NY), and fungizone from Gibco BRL, Division of Life Technologies (Gaithersburg, MD).

Animals and Primary Culture. 2-day-old crossbred pigs were obtained from a commercial producer and sacrificed by CO_2 asphyxiation in a manner approved by the Animal Care and Use Committee at Oregon State University. Stromal-vascular (S-V)

cells were harvested by a collagenase digestion procedure as previously described (Suryawan and Hu, 1997). Aliquots of S-V cells were counted using a hemacytometer and seeded in either 35-mm or 100-mm culture dishes at a density of 5 x 10^4 cells/cm^2 and incubated at 37° C in 5 % CO_2 in air. Plating medium consisted of DME/F12 (v 1:1) containing 15 mmol/L $NaHCO_3$, 15 mmol/L HEPES buffer and 50 mg/L gentamicin sulfate supplemented with 10% fetal calf serum (FCS). After 24 h, attached cells were washed three times using plating medium to remove unattached cells and cellular debris. After washing, cells were maintained in various test medium containing 10% FCS. Culture media was changed every three days until d 10 (except where stated otherwise) when cells were subjected to GPDH assays, oil red O staining, or gene expression analysis. By d 10 in culture medium greater than 70% of the cells accumulated multilocular lipid droplets.

Histochemistry. In order to qualitatively assess stromal-vascular cell differentiation by microscopy, cells were exposed to induction media from day 0 to d 8 then fixed in 10% formalin, stained with oil red O (ORO) for lipid and counterstained with Harris hematoxylin as described by Suryawan and Hu (1993). Briefly the wells were fixed with Baker's formalin for 15 minutes, rinsed with distilled water, equilibrated in 100% propylene glycol for 2 min, and then stained with ORO for 10 min. Wells were then treated with 60% propylene glycol for 1 min to remove free ORO, rinsed with distilled water, and counterstained for 6 minutes in Harris Hematoxylin. Finally cells were mounted in glycerin jelly.

Glycerol-3-phosphate dehydrogenase (GPDH) activity. The Sn-glycerol-3-phosphate dehydrogenase (GPDH; EC 1.1.1.8) activity was determined by spectrophotometrically measuring the disappearance of NADH during the GPDH-catalyzed reduction of dihydroxyacetone phosphate under zero-order conditions by the method of Kozak and Jensen (1974) as modified by Wise and Green (1979). GPDH activity is expressed as units per mg of protein where one unit of activity is defined as the oxidation of 1 nmol NADH/min. Protein was measured according to Bradford (1976).

Cell number assay. The colorimetric assay for quantitation of cell number and cell viability, based upon the cleavage of the tetrazolium salt WST-1 {4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate} by mitochondrial dehydrogenases, was performed according to the manufacturer (Boehringer Mannheim #1644 807). The WST-1 assay was validated for the primary stromal-vascular cell system by verifying that increased stromal-vascular cell plating density correlated with increased formazan formation.

RNA Isolation. Cells were harvested with a cell scraper and total RNA was extracted using the quanidinium-acid phenol method (Chomczynski and Sacchi, 1987). Total RNA concentration was determined spectrophotometrically using A_{260} and A_{280} measurements. The ratio of A_{260} / A_{280} was between 1.9 and 2.1 for all samples. Five micrograms of total RNA from each sample was separated on a 1.2% denaturing formaldehyde gel and stained with ethidium bromide. The RNA integrity was assessed visually by judging the quality of 18 and 28s rRNA bands.

Semiquantitative RT-PCR. Reverse transcription (RT) reactions (20 μL) consisted of 4 μg of total RNA, 50 U SuperScript II reverse transcriptase (Invitrogen/Life Technologies), 40 U of an RNAse inhibitor (Invitrogen/Life Technologies), 0.5 mmol/L d NTP, and 100 ng random hexamer primers. Polymerase chain reaction (PCR) was performed in 50 μL containing 20 mmol/L Tris-HCL, pH 8.4, 50 mmol/L KCL, 1.0 μL of RT reaction, 2.5 U of Platinum Taq DNA polymerase (Hot Start, Invitrogen/Life Technologies), 0.2 mmol/L dNTP, 2 mmol/L Mg^{2+} (Invitrogen/Life Technologies), 10 pmol each of gene specific primers and 10 pmol each of primers specific for either β-actin or 36B4. Thermal cycling parameters were as follows: 1 cycle 94°C for 4 min, followed by 26-30 cycles, 94°C for 1 min, 56°C for 2 min, 72 °C for 2 min with a final extension at 72°C for 8 min. Primers were synthesized at the Center for Gene Research at Oregon State University. Identity of PCR products was verified either by restriction digest analysis or via DNA sequencing. Cycle number for each multiplex PCR reaction was selected by experimentally determining the highest cycle number in which the amplification of both cDNA products was within a linear range. RT-PCR products were visualized by separating DNA on a 3% agarose gel and staining with CyberGreen according to the manufacturer's directions (Molecular BioProbes, Eugene, OR). The optimal cycle number was then considered to be two cycles lower than the highest cycle of linearity. Primer sequences, product size and cycle length are listed in Table 1. Data for each replicate represented the mean of three individual PCR reactions.

Northern Blot Analysis. In order to verify results of semiquantitative RT-PCR, twenty micrograms of total RNA was separated, blotted and probed for C/EBPβ and RXRα and normalized to 18S mRNA abundance as described by Brodie et al., (1999) in order to verify results of semiquantitative RT-PCR. The non-radioactive labeling of probes with digoxigenin-dUTP was performed using the PCR Dig Probe Synthesis Kit as described by the manufacturer (Roche Diagnostics, Indianapolis, IN). Blots were prehybridized and hybridized at 50 ° C for 16 hours and washed two times at 50° C with 1 X SSPE/ .1 % SDS for 50 minutes and one time with .1 X SSPE / .1 % SDS at 60° C for 30 minutes. Chemiluminescent detection using the CSPD alkaline phosphatase substrate was performed as recommended by the manufacturer using the Dig Detection kit II (Roche Diagnostic, Indianapolis, IN) followed by autoradiography.

Generation of pRevTRE-COUPTF1AS construct. A cDNA encoding the entire 423 amino acid coding sequence of human COUP-TF1 (10 bp of 5' untranslated region, 1269 bp of coding sequence and 207 bp of untranslated region) was generously provided by Dr. Ming-Jer Tsai, Baylor College of Medicine, Houston, TX. A smaller COUP-TF1 cDNA was generated (spanning base pairs 458-1755 and encompassing 20 base pairs upstream from the first ATG to 6 base pairs past the stop codon) and subcloned into the SmaI site of Bluescript II SK+. Sense and antisense constructs relative to the T7 promoter were obtained, cut with EcoRI and BamHI, and subcloned into pTRE also cut with EcoRI and BamHI. Insertion and orientation were determined

by restriction digest analysis. These two plasmids were designated pTRE-TF1AS and pTRE-TF1S.

Retroviral infections. Retroviruses were packaged by individually transfecting PT67 cells (BD Biosciences, San Jose, CA.) with either pRevTet-On (regulatory vector) or pRevTRE-COUPTF1AS (COUP antisense construct under control of tet-responsive element) and selecting for transfected cells with either G418 or hygromycin respectively. Supernatants were collected after 48 h and were either used immediately to infect 3T3-L1 preadipocytes or were frozen at -80° C for later use.

Stable transfections. The stable COUP-TF1 antisense cell line was generated using the Tet-On gene expression system (Clontech, Palo Alto, CA). First, 3T3-L1 cells were stably transfected with pUHD 17-2 neo (pTet-On), a plasmid encoding a transcriptional activator that is functional in the presence of doxycycline. Two days post transfection, cells were plated, and 24 hours later cells were selected for stable integration of the plasmid with 500 μg/ml G418 (a neomycin resistance gene being encoded on the pUHD 17-2neo). Resulting clones were labeled L1-Tet-On and screened for rtTA expression by transiently transfecting pUHG 16-3 (pTRE-β-gal) and measuring β-galactosidase activity in response to increasing concentrations of doxycycline. Doxycycline-inducible cell lines were then screened for their ability to undergo adipogenesis in the presence of differentiation medium (see below) in the presence or absence of doxycycline by visualizing the accumulation of fat droplets following Oil Red O staining on d 8 post-induction.

Stable L1-Tet-On cells were transfected with pRevTRE-COUPTF1AS and pTK-Hyg at 20:1 ratio. Stable transformants were selected using 200 μg/mL hygromycin B (Sigma Chemicals, St Louis, MO). G418 was also included in the selection medium to keep pressure upon the transformants to retain inducer DNA. Resulting clones were screened for the expression of antisense COUP-TF1 by RT-PCR in response to increasing concentrations of doxycycline. Clones that demonstrated doxycycline-inducible expression of antisense COUP-TF1 constructs were screened for their ability to undergo adipogenesis as described above.

Stable antisense COUP-TF 3T3-L1 preadipocyte culture conditions. Cells were cultured in DME with 10% FBS at 5% CO_2. After retroviral infection and selection, cells were allowed to grow to confluence on six well (35 mm) tissue culture plates (d –2). On d 0, cells were induced to differentiate by exposure to insulin (10 μg/L), dexamethasome (1μmol/L), and isobutylmethylxanthine (.5 mmol/L). After 2 days, cells were maintained in medium containing insulin until cells were harvested for histochemical analysis on d 8. Expression of the antisense COUP-TF transgene was induced by addition of 2 μmol/L doxycycline in the medium. Expression of the antisense COUP-TF construct knocked down COUP-TF mRNA by greater than 70% as measured by RT-PCR.

Statistical Analysis. Data are expressed as the mean +/- SEM. Each replicate consisted of a single batch of S-V cells harvested from the subcutaneous adipose tissue of an individual pig. Data were analyzed by using one-way ANOVA followed by multiple comparisons of means with Fisher's least significant difference using SAS

(SAS institute, Cary, NC). Gene expression data was analyzed using two-way ANOVA with day and treatment as main effects. Differences were considered significant if $P < .05$

Table 3.1
Oligonucleotide PCR primers

Gene	Accession #	Primer Sequence (5'→3')	Orientation	Product size (bp)	Multiplex PCR Cycle
1. β-actin	AF054837	CGTGGGCCGCCCTAGGCACCA TTGGCCTTAGGGTTCAGGGGGG	Forward Reverse	210	-
2. 36B4	BC011291	GCACTCTCGCTTTCTGGAGGGTGTC TGACTTGGTTGCTTTGGCGGGATTAG	Forward Reverse	292	-
3. ADD1	U00968	TGTGACCTCGCAGATCCAGC GCGAATGTAGTCGATGGCCT	Forward Reverse	433	29
4. C/EBPα	U34070	GGTGGACAAGAACAGCAACG AGGCACCGGAATCTCCTAGT	Forward Reverse	370	29
5. COUP-TF	X16155.1	CCCACTTTGAGGCACTTCT ACATCGAGTGCGTGGTGT	Forward Reverse	201	30
6. PPARγ	L40904	CACAGGCCGAGAAGGAGAAG ATCTCCTGCACAGCCTCCAC	Forward Reverse	330	28

Results

Differentiation

Sn-glycerol-3-phosphate dehydrogenase (GPDH; EC 1.1.1.8) activity was used as a marker of differentiation in our primary culture system since GPDH activity is expressed in terminally differentiated, mature fat cells but not in preadipocytes. GPDH activity was inhibited in a dose-dependent fashion by continually treating porcine SV cells with the CLA mixture (25-100 μmol/L) from d 0 to d 10 ($p < .01$) (Figure 3.1). Linoleic acid increased GPDH activity at all concentrations administered ($p < .01$). In order to determine the active CLA isomer responsible for the anti-adipogenic action of CLA, the cis-9, trans-11 CLA and the trans-10, cis-12 CLA isomers were tested for their ability to inhibit differentiation. The cis-9, trans-11 isomer failed to inhibit GPDH activity at any concentration. Since the trans-10, cis-12 CLA isomer was toxic to cells in this system at concentrations greater than 25 μmol/L, it was administered at concentrations of 6.25, 12.5, and 25 μmol/L. Trans-10, cis-12 CLA dramatically inhibited GPDH activity in a dose-dependent fashion ($p < .0001$) with inhibition of greater than 80% occurring at the lowest dose administered (Figure 3.1). In agreement with the GPDH activity data, both the CLA mixture and the trans-10, cis-12 CLA isomer dramatically reduced the number of lipid-filled adipocytes present in culture while cis-9, trans-11 CLA isomer had no affect upon the number of lipid-filled fat cells versus the control (Figure 3.2).

Since it has been suggested that cis-9, trans-11 CLA might increase the differentiation of pig preadipocytes the possibility exists that cis-9, trans-11 CLA may

antagonize the action of trans-10, cis-12 CLA in pig preadipocytes. Thus, we measured the effectiveness of trans-10, cis-12 CLA in the presence of increasing concentrations (12.5-150 μM) of cis-9, trans-11 CLA (Figure 3.3). While a dose response effect was not observed, presence of cis-9, trans-11 CLA tended to blunt the ability of trans-10, cis-12 CLA to inhibit differentiation of the adipose S-V cells by 35% ($p < .09$).

Proliferation

In order to study the effect of CLA on the proliferation of porcine stromal-vascular cells, the cleavage of the tetrazolium salt, WST-1, by mitochondrial dehydrogenases was measured on d 2 following induction and treatment 25-100 μmol/L of either CLA mixture, cis-9, trans-11 CLA isomer or linoleic acid (Figure 3.4). The proliferation of porcine SV cells was not affected by any treatment regardless of concentration. The trans-10, cis-12 CLA isomer was administered at concentrations of 6.25, 12.5, and 25 μmol/L. Proliferation was reduced by the trans-10, cis-12 CLA isomer only at 25 μmol/L ($p<.05$).

Gene Expression

In order to further study the effect of CLAs upon the differentiation of porcine preadipocytes, the isomer-specific impact of CLAs on the expression of genes known to encode proteins that regulate adipocyte differentiation was examined. Time courses of expression of these genes from d 0 to d 8 are shown in Figure (3.5a-d). The abundance of PPARγ mRNA was significantly decreased by both the crude CLA mix and the trans-10, cis-12 CLA isomer ($p < .003$; Figure 3.5a). Expression was higher

on d 8 and interestingly, the crude CLA mix failed to decrease PPARγ mRNA abundance versus controls on this day ($p < .002$). Neither treatment nor day altered the expression of C/EBPα mRNA verses controls (Figure 3.5b). Meanwhile, both the CLA mix and the 10t12c isomer markedly decreased ADD1 mRNA regardless of day in culture ($p < .05$; Figure 3.5c). Both the crude CLA mix and tran-10, cis-12 CLA increased the mRNA abundance of COUP-TF ($p < .002$). Interestingly, in a pattern paralleling the effect upon PPARγ, the crude CLA mix increased the abundance of COUP-TF mRNA on d 2 but not on d 8 of culture (Figure 3.5d). The 10t12c isomer increased COUP-TF mRNA on both d 2 and 8 (Figure 3.5d).

Antisense Experiments

In order to further study the role of COUP-TF1 in mediating the inhibitory effect of CLAs upon adipogenesis, the ability of the CLA mix to inhibit differentiation of 3T3-L1 preadipocytes that have been stably transfected with antisense COUP-TF under control of a doxycycline- inducible promoter was investigated (Figure 3.6). The antisense COUP-TF cells differentiated robustly both in the absence of doxycycline and when the antisense COUP-TF was induced indicating that the neither the stable integration nor expression of transgenes effected the normal behavior the 3T3-L1 preadipocytes (Figure 3.6a,b). Differentiation of the antisense COUP-TF1 cells was significantly inhibited by the crude CLA mix in the absence of doxycycline (Figure 3.6c). The induction of antisense COUP-TF1 by doxycycline blocked the ability of the CLA mix to inhibit differentiation of the antisense COUP-TF cells (Figure 3.6d).

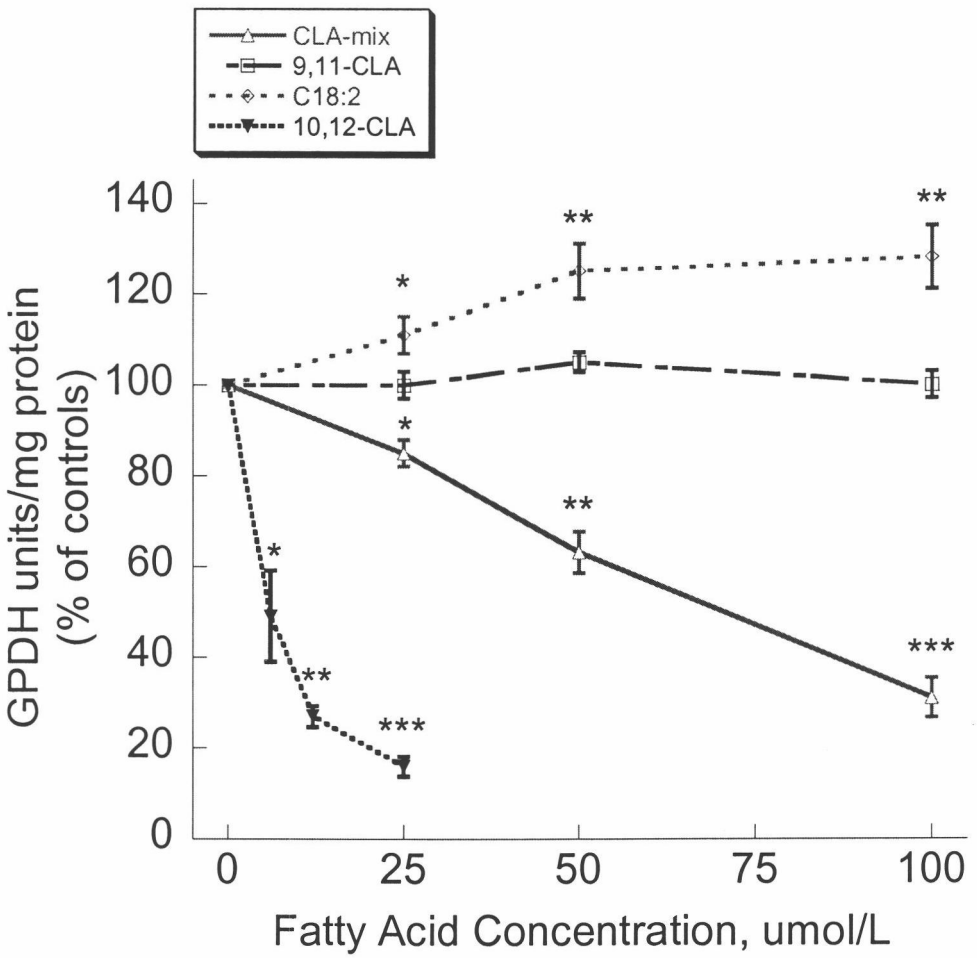


Figure 3.1. Conjugated linoleic acids (CLAs) inhibit porcine preadipocyte differentiation in an isomer-specific manner indicated by glycerol-3-phosphate dehydrogenase (GPDH) activity at d 10 post-induction in primary cultures of porcine S-V cells. Stromal-vascular cells were isolated and cultured as indicated in the Methods section. Stromal-vascular cells were treated with 0 to 100 μmol/L of a crude CLA mixture, the pure cis-9, trans11 CLA, or linoleic acid (C18:2) from d 0 to 10. S-V cells were treated with 0-25 μmol/L tran-10, cis-12 CLA because concentrations greater than 25 μmol/L were toxic to the cells. Values are means +/- SEM, n=6. Data was analyzed using analysis of variance with treatment as the main effect. Values within a treatment that do not share a common designator are significantly different, $P < .05$.

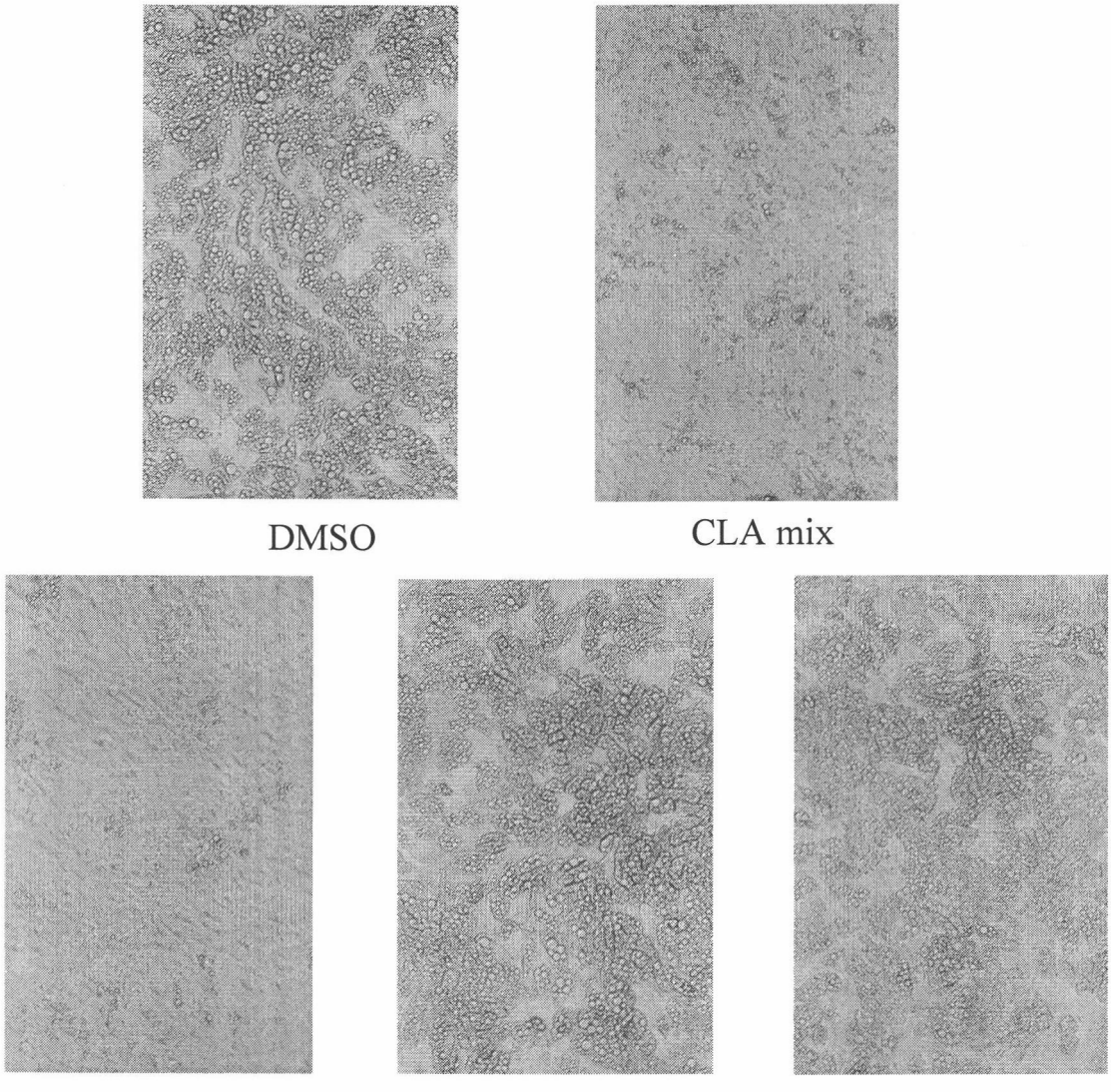


Figure 3.2. Conjugated linoleic acids (CLAs) inhibit porcine preadipocyte differentiation in an isomer-specific manner indicated by the number of lipid-filled adipocytes present at d 10 post-induction in primary cultures of porcine S-V cells. Stromal-vascular cells were treated with either carrier (DMSO), 100 μmol/L CLA mix, 100 μmol/L cis-9, trans-11 CLA, 100μmol/L linoleic acid (C18:2), or 25 μmol/L trans-10,cis-12 CLA from d 0 to 10. Cells were photographed at 10X.

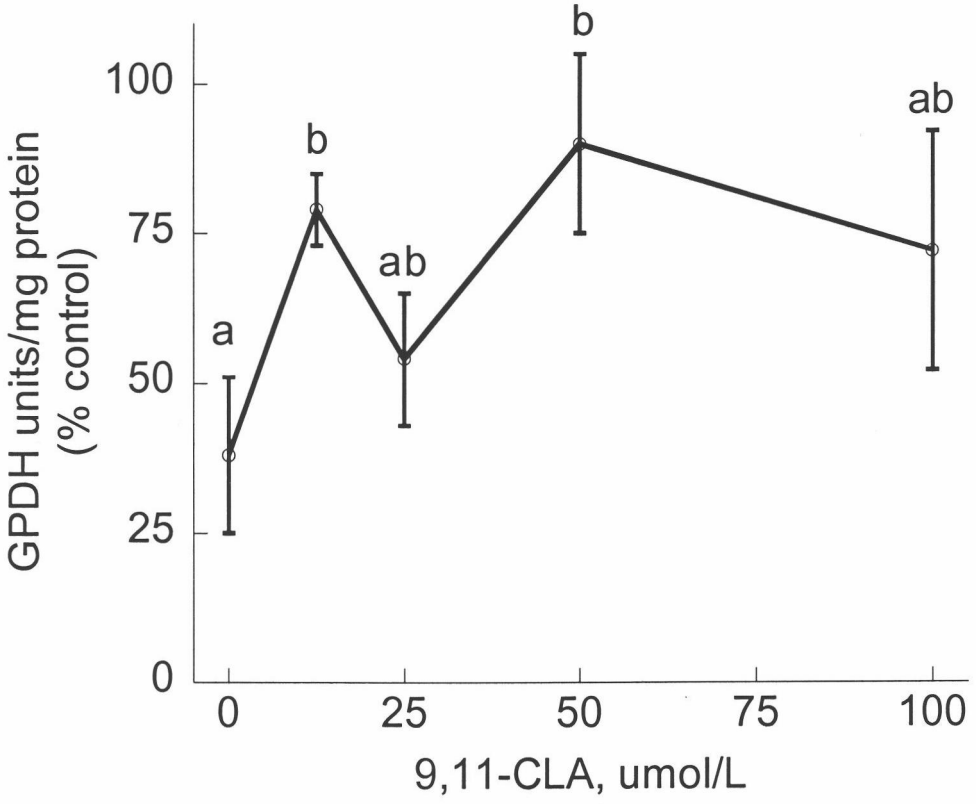


Figure 3.3. Cis-9, trans-11 CLA antagonizes the action of trans-10, cis-12 CLA upon porcine preadipocyte differentiation indicated by glycerol-3-phosphate dehydrogenase (GPDH) activity at d 10 post-induction in primary cultures of porcine S-V cells. Stromal-vascular cells were treated with 12.5 μmol/L trans-10, cis-12 CLA in the presence of the indicated concentrations of cis-9, trans-11 CLA from d 0 to 10. Values are means +/- SEM, n=4. Values without a common letter are significantly different, $P < .05$.

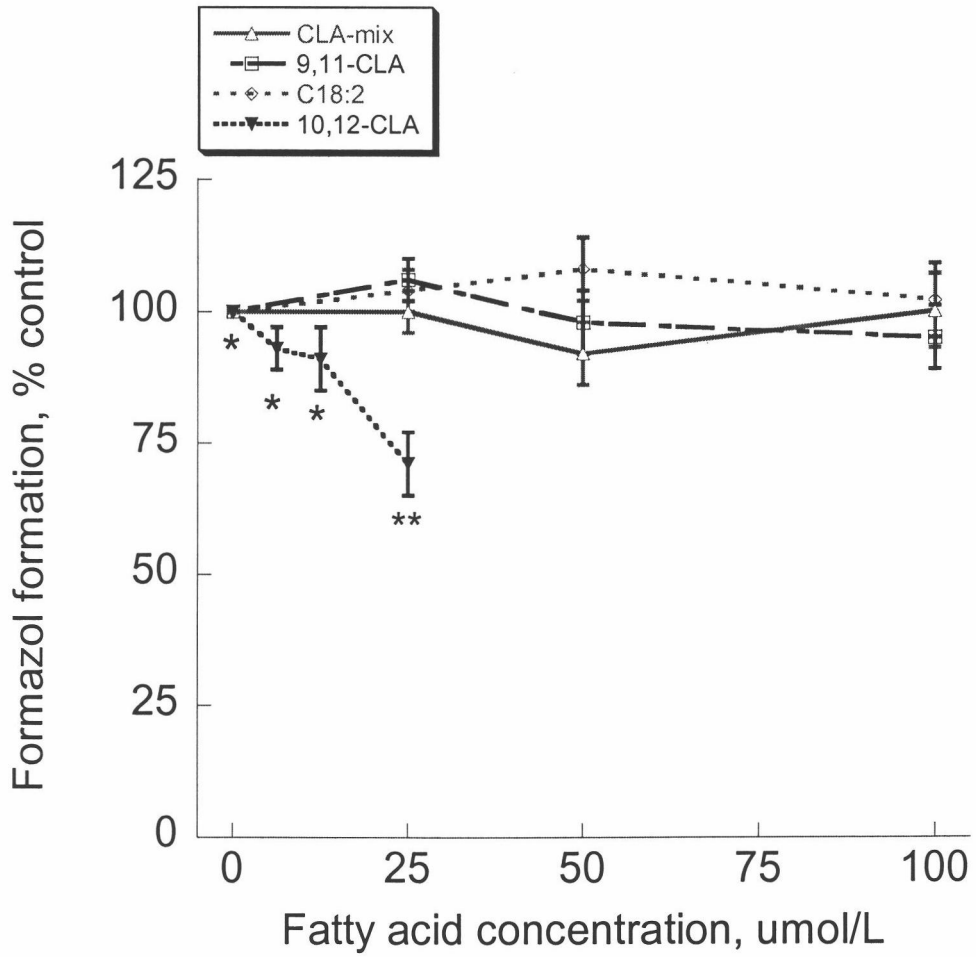


Figure 3.4. Cell number is reduced by conjugated linoleic acids (CLAs) in an isomer-specific manner. Cells were treated for 2 d with either DMSO, 25 to100 μmol/L of either a crude CLA mix, cis-9,trans-11 CLA, or linoleic acid (C18:2) or 6.25, 12.5, and 25 μmol/L trans-10, cis-12 CLA. Cell number was determined based on the formation of formazan after 4 h incubation with the tetrazolium salt WST-1 (see the Materials and Methods section). Values are means +/- SEM, n=4. Data was analyzed using analysis of variance with treatment as the main effect. Values within a treatment that do not share a common designator are significantly different, $P < .05$.

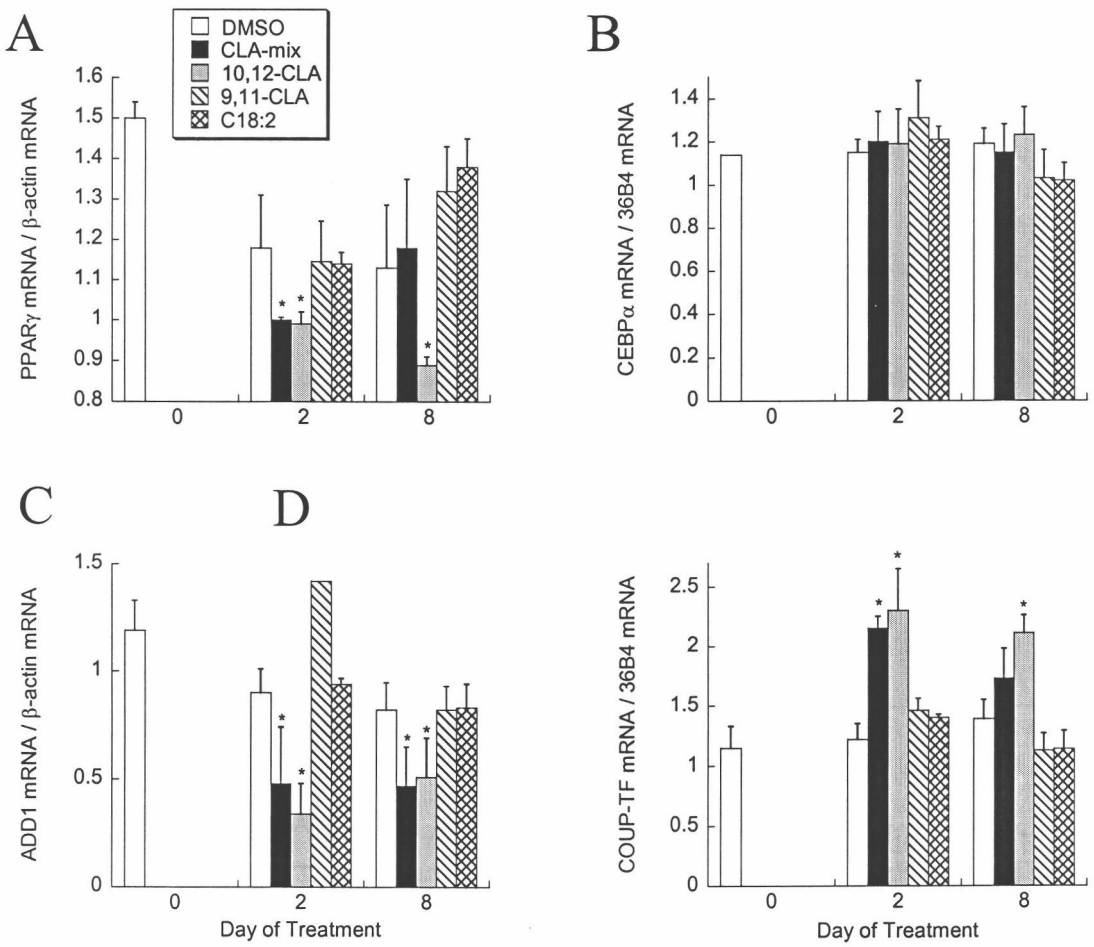


Figure 3.5. Isomer-specific effect of CLAs on the expression of A. PPARγ, B. C/EBPα, C. ADD1, D. COUP-TF mRNA on d 0, 2, and 8 post-induction in primary cultures of porcine S-V cells. Stromal-vascular cells were treated with either DMSO, 100 μmol/L of either cis-9, trans11 CLA or linoleic acid (C18:2) or 12.5 μmol/L of trans-10, cis-12 CLA from d 0 to d8. Total RNA was isolated on the indicated days and mRNA expression was measured using semiquantitative RT-PCR (see Materials and Methods section for details). Values are means +/- SEM, n=3. Data was analyzed using two-way analysis of variance with treatment and day as the main effects. Day was not significant for any gene. Treatments without a common designator are significantly different, $P < .05$.

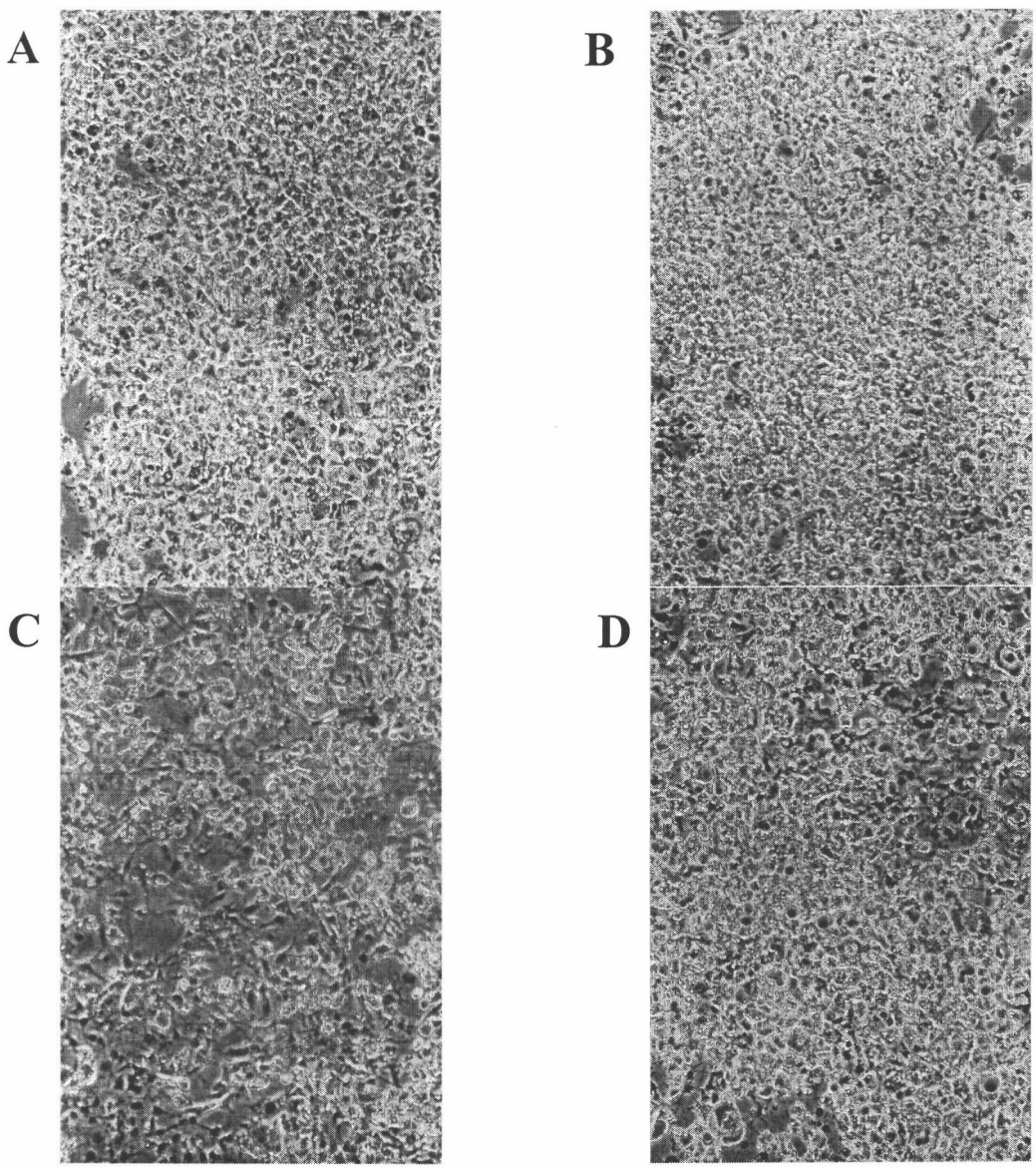


Figure 3.6. Antisense COUP-TF blocks the ability of CLAs to inhibit differentiation of 3T3-L1 preadipocytes indicated by Oil-red-O staining. 3T3-L1 preadipocytes that were stably transfected with a doxycycline-inducible COUP-TF antisense construct were seeded at a density of 3 x 10^4 cells/cm^2 and cultured as described in the Materials and Methods. Cells were treated continuously upon induction of differentiation with either A. vehicle, B. 2 μM doxycycline, C. 100 μM crude mix CLA, or D. 2 μM doxycycline plus 100 μM of crude CLA mix. On day 8 post-induction, differentiation was evaluated via Oil-red-O staining of stored triglyceride (magnification=10X). Representative fields are shown.

Discussion

The present study provides evidence that CLAs can limit adipogenesis in the pig as suggested by the ability of CLAs to potently inhibit the differentiation of porcine preadipocytes in primary culture. This inhibitory action is isomer-specific as trans-10, cis-12 CLA potently decreased several indices of differentiation while cis-9, trans-11 CLA had no effect. The mechanism underlying this inhibitory action involves the down-regulation of PPARγ and ADD1 gene expression through a pathway that appears to involve the up-regulation of COUP-TF1.

Our observation that CLAs potently inhibited the differentiation of pig preadipocytes contrasts with the findings of the few published studies that have directly addressed the effect of CLAs upon the differentiation of porcine stromal vascular cells in primary culture. Ding et al. (2000) treated porcine adipose-derived S-V cells with CLAs for 24 h in a serum-free culture system and observed that cis-9, trans-11 CLA increased triglyceride accumulation compared to linoleic acid, but neither the cis-9, trans-11 nor trans-10, cis-12 CLA isomers had an effect on the mRNA abundance of the PPARγ, CEBPα, or aP2 genes. Their study was confounded, however, by major cell losses due to the addition of polyunsaturated fatty acids in the serum-free medium necessitating that differentiation be measured after only 24 hours of culture. The use of triglyceride accumulation as an acute marker of adipogenesis in primary cultures is problematic for several reasons. First, little lipid accumulation is observable at 24 h post-induction making it difficult to detect treatment effects upon differentiation. Secondly, primary cultures of adipose-derived S-V cells contain a

heterogeneous population of preadipocytes that are in various stages of differentiation. Because a confounding subpopulation of unfilled, terminally differentiated adipocytes is present in culture before induction of differentiation, it is impossible to determine if acute triglyceride accumulation is due to the lipid filling of cells that have been induced to differentiate or the filling of pre-existing adipocytes that were previously unidentifiable as terminally differentiated. More recently, McNeel and Mersmann (2003) used a serum-containing primary culture system to investigate the effect of CLAs upon the differentiation of porcine S-V cells and they failed to observe an effect of cis-9, trans-11, or trans-10, cis-12 CLA upon the triglyceride accumulation of porcine preadipocytes. However, their ability to detect an effect of CLAs upon differentiation may have been obscured by a confounding low differentiation response in control cells which would be expected to increase the contribution of preexisting, unfilled adipocytes to the ORO data. In the present study, we observed that 50 to 70% of control S-V cells consistently differentiated in our primary culture system. Furthermore, preadipocyte differentiation was evaluated by measuring morphological data, GPDH activity (functional assay), and marker gene expression. The effect of CLAs was consistent and dose-dependent for all indices examined. Our results indicate that CLAs can potently inhibit the differentiation of pig preadipocytes in primary culture.

We are the first to demonstrate a direct, isomer-specific effect of CLAs upon pig preadipocytes. CLAs naturally occur in dairy and meat products derived from ruminating animals. Cis-9, trans-11 CLA is the predominant isomer present in

foodstuffs while the cis-9, trans-11 and trans-10, cis-12 isomers are found in roughly equamolar concentrations in most commercial preparations of crude CLA mixtures through the ratio of these two isomers can vary depending upon the commercial supplier. Our data indicate that the trans-10, cis-12 isomer, and not the cis-9, trans-11 CLA isomer is responsible for the ability of crude CLA mixtures to inhibit the differentiation of porcine adipose S-V cells. In agreement with our findings, trans-10, cis-12 CLA potently inhibits the differentiation of 3T3-L1 preadipocytes and is now considered the active anti-adipogenic isomer in clonal preadipocyte cell lines (Brown et al., 2001; Kang et al., 2003). Brown et al. (2003) reported that trans-10, cis-12 and not cis-9, trans-11 CLA inhibited the differentiation of human preadipocytes in serum-containing primary culture. Importantly, they reported striking inhibition of triglyceride accumulation in these cultures in response to trans-10, cis-12 but not cis-9, trans-11 CLA. In agreement with their results, we observed very similar isomer-specific effects upon morphology in our porcine S-V cell culture system as well as significant isomer-specific effects upon GPDH activity and gene expression. Interestingly, some in vitro data has suggested that the cis-9, trans-11 CLA isomer might increase the differentiation of pig preadipocytes (Ding et al., 2000). Thus, the possibility exists that the cis-9, trans-11 CLA isomer may actually antagonize the action of the tran-10, cis-12 CLA in pig preadipocytes. While a stimulatory action of cis-9, trans-11 CLA was not observed in the current study, presence of cis-9, trans-11 CLA tended to blunt the ability of tran-10, cis-12 CLA to inhibit differentiation of the adipose-derived S-V cells in primary cultures. This suggests that the presence of cis-

9, trans-11 CLA may antagonize the action of trans-10, cis-12 CLA and may reduce the potency of crude preparations of CLA. Such an effect may explain why growth trials using crude CLA preparations have produced mixed results in growing pigs. Collectively, our data supports a role for trans-10, cis-12 CLA as an active isomer that inhibits the differentiation of pig preadipocytes.

In the current study, we demonstrate that trans-10, cis-12 CLA inhibited the differentiation of pig preadipocytes by a mechanism that involved the down regulation of PPARγ and ADD1expression. Interestingly, controversy exists concerning the effect of CLAs upon the expression of adipogenic transcription factors. Using 3T3-L1 preadipocytes, Brodie et al. (1999) observed that a crude CLA mix decreased the mRNA abundances of C/EBP-α, PPAR,γ and aP2. Other laboratories have confirmed these results in 3T3-L1 preadipocytes (Evans et al., 2001, Kang et al., 2003). However, Choi et al. (2000) reported that while a crude CLA mix decreased both PPARγ2 and aP2 mRNA abundance in a dose dependent fashion trans-10, cis-12 CLA failed to decrease the mRNA abundances for either C/EBP-α or PPARγ. Using S-V cells derived from human adipose tissue, Brown et al. (2003) reported that trans-10, cis-12 CLA decreased PPARγ, CEBP-α, and aP2 mRNA abundance. Feeding trans-10, cis-12 CLA to mice has consistently decreased PPARγ mRNA (Kang and Pariza, 2001; Takahashi et al., 2002). Thus while contradictory effects of CLAs upon adipogenic transcription factors exists in the literature, taken together there is a consensus that CLAs decreases the expression of PPARγ mRNA abundance. Data from our study is consistent with this conclusion.

It is somewhat surprising that no affect of CLAs were observed upon C/EBP-α expression in the current study. However, given the emerging role of PPARγ as the "master regulator" of adipogenesis (Farmer et al., 2002; Rosen et al., 2002), effects upon C/EBP-α may not be necessary in order to significantly inhibit fat cell differentiation. This is especially true given the observation that other proteins that play a role in regulating differentiation (ADD1 and COUP-TF) had altered gene expression in the current study. ADD1 has been shown to regulate adipogenesis both through positive effects upon PPARγ expression and activity as well as through up regulating the expression of several genes involved in adipocyte metabolism (Kim and Spiegelman, 1996; Kim et al., 1998; Rosen et al., 2000). Thus, the down regulation of ADD1 would be expected to have an inhibitory effect upon fat cell differentiation. Importantly, in the current study ADD1 mRNA was decreased in an isomer-specific pattern that mirrored the effect of CLAs upon GPDH activity suggesting that ADD1 played a role in the mechanism by which CLAs inhibited the differentiation of pig preadipocytes. Finally, species-specific differences in the regulation of adipogenesis may exist which explain the lack of effect upon C/EBP-α in pig preadipocytes versus human and rodent models of fat cell differentiation.

To our knowledge, this is the first study to suggest that COUP-TF1 may play a role in the anti- adipogenic action of CLAs or that COUP-TF1 may regulate the differentiation of pig preadipocytes. COUP-TF1 is a member of the steroid/thyroid hormone superfamily of nuclear receptors and it is well known that COUP-TF1 can compete with PPARγ for both dimerization with RXR receptors and for binding to

sites (DR-1) in the promoter region of PPARγ-regulated genes (Tsai and Tsai, 1997). Thus, the up-regulation of COUP-TF1 would be expected to significantly decrease the transcriptional activity of PPARγ. Interestingly, CLAs have recently been shown to antagonize the ability of the PPARγ ligand, BRL49653, to activate transcription of a reporter gene attached to PPRE elements during transient transfection studies in 3T3-L1 preadipocytes (Brown et al., 2003). In the present study, trans-10, cis-12 CLA increased the expression of COUP-TF1 while also decreasing the expression of PPARγ mRNA. Further supporting a role of COUP-TF1 in the inhibitory action of CLAs, the expression of antisense COUP-TF1 mRNA blocked the ability of CLAs to inhibit the differentiation of 3T3-L1 preadipocytes. Brodie et al. (1996) demonstrated that retinoic acid (RA), another potent inhibitor of adipogenesis, also induced the expression of COUP-TF1. In that study, the authors further demonstrated the RA treatment increased the binding of COUP-TF1 to a PPARγ DNA response element that had been transfected into 3T3-L1 preadipocytes. Thus, we speculate that CLAs may inhibit the differentiation of pig preadipocytes in primary culture through both decreasing transcription of the PPARγ gene (possibly through the down regulation of ADD1) and by inhibiting the transcriptional activity of existing PPARγ protein through the up regulation of COUP-TF1.

In summary, our data suggest that CLAs can inhibit the differentiation of pig preadipocytes in an isomer-specific manner as trans-10, cis-12 CLA but not cis-9, trans-11 CLA possesses this inhibitory activity in pig preadipocytes. The mechanism underlying this antiadipogenic action appears to involve both the down-regulation of

PPARγ and ADD1 and the up-regulation of COUP-TF gene expression. In this model, trans-10, cis-12 CLA decreases expression of the PPARγ gene either through direct effects upon the PPARγ gene (possibly mediated by COUP-TF1) or indirectly through the down-regulation of ADD1. CLAs further inhibit adipogenesis through the up regulation of COUP-TF which competes with existing PPARγ protein for dimerization with RXR and the binding to DR-1 sites within the promoters of PPARγ target genes. These results have important implications for the animal industry as they confirm that CLAs can inhibit fat cell differentiation as well as identify a novel potential regulator of adipogenesis.

CHAPTER 4
REGULATION OF DIFFERENTIATING PIG PREADIPOCYTES BY RETINOIC ACID

Abstract

All-trans retinoic acid (ATRA) potently inhibits porcine preadipocyte differentiation in primary culture. However, the mechanism by which ATRA exerts this effect is poorly understood. The objective of this study was to investigate the molecular mechanism underlying the anti-adipogenic action of retinoids in cultured pig preadipocytes. Stromal-vascular cells were harvested from porcine adipose tissue and cultured in serum-free medium (DME/F12 medium containing 100 n*M* insulin, 10 μg/mL transferrin, and 50 ng/mL hydrocortisone). Addition of increasing amounts of the RAR receptor ligands, ATRA and TTNPB, (10, n*M* to 10 μ*M*) to the medium reduced glycerol-3-phoshphate dehydrogenase (GPDH) activity, a late marker of preadipocyte differentiation. Addition of Ro-61, a RAR-specific antagonist (10 n*M* to 10 μ*M*) reversed the ability of ATRA and TTNBP to inhibit GPDH activity. Addition of methoprene acid, an RXR-specific agonist, increased GPDH activity. Preadipocytes were then continuously treated with 10 n*M* of TTNPB +/- 1 μ*M* Ro-61 and mRNA was isolated on days 2 and 8. TTNPB decreased the expression of PPARγ, ADD1/SREBP, RXRα and aP2 mRNA transcripts while these effects were prevented by the presence of Ro-61. TTNBP increased the mRNA abundance of the orphan nuclear receptor COUP-TF1 while Ro-61 prevented this increase. These changes were independent of alterations in the mRNA abundances of the RARα, C/EBPβ and C/EBPα genes. These results suggest that retinoic acid inhibits porcine

preadipocyte differentiation by a mechanism that involves activation of RAR receptor and down regulation of PPARγ, RXRα and ADD1 mRNA but is independent of changes in CAAT-Enhancer Binding Protein (CEBP) mRNA abundance.

KEY WORDS: Adipocyte, Cell Culture, Retinoids, Pigs

Introduction

Vitamin A, acting through metabolites such as retinoic acid (ATRA), is essential for biological processes such as reproduction, and the growth and differentiation of various tissues (Mangelsdorf et al., 1994). Interestingly, ATRA potently inhibits the differentiation of 3T3-L1 preadipocytes in culture (Chawla and Lazar, 1994; Xue et al., 1996). Kawada et al. (2000) reported that provitamin A carotenoids also markedly inhibit the differentiation of 3T3-L1 preadipocytes. Suryawan and Hu (1997) were the first to examine the effect of retinoids on preadipocytes from meat producing animals. They reported that ATRA effectively inhibited the differentiation of porcine preadipocytes in primary culture suggesting that retinoids may inhibit adipogenesis in growing animals.

The mechanism by which retinoids inhibit the differentiation of pig preadipocytes is unclear. In the current model of adipogenesis, sequential induction of C/EBPβ, PPARγ and C/EBPα in preadipocytes results in transactivation of adipocyte-specific genes leading to terminal differentiation of the adipocyte. PPARγ acts as a master regulator in this process while C/EBPα potentiates adipogenesis through up regulating genes that confer insulin sensitivity upon the adipocyte (Farmer et al., 2002;

Rosen et al., 2002). Studies using 3T3-L1 preadipocytes suggest that ATRA inhibits adipogenesis in the mouse by down regulating the expression of PPARγ and C/EBPα (Xue et al., 1994; Kawada et al., 2000). The effect of retinoids upon the expression of these transcription factors in pig preadipocytes has not been explored.

Better understanding how retinoids inhibit the differentiation of pig preadipocytes may provide insight into the regulation of adipose tissue development in vivo. Therefore, the objective of this study was to utilize retinoid receptor-specific compounds to investigate the underlying mechanism by which retinoids inhibit the differentiation of porcine preadipocytes in primary culture.

Materials and Methods

Materials. Dulbecco's modified Eagle's medium, nutrient mixture F-12, dihydroxyacetone phosphate (DHAP), reduced nicotinamide adenine dinucleotide (NADH), gentamicin sulfate, HEPES buffer, hydrocortisone, insulin, transferrin, and (E)-4-[2-(5,6,7, 8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl-1-propenyl]benzoic acid (TTNPB), were purchased from Sigma Chemical (St. Louis, MO). All-trans-retinoic acid, 9-cis-retinoic acid and methoprene acid were purchased from BIOMOL (Plymouth Meeting, PA). Collagenase (type I) was purchased from Worthington Biochemical (Freehold, NJ), fetal calf serum from Intergen (Purchase, NY), and fungizone from Gibco BRL, Division of Life Technologies (Gaithersburg, MD).

Animals and Cell Culture. 2-day-old crossbred pigs were obtained from a commercial producer (Drahn Acre Farms, Corvallis, OR) and sacrificed by CO_2 asphyxiation in a manner approved by the Animal Care and Use Committee at Oregon

State University. Stromal-vascular (S-V) cells were harvested by a collagenase digestion procedure as previously described (Suryawan and Hu, 1997). Aliquots of S-V cells were counted using a hemacytometer and seeded in either 35-mm or 100-mm culture dishes at a density of 5 x 10^4 cells/cm^2 and incubated at 37° C in 5 % CO_2 in air. Plating medium consisted of DME/F12 (v 1:1) containing 15 mM $NaHCO_3$, 15 mM HEPES buffer and 50 mg/L gentamicin sulfate supplemented with 10% fetal calf serum (FCS). After 24 h, attached cells were washed three times using plating medium to remove unattached cells and cellular debris. After washing, cells were maintained in serum-free DME/F12 medium containing 100 n*M* insulin, 10 μg/mL transferrin, and 50 ng/mL hydrocortisone. Culture media was changed every three days until d 8 (except where stated otherwise). Cells were subjected to GPDH assays and oil red O staining on d 8 while subsets of cells were harvested on d 2 and d 8 for gene expression analysis. By d 8 in culture medium more than 70% of the cells accumulated multilocular lipid droplets.

Histochemistry. In order to qualitatively assess stromal-vascular cell differentiation by microscopy, cells were exposed to induction media from day 0 to d 8 then fixed in 10% formalin, stained with oil red O (ORO) for lipid and counterstained with Harris hematoxylin as described by Suryawan and Hu (1993). Briefly the wells were fixed with Baker's formalin for 15 minutes, rinsed with distilled water, equilibrated in 100% propylene glycol for 2 min, and then stained with ORO for 10 min. Wells were then treated with 60% propylene glycol for 1 min to remove

free ORO, rinsed with distilled water, and counterstained for 6 minutes in Harris Hematoxylin. Finally cells were mounted in glycerin jelly.

Glycerol-3-phosphate dehydrogenase (GPDH) activity. The Sn-glycerol-3-phosphate dehydrogenase (GPDH; EC 1.1.1.8) activity was determined by spectrophotometrically measuring the disappearance of NADH during the GPDH-catalyzed reduction of dihydroxyacetone phosphate under zero-order conditions by the method of Kozak and Jensen (1974) as modified by Wise and Green (1979). GPDH activity is expressed as units per mg of protein where one unit of activity is defined as the oxidation of 1 nmol NADH/min. Protein was measured according to Bradford (1976).

RNA Isolation. Cells were harvested with a cell scraper and total RNA was extracted using the quanidinium-acid phenol method (Chomczynski and Sacchi, 1987). Total RNA concentration was determined spectrophotometrically using A_{260} and A_{280} measurements. The ratio of A_{260} / A_{280} was between 1.9 and 2.1 for all samples. Five micrograms of total RNA from each sample was separated on a 1.2% denaturing formaldehyde gel and stained with ethidium bromide. The RNA integrity was assessed visually by judging the quality of 18 and 28s rRNA bands.

Semiquantitative RT-PCR. Reverse transcription (RT) reactions (20 μL) consisted of 4 μg of total RNA, 50 U SuperScript II reverse transcriptase (Invitrogen/Life Technologies), 40 U of an RNAse inhibitor (Invitrogen/Life Technologies), 0.5 mmol/L d NTP, and 100 ng random hexamer primers. Polymerase chain reaction (PCR) was performed in 50 μL containing 20 mmol/L Tris-HCL, pH

8.4, 50 mmol/L KCL, 1.0 μL of RT reaction, 2.5 U of Platinum Taq DNA polymerase (Hot Start, Invitrogen/Life Technologies), 0.2 mmol/L dNTP, 2 mmol/L Mg^{2+} (Invitrogen/Life Technologies), 10 pmol each of gene specific primers and 10 pmol each of primers specific for either β-actin or 36B4. Thermal cycling parameters were as follows: 1 cycle 94°C for 4 min, followed by 26-30 cycles, 94°C for 1 min, 56°C for 2 min, 72 °C for 2 min with a final extension at 72°C for 8 min. Primers were synthesized at the Center for Gene Research at Oregon State University. Identity of PCR products was verified either by restriction digest analysis or via DNA sequencing. Cycle number for each multiplex PCR reaction was selected by experimentally determining the highest cycle number in which the amplification of both cDNA products was within a linear range. The optimal cycle number was then considered to be two cycles lower than the highest cycle of linearity. RT-PCR products were visualized by separating DNA on a 3% agarose gel and staining with CyberGreen according to the manufacturer's directions (Molecular BioProbes, Eugene, OR). Primer sequences, product size and cycle length are listed in Table 4.1. Data for each replicate represented the mean of three individual RT-PCR reactions.

Northern Blot Analysis. In order to verify results of semiquantitative RT-PCR, twenty micrograms of total RNA was separated, blotted and probed for C/EBPβ and RXRα and normalized to 18S mRNA abundance as described by Brodie et al., (1999). The non-radioactive labeling of probes with digoxigenin-dUTP was performed using the PCR Dig Probe Synthesis Kit as described by the manufacturer (Roche Diagnostics, Indianapolis, IN). Blots were prehybridized and hybridized at 50

° C for 16 hours and washed two times at 50° C with 1 X SSPE/ .1 % SDS for 50 minutes and one time with .1 X SSPE / .1 % SDS at 60° C for 30 minutes. Chemiluminescent detection using the CSPD alkaline phosphatase substrate was performed as recommended by the manufacturer using the Dig Detection kit II (Roche Diagnostic, Indianapolis, IN) followed by autoradiography.

Statistical Analysis. Data are expressed as the mean +/- SEM. Each replicate consisted of a single batch of S-V cells harvested from the subcutaneous adipose tissue of an individual pig. Data were analyzed by using one-way ANOVA followed by multiple comparisons of means with Fisher's least significant difference using SAS (SAS institute, Cary, NC). Gene expression data was analyzed using two-way ANOVA with day and treatment as main effects. Differences were considered significant if $P < .05$

Table 4.1
Oligonucleotide PCR primers

Gene	Accession #	Primer Sequence (5'→3')	Orientation	Product size (bp)	Multiplex PCR Cycle
β-actin	AF054837	CGTGGGCCGCCCTAGGCACCA TTGGCCTTAGGGTTCAGGGGGG	Forward Reverse	210	-
36B4	BC011291	GCACTCTCGCTTTCTGGAGGGTGTC TGACTTGGTTGCTTTGGCGGGATTAG	Forward Reverse	292	-
ADD1	U00968	TGTGACCTCGCAGATCCAGC GCGAATGTAGTCGATGGCCT	Forward Reverse	433	29
aP2	AF102872	GGCTTTGCTACCAGGAAAGT CGCAGTGACACCATTCATGAC	Forward Reverse	292	29
C/EBPα	U34070	GGTGGACAAGAACAGCAACG AGGCACCGGAATCTCCTAGT	Forward Reverse	370	29
C/EBPβ	AF103945	GCTTGAACAAGTTCCGCAGG CAAGAAGACGGTGGACAAGC	Forward Reverse	209	27
COUP-TF	X16155.1	CCCACTTTGAGGCACTTCT ACATCGAGTGCGTGGTGT	Forward Reverse	201	30
PPARγ	L40904	CACAGGCCGAGAAGGAGAAG ATCTCCTGCACAGCCTCCAC	Forward Reverse	330	28
RARα	U82260	GCATCCAGAAGAACATGGTGT CCTGCTTGGCGAACTCCACAGT	Forward Reverse	392	26
RXRα	X52773	GATTGACAAGCGGCAGCA CGGAGAAGGAGGCGATGAGCA	Forward Reverse	409	27

Results

Differentiation

Glycerol-3-phosphate dehydrogenase (GPDH) activity was used as a marker of differentiation since GPDH activity is expressed in terminally differentiated, mature fat cells but is not present in undifferentiated stromal-vascular cells. Continually treating S-V cells with increasing amounts of either ATRA or 9c-RA (10 n*M* to 10 μ*M*) inhibited GPDH activity in a dose-dependent fashion ($P < .01$; Figure 4.1). In order to determine which retinoid receptor mediated this effect, receptor specific-retinoids were tested for their ability to inhibit differentiation. The RAR-specific retinoid, TTNPB, (10 p*M* to 10 μ*M*) potently reduced GPDH activity with as little as 100 p*M* ($P < .001$). Addition of methoprene acid (10 n*M* to 10 μ*M*), an RXR-specific agonist, increased GPDH activity ($P < .01$; Figure 4.1). Addition of Ro-61, a RAR-specific antagonist (10 p*M* to 10 μ*M*) reversed the ability of both ATRA and TTNBP to inhibit GPDH activity (Figure 4.2).

In agreement with the GPDH activity data, as little as 10 nM TTNPB dramatically reduced the number of lipid-filled cells versus the control (Figure 4.3) while the addition of Ro-61 completely reversed this effect. Methoprene acid increased the number of lipid-filled cells in culture relative to the control.

Gene Expression

Total mRNA was extracted from cells following either 0, 2 or 8 days of treatment by either DMSO (carrier), 10 nM TTNPB or 10nM TTNPB plus 1 uM Ro-61. Using semiquantitative RT-PCR, mRNA was detected for all genes measured as

early as d 0. TTNPB significantly decreased the mRNA abundance for the aP2 gene relative to the control on both d 2 and 8 ($P < .008$; Figure 4.4A) with the effect being greater on day 8 ($P < .001$). The addition of Ro-61 reversed this effect. TTNPB significantly decreased the mRNA abundance for the PPARγ gene relative to the control on both d 2 and 8 ($P < .0001$; Figure 4.4b) and this effect was reversed by the addition of Ro-61treatment. TTNPB significantly decreased the mRNA abundance for RXRα relative to the control on both d 2 and 8 ($P < .0002$; Figure 4.4C) while the addition of Ro-61 reversed this effect. TTNPB significantly decreased the mRNA abundance for ADD1 relative to the control on both d 2 and 8 ($P < .0001$; Figure 4.4D) while the addition of Ro-61 reversed this effect. TTNPB had no effect upon the expression of RARα mRNA abundance ($P < .8$; Figure 4.4E) although the expression level of RARα mRNA was lower on each successive day tested ($P < .01$). Interestingly, TTNPB significantly increased the mRNA abundance for the COUP-TF1 gene on both d 2 and 8 ($P < .0002$; Figure 4.4F). Addition of Ro-61 reversed this effect. TTNPB failed to affect the mRNA abundance for the C/EBPβ, and C/EBPα, genes (Figures 4.5a,b) for any day measured. Likewise, the addition of Ro-61 had no effect on the mRNA for these genes. There were no significant interactions between day and treatment for any gene measured.

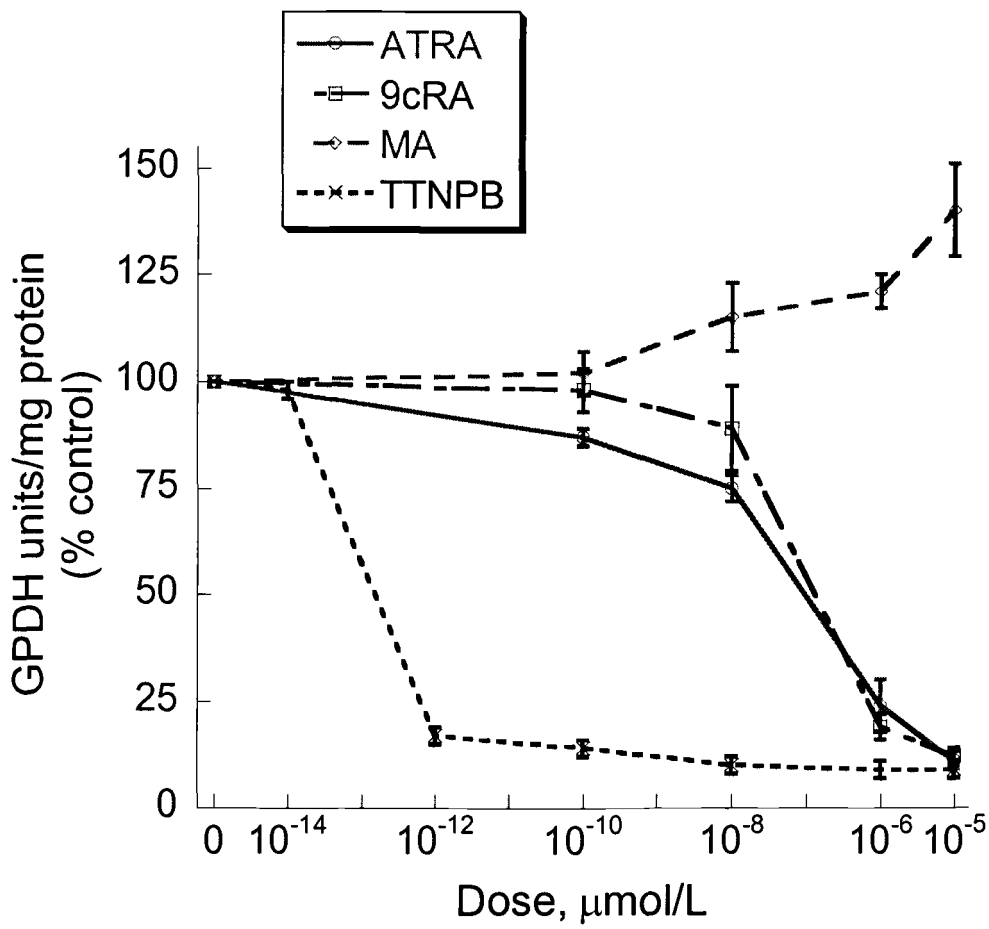
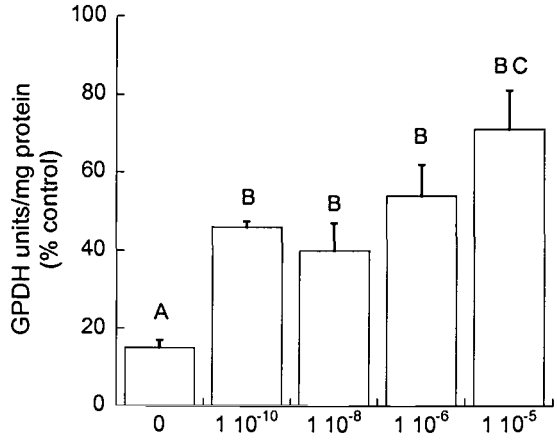


Figure 4.1. Retinoids inhibit porcine preadipocyte differentiation through binding the RAR receptor as indicated by glycerol-3-phosphate dehydrogenase (GPDH) activity at d 10 post-induction in primary cultures of porcine S-V cells. Stromal-vascular cells were isolated and cultured as indicated in the Methods section. Stromal-vascular cells were treated with 0 to 10 µmol/L of all-trans retinoic acid (ATRA), 9-cis retinoic acid (9cRA), methoprene acid (MA; RXR-selective) or TTNPB (RAR-selective) from d 0 to 10. Values are means +/- SEM, n=6. Data was analyzed using analysis of variance with treatment as the main effect.

A



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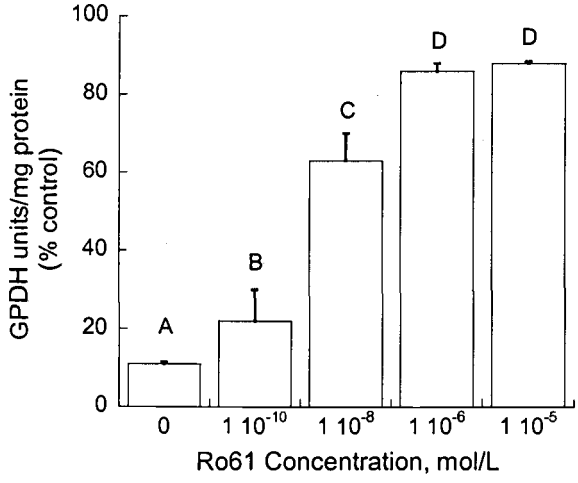


Figure 4.2. Ro61, a potent RAR receptor antagonist, blocks the inhibitory action of retinoids upon porcine preadipocyte differentiation indicated by glycerol-3-phosphate dehydrogenase (GPDH) activity at d 10 post-induction in primary cultures of porcine S-V cells. Stromal-vascular cells were treated with 0 to 10 μmol/L Ro61 in the presence of either A) 1 μmol/L ATRA or B) .1 nmol/L TTNPB from d 0 to 10. Values are means +/- SEM, n=6. Values without a common letter are significantly different, $P < .05$.

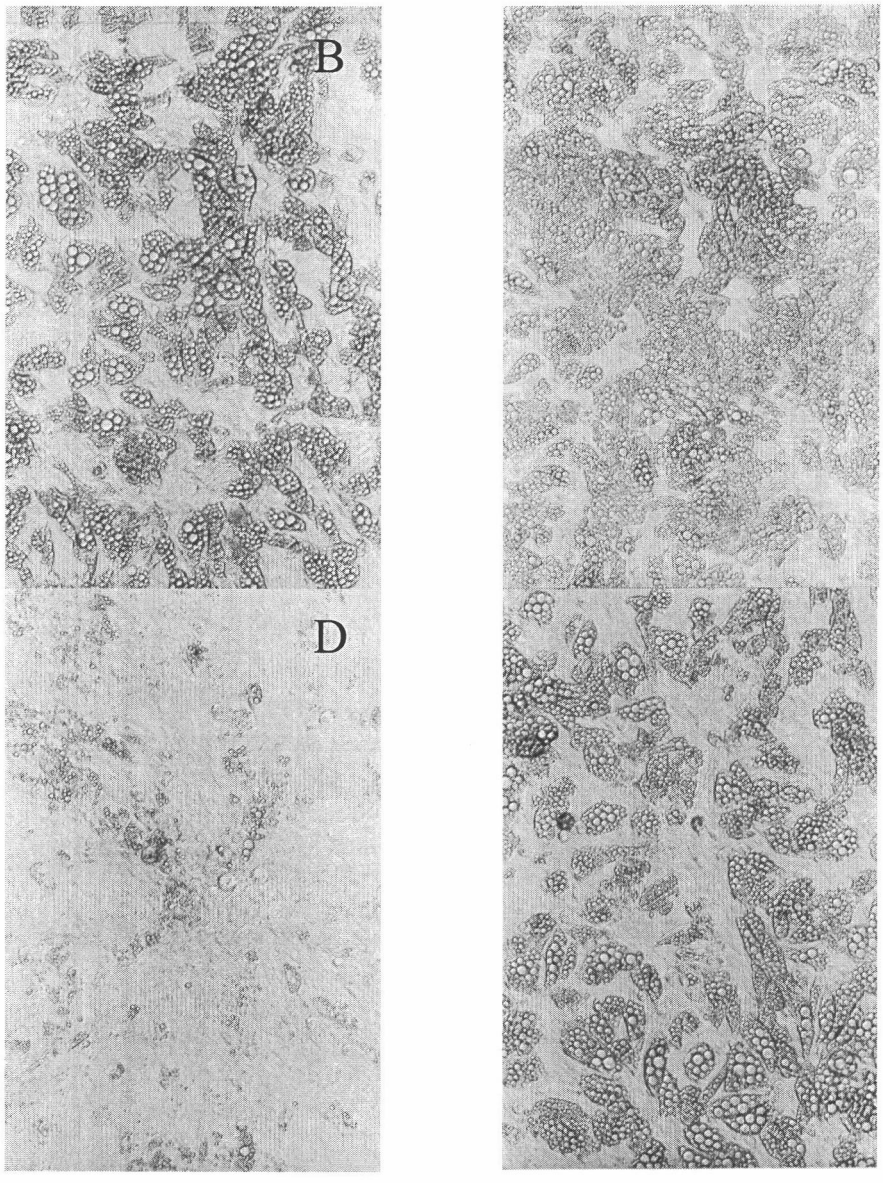


Figure 4.3. Retinoids inhibit porcine preadipocyte differentiation through binding the RAR receptor as indicated by the number of lipid-filled adipocytes present at d 10 post-induction in primary cultures of porcine S-V cells. Stromal-vascular cells were treated with either A. carrier (DMSO), B. 10 μmol/L methoprene acid, C. 10 nmol/L TTNPB, or D. 10 nmol/L TTNPB + 10 μmol/L Ro61 from d 0 to 10. All cells were photographed at 10X.

Figure 4.4. Effect of TTNPB on the expression of A. aP2A, B. PPARγ, C. RXRα, D. ADD1, E. RARα, F. COUP-TF mRNA on d 0, 2, and 8 post-induction in primary cultures of porcine S-V cells. Stromal-vascular cells were treated with either DMSO, .1 nmol/L TTNPB, or .1 nmol/L TTNPB plus 1 μmol/L Ro61 from d 0 to d8. Total RNA was isolated on the indicated days and mRNA expression was measured using either northern blotting or semiquantitative RT-PCR (see Materials and Methods section for details). Values are means +/- SEM, n=3 (each replicate for RT-PCR data represents the mean of 3 repeated PCR reactions). Data was analyzed using two-way analysis of variance with treatment and day as the main effects. Day was not significant for any gene. Treatments with an asterisk were significantly different that control, $P < .05$.

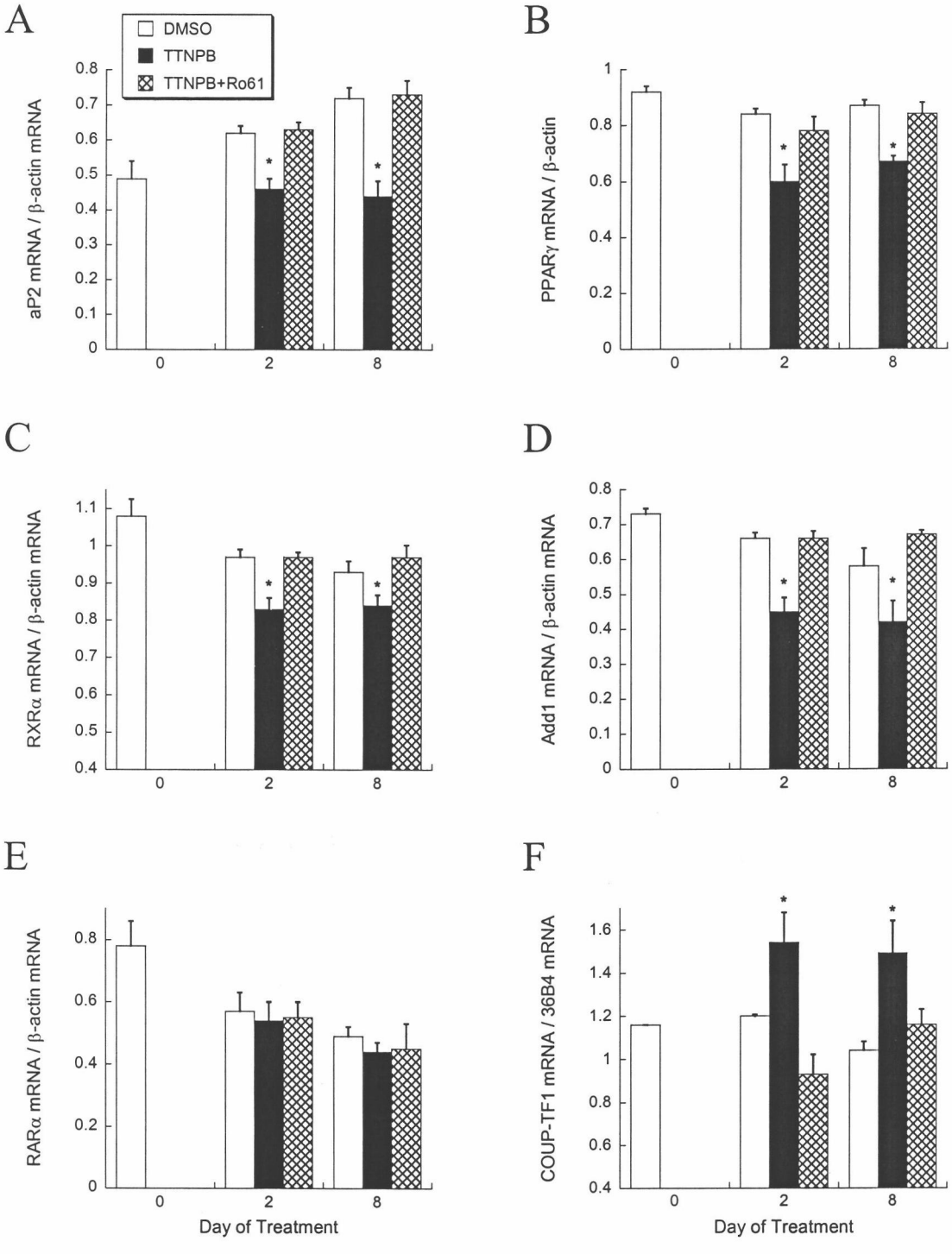
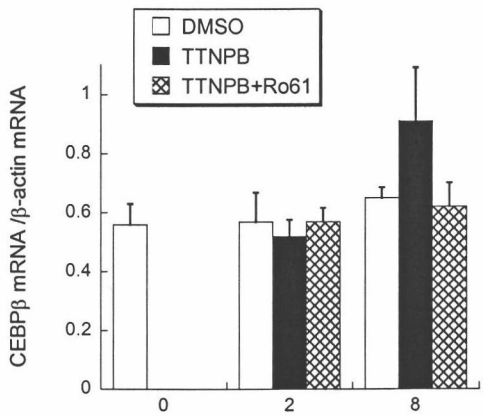

A
DMSO
TTNPB
TTNPB+Ro61
aP2 mRNA / β-actin mRNA
B
PPARγ mRNA / β-actin
C
RXRα mRNA / β-actin mRNA
D
Add1 mRNA / β-actin mRNA
E
RARα mRNA / β-actin mRNA
F
COUP-TF1 mRNA / 36B4 mRNA
Day of Treatment
Day of Treatment

Figure 4.4

A



B

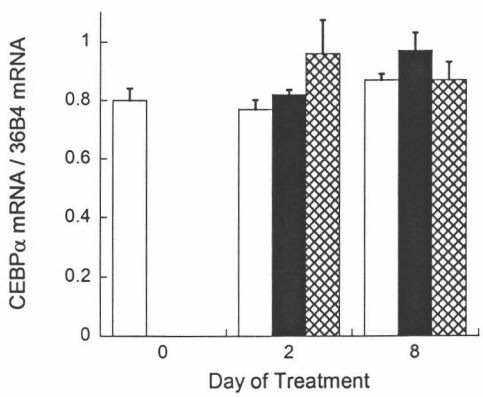


Figure 4.5. Effect of TTNPB on the expression of A. C/EBPβ, and B. C/EBPα mRNA on d 0, 2, and 8 post-induction in primary cultures of porcine S-V cells. Stromal-vascular cells were treated with either DMSO, .1 nmol/L TTNPB, or .1 nmol/L TTNPB plus 1 μmol/L Ro61 from d 0 to d8. Total RNA was isolated on the indicated days and mRNA expression was measured using either northern blotting or semiquantitative RT-PCR (see Materials and Methods section for details). Values are means +/- SEM, n=3 (each replicate for RT-PCR data represents the mean of 3 repeated PCR reactions). Data was analyzed using two-way analysis of variance with treatment and day as the main effects. Day was not significant for any gene. Treatments with an asterisk were significantly different that control, $P < .05$.

Discussion

Suryawan and Hu (1997) determined that retinoic acid was a potent inhibitor of pig preadipocyte differentiation in primary culture however the underlying mechanism was not elucidated. The present study provides direct evidence that retinoids inhibit porcine preadipocyte differentiation by a mechanism that involves activation of the RAR receptor and the down regulation of PPARγ, RXRα, and ADD1 mRNA but is independent of changes in CAAT-Enhancer Binding Protein (CEBP) mRNA abundance. Furthermore, an up regulation of COUP-TF1 mRNA abundance is correlated with retinoid-induced inhibition of adipogenesis suggesting that COUP-TF1 may play a role in the antiadipogenic action of retinoids.

Using receptor-specific ligands, we determined that RAR and not RXR receptors mediate the inhibitory action of ATRA upon the differentiation of pig preadipocytes in primary culture. Retinoid receptors can be classified into two families consisting of the retinoic acid receptors (RAR), and the retinoid X receptors (RXR; Leid et al., 1992). We have demonstrated that RARα is expressed in porcine adipocytes for the first time. Since RXRα mRNA is also expressed in porcine adipose tissue (McNeel et al., 2000), the possibility exists that ATRA may signal through either receptor system. Thus, RAR- or RXR-specific retinoids were utilized in order to identify which retinoid receptor pathway mediated the antiadipogenic activity of retinoids. The RAR-specific retinoid, TTNPB, was much more effective at inhibiting adipocyte differentiation compared to either ATRA or 9cis-RA as determined by

GPDH activity (Figure 4.1). Furthermore, the antiadipogenic activity of TTNPB was blocked by addition of the RAR-selective antagonist, Ro-61 (Figure 4.2). In contrast to RAR agonists, the RXR-specific retinoid, methoprene acid, stimulated the differentiation of pig preadipocytes (Figure 4.1). These data indicate that the antiadipogenic action of retinoids is likely mediated by the RAR receptor system. This observation is consistent with a previous study demonstrating that RAR receptors mediated the antiadipogenic effect of ATRA in mouse-derived 3T3-L1 preadipocytes (Xue et al., 1996). Importantly, these results implicate genes with RAR-responsive promoters as a new class of genes that may negatively regulate adipose tissue development in the pig.

Our second objective was to investigate the effect of retinoid administration upon the expression of adipogenic transcription factors that are known to regulate adipogenesis. In the current model, the sequential induction of C/EBPβ, PPARγ and C/EBPα in preadipocytes results in transactivation of adipocyte-specific genes leading to terminal differentiation of the adipocyte (Lazar, 2002). During this process, ligand-activated PPARγ forms a heterodimer with RXRα resulting in the induction of PPARγ-responsive genes. Work in 3T3-L1 preadipocytes indicates that ATRA inhibits adipogenesis by down regulating PPARγ and C/EBPα protein while not preventing the initial induction of C/EBPβ (Schwarz et al., 1997). These changes seem to be the result of the down regulation of gene expression as subsequent studies have indicated that ATRA treatment decreases the mRNA abundance of the PPARγ, C/EBPα, RXRα and RARα but not C/EBPβ genes in 3T3-L1 preadipocytes (Chen et

al., unpublished data; Kawada et al., 2000). In the present study, TTNPB decreased the expression of PPARγ, ADD1/SREBP, RXRα and aP2 mRNA transcripts in pig preadipocytes but surprisingly these changes were independent of alterations in the mRNA abundances of the RARα and C/EBPα genes. Murine-derived 3T3-L1 cells represent a homogeneous population of preadipocytes that are uniformly at the same stage of commitment. Primary cultures of S-V cells represent a heterogeneous population of cells at varying stages of differentiation. Thus, differences between the two culture systems might be responsible for the divergent regulation that was observed in response to ATRA in 3T3-L1 preadipocytes and primary cultures of porcine preadipocytes. Alternatively, species-specific differences in the regulation of adipocyte differentiation may exist.

The down-regulation of PPARγ and ADD1 in response to retinoids is consistent with the current model of adipogenesis. PPARγ is now considered the master regulator of adipocyte differentiation while C/EBPα is thought to potentiate differentiation by up regulating genes that confer insulin sensitivity upon the adipocyte (Hamm et al., 1999; Lazar, 2002; Rosen et al., 2002; Wu et al., 1999). ADD1 is believed to potentiate adipogenesis both by up regulating PPARγ expression and indirectly by increasing availability of ligands for PPARγ through up regulating genes involved in lipid metabolism (Kim et al., 1998; Fajas et al., 1999). Thus, it is conceivable that adipogenesis could be effectively inhibited through a mechanism that is independent of effects upon the expression of either C/EBPβ or C/EBPα especially if the mechanism targets PPAR-γ. Given that the differentiation of pig preadipocytes

was dramatically inhibited in the absence of significant changes in expression of C/EBPα, these results suggest that C/EBPα may play only a minor role in regulating adipogenesis in the pig while PPARγ and ADD1 likely play more significant roles.

In the present study, TTNPB increased the expression of COUP-TF mRNA concomitant with the inhibition of preadipocyte differentiation suggesting for the first time that COUP-TF1 may play a role in the antiadipogenic action of retinoids in pig preadipocytes. Chicken Ovalbumin Upstream Promoter Transcription Factor (COUP-TF) is an orphan nuclear receptor that has been implicated in the negative regulation of differentiation in multiple cell types (Tsai and Tsai, 1997). Importantly, COUP-TF can compete with PPARγ for both dimerization with RXR receptors and for binding to sites (DR-1) in the promoter regions of target genes (Tsai and Tsai, 1997). Given that PPARγ must bind to RXR in order to be transcriptionally active, even slight changes in the kinetics of RXR binding would be expected to significantly impact the transcriptional activity of PPARγ. Furthermore, initiation is the rate-limiting step in transcription. Thus, DNA binding of PPARγ to response elements in target genes represents a critical point in the regulation of gene transcription by PPARγ and competition for DNA binding sites could be expected to significantly decrease the transcriptional activity of PPARγ. In this regard, Brodie et al. (1996) observed that ATRA both induced the expression of COUP-TF and increased the binding of COUP-TF to a PPARγ binding sequence transfected into 3T3-L1 preadipocytes. In the present study, TTNPB increased the expression of COUP-TF mRNA and this effect was blocked by the addition of the RAR receptor antagonist, Ro-61, in a manner that

was parallel to the ability of retinoids to inhibit the differentiation of pig preadipocytes. Given the importance of PPARγ in regulating adipogenesis and the fact that COUP-TF can compete with PPARγ both for dimerization with RXR and for the binding to DNA binding sites, it seems likely that the up regulation of COUP-TF plays a role in the mechanism by which retinoids inhibit the differentiation of porcine preadipocytes.

These results collectively suggest a mechanism for the antiadipogenic action of retinoids in pig preadipocytes. In this model, retinoids with antiadipogenic activity activate RAR receptors leading to the direct inhibition of adipogenesis through the down regulation of PPARγ, ADD1and RXRα gene expression. Retinoids also indirectly inhibit differentiation through the up regulation of COUP-TF1 which competes with existing PPARγ protein for dimerization with RXRα and the binding to DR-1 sites within the promoters of target genes.

CHAPTER 5
EFFECT OF FEEDING RETINOIC ACID ON GROWTH AND CARCASS ADIPOSITY OF BROILERS

Abstract

All-trans retinoic acid (ATRA) can potently inhibit adipocyte differentiation in vitro. However, the ability of RA to limit adipose tissue accretion in meat producing animals has not been investigated. Thus, the objective of this study was to determine the effect of feeding ATRA on adiposity in growing broiler chickens. In the first experiment, 48 one-day-old birds were fed either a commercial starter-grower diet or the commercial diet supplemented with 14 mg/kg ATRA for six weeks. Retinoic acid decreased weight gain (13%; $P < .001$), feed intake (10%; .0003), average daily gain (12%; .0001), and carcass weight (13%; $P < .0001$). While ATRA only tended to decrease abdominal fat mass (12%; $P < .07$), total carcass lipid was significantly decreased by 14% versus control birds ($P < .008$). In the second experiment, 36 one-day-old chicks were randomly assigned to either the control or ATRA-supplemented diet (14 mg/kg) and six chicks from each treatment were sacrificed on days 14, 28, and 42. Plasma, liver and adipose tissue retinoic acid concentrations were determined using HPLC. Liver retinoic acid concentrations were three-fold higher in birds consuming the ATRA-supplemented diet ($P < .02$) versus birds fed the control diet. Retinoic acid supplementation did not significantly affect RA concentration in adipose tissue ($P < .21$). However, retinoic acid concentrations were numerically lower in birds fed the ATRA-supplemented diet on each day measured. These results suggest that feeding ATRA can reduce carcass adiposity. However this approach may be

limited by physiological mechanisms that constrain retinoic acid accumulation in adipose tissue. Better characterization of these mechanisms may reveal targets that could be useful for reducing carcass fat in growing broilers either through selection programs or the administration of exogenous effectors.

KEY WORDS: Retinoic Acid, Broiler, Carcass Fat

Introduction

Worldwide there has been a proportional decline in the number of processed whole birds and an increase in the amount of further-processed broilers. With increasing demand for further-processed poultry products, there is now considerable pressure to reduce fat content in the broiler carcass and to increase lean meat yield. Traditional selection practices have emphasized rapid growth (i.e. total pounds of meat) without consideration of carcass composition. Since abdominal fat increases with body weight in broilers, blind selection for increased growth rate has also inadvertently led to birds that contain considerable amounts of carcass fat. As the industry has changed to emphasize percentages of parts (i.e. breast meat yield), carcass fat has become a less valued commodity. Since increased body fat is usually associated with poorer feed conversion, it is now estimated that excess fat production in the modern broiler industry accounts for an annual loss to the poultry industry of 800 to 950 million dollars annually (Rosebrough et al., 1999).

In growing broilers, adipose tissue develops in discreet anatomical locations of the carcass and the deposition of fat in these locations is related to the age and

growth rate of the bird. Chickens have relatively low amounts of intramuscular fat compared to other economically important production animals. While the leg and neck depots are considerably more developed than abdominal fat in broilers posthatch, the abdominal fat pad has a greater growth rate than the neck and leg depots during the growing period and this depot accounts for the greatest percentage of carcass fat at market weight (Butterworth, 1989). Based upon [^{3}H] Thymidine incorporation studies, it is thought that adipocyte hyperplasia is a major contributor to the growth of abdominal fat up to 7 weeks post-hatch (Butterworth, 1996). Therefore strategies that inhibit adipogenesis may be effective at limiting carcass adiposity in growing broilers.

Several studies have now demonstrated that RA potently inhibits the differentiation of both clonal 3T3-L1 preadipocytes (Chawla and Lazar, 1994; Chen 1996; Xue et al., 1996) and pig preadipocytes in primary culture (Suryawan and Hu, 1997; Brandebourg and Hu, 2003). This inhibitory action on porcine-derived preadipocytes suggests that feeding RA may inhibit fat cell differentiation in growing animals. Though the potential of RA to limit adipose tissue accretion in meat producing animals is largely unexplored, this possibility is supported by a unique practice in the Japanese beef industry where marbling is increased in steers that are deliberately fed vitamin A-deficient diets (Takeyama et al., 1996). Thus, the objective of this study is to determine if feeding RA can effectively reduce adiposity in growing broilers.

Materials and Methods

Animals and Design. All animals were handled according to guidelines approved by the Animal Care and Use Committee at Oregon State University. Ninety

one-day-old chicks were purchased from a commercial supplier (Jenks Hatchery Inc, Tangent, OR.) and housed in groups for seven days with ad libitum access to water and Standard Purina Mills Start and Grow MP (Table 5.1; Purina Mills, St Louis, MO). On day 8, chicks were then weighed, housed in individual cages, and randomly assigned to either treatment 1 (commercial diet) or treatment 2 (the commercial diet supplemented with 14 mg/kg all-trans retinoic acid). Room temperature was maintained at 90° F during week 1 and lowered 5 ° F each week until finally maintained at 75 F. Birds were maintained in continuous light (23 hours light: 1 hour dark per day) for weeks 1 and 2 and switched to 12 hours of light per day from weeks 3 through 6. Retinoic acid was purchased from BIOMOL (Plymouth Meeting, PA). Retinoic acid was handled under yellow light and dissolved in 100 ml corn oil and then mixed into the commercial ration. Since Retinoic acid is highly labile, rations were mixed weekly and stored in the absence of light at 0° C until distributed to birds. In the first experiment, 48 chicks were randomly assigned to either treatment 1 or 2 (n = 24). Birds were given ad libitum access to water and feed. Body weight gain and feed intake will be recorded biweekly until day 42 when all birds were stunned via electric shock and sacrificed. Upon death, birds were scalded for 1 minute and defeathered. Abdominal fat (considered to be fat extending within the ischium, surrounding the cloaca, and adjacent to the abdominal muscle) was immediately dissected from the viscera and abdominal fat mass was recorded. Internal organs were removed from the viscera and examined for potential lesions related to retinoic acid toxicity. Birds were sexed based upon the presence or absence of testis. The feet and

head were removed from the carcass and carcass weight was recorded. Abdominal fat and organs were replaced in the carcass and the bird was stored at -20° C until lipids were extracted for determination of total carcass lipid. In the second experiment, 36 chicks were randomly assigned to either treatment 1 or 2. A serial slaughter protocol was utilized in which six chicks from each treatment were sacrificed as described above on days 14, 28 and 42 (n=12 per time point). Six chicks were sacrificed on day 7 for establishment of baseline values. Feed intake and body weight were recorded for each time point. Blood plasma was collected prior to death under yellow light. Liver and adipose tissues were rapidly collected following death, minced, quickly frozen in liquid nitrogen, and subsequently stored at -80° C.

HPLC Procedures. Retinoic acid was analyzed using reverse-phase HPLC following the procedures of Wang et al. (1991). Plasma, liver and adipose tissues were extracted under yellow light without saponification using the following method. One hundred milligrams of tissue was extracted by adding 150 µl water, 107 µl 2 M NaOH, 50 µL of the internal standard, Ro 13-6307 (100 ng), and 500 µl hexane and subsequently vortexing for ten minutes. Samples were then centrifuged for 5 minutes at 1500 X g. The organic layer was re-extracted by adding 150 µL 2 M HCL, 215 µL ETOH, and 500 µL hexane then vortexing for ten minutes followed by centrifugation for 5 minutes at 1500X g. The organic layer was evaporated to dryness under N_2 at 37° C and the residue was re-dissolved in 200 µl methanol. Three ml of plasma were extracted by scaling the above procedure up by a factor of 6 while re-dissolving the final residue in 200 µl methanol. A 50-µl aliquot of the final extract was injected into

the HPLC system. The HPLC system consists of a Waters 2690 Separation Module (Waters Chromatography, Milford, MA), a Waters Nova-Pak C18 3.9 x 150 mm steel column and a Waters 996 Photodiode array detector set at wavelength of 350 nm. The column was a Waters Nova-Pak C18. The mobile phase was 100% methanol (solvent B) and H_2O (solvent A). The gradient procedure was as follows: flow rate was .8 ml/min with 90% solvent B and 10% solvent A followed by a one minute linear gradient to 1.5 ml/min 100% solvent B, a 18 minute hold at 100% solvent B then a one minute linear gradient back to 10% solvent A, 90% solvent B and a final 5 minute hold. Column temperature was 35 ° C. Retinoic acid was quantified by determining peak areas calibrated against known amounts of standards as calculated by the Millenium[32] software (version 3.05.01). The lowest limit of detection was .2 pmol. Representative chromatographs are depicted in Figure 5.1.

Total Carcass Lipid. Carcasses were thawed and placed in a 3 liter beaker containing 1 liter of water. The beaker was tightly sealed and autoclaved at 121°C (15psi) for 2.5 h. When cooled, the contents were homogenized in a 4 L Waring Heavy Duty Laboratory blender at 20,000 rpm for 5 min. The resulting slurry was stored at stored at -20°C until lipid analysis. Approximately 2-g samples were accurately weighed into a 50-mL test tube. After adding 18 ml of Folch 1 (chloroform:methanol=2:1 w/v, Folch et al., 1957), the samples were vortexed for thirty seconds and held at room temperature for 6 hours. Then 4 ml 0.88% NaCl was added and tubes were gently inverted followed by centrifugation at 2000 rpm for ten minutes. Following centrifugation, 3 ml of the chloroform layer was pipetted onto

pre-weighed aluminum pans in duplicate. The chloroform was gently evaporated off and pans reweighed. Percent total lipid was calculated as follows [(pan +sample weight-pan weight) * 12 ml chloroform* 100]/ [3 ml *sample weight].

RNA Isolation. Cells were harvested with a cell scraper and total RNA was extracted using the quanidinium-acid phenol method (Chomczynski and Sacchi, 1987). Total RNA concentration was determined spectrophotometrically using A_{260} and A_{280} measurements. The ratio of A_{260} / A_{280} was between 1.9 and 2.1 for all samples. Five micrograms of total RNA from each sample was separated on a 1.2% denaturing formaldehyde gel and stained with ethidium bromide. The RNA integrity was assessed visually by judging the quality of 18 and 28s rRNA bands.

Semiquantitative RT-PCR. Reverse transcription (RT) reactions (20 μL) consisted of 4 μg of total RNA, 50 U SuperScript II reverse transcriptase (Invitrogen/Life Technologies), 40 U of an RNAse inhibitor (Invitrogen/Life Technologies), 0.5 mmol/L d NTP, and 100 ng random hexamer primers. Polymerase chain reaction (PCR) was performed in 50 μL containing 20 mmol/L Tris-HCL, pH 8.4, 50 mmol/L KCL, 1.0 μL of RT reaction, 2.5 U of Platinum Taq DNA polymerase (Hot Start, Invitrogen/Life Technologies), 0.2 mmol/L dNTP, 2 mmol/L Mg^{2+} (Invitrogen/Life Technologies), 10 pmol each of gene specific primers and 10 pmol each of primers specific for either β-actin or 36B4. Thermal cycling parameters were as follows: 1 cycle 94°C for 4 min, followed by 26-30 cycles, 94°C for 1 min, 56°C for 2 min, 72 °C for 2 min with a final extension at 72°C for 8 min. Primers were synthesized at the Center for Gene Research at Oregon State University. Identity of

PCR products was verified either by restriction digest analysis or via DNA sequencing. Cycle number for each multiplex PCR reaction was selected by experimentally determining the highest cycle number in which the amplification of both cDNA products was within a linear range. The optimal cycle number was then considered to be two cycles lower than the highest cycle of linearity. RT-PCR products were visualized by separating DNA on a 3% agarose gel and staining with CyberGreen according to the manufacturer's directions (Molecular BioProbes, Eugene, OR). Primer sequences, product size and cycle length are listed in Table 5.2. Data for each replicate represented the mean of three individual RT-PCR reactions.

Statistical Analysis. Data are expressed as the mean +/- SEM. Birds were individually housed in cages. Thus, the experimental unit is individual bird. Data were analyzed by using the analysis of variance procedure followed by multiple comparisons of means with Fisher's least significant difference using SAS (SAS institute, Cary, NC). Differences were considered significant if $P < .05$.

TABLE 5.1

Composition of broiler diets[1,2]

Ingredient	g/100g diet
Crude Protein	17.00
Lysine	.85
Methionine	.30
Crude Fat	3.00
Crude Fiber	5.00
Calcium (Ca)	1.00
Phosphorus (P)	.60
Salt (NaCl)	.60

[1] Standard Purina Mills® Start & Grow MP 0.0125%

[1] Medicated with .0125% Amprolium as an aid in prevention of Coccidiosis

[2] Ration processed as a crumble

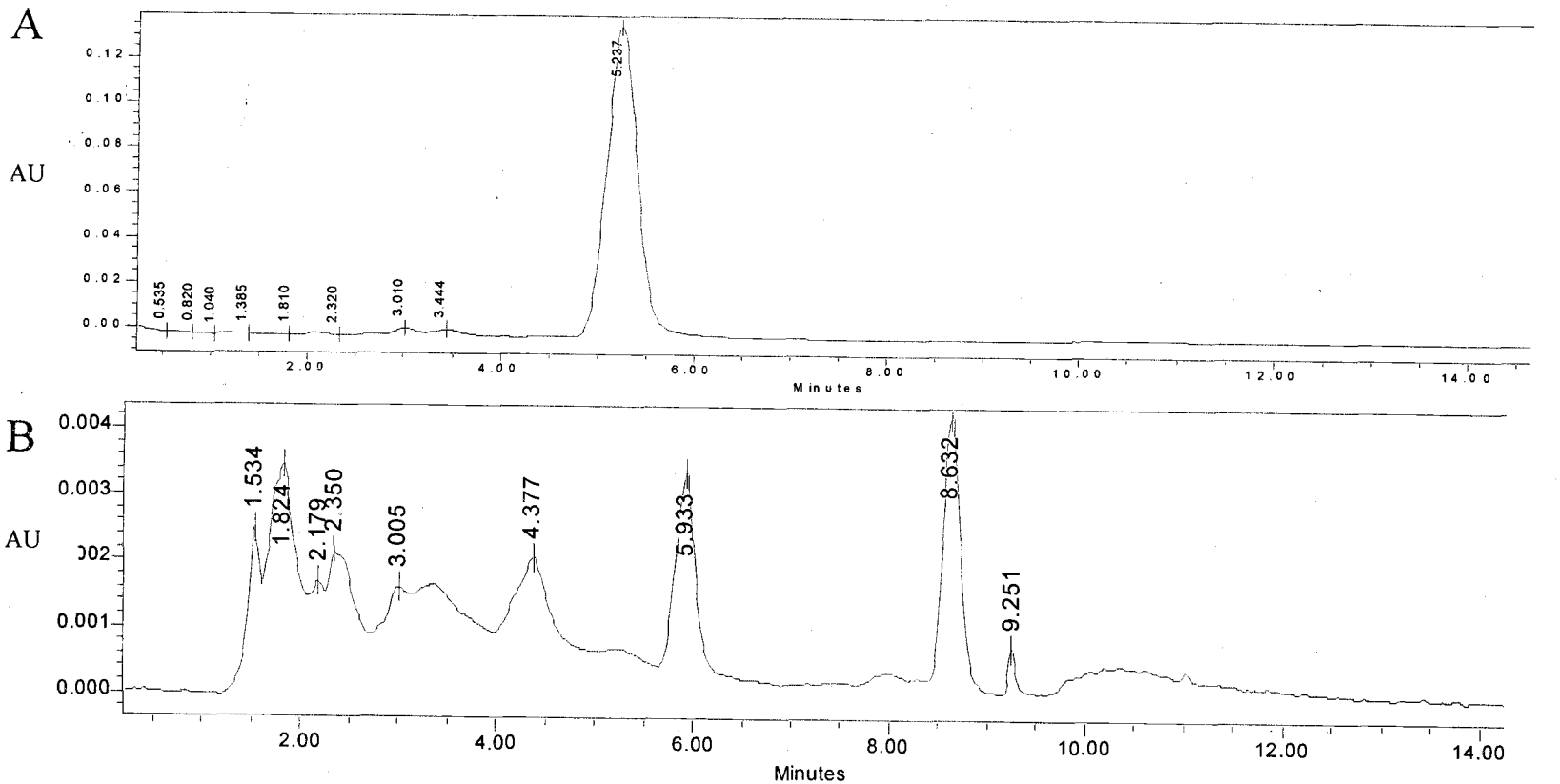


Figure 5.1. Representative chromatographs depicting elution time of A. TTNPB standard, B. retinoic acid extracted from liver sample. Extraction and detection were performed as indicated in the Materials and Methods.

Table 5.2
Oligonucleotide PCR primers

Gene	Accession #	Primer Sequence (5'→3')	Orientation	Product size (bp)	Multiplex PCR Cycle
β-actin	AF054837	CGTGGGCCGCCCTAGGCACCA TTGGCCTTAGGGTTCAGGGGGG	Forward Reverse	210	-
36B4	BC011291	GCACTCTCGCTTTCTGGAGGGTGTC TGACTTGGTTGCTTTGGCGGGATTAG	Forward Reverse	292	-
COUP-TF	X16155.1	CCCACTTTGAGGCACTTCT ACATCGAGTGCGTGGTGT	Forward Reverse	201	30
RARα	U82260	GCATCCAGAAGAACATGGTGT CCTGCTTGGCGAACTCCACAGT	Forward Reverse	392	26

Results and Discussion

Retinoic acid potently inhibits preadipocyte differentiation in both murine-derived clonal preadipocytes (Chawla and Lazar, 1994; Xue et al., 1996) and primary cell culture models encompassing sheep (Torii et al., 1995), pigs (Suryawan and Hu, 1997), and cattle (Ohyama et al., 1998). These results suggest that feeding retinoic acid may inhibit fat cell differentiation in growing animals. However, the effect of RA supplementation upon adiposity in production animals has never been directly investigated. Thus, the primary objective in this study was to determine if feeding ATRA could decrease adiposity in growing broilers.

Since the effect of feeding ATRA to broilers had never been investigated, the first objective in this study was to establish a dose-response curve to identify the optimal dose of ATRA that, when fed to growing broilers from d7 to d 42, could affect adiposity while avoiding confounding effects of toxicity. Forty broilers were randomly assigned to one of 5 levels of ATRA (0, 3.5, 14, 28 mg/kg; n=8) based upon the NRC recommendations for vitamin A supplementation in broiler rations. As shown in Table 5.3, when female broilers were exposed to increasing concentrations of ATRA, there was a numeric trend for abdominal fat mass to be decreased as birds chronically fed the highest dose of ATRA had 50% less abdominal fat than broilers fed the control diet. This trend was less dramatic in male broilers (Table 5.4). Importantly, though weight gain, feed intake, and ADG numerically decreased at doses higher than 7 mg/kg, feed conversion was not dramatically higher suggesting that ATRA may decrease adiposity and toxicity would not problematic at doses lower

than 14 mg/kg (Tables 5.3, 5.4). Using these data, a level of 14 mg/kg ATRA was selected to conduct the subsequent growth trials.

In order to further test the possibility that feeding ATRA to growing broilers could limit adiposity, 48 1-day-old broilers were randomly assigned to either the control basal ration (Table 5.1) or the basal ration supplemented with 14 mg/kg ATRA. Diets were fed until d 42 and growth performance summarized in Table 5.5. Female broilers are known to have a higher percentage of carcass fat and greater quantities of abdominal fat compared to male broilers at a given age. As expected, female birds had more abdominal fat than their male counterparts (21%; $P < .003$) and abdominal fat was a higher percentage of carcass weight in female birds versus males (25%; $P < .0003$). Feeding 14 mg/kg ATRA decreased weight gain (13%; $P < .001$), feed intake (10%; .0003), average daily gain (12%; .0001), and carcass weight (13%; $P < .0001$) irrespective of sex. While ATRA only tended to decrease abdominal fat mass (12%; $P < .07$), total carcass lipid was significantly decreased by 14% versus control birds ($P < .008$; Figure 5.2). There was no effect of RA upon feed conversion though females fed RA converted feed more efficiently than untreated females. Results of our study suggest that feeding ATRA can significantly decrease carcass adiposity as total carcass lipid was decreased 14% and abdominal fat mass was decreased 10%.

These results are consistent with several studies that have examined the effect of vitamin A upon body adiposity in mammals. Feeding elevated levels of vitamin A decreases adiposity. A 9% decrease of adiposity was reported in F-344xBN rats

following supplementation of their diets with 50 fold higher levels of vitamin A (Kumar et al. 1999). A 46% decrease in body fat content was observed in male NMRI mice fed 100 mg ATRA/kg body weight for 4 days versus control mice (Ribot et al., 2001) and these changes were correlated with the downregulation of PPARγ mRNA in white adipose tissue depots. Several studies have also examined the effect of vitamin A upon body adiposity by feeding vitamin A deficient diets. Ribot et al. (2001) reported that mice fed a diet containing vitamin A at less than 7% of daily minimum requirements had 63% greater epididymal and inguinal WAT masses versus control animals. In Japan, beef cattle that have been fed vitamin-A deficient diets have greater marbling (Takeyama et al., 1996) and low serum retinol concentrations have been correlated with higher marbling in beef (Nakai et al., 1992; Oka et al., 1992; Tori et al., 1996). Given the implication that ATRA can inhibit preadipocyte differentiation in both clonal and primary culture cell models and hypertrophy of adipose tissue is associated with consumption of vitamin A-deficient, it seems likely that vitamin A regulates body adiposity through direct effects upon adipose tissue development (i.e. adipogenesis). Our results suggest for the first time that feeding elevated levels of ATRA to growing broilers can decrease carcass adiposity.

The depression of several indices of growth performance (Table 5.5) suggest that chronically feeding 14 mg/kg ATRA was associated with toxicity though no gross signs of severe toxicity were observed. The decrease in economically important parameters of growth performance is undesirable and any feed additive that has such an effect would be avoided by the broiler industry. Given the ability of 14 mg/kg of

ATRA to decrease adiposity in broilers and the indication that 7 mg/kg decreased abdominal fat mass without undesirable impacts upon performance (Table 5.3), it is very possible that feeding a dose of ATRA lower than 14 mg/kg could effectively reduce carcass adiposity while avoiding negative side effects. Thus, further trials investigating the impact of ATRA in growing broilers are warranted.

Given that the efficiency of ATRA absorption is poorly characterized in growing broilers, the second objective of this study was to determine how supplementing diets with ATRA effected the concentrations of retinoic acid in plasma, liver, and adipose tissue. Retinoic acid primarily acts through regulating gene expression in a process involving activation of nuclear retinoic acid receptors which then bind DNA and regulate gene transcription (Sporn et al., 1984; Green and Champon, 1988; Mangelsdorf et al, 1994). Thus in order for retinoic acid to exert an effect upon a tissue, it is essential that retinoic acid be present in the target cell. Studies in ruminants have indicated that the efficiency by which vitamin A is absorbed can dramatically decrease as levels of vitamin A increases in a ration (Wing et al., 1969; Donoghue et al., 1983). This suggests the possibility that the absorption of retinoic acid may have been a limiting factor in the present study. However, as shown in Table 5.6, overall retinoic acid concentrations were three-fold higher in livers of birds consuming the ATRA-supplemented diet versus birds fed the control diet ($P < .02$). Since droppings were not collected, it is not possible to directly quantify the absorption efficiency of retinoic acid in the present study. However, the three-fold enrichment of the liver indicated that birds fed the supplemented diets indeed absorbed

significant amounts of ATRA suggest that absorption was not a limiting factor. Furthermore, the significant effects of supplementation upon growth performance observed in the present study supports the conclusion that birds were able to absorb significant amounts of ATRA from the supplemented diet. Interestingly, while retinoic acid levels were elevated in the livers of treated birds, adipose tissue was not enriched by ATRA supplementation ($P < .21$; Table 5.7). In fact, retinoic acid concentrations were numerically lower in birds fed the ATRA-supplemented diet on each day measured. ATRA supplementation also failed to effect to mRNA abundances for the RARα or COUP-TF1 genes in the adipose tissue of treated broilers (Figure 5.3). This suggests that the potential for ATRA to inhibit fat accretion in growing broilers may have been limited by a physiological mechanism that regulates retinoic acid levels in adipose tissue.

Clinical studies suggest that barriers may exist which could limit the effect of supplementing retinoic acid in broiler diets by creating a state of "retinoic acid resistance" in adipose tissue. The activity of retinoic acid in a given tissue is determined at a number of levels (Luu et al., 2001). First, the expression of retinoic acid receptor levels is vital to the activity of retinoic acid. Alterations in the levels of retinoic acid receptors could have dramatic effects on the ability of retinoic acid to regulate processes in a given tissue since binding of retinoic acid to its receptor is an essential first step in retinoic acid action. Second, cellular retinol-binding proteins (CRBPs) are required for the efficient conversion of retinol to retinoic acid in peripheral tissues. Once inside the cell, CRBP proteins rapidly bind retinol. Noy and

Blaner (1991) have demonstrated that cellular uptake is actually dictated by the intracellular content of apo-CRBP (CRBP which is free of retinol). In their model, retinol uptake would increase in response to anything that increased the amount of apo-CRBP content. Thus a decrease in CRBP expression would limit tissue uptake of retinol and ultimately limit tissue retinoic acid levels. Furthermore, the control of RA levels in a peripheral tissue is apparently regulated by a balance between RA synthesizing and catabolizing enzymes. Since retinol is irreversibly oxidized to retinoic acid in cells, retinoic acid catabolism governs tissue sensitivity to retinoic acid. Several studies indicate that retinoic acid catabolism is induced by high levels of retinoic acid and this can result in the development of retinoic acid resistance in peripheral tissues and cell lines such as 3T3-L1 preadipocytes (Frolik et al., 1979; Roberts et al., 1979; Duell et al., 1992; Luu et al., 2001). This effect is primarily believed to occur via a retinoic acid-induced increase in CYP26 (cytochrome P450 enzyme; P450AI) expression and activity (Luu et al., 2001).

Better characterization of the mechanisms that limit retinoic acid accumulation in the adipose tissue of growing broilers may reveal targets that could be useful for reducing carcass fat in growing broilers either through selection programs or the administration of exogenous effectors. Interestingly, a CYP26 homolog has been characterized in chicks (White et al., 1997). It may be possible to limit carcass adiposity through formulating rations containing CYP enzyme inhibitors thus increasing the impact of dietary vitamin A upon adiposity. Furthermore, CYP

enzymes may prove to be useful markers that can be exploited to select for leaner birds through

This study demonstrates for the first time that feeding ATRA to growing broilers can reduce carcass adiposity and suggests that the further study of ATRA supplementation in broiler diets is warranted while identifying potential new strategies for limiting adiposity in growing animals.

TABLE 5.3

Growth performance of female broilers fed control and retinoic acid supplemented diets[1]

Variable	Control	3.5 mg/kg	7 mg/kg	14 mg/kg	28 mg/kg	P-value
Initial BW[2], kg	.148±.008	.153± .007	.160±.008	.151±.009	.136±.009	.46
Weight Gain, kg	1.83±.13	1.79±.10	1.81±.12	1.44±.13	1.43±.17	.14
Feed Intake, kg	3.50±.23	3.55±.18	3.56±.21	3.03±.23	2.83±.31	.25
ADG[3], kg	.052±.004	.051±.003	.052±.003	.041±.004	.048±.004	.13
Feed conversion[4]	1.44±.04	1.49±.03	1.50±.02	1.50±.02	1.51±.03	.57
Abdominal Fat, kg	.031±.005	.027±.004	.023±.005	.020±.005	.015±.007	.40
Carcass Weight	1.37±.11	1.35±.08	1.38±.10	1.07±.02	1.05±.15	.16
% abdominal fat[5]	2.19±.31	1.87±.24	1.59±.29	1.84±.31	1.43±.42	.05
n	4	6	4	4	3	-

[1] Values are group mean ± SEM
[2] Initial body weight
[3] Average daily gain
[4] Feed conversion expressed as feed intake divided by weight gain
[5] Percent abdominal fat expressed is abdominal fat mass expressed as a percent of carcass weight

TABLE 5.4

Growth performance of male broilers fed control and retinoic acid supplemented diets[1]

Variable	Control	3.5 mg/kg	7 mg/kg	14 mg/kg	28 mg/kg	P-value
Initial BW[2], kg	.163±.01	.135± .01	.156±.008	.156±.009	.155±.011	.40
Weight Gain, kg	1.99±.16	1.70±.18	1.95±.12	1.75±.14	1.60±.11	.21
Feed Intake, kg	3.68±.2	3.71±.23	3.81±.15	3.65±.17	3.23±.13	.07
ADG[3], kg	.057±.03	.048±.005	.056±.003	.050±.004	.046±.003	.20
Feed conversion[4]	1.47±.03	1.48±.03	1.48±.02	1.54±.02	1.46±.03	.39
Abdominal Fat, kg	.021±.005	.021±.005	.023±.003	.020±.004	.015±.003	.39
Carcass Weight	1.46±.12	1.27±.14	1.51±.09	1.35±.11	1.22±.09	.27
% abdominal fat[5]	1.45±.22	1.55±.25	1.51±.17	1.41±.19	1.18±.15	.05
n	3	2	4	4	5	-

[1] Values are group mean ± SEM
[2] Initial body weight
[3] Average daily gain
[4] Feed conversion expressed as feed intake divided by weight gain
[5] Percent abdominal fat expressed is abdominal fat mass expressed as a percent of carcass weight

TABLE 5.5

Growth performance of broilers fed control and retinoic acid supplemented diets (14 mg/kg) [1]

	Male		Female			P-value		
Variable	Control	RA	Control	RA	SE	sex	trt	Sex*trt
Initial BW[2], kg	.12	.109	.118	.116	.003	.48	.07	.27
Weight Gain, kg	1.43	1.29	1.42	1.22	.03	.19	.0001	.33
Feed Intake, kg	2.1	1.91	2.09	1.87	.05	.68	.0003	.78
ADG[3], kg	.041	.037	.041	.035	.001	.29	.0001	.47
Feed conversion[4]	1.47	1.48	1.48	1.54	.022	.21	.15	.32
Abdominal Fat, kg	.027	.023	.033	.03	.002	.003	.07	.86
Carcass Weight	1.49	1.31	1.44	1.25	.03	.07	.0001	.96

[1] Values are group mean, n=12 for male control, n=7 for male RA, n= 11 for female control, n=12 for female RA

[2] Initial body weight

[3] Average daily gain

[4] Feed conversion expressed as feed intake divided by weight gain

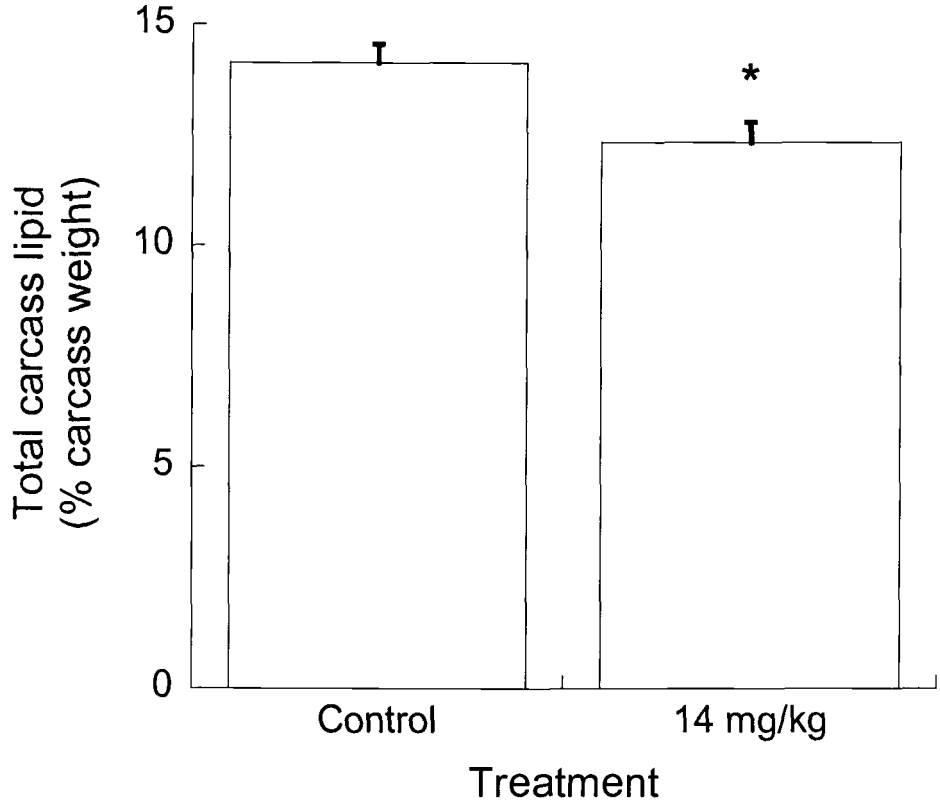


Figure 5.2. Feeding rations supplemented with 14 mg/kg ATRA daily decreases total carcass lipid (TCL) by 14% in broilers after d 42 of treatment. Total carcass lipid was determined as described in Materials and Methods and expressed as a percentage of final carcass weight. Values are means +/- SEM, n= 12. Data was analyzed using one-way analysis of variance with treatment as the main effect. Treatments with an asterisk were significantly different than control, $P < .01$.

TABLE 5.6

Concentration of retinoic acid in liver of broilers fed control and retinoic acid supplemented diets[1,2]

	Control	Retinoic Acid	P-value
Day 14	165 ± 41	128 ± 45	.61
Day 28	459 ± 168	1088 ± 168	.03
Day 42	610 ± 521	2239 ± 672	.1
Overall Response	429 ± 223	1241 ± 264	.02

[1] Values are ng per g tissue

[2] Values are mean ± SE, n=6 for day 14, n=12 for day 28, n= 8 for day 42 and n=26 for Overall Response

TABLE 5.7

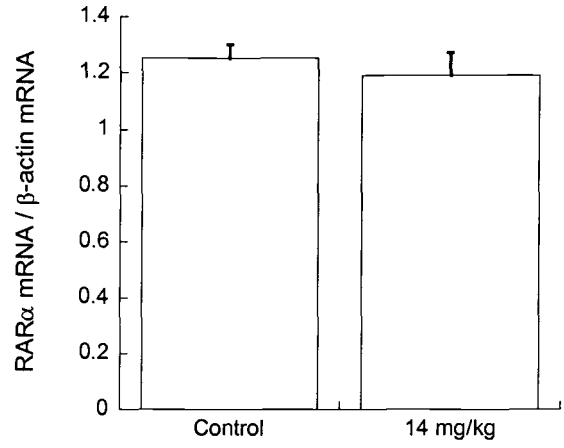
Concentration of retinoic acid in adipose tissue of broilers fed control and retinoic acid supplemented diets[1,2]

	Control	Retinoic Acid	P-value
Day 14	205 ± 41	193 ± 45	.84
Day 28	110 ± 20	78 ± 21	.30
Day 42	231 ± 46	142 ± 50	.22
Overall Response	182 ± 23	138 ± 26	.21

[1] Values are ng per g tissue

[2] Values are mean ± SE, n=12 for day and n=36 for Overall Response

A



B

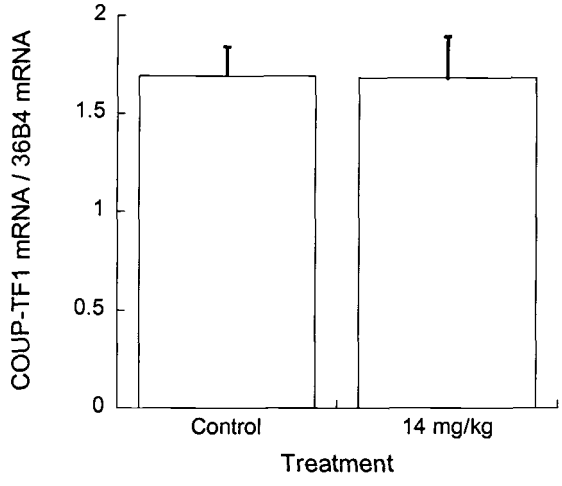


Figure 5.3. Effect of feeding 14 mg/kg ATRA on the expression of A. RARα and B. COUP-TF1 mRNA in adipose tissue of broilers after d 42 of treatment. Total RNA was isolated on d 42 and mRNA expression was measured using semiquantitative RT-PCR (see Materials and Methods section for details). Values are means +/- SEM, n=5 for control and n=4 for treated group (each replicate for RT-PCR data represents the mean of 3 repeated PCR reactions). Data was analyzed using one-way analysis of variance with treatment as the main effect. Treatments with an asterisk were significantly different than control, $P < .05$.

CHAPTER 6
CONCLUSION

This study was designed to investigate the nutritional regulation of adipocyte differentiation in production animals. The objectives were two-fold. First, primary cultures of pig preadipocytes were used both to screen for nutrients with antiadipogenic activity and as a model system to investigate the underlying regulatory mechanisms triggered by such inhibitors. It was hypothesized that negative regulators of adipogenesis would act upon similar regulatory pathways thus allowing the identification of a common protein that might act as a master regulator. Such a protein would represent a potential novel target for strategies aimed at limiting adipose tissue development in growing animals. Our second objective was to conduct a growth trial to test the ability of nutrients identified as antiadipogenic to limit carcass adiposity in growing broilers.

In the first experiment, it was determined that CLAs can inhibit the differentiation of pig preadipocytes in an isomer-specific manner as trans-10, cis-12 CLA but not cis-9, trans-11 CLA possessed antiadipogenic activity. The mechanism underlying this antiadipogenic action appears to involve both the down-regulation of PPARγ and ADD1 and the up-regulation of COUP-TF1 gene expression in pig preadipocytes. Furthermore, it was observed that cis-9, trans-11 CLA dampened the inhibitory activity of trans-10, cis-12 CLA. Collectively these data suggest that CLAs can limit carcass adiposity in part through inhibiting fat cell differentiation. Furthermore, supplements containing pure trans-10, cis-12 CLA would be more effective at limiting carcass adiposity. Currently CLAs are added to pig rations as a

crude CLA mix containing roughly equal amounts of trans-10, cis-12 CLA and cis-9, trans-11 CLA.

In the second experiment, the underlying molecular mechanism governing the anti-adipogenic action of retinoic acid was investigated using retinoic receptor-specific compounds. It was determined that retinoic acid activated the RAR family of receptors and subsequently downregulated the message abundances of PPARγ, RXRα and ADD1 while upregulating COUP-TF message expression. These changes occurred independent of changes in CAAT-Enhancer Binding protein expression.

This is the first study to identify COUP-TF1 as a potential regulator of adipogenesis in meat producing animals. These results collectively suggest a central regulatory mechanism which controls the differentiation of pig preadipocytes. In this model, antiadipogenic compounds down regulate of PPARγ and ADD1 gene expression. Such compounds likely also inhibit differentiation through the up regulation of COUP-TF1 which competes with existing PPARγ protein for dimerization with RXR-α and the binding to DR-1 sites within the promoters of target genes. C/EPB-α and -β do not appear to play a role in the regulatory mechanism which controls the differentiation of fat cells in pigs.

Finally this study tested the hypothesis that retinoic acid is a potent inhibitor of adipogenesis in vivo by feeding ATRA-supplemented rations to growing broilers and measuring the affect upon carcass adiposity and growth performance. Retinoic acid significantly decreased total carcass lipid by 14% and tended to decrease abdominal fat mass by 10% suggesting the feeding retinoids to growing broilers can reduce

carcass adiposity. Furthermore, mechanisms appear to exist which limit the ability to enrich adipose tissue with retinoic acid. Better understanding this mechanism could suggest genetic and pharmacological strategies to limit adipogenesis (i.e. yield targets manipulated through inhibitors or used as selection criteria in breeding programs).

In summary, using unrelated compounds each possessing antiadipogenic activity, these studies identified a common regulatory pathway governing the differentiation of pig preadipocytes that involves PPARγ and ADD1 but is independent of the C/EBP family of transcription factors. Furthermore, these studies are the first to identify COUP-TF1 as a novel negative regulator of adipogenesis in the pig. Finally, it was demonstrated that feeding ATRA (a compound that potently inhibits adipogenesis in vitro) to growing broilers likewise decreases carcass adiposity in vivo. Collectively these studies have suggested several new targets as potential regulators of adipose tissue development in growing animals.

BIBLIOGRAPHY

Ailhaud, G., Grimaldi, P., and Negrel, R. (1992) *Int J Obes Relat Metab Disord* **16 Suppl 2,** S17-21.

Ailhaud, G. (1992) *Diabetes Metab Rev* **8**(1), 3-7.

Ailhaud, G., Grimaldi, P., and Negrel, R. (1992) *Annu Rev Nutr* **12,** 207-33

Allee, G. L., Baker, D. H., and Leveille, G. A. (1971) *J Anim Sci* **33**(6), 1248-54.

Allee, G. L., Baker, D. H., and Leveille, G. A. (1971) *J Nutr* **101**(10), 1415-21.

Allee, G. L., O'Hea, E. K., Leveille, G. A., and Baker, D. H. (1971) *J Nutr* **101**(7), 869-78.

Allee, G. L. (1997) in *World Pork Symposium and Tour*, National Pork Producers Council, Indianapolis, Indiana

Allen, C. E., Beitz, C.D., Cramer, D.A. and R.G. Kauffman. (1976), North Central Regional Research Publications, University if Wisconsin-Madison

Anderson, D. B., and Kauffman, R. G. (1973) *J Lipid Res* **14**(2), 160-8.

Bachmeier, M., and Loffler, G. (1997) *Eur J Biochem* **243**(1-2), 128-33.

Barua, A. B., and Olson, J. A. (1991) *Int J Vitam Nutr Res* **61**(3), 258-63

Barua, A. B., Gunning, D. B., and Olson, J. A. (1991) *Biochem J* **277**(Pt 2), 527-31.

Berlohr, D. A. a. M. A. S. (1996) in *Biochemistry of Lipids, Lipoproteins and Membranes* (Vance, J. E., ed), pp. 257-281, Elsevier Science, New York

Berlohr, D. A., Ribarik-Coe, N, Simpson, M.A., and A.V. Hertzel. (1997) in *Dietary fat and cancer* (AICR, ed), pp. 145-156, Plenum Press, New York

Berne, R. M., M.N. Levy. (1996) *Principles of Physiology*, 2nd Ed., Mosby, St Louis, MO.

Blaner, W. S., Dixon, J. L., Moriwaki, H., Martino, R. A., Stein, O., Stein, Y., and Goodman, D. S. (1987) *Eur J Biochem* **164**(2), 301-7.

Blaner, W. S., and J.A. Olson. (1994) in *The Retinoids: Biology, Chemistry, and Medicine* (Sporn, M. S., Roberts, A.B., and D.S. Goodman, ed), Raven Press, New York

Blomhoff, R., Norum, K. R., and Berg, T. (1985) *J Biol Chem* **260**(25), 13571-5.

Blomhoff, R. (1994) in *Vitamin A in Health and Disease* (Blomhoff, R., ed), pp. 1-37, Marcel Dekkar Inc., New York

Braissant, O., Foufelle, F., Scotto, C., Dauca, M., and Wahli, W. (1996) *Endocrinology* **137**(1), 354-66.

Brodie, A. E., Manning, V. A., and Hu, C. Y. (1996) *Biochem Biophys Res Commun* **228**(3), 655-61.

Brodie, A. E., Azarenko, V. A., and Hu, C. Y. (1996) *Toxicol Lett* **84**(1), 55-9.

Brodie, A. E., Azarenko, V. A., and Hu, C. Y. (1997) *Toxicol Lett* **90**(2-3), 91-5.

Brodie, A. E., Manning, V. A., Ferguson, K. R., Jewell, D. E., and Hu, C. Y. (1999) *J Nutr* **129**(3), 602-6.

Brun, R. P., Kim, J. B., Hu, E., Altiok, S., and Spiegelman, B. M. (1996) *Curr Opin Cell Biol* **8**(6), 826-32.

Butterwith, S.C, Donnelly, LE, and A. Cryer. (1989) *Br. Poult Sci.* **30**(2), 371-8.

Butterwith, S.C. (1997) *Poutry Sci.* **76**(1), 188-23.

Camp, H. S., and Tafuri, S. R. (1997) *J Biol Chem* **272**(16), 10811-6.

Cao, Z., Umek, R. M., and McKnight, S. L. (1991) *Genes Dev* **5**(9), 1538-52.

Chawla, A., and Lazar, M. A. (1994) *Proc Natl Acad Sci U S A* **91**(5), 1786-90.

Chawla, A., Schwarz, E. J., Dimaculangan, D. D., and Lazar, M. A. (1994) *Endocrinology* **135**(2), 798-800.

Chawla, A., and Lazar, M. A. (1994) *Proc Natl Acad Sci U S A* **91**(5), 1786-90.

Chen, C. L. (1996) in *Animal Sciences*, Oregon State University, Corvallis

Chen, C. L., Brodie, A. E., and Hu, C. Y. (1997) *Obes Res* **5**(2), 146-52.

Cheneval, D., Christy, R. J., Geiman, D., Cornelius, P., and Lane, M. D. (1991) *Proc Natl Acad Sci U S A* **88**(19), 8465-9.

Christy, R. J., Yang, V. W., Ntambi, J. M., Geiman, D. E., Landschulz, W. H., Friedman, A. D., Nakabeppu, Y., Kelly, T. J., and Lane, M. D. (1989) *Genes Dev* **3**(9), 1323-35.

Christy, R. J., Kaestner, K. H., Geiman, D. E., and Lane, M. D. (1991) *Proc Natl Acad Sci U S A* **88**(6), 2593-7.

Clarke, S. D., Armstrong, M. K., and Jump, D. B. (1990) *J Nutr* **120**(2), 218-24.

Clarke, S. D., Armstrong, M. K., and Jump, D. B. (1990) *J Nutr* **120**(2), 225-31.

Clarke, S. D., and Hembree, J. (1990) *J Nutr* **120**(6), 625-30.

Clarke, S. D. (1993) *J Anim Sci* **71**(7), 1957-65.

Clarke, S. D., and Jump, D. B. (1993) *Prog Lipid Res* **32**(2), 139-49

Clarke, S. D., and Jump, D. B. (1994) *Annu Rev Nutr* **14**, 83-98

Clarke, S. D., and Jump, D. (1997) *Prostaglandins Leukot Essent Fatty Acids* **57**(1), 65-9.

Clarkson, R. W., Chen, C. M., Harrison, S., Wells, C., Muscat, G. E., and Waters, M. J. (1995) *Mol Endocrinol* **9**(1), 108-20.

Cognet, M. V. a. M. D. L. (1993) *Curr. Opin. Gen. Dev.* **3**(238-245)

Cornelius, P., MacDougald, O. A., and Lane, M. D. (1994) *Annu Rev Nutr* **14**, 99-129

de la Brousse, F. C., Shan, B., and Chen, J. L. (1996) *Proc Natl Acad Sci US A* **93**(9), 4096-101.

De Leenheer, A. P., Lambert, W. E., and Claeys, I. (1982) *J Lipid Res* **23**(9), 1362-7.

Donoghue, S., Kronfeld, D. S., and Sklan, D. (1983) *Br J Nutr* **50**(2), 235-48.

Dunshea , F. R., Harris, D.M., Bauman, D.E., Boyd, R.D., and A. W. Bell. (1992) *J. Anim. Sci.* **70**, 141-151

Eckhoff, C., and Nau, H. (1990) *Arch Toxicol* **64**(6), 502-3

Eckhoff, C., Collins, M. D., and Nau, H. (1991) *J Nutr* **121**(7), 1016-25.

Erdman, J. W., Jr., Thatcher, A. J., Hofmann, N. E., Lederman, J. D., Block, S. S., Lee, C. M., and Mokady, S. (1998) *J Nutr* **128**(11), 2009-13.

Ezaki, O., Flores-Riveros, J. R., Kaestner, K. H., Gearhart, J., and Lane, M. D. (1993) *Proc Natl Acad Sci U S A* **90**(8), 3348-52.

Flores-Riveros, J. R., Kaestner, K. H., Thompson, K. S., and Lane, M. D. (1993) *Biochem Biophys Res Commun* **194**(3), 1148-54.

Flores-Riveros, J. R., McLenithan, J. C., Ezaki, O., and Lane, M. D. (1993) *Proc Natl Acad Sci U S A* **90**(2), 512-6.

Forman, B. M., Tontonoz, P., Chen, J., Brun, R. P., Spiegelman, B. M., and Evans, R. M. (1995) *Cell* **83**(5), 803-12.

Forman, B. M., and Evans, R. M. (1995) *Ann N Y Acad Sci* **761**, 29-37.

Forman, B. M., Goode, E., Chen, J., Oro, A. E., Bradley, D. J., Perlmann, T., Noonan, D. J., Burka, L. T., McMorris, T., Lamph, W. W., and et al. (1995) *Cell* **81**(5), 687-93.

Forman, B. M., Umesono, K., Chen, J., and Evans, R. M. (1995) *Cell* **81**(4), 541-50.

Freytag, S. O., Paielli, D. L., and Gilbert, J. D. (1994) *Genes Dev* **8**(14), 1654-63.

Friedman, A. D., Landschulz, W. H., and McKnight, S. L. (1989) *Genes Dev* **3**(9), 1314-22.

Geison, R. L., and Johnson, B. C. (1970) *Lipids* **5**(4), 371-8.

Giguere, V., Ong, E. S., Segui, P., and Evans, R. M. (1987) *Nature* **330**(6149), 624-9.

Gjoen, T., Bjerkelund, T., Blomhoff, H. K., Norum, K. R., Berg, T., and Blomhoff, R. (1987) *J Biol Chem* **262**(23), 10926-30.

Goodman, D. S. (1984) *J Natl Cancer Inst* **73**(6), 1375-9.

Goodman, D. S. (1984) *N Engl J Med* **310**(16), 1023-31.

Gregoire, F. M., Smas, C.M., and H.S. Sul. (1998) *Physiological Reviews* **78,** 783-809

Hanson, R. W. (1998) *J Biol Chem* **273**(44), 28543.

Harris, W. S. (1986) *J. Lipid Res.* **30,** 785-794

Hausman, D. B., Hausman, G. J., and Martin, R. J. (1993) *Biol Neonate* **64**(2-3), 116-26

Hausman, D. B., Hausman, G. J., and Martin, R. J. (1996) *Biol Neonate* **70**(1), 41-51

Hausman, G. J., and Wright, J. T. (1996) *J Anim Sci* **74**(2), 354-62.

Hausman, G. J., and Richardson, R. L. (1998) *J Anim Sci* **76**(1), 48-60.

Hausman, G. J., and Yu, Z. K. (1998) *Growth Dev Aging* **62**(3), 107-18.

Hemati, N., Ross, S. E., Erickson, R. L., Groblewski, G. E., and MacDougald, O. A. (1997) *J Biol Chem* **272**(41), 25913-9.

Hendricks-Taylor, L. R., and Darlington, G. J. (1995) *Nucleic Acids Res* **23**(22), 4726-33.

Hendriks, H. F., Brekelmans, P. J., Buytenhek, R., Brouwer, A., de Leeuw, A. M., and Knook, D. L. (1987) *Lipids* **22**(4), 266-73.

Heyman, R. A., Mangelsdorf, D. J., Dyck, J. A., Stein, R. B., Eichele, G., Evans, R. M., and Thaller, C. (1992) *Cell* **68**(2), 397-406.

Houseknecht, K. L., Bidwell, C. A., Portocarrero, C. P., and Spurlock, M. E. (1998) *Gene* **225**(1-2), 89-96.

Houseknecht, K. L., and Portocarrero, C. P. (1998) *Domest Anim Endocrinol* **15**(6), 457-75.

Houseknecht, K. L., Baile, C. A., Matteri, R. L., and Spurlock, M. E. (1998) *J Anim Sci* **76**(5), 1405-20.

Houseknecht, K. L., Vanden Heuvel, J. P., Moya-Camarena, S. Y., Portocarrero, C. P., Peck, L. W., Nickel, K. P., and Belury, M. A. (1998) *Biochem Biophys Res Commun* **244**(3), 678-82.

Hu, E., Tontonoz, P., and Spiegelman, B. M. (1995) *Proc Natl Acad Sci U S A* **92**(21), 9856-60.

Hu, E., Kim, J. B., Sarraf, P., and Spiegelman, B. M. (1996) *Science* **274**(5295), 2100-3.

Hwang, C. S., Mandrup, S., MacDougald, O. A., Geiman, D. E., and Lane, M. D. (1996) *Proc Natl Acad Sci U S A* **93**(2), 873-7.

Ibrahimi, A., Bonino, F., Bardon, S., Ailhaud, G., and Dani, C. (1992) *Biochem Biophys Res Commun* **187**(3), 1314-22.

Ibrahimi, A., Teboul, L., Gaillard, D., Amri, E. Z., Ailhaud, G., Young, P., Cawthorne, M. A., and Grimaldi, P. A. (1994) *Mol Pharmacol* **46**(6), 1070-6.

Inagami, S., and Ong, D. E. (1992) *J Nutr* **122**(3), 450-6.

IUPAC-IUB. (1982) *Eur. J. Biochemistry* **129**, 1-5

Johnson, E. J., Qin, J., Krinsky, N. I., and Russell, R. M. (1997) *J Nutr* **127**(10), 1993-9.

Jump, D. B., Clarke, S. D., Thelen, A., and Liimatta, M. (1994) *J Lipid Res* **35**(6), 1076-84.

Jump, D. B., Ren, B., Clarke, S., and Thelen, A. (1995) *Prostaglandins Leukot Essent Fatty Acids* **52**(2-3), 107-11.

Jump, D. B., Clarke, S. D., Thelen, A., Liimatta, M., Ren, B., and Badin, M. V. (1997) *Adv Exp Med Biol* **422,** 167-76

Jump, D. B., Badin, M. V., and Thelen, A. (1997) *J Biol Chem* **272**(44), 27778-86.

Kaestner, K. H., Christy, R. J., and Lane, M. D. (1990) *Proc Natl Acad Sci U S A* **87**(1), 251-5.

Kamei, Y., Kawada, T., Kazuki, T., Ono, T., and E. Sugimoto. (1993) *Biochem. Biophys. Res. Commun.* **193**:948-55.

Kawada, T., Kamei, Y., and Sugimoto, E. (1996) *Int J Obes Relat Metab Disord* **20 Suppl 3,** S52-7.

Kawada, T., Kamei, Y., Fujita, A., Hida, Y., Takahashi, N., Sugimoto, E., and Fushiki, T. (2000) *Biofactors* **13**(1-4), 103-9

Klaus, S. (1997) *Bioessays* **19**(3), 215-23.

Kletzien, R. F., Foellmi, L. A., Harris, P. K., Wyse, B. M., and Clarke, S. D. (1992) *Mol Pharmacol* **42**(4), 558-62.

Kletzien, R. F., Clarke, S. D., and Ulrich, R. G. (1992) *Mol Pharmacol* **41**(2), 393-8.

Kliewer, S. A., Umesono, K., Noonan, D. J., Heyman, R. A., and Evans, R. M. (1992) *Nature* **358**(6389), 771-4.

Kliewer, S. A., Umesono, K., Heyman, R. A., Mangelsdorf, D. J., Dyck, J. A., and Evans, R. M. (1992) *Proc Natl Acad Sci U S A* **89**(4), 1448-52.

Kliewer, S. A., Forman, B. M., Blumberg, B., Ong, E. S., Borgmeyer, U., Mangelsdorf, D. J., Umesono, K., and Evans, R. M. (1994) *Proc Natl Acad Sci U S A* **91**(15), 7355-9.

Kliewer, S. A., Lenhard, J. M., Willson, T. M., Patel, I., Morris, D. C., and Lehmann, J. M. (1995) *Cell* **83**(5), 813-9.

Landschulz, W. H., Johnson, P. F., and McKnight, S. L. (1989) *Science* **243**(4899), 1681-8.

Lane, M. D., Flores-Riveros, J. R., Hresko, R. C., Kaestner, K. H., Liao, K., Janicot, M., Hoffman, R. D., McLenithan, J. C., Kastelic, T., and Christy, R. J. (1990) *Diabetes Care* **13**(6), 565-75.

Lee, K., Hausman, G. J., and Dean, R. G. (1998) *Mol Cell Biochem* **178**(1-2), 269-74.

Legraverend, C., Antonson, P., Flodby, P., and Xanthopoulos, K. G. (1993) *Nucleic Acids Res* **21**(8), 1735-42.

Leid, M., Kastner, P., and Chambon, P. (1992) *Trends Biochem Sci* **17**(10), 427-33.

Leo, M. A., Iida, S., and Lieber, C. S. (1984) *Arch Biochem Biophys* **234**(1), 305-12.

Levin, A. A., Sturzenbecker, L. J., Kazmer, S., Bosakowski, T., Huselton, C., Allenby, G., Speck, J., Kratzeisen, C., Rosenberger, M., Lovey, A., and et al. (1992) *Nature* **355**(6358), 359-61.

Levin, A. A., Sturzenbecker, L. J., Kazmer, S., Bosakowski, T., Huselton, C., Allenby, G., Speck, J., Kratzeisen, C., Rosenberger, M., Lovey, A., and et al. (1992) *Ann N Y Acad Sci* **669**, 70-85; discussion 85-6.

Lin, F. T., and Lane, M. D. (1992) *Genes Dev* **6**(4), 533-44.

Lin, F. T., MacDougald, O. A., Diehl, A. M., and Lane, M. D. (1993) *Proc Natl Acad Sci U S A* **90**(20), 9606-10.

Lin, F. T., and Lane, M. D. (1994) *Proc Natl Acad Sci U S A* **91**(19), 8757-61.

Loftus, T. M., and Lane, M. D. (1997) *Curr Opin Genet Dev* **7**(5), 603-8.

Luu, L., Ramshaw, H., Tahayato, A., Stuart, A., Jones, G., White, J., and Petkovich, M. (2001) *Adv Enzyme Regul* **41**, 159-75

MacDougald, O. A., Cornelius, P., Lin, F. T., Chen, S. S., and Lane, M. D. (1994) *J Biol Chem* **269**(29), 19041-7.

Mahley, R. W., Weisgraber, K. H., Innerarity, T. L., and Rall, S. C., Jr. (1991) *Jama* **265**(1), 78-83.

Malasanos, T. H., and Stacpoole, P. W. (1991) *Diabetes Care* **14**(12), 1160-79.

Mangelsdorf, D. J., Borgmeyer, U., Heyman, R. A., Zhou, J. Y., Ong, E. S., Oro, A. E., Kakizuka, A., and Evans, R. M. (1992) *Genes Dev* **6**(3), 329-44.

Mangelsdorf, D. J., Umesono, K, and R.M. Evans. (1994) in *The Retinoids: Biology, Chemistry, and Medicine* (Sporn, M. S., Roberts, A.B., and D.S. Goodman, ed), Raven Press, New York

Matsuno, F., Chowdhury, S., Gotoh, T., Iwase, K., Matsuzaki, H., Takatsuki, K., Mori, M., and Takiguchi, M. (1996) *J Biochem (Tokyo)* **119**(3), 524-32.

McDonald, P., Edwards, R.A., Greenhalgh, J.F., and C.A. Morgan. (1995) *Animal Nutrition*, 5th Ed., Longman Scientific and Technical, Burnt Mill

McKeon, C., and Pham, T. (1991) *Biochem Biophys Res Commun* **174**(2), 721-8.

Mersmann, H. J. (1990) in *Reducing fat in meat animals* (Fischer, A. V., ed), pp. 101-136, Elsevier Applied Sciences, London and New York

Moran, E. T., Jr. (1989) *Poult Sci* **68**(8), 1141-7.

Moriwaki, H., Blaner, W. S., Piantedosi, R., and Goodman, D. S. (1988) *J Lipid Res* **29**(11), 1523-34.

Murray, R. K., Granner, D.K., Mayes, P.A., and V.W. Rodwell. (1996) *Harper's Biochemistry*, Appleton and Lange, Stamford, CT

Muscat, G. E., Downes, M., and Dowhan, D. H. (1995) *Bioessays* **17**(3), 211-8.

Nakai, A., Hasegawa, K., and M., Hiramitsu. (1992) *J. Clin. Vet Med.* **10**: 2142-2148.

Napoli, J. L., Pramanik, B. C., Williams, J. B., Dawson, M. I., and Hobbs, P. D. (1985) *J Lipid Res* **26**(3), 387-92.

Nerlov, C., and Ziff, E. B. (1994) *Genes Dev* **8**(3), 350-62.

Nerlov, C., and Ziff, E. B. (1995) *Embo J* **14**(17), 4318-28.

Nilsson-Ehle, P., Garfinkel, A. S., and Schotz, M. C. (1976) *Biochim Biophys Acta* **431**(1), 147-56.

Noy, N., and Xu, Z. J. (1990) *Biochemistry* **29**(16), 3883-8.

Noy, N., and Blaner, W. S. (1991) *Biochemistry* **30**(26), 6380-6.

Noy, N., Slosberg, E., and Scarlata, S. (1992) *Biochemistry* **31**(45), 11118-24.

NRC. (1994), National Academy of Science Press, Washington, DC.

NRC. (1998), National Academy of Science Press, Washington, DC.

O'Hea, E. K., and G.A. Levielle. (1969) *J. Nutr.* **99**, 338-347

Ohyama, M., Matsuda, K., Torii, S., Matsui, T., Yano, H., Kawada, T., and T. Ishihara. (1998) *J. Anim. Sci.* **76**, 61-65.

Oka, A., Hasegawa, K., and M., Hiramitsu. (1992) *J. Clin. Vet Med.* **10**: 2152-2158.

Okuno, M., Caraveo, V. E., Goodman, D. S., and Blaner, W. S. (1995) *J Lipid Res* **36**(1), 137-47.

Ong, D. E., Lucas, P. C., Kakkad, B., and Quick, T. C. (1991) *J Lipid Res* **32**(9), 1521-7.

Ong, D. E. (1993) *J Nutr* **123**(2 Suppl), 351-5.

Pan, D. A., Hulbert, A. J., and Storlien, L. H. (1994) *J Nutr* **124**(9), 1555-65.

Pariza, M. W., Park, Y., and Cook, M. E. (2000) *Proc Soc Exp Biol Med* **223**(1), 8-13.

Petkovich, M., Brand, N. J., Krust, A., and Chambon, P. (1987) *Nature* **330**(6147), 444-50.

Potter, J. J., Mezey, E., Christy, R. J., Crabb, D. W., Stein, P. M., and Yang, V. W. (1991) *Arch Biochem Biophys* **285**(2), 246-51.

Power, R. F., Conneely, O. M., and O'Malley, B. W. (1992) *Trends Pharmacol Sci* **13**(8), 318-23.

Quick, T. C., and Ong, D. E. (1990) *Biochemistry* **29**(50), 11116-23.

Ramsay, T. G., Hausman, G. J., and Martin, R. J. (1987) *Cell Tissue Res* **250**(1), 185-90.

Ramsay, T. G., Hausman, G. J., and Martin, R. J. (1987) *J Anim Sci* **64**(3), 735-44.

Ramsay, T. G., Hausman, G. J., and Martin, R. J. (1987) *J Anim Sci* **64**(3), 745-51.

Ren, B., Thelen, A., and Jump, D. B. (1996) *J Biol Chem* **271**(29), 17167-73.

Ren, B., Thelen, A. P., Peters, J. M., Gonzalez, F. J., and Jump, D. B. (1997) *J Biol Chem* **272**(43), 26827-32.

Ribot J., Felipe, F., Bonet, M.L. and A. Palou. (2001), *Obes Res* **9**:500-09

Rigtrup, K. M., and Ong, D. E. (1992) *Biochemistry* **31**(11), 2920-6.

Rosebrough, RW, McMurtry, JP and R. Vasilatos-Younken. (1999) *Poultry Sci.* **78**:992-8.

Ross, A. C. (1993) *J Nutr* **123**(2 Suppl), 346-50.

Ross, A. C. (1993) *Faseb J* **7**(2), 317-27.

Ross, A. C., and Ternus, M. E. (1993) *J Am Diet Assoc* **93**(11), 1285-90; quiz 1291-2.

Salati, L. M. a. A. G. G. (1996) in *Biochemistry of Lipids, Lipoproteins and Membranes* (Vance, J. E., ed), pp. 257-281, Elsevier Science, New York

Sandouk, T., Reda, D., and Hofmann, C. (1993) *Endocrinology* **133**(1), 352-9.

Sandouk, T., Reda, D., and Hofmann, C. (1993) *Am J Physiol* **264**(6 Pt 1), C1600-8.

Schoonjans, K., Staels, B., and Auwerx, J. (1996) *Biochim Biophys Acta* **1302**(2), 93-109.

Schwarz, E. J., Reginato, M. J., Shao, D., Krakow, S. L., and Lazar, M. A. (1997) *Mol Cell Biol* **17**(3), 1552-61.

Smith, J. E., Milch, P. O., Muto, Y., and Goodman, D. S. (1973) *Biochem J* **132**(4), 821-7.

Sporn, M. S. a. A. B. R. (1994) in *The Retinoids: Biology, Chemistry, and Medicine* (Sporn, M. S., Roberts, A.B., and D.S. Goodman, ed), Raven Press, New York

Stahl, W., Schwarz, W., von Laar, J., and Sies, H. (1995) *J Nutr* **125**(8), 2128-33.

Sul, H. S., Smas, C.M. and N. Moustaid. (1993) *J. Nutr. Biochem.* **4**, 554-562

Suryawan, A., and Hu, C. Y. (1997) *J Anim Sci* **75**(1), 112-7.

Szymczyk, B., Pisulewski, P. M., Szczurek, W., and Hanczakowski, P. (2001) *Br J Nutr* **85**(4), 465-73.

Tae, H. J., Luo, X., and Kim, K. H. (1994) *J Biol Chem* **269**(14), 10475-84.

Takyi, E. E. (1999) *J Nutr* **129**(8), 1549-54.

Tang, G. W., and Russell, R. M. (1990) *J Lipid Res* **31**(2), 175-82.

Timchenko, N., Wilson, D. R., Taylor, L. R., Abdelsayed, S., Wilde, M., Sawadogo, M., and Darlington, G. J. (1995) *Mol Cell Biol* **15**(3), 1192-202.

Tontonoz, P., Hu, E., and Spiegelman, B. M. (1994) *Cell* **79**(7), 1147-56.

Tontonoz, P., Graves, R. A., Budavari, A. I., Erdjument-Bromage, H., Lui, M., Hu, E., Tempst, P., and Spiegelman, B. M. (1994) *Nucleic Acids Res* **22**(25), 5628-34.

Tontonoz, P., Hu, E., Graves, R. A., Budavari, A. I., and Spiegelman, B. M. (1994) *Genes Dev* **8**(10), 1224-34.

Tontonoz, P., Hu, E., and Spiegelman, B. M. (1995) *Curr Opin Genet Dev* **5**(5), 571-6.

Tontonoz, P., Hu, E., Devine, J., Beale, E. G., and Spiegelman, B. M. (1995) *Mol Cell Biol* **15**(1), 351-7.

Torii,S., Matsui, T., and H. Yano. (1996) *Anim. Sci.* **63**:73-8.

Torii, S. Matsumoto, K., Matsui, T. and H. Yano. (1995), *Anim. Sci. Technol.* **66**:1039-42.

Tso, P. (1985) *Adv Lipid Res* **21**, 143-86

Tsutsumi, C., Okuno, M., Tannous, L., Piantedosi, R., Allan, M., Goodman, D. S., and Blaner, W. S. (1992) *J Biol Chem* **267**(3), 1805-10.

van den Berg, H. (1999) *Nutr Rev* **57**(1), 1-10.

Vasseur-Cognet, M., and Lane, M. D. (1993) *Proc Natl Acad Sci U S A* **90**(15), 7312-6.

Vasseur-Cognet, M., and Lane, M. D. (1993) *Curr Opin Genet Dev* **3**(2), 238-45.

Vidal, H., Auboeuf, D., De Vos, P., Staels, B., Riou, J. P., Auwerx, J., and Laville, M. (1996) *J Clin Invest* **98**(2), 251-5.

Vidal-Puig, A., Jimenez-Linan, M., Lowell, B. B., Hamann, A., Hu, E., Spiegelman, B., Flier, J. S., and Moller, D. E. (1996) *J Clin Invest* **97**(11), 2553-61.

Walton, P. E. a. T. D. E. (1986) *J. Anim. Sci.* **62**, 1584-1593

Wang, D., and Sul, H. S. (1995) *J Biol Chem* **270**(48), 28716-22.

Warden, C. H., Langner, C. A., Gordon, J. I., Taylor, B. A., McLean, J. W., and Lusis, A. J. (1989) *J Biol Chem* **264**(36), 21573-81.

Williams, P. M., Chang, D. J., Danesch, U., Ringold, G. M., and Heller, R. A. (1992) *Mol Endocrinol* **6**(7), 1135-41.

Wolf, G. (1995) *Nutr Rev* **53**(4 Pt 1), 98-9.

Wouters, W., van Dun, J., Dillen, A., Coene, M. C., Cools, W., and De Coster, R. (1992) *Cancer Res* **52**(10), 2841-6.

Xing, H., Northrop, J. P., Grove, J. R., Kilpatrick, K. E., Su, J. L., and Ringold, G. M. (1997) *Endocrinology* **138**(7), 2776-83.

Xue, J. C., Schwarz, E. J., Chawla, A., and Lazar, M. A. (1996) *Mol Cell Biol* **16**(4), 1567-75.

Yamada, M., Blaner, W. S., Soprano, D. R., Dixon, J. L., Kjeldbye, H. M., and Goodman, D. S. (1987) *Hepatology* **7**(6), 1224-9.

Zhang, B., Graziano, M. P., Doebber, T. W., Leibowitz, M. D., White-Carrington, S., Szalkowski, D. M., Hey, P. J., Wu, M., Cullinan, C. A., Bailey, P., Lollmann, B., Frederich, R., Flier, J. S., Strader, C. D., and Smith, R. G. (1996) *J Biol Chem* **271**(16), 9455-9.

Zhang, B., Berger, J., Hu, E., Szalkowski, D., White-Carrington, S., Spiegelman, B. M., and Moller, D. E. (1996) *Mol Endocrinol* **10**(11), 1457-66.

Zhu, Y., Qi, C., Korenberg, J. R., Chen, X. N., Noya, D., Rao, M. S., and Reddy, J. K. (1995) *Proc Natl Acad Sci U S A* **92**(17), 7921-5.

APPENDICES

Appendix A. Primary Culture Materials

A. Equipment

1. Dessicator
2. Dish Pan
3. Surgical Tray
4. Scrub Brush
5. Ethanol Spray Bottle
6. Bunsen Burner
7. Disection Kit
   A. Autoclavable Surgical pack or Stainless Steel Utility Tray w/lid
   B. #10 scalpel handle
   C. Forceps
   D. Scissors
   E. Flexible Plastic Rod
   F. 15 ml Flask (Nalgene)
   G. Rubber Stopper
8. Sectioned Petri Dish (Parbur Medical Products; 100x15mm)
9. 15 ml Falcon tubes (cat. 352099)
10. 50 ml Falcon Tubes (cat. 352070)
11. Chiffon filters (250 μM, 75 μM pore sizes)
12. Costar 6 well plates (cat 072083 **Note**: We had problems with cells pulling off of the well when recently trying cat # 0720080. I suggest you avoid using cat # 0720080.)
13. Corning sterile culture petri dishes (cat. 25026)
14. Pipettes (Falcon)
    A. 1ml (cat. 7506)
    B. 5ml (cat. 357543)
    C. 10ml (cat. 357551)
    D. 25ml (cat. 357515)

B. Reagents

1. Fetal Bovine Serum (ATCC Vitacell cat. 30-2020)
2. DME (Sigma cat. D5523)
3. Ham's F12 (Sigma cat. N6760)
4. Insulin (Sigma cat. 16634)
5. Transferrin (Sigma cat. T1428)
6. Hydrocortisone (Sigma cat. H0396)
7. Collagenase Type 1 (Worthington type 1 CLS 1 #4196)
8. Gentamycin Sulfate (Sigma G1264)
9. Fungizone (Gibco cat. 600-5295AE)
10. Absolute Ethanol

Appendix B. Porcine stromal vascular cell isolation

1. Pre-surgery day preparation:

   A. prepare and sterilize: forceps, scissors, plastic rods, scalpel handle, funnels, culture flasks, stoppers, and filter materials.

2. Dissecting fat pads:
   A. pigs are 2-3 days old.
   B. turn on water bath and hood.
   C. prepare 100 mLs sterile KRB+antibiotics—add 1mL gentamycin (40 μg/mL from stock 4000 μg/mL) and 12.5μL fungizone (2.5 ng/mL from stock 250 ng/mL) to 98 mLs KRB. Put in water bath.
   D. weigh one empty petri plate and record.
   E. check seal on dessicator for lubricant. Turn off small valve and hook tube up to jar.
   F. euthanize w/ CO_2
   G. wash: First in soapy water
      Scrub with iodine, rinse in soapy water
      Scrub with iodine and rinse again
      Scrub again with iodine and then rinse iodine off neck with 70% EtOH
   H. transfer to hood and assemble scalpel blade.
   I. make dorsal incision from just above base of head, down and then across to form right angle (at either top or base of dorsal incision.)
   J. pull skin up and cut fat off of skin using scalpel.
   K. make incision off other side of dorsal cut—incisions end up looking like a T.
   L. completely cut fat pad away from skin, then separate from muscle underneath.
   M. transfer fat tissue to petri plate and weigh. Put in incubator while calculations are done.

3. Isolating cells:
   A. at any time of not doing something specific with cells, keep them warm either in incubator (petri plates) or in 37° water bath (culture tubes).
   B. add 6.3 mg collagenase per gram of tissue to 3 mLs KRB+antibiotics per gram of tissue. Filter sterilize into centrifuge tube and add to tissue in petri plate.
   C. dice tissue up into very small pieces, the smaller the better, using scissors.
   D. put into culture flask with stopper, and incubate for one hour in giratory water bath (at about 2.5 rpm) to let digestion occur.
   E. remove culture flask from water bath and prepare to filter. Set up chiffon inside of funnel, inside of 50 mL centrifuge tube.

F. pour cell culture into chiffon, and fold the separated side of chiffon over to other side.
G. moosh cells through with plastic rod, using downward sweeping motion. Rinse tissue left in chiffon with about 10 mLs of sterile KRB+antibiotics and moosh. Do this two times.
H. let cells in centrifuge tube sit in water bath as cells rise to top.
I. centrifuge at 800g for about 10 mins. (setting @ ¾ on our old centrifuge in spec/lunch room)
J. carefully decant supernatant into waste beaker.
K. add 10mLs KRB+antibiotics to pellet and mix with pipette. Centrifuge again.
L. carefully and slowly suck supernatant off with pipette and dispose into waste beaker.
M. add 5 mLs of DME+10%FBS and re-suspend pellet evenly.
N. filter cell suspension (using same procedure as before) through 75 micron filter into clean, sterile centrifuge tube.
O. rinse filter with 5 or 10 mLs (depending on estimated amount of cells) of DME+10%FBS.
P. do cell count and aliquot cells into plates at desired density.

4. Cell Counts:
A. If we got three counts of 69, 75, and 70, for an average of 71:

71×10^4 cells times 10 mLs (or by amount that cells were resuspended in) $= 7.1 \times 10^6$ cells total

if desired plating density is 5×10^5 cells/well,
total well # is:

$$\frac{7.1 \times 10^6 \text{ cells}}{3 \times 10^5 \text{ cells}} = 23.66 \text{ , about 24 wells.}$$

B. Add enough media to the cell suspension to make 24 wells at 2 mLs/well, mix well, and plate cells.

Appendix C. Day 0: Wash and Induction of Primary Cells

The culture system is a serum free one. The presence of serum will inhibit differentiation for unknown reasons. Thus it is essential to wash FBS from cells as thoroughly as possible.

1. Wash

A. Aspirate overnight media completely from monolayer.
B. Add appropriate volume DME/F12 media (prewarmed to 37° C and free of FBS) and gently swirl. Place in incubator for 5 minutes.
C. Repeat steps A & B (for total of two 5 minute washes).
D. Incubate third wash for 1 hr at 37° C.
E. Induce and treat as below.

2. Induction

A. Mix ITC media (10 μg/ml transferrin, 100nM insulin, 50 ng/ml HC)

100 ml volume: (all stocks should be sterilized via filtration)

1. 100 μl insulin (100 μM stock)
2. 100 μl transferrin (10 mg/ml stock)
3. 5 ml hydrocortisone (1 μg/ml stock; make sure its in serum free media!)
4. 95 ml DME/F12 media

B. Add 2 ml per well (6 well plate) or 10 ml per petri dish.
C. Change media every third day until experiment ends on d 10 with GPDH assay and Bradford protein analysis.

Appendix D. Making DME-F12

2 liters:

A. Add one bottle of DME and one bottle of F12 to 1000 ml of ddH2O.
B. Rinse each bottle out with extra 400 ml of water.
C. Stir until all media powder has dissolved.
D. Add 550 ml of ddH2O.
E. Add 4.88g NaHCO3.
F. Stir again for several minutes.
G. Add 100 mg Gentamycin.
H. Adjust pH to 7.3 with 5M HCl.
I. Filter sterilize.

With Hepes:
A. Add one bottle of DME and one bottle of F12 to 1000 ml of ddH2O.
B. Rinse each bottle out with extra 400 ml of water.
C. Stir until all media powder has dissolved.
D. Add 550 ml of ddH2O.
E. Add 50 ml of HEPES buffer (1 M pH 7.2), kept in refrigerator.
F. Add 3.2g of NaHCO3.
G. Stir again for several minutes.
H. Add 100 mg Gentamycin.
I. No need to adjust pH.
J. Filter sterilize.

Appendix E. Preparation of KRB Buffer

For 1.5 Liters:

Content:	**Grams:**
H2O	1200.0
.770 M NaCl	10.665
.770 M KCl	0.5425
.770 M KH2PO4	0.2435
.770 M MgSO4	0.4415
.770 M NaHCO3	3.2220
.275 M CaCl2H2O	0.2910
20mM Hepes	7.15

Mix contents together thoroughly. Bring up to 1.5 L.

Filter sterilize and keep refrigerated til use.
Do not autoclave, salts will precepitate.

Appendix F. Preparation of Insulin Stock

Stock: 100 μM Insulin for cell culture
6 mg Insulin (MW approx. 6000)
100 μl glacial acetic acid
10 ml dd H_2O

Filter sterilize (.22 micron filter)

Aliquot insulin in 1 ml volumes into eppendorfs that are wrapped in tinfoil. Limit exposure to light and minimize the number of times stocks are thawed and refrozen.

Appendix G. I.T.C. Media

Hormone cocktail used in serum free primary culture system

Per 100 mls:

(100 nM Insulin, 10 μg/ml Transferrin, 50 ng/ml Hydrocortisone)

100 μl Insulin (100 μM stock)

100 μl Transferrin (10 mg/ml stock)

5 ml Hydrocortisone (1 μg/ml stock)

95 ml DME/Ham's F12 **(NO FBS)**

Appendix H. Oil Red O Staining of primary adipocytes

Purpose: Oil red O is a specific neutral lipid marker thus ORO staining indicates the presence of lipid in fresh tissue sections or cell cultures.

Principle: Staining with oil-soluble dyes is based upon the greater solubility of the dye in the lipid droplets than in the usual hydroalcoholic dye solvents.

Expected Result: Cytosolic fat is stained red and nuclei are stained blue

A. Solutions:

1. 10 % buffered neutral formalin

100 ml	37% formaldehyde solution
900 ml	distilled H_2O
4 g	Sodium Phosphate (mono H_2O)
6.5g	Anhydrous disodium phosphate

2. .5% Oil red O solution in 100% propylene glycol

.5 g	Oil red O
100 ml	propylene glycol

Comments: Add a small amount of propylene glycol to ORO in a beaker and mix well (crush larger pieces if necessary). Gradually add the remaining propylene glycol while stirring occasionally. Heat gently with stirring until solution reaches 95-100° C for a few minutes as needed (do not let temperature go over 110°). Filter the solution through Whatman #541 (or similar) paper while solution is still warm. Allow solution to stand overnight at room temperature.

3. 100% propylene glycol

4. 60% propylene glycol in distilled water

5. Harris Hematoxylin solution

6. Glycerin-gelatin (heat 40g gelatin and 120 ml glycerin in 210 ml distilled water until dissolved).

B. Procedure

1. Fix cells in 10% buffered neutral formalin (we use 1 ml per well and let stand 15 min.)

note: I have seen labs incubate cells in formalin for as long as 2 hr.

2. Aspirate wells and rinse with distilled water to thoroughly remove formalin.

3. Bathe in 100% propylene glycol for 2 min (we use .5 ml per well for 6 well plates).

note: The ORO is dissolved in propylene glycol so this step essentially equilibrates the system for the carrier. The principle is that ORO is lipid soluble and will preferentially aggregate with neutral lipid rather than the carrier. Perhaps one could also envision this step as a dehydration of the fixed cells?

4. Stain in Oil red O for 10 min (we use .5 ml per well for 6 well plates)

5. Differentiate in 60% propylene glycol for 1 min. (again .5 ml per well)

note: This step removes any ORO that did not bind to lipid as unbound ORO will associate with the propylene glycol. Lipid should be stained red.

6. Rinse thoroughly with distilled water.

7. Stain in Harris Hematoxylin solution for 6 min (again use .5 ml per well).

note: This step stains the nuclei blue.

8. Rinse thoroughly in water (we wash 10 minutes in running water).

9. Mount in glycerin jelly

note: Allow wells to air dry . Then add warm glycerol gel to the well so that the surface is covered. Allow to air dry.

C. General Comments:

Procedure referenced by Suryawan and Hu, 1993. (Comp. Biochem. Physiol. 105A:485-492).

Formalin used is similar to Lilly's buffered neutral formalin and is not buffered with $CaCO_3$).

Some protocols vary from the above in that ORO is dissolved in isopropanol rather than propylene glycol. In this case, isopropanol rather than propylene glycol should be used in steps 3 and 5 of the above procedure.

PBS may also be used to rinse cells rather than distilled water. If the fixed cells will be probed with antibodies (for detection of something via fluorescence) then PBS is likely a better choice. Detection of lipid in my primary cultures with ORO is a rather blunt process and I have found PBS is not necessary for this purpose.

Appendix I. Phosphate-Buffered Saline Solution (PBS) 20X Concentration

Chemical	*Amount*	*MW*
NaCl	142g	58.44
Na_2HPO_4	23g	141.96
KH_2PO_4	4g	136.09
KCl	4g	74.55

Bring volume up to 1Liter with ddH_2O, filter sterilize under laminar flow hood.

*For use, dilute to 1X concentration with ddH_2O

Appendix J. HEPES (1M pH 7.2)

1. Weigh 119.15g HEPES (no sodium, Sigma H9136 or Fisher BP310-500 or BP310-1) in 500ml beaker.

2. Add 400ml ddH_2O.

3. Stir with stir bar over magnetic plate. Bring pH to 7.2 with Sodium Hydroxide (start with pellets, then liquid) – use ~20 pellets.

4. Bring volume up to 500ml.

5. Sterilize solution by filtering with a 150ml Nalgene disposable bottle topfilter (0.2u), under the laminar flow hood.

6. Label bottle and place in the refrigerator.

Appendix K. The Bradford Dye-Protein Assay

Reference: Bradford, Marion M. 1976. Anal. Biochem. 72, 248-254. A rapid and sensitive method for the quantitative of microgram quantities of protein utilizing the principles of protein-dye binding.

Reagents: Coomassie Brilliant Blue G (Sigma B0770)
Phosphoric acid, reagent grade (85% w/v)
95% Ethanol

Preparation of dye reagent:

1. ***Solution A***: Dissolve 100mg of Coomassie blue dye in 50ml 95% ethanol (use beaker and stirring bar; add dye powder to ethanol while stirring; expect some undissolved dye residue to remain.) Keep in dark brown bottle, in refrigerator.
2. ***Solution B***: Mix 50ml dye solution A with 100ml undiluted reagent grade phosphoric acid. Store solution B in dark brown bottle, refrigerator until needed.
3. ***Solution C***: To prepare working reagent (solution C) dilute 15ml dye solution B to 100ml with ddH_2O and filter through Whatman #1 filter paper. Do this in the dark (in a cupboard) as the protein solution is light sensitive.

Preparation of Standard:

1. Prepare an appropriate protein standard (BSA or IgG) containing 1000ug protein/ml. From this, prepare a series of dilutions containing 0, 50, 100, 250, 500, and 750 ug protein/ml.

Assay:

1. Make sure you have plenty of protein reagents A and B (both kept in the –20 refrigerator). Get protein reagent B.
2. Get enough disposable cuvettes for all your samples plus the six protein standards.
3. Figure out how much protein reagent C you will need. For each cuvette, you need 8ml of reagent C. **Always** make extra reagent, as you will ruin your experiment if you run out of protein reagent C before allocating .8mls to all your cuvettes. It is recommended to make almost double the amount that you need.
4. Make protein solution C and leave to filter while preparing your samples and standards.
5. If the protein standards and samples were frozen, thaw them out and vortex briefly to mix up the suspension. You may need to pulse the samples in the centrifuge in order to spin down enough suspension to use.
6. Alloquot 30uls of each protein standard to a cuvette. Depending on how much sample suspension you have, alloquot 5-30uls to appropriate cuvettes. (After reading on the spec, you will multiply the value to the equivalent of 30uls. ie, if you use 10uls of your samples, multiply the absorption value by 3, so that it is comparable to the standard)

7. Add .8ml of protein solution C to each cuvette and let sit for six minutes before reading. During this time, turn on the spectrophotometer and let warm up. When it is ready, make the following adjustments to the parameters:
   Select "5. Quantitative" and enter.
   Enter "yes" for parameter change.
   Select "1. Wave Length" and enter. Choose "1" and enter. Change wavelength to 595 nm and enter.
   Select "2. Number of Standards" and enter. Change to 6 and enter.
   Select "5. Data print" and enter. "no" will toggle to "yes".
   Select "no" parameter change and enter.
   Enter concentrations of standards (0,50,100,250,500,750) and enter after each.
   Select "no" and enter for the next two options.
   Scan standards.

8. View linear plot of standards to make sure none of the standards deviate greatly from a straight line. If fit is good, proceed. If one or more deviates greatly, make a new cuvette of the deviating standard(s) and repeat procedure above.
9. Proceed with samples. Data should print out after each sample.
10. Once complete and all data seems reasonable (if one does not seem reasonable, make another cuvette with sample and Solution C, run again in spec.), discard all cuvettes and turn off spectrophotometer.
11. Clean up bench.

Remember to multiply the values for the protein samples by the appropriate factor in order to make the sample values comparable to the standards